Heat Shock Proteins 7
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Moonlighting Cell Stress Proteins in Microbial Infections

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HEAT SHOCK PROTEINS

Volume 7

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About the Editor

Brian Henderson is a professor of biochemistry at University College London. He started his research career as a cell biologist, studying the chronic inflammatory disease, rheumatoid arthritis. This was followed by some years in the pharmaceutical industry developing inhibitors of cytokines and matrix metalloproteinases. He then rejoined academia and became interested in the study of bacteria–host interactions, publishing the first book on the new science of cellular microbiology. His research led to the discovery of the extracellular actions of bacterial molecular chaperones and the realisation that these proteins were functioning as moonlighting proteins and that such bacterial moonlighting proteins could function as virulence factors. Currently, his research is focused on the biological actions of secreted cell stress proteins in infection and cardiovascular disease.

Preface

It is now clearly recognised that infection of metazoans by microbial pathogens involves significant molecular and cellular interactions between host and pathogen. It is also recognised that microbial infection is a major cause of stress, both for the invading microbe and the invaded host. Molecular chaperones and protein-folding catalysts are essential proteins in all three Kingdoms of life. Many of the genes encoding these proteins are up-regulated when cells are stressed, and so these molecules are often referred to, collectively, as cell stress proteins. An older term is heat shock protein. The initial purpose identified for cell stress proteins was as a system to control protein misfolding and protein aggregation (proteotoxic stress) within the cell. Such systems will clearly be required in the complex processes that are infections. However, over the past 20 years, it has also emerged that cell stress proteins have additional (non-folding) biological functions, which often require the movement of these proteins from their normal intracellular milieu to other sites such as the cell wall/plasma membrane or, indeed, into the extracellular space.

There is now a substantial literature on the non-folding biological actions of eukaryotic cell stress proteins, and it is now recognised that these proteins can function, in different cell compartments, on the cell surface, as receptors, and, in the extracellular milieu, as intercellular signalling molecules. These additional biological actions of the cell stress proteins mark them out as being important moonlighting proteins – i.e. proteins with more than one biological function. As an example of this, it is now established that many human cell stress proteins can enter into the blood, and there is growing evidence that these molecules can act as important biomarkers of human disease and be predictors of the response to therapy. The importance of the moonlighting actions of these proteins can be judged by the fact that at least five human cell stress proteins are currently in clinical trial, as therapeutics, for various diseases.

Bacteria encode homologues of many of the eukaryotic cell stress proteins, and there is evidence that these microbial proteins have moonlighting functions – for example – acting as cell surface adhesins for selected host components. There is growing evidence that the interactions of microbes, with their multicellular hosts, involve the participation of both bacterial and eukaryotic cell stress proteins. Indeed, the available evidence suggests that cell stress proteins contribute to the virulence

behaviour that can occur when microbes interact with their hosts. Thus microbial cell stress proteins can act with a surprisingly large array of biological mechanisms to modulate host immunity, to aid binding and invasion of cells, and even to modulate the movement of intracellular mitochondria. In turn, it appears that microbes have also evolved mechanisms to utilise host cell stress proteins to aid in the infectious process. Thus *Listeria monocytogenes* can use one moonlighting protein, alcohol acetaldehyde dehydrogenase, found on the bacterial cell surface, to bind to a host cell surface chaperonin (Hsp)60 in order to enable the bacterium to bind and invade target cells. In another example, it is now recognised that host BiP and gp96 are, individually, important targets of a growing number of bacteria and bacterial toxins – being required to induce the biological actions of the said toxins. BiP is also a receptor for a number of viruses.

The volume is divided into four parts. In Part I, the reader is introduced to both prokaryotic and eukaryotic molecular chaperones and protein-folding catalysts (collectively, cell stress proteins) with an explanation of the role that these proteins play within the cell to control proteostasis (protein homeostasis), including descriptions of their protein-folding mechanisms and with a brief discussion of their key non-folding biological actions. In Chap. 3, Connie Jeffery, who coined the term 'protein moonlighting', provides a current view of protein moonlighting and of recent discoveries in this field. Part II contains a collection of chapters on the various microbial cell stress proteins including: chaperonin 10, peptidylprolyl isomerases, chaperonin 60, Hsp70 and Hsp90, which contribute to the virulence behaviour of microbes or form part of the microbial-host dialogue. Of importance, some of these moonlighting cell stress proteins are likely to be therapeutic targets in selected infections. Part III switches attention away from the role of prokaryotic cell stress proteins in microbial virulence to a consideration of the roles that host cell stress proteins play in microbial infection. The final part introduces the reader to possible future aspects of the cell stress response as it applies to host–pathogen interactions. In Chap. 24, the novel discovery that the major cytoplasmic protein machinery in bacteria, ClpP, which, along with diverse Clp-ATPases and adaptor proteins, is responsible for the removal of misfolded and aggregated proteins from the bacterial cytoplasm, is a target for bacterial acyldepsipeptides – a novel form of antibiotic. The final chapter deals with a novel form of host–pathogen interactions in terms of the ability that certain bacteria have to identify and respond to host neuroendocrine hormones such as adrenaline and noradrenaline. This may bring the cell stress response full circle and show how fully integrated is the interaction between infecting microorganisms and the host in terms of the stress response.

This book brings together leading experts in this field of microbiology, cell biology, immunology and cell stress protein biology to describe, in detail, the molecular and cellular roles of cell stress proteins in bacteria–host interactions and in microbial virulence. This volume will be of interest to medical and non-medical microbiologists, biochemists, cell biologists, immunologists, molecular biologists, pharmacologists and pathologists.

University College, London, UK Brian Henderson

Notes on Cell Stress Protein Nomenclature

Editing a book on cell stress proteins, particularly one which has contributions, as this volume has, from 25 different laboratories, generates certain problems. The major one perceived by the editor is the nomenclature employed to describe the cell stress proteins used throughout the chapters. It would be sensible to try and maintain a standard nomenclature throughout the volume. However, this has certain practical difficulties, which has required the writing of this section. The term, cell stress protein, refers to any protein whose rate of gene transcription is significantly altered in a stressed cell. In this volume, the focus is specifically on the cell stress proteins that are also molecular chaperones and protein-folding catalysts. These are proteins involved in the folding and re-folding of client proteins and in the solubilisation of aggregated proteins within the cell and, more than likely, also within the extracellular fluids of the body. The history of these proteins led to the term heat shock proteins (Hsp/HSP) being applied to them, and thus, we have a range of cell stress proteins known by a number: Hsp/HSP10, Hsp/HSP27, Hsp/HSP60, Hsp/HSP70 and so on. Other proteins were identified as interacting with Hsps both in prokaryotes and eukaryotes and were given different nomenclatures. Thus, the large family of 40 kDa proteins that interact with Hsp70 are known as Hsp40 in eukaryotes but DnaJ in prokaryotes. Indeed, Hsp70 is known as DnaK in prokaryotes. However, to confuse the reader further, a recent attempt to bring order to the nomenclature of cell stress proteins has used the term DNAJ as the basis of the naming of all Hsp40 proteins currently known. Thus, Hsp40 (also known as HSPF1) is now known as DNAJB1 with the gene encoding this protein being named *DNAJB1* (Kampinga et al. 2009).

The Hsp60 and Hsp10 proteins are cytosolic proteins in bacteria and mitochondrial/chloroplast proteins in eukaryotes. The correct terminology for this type of protein is chaperonin which is abbreviated to Cpn10/Cpn60. The first pair of such proteins identified was from *Escherichia coli* in which these proteins were known as GroEL/Cpn60 and GroES/Cpn10. So we now have the possibility of the molecular chaperones of 10 and 60 kDa being variously termed GroES/GroEL, Hsp10/Hsp60 and Cpn10/Cpn60. Certain bacteria, and even insects, can synthesise more than one of these proteins, so the nomenclature can become even more complicated with Hsp60.1,

GroEL1 and Cpn60.1, all being synonymous if referring to the same protein from the same organism.

Given the range of alternative nomenclatures and the fact that in different areas of research particular forms of nomenclature have become established, the editor has opted to not shoehorn each chapter into a fixed nomenclatural basis but to explain at the start of the chapter the nature of the nomenclatural confusion and let the contributor use whatever nomenclature she/he feels comfortable with, provided it is an accepted nomenclature in the field of study involved.

As we are in a dynamic state vis-à-vis changes in cell stress protein nomenclature, the following is the old and newly recommended nomenclature for a small number of eukaryotic cell stress proteins taken from Kampinga et al. (2009).

New gene name	New protein name	Older names
HSPB1	HSPB1	HSP27, HSP28, HSP25, CMT2F, HMN2B, plus many more examples
DNAJA1	DNAJA1	DJ-2, DjA1, HDJ2, HSDJ, HSF4
DNAJB1	DNAJB1	HSP40, HSPF1, plus many more examples
HSPD1	HSPD1	Chaperonin (Cpn)60, HSP60, GroEL
HSPE1	HSPE1	Cpn10, Hsp10, GroES
HSPA1A	HSPA1A	HSP70-1, HSP72, HSPA1
HSPA5	HSPA5	BiP, GRP78, MIF2
HSPA9	HSPA9	GRP75, HSPA9B, MOT, MOT2, PBP74, mot-2, plus more examples
HSPC1	HSPC1	HSP90, HSP90A, HSP90AA1, HSPN, LAP2, HSP86, plus five more examples

Clearly, we are just at the start of dealing with the nomenclatural problem, and it is hoped that within the next few years a more consistent nomenclature can be introduced and used to make reading this book much less confusing. Readers should refer to Kampinga et al. (2009) for further discussion of nomenclature.

Reference

Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE (2009) Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 14:105–111

Contents

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Part I
Cell Stress, Cell Stress Proteins and Protein Moonlighting

Chapter 1
Bacterial Stress Responses

Peter A. Lund

Abstract The bacterial stress response is an early-evolved system of protein-folding proteins, both molecular chaperones and protein-folding catalysts, linked to appropriate transcription factors for generating appropriate cellular responses to external and internal stress. Two key requirements of this vital stress response system are *robustness* and *resilience.* Most of our understanding of the bacterial stress response comes largely from study of the K12 laboratory strain of *E. coli.* The essential elements of the cytoplasmic stress response are the protein pairs: chaperonin 60/chaperonin 10 and DnaK/DnaJ whose mechanisms of action are distinct but which work together to maintain cytoplasmic proteostasis. Other cytoplasmic stress proteins include the small heat shock proteins and bacterial Hsp90. The other major cell volume in bacteria is the periplasmic space which, in contrast the cytoplasm, has an oxidising environment. This requires another set of stress proteins including the protein disulphide isomerases and the peptidylprolyl isomerases. Essential to our understanding of the bacterial cell stress response is the realisation that it is a *biological control system* whose overall role is to enable prokaryotes to cope with any stresses that the environment throws at them – such as the infectious process. It is unclear how the moonlighting actions of certain of these proteins integrates with their protein-folding/stress relieving actions.

1.1 The Concept of the Stress Response

If we define a stress as being any event that compromises the normal function of an organism, it is obvious that there are two basic strategies that can protect against that stress. One is to have a pre-existing capacity to resist the stress. This works, up to a

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point, but its maintenance requires the continuous use of resources that could be devoted to some other activity. The other is to detect the stress and respond to it, perhaps by moving to a less stressful location, or by changing properties so that the stress is no longer so damaging. This also requires resources, but they are only used when needed. The two strategies can be described as *robustness* and *resilience*. Organisms have to find the correct balance between the two. Those that fail to do so will not reproduce, and so will be selected against. Modern day organisms are examples of systems that have evolved to have an optimal balance between efficiency on the one hand, in terms of being able to use resources (energy, food, and the environment in which they live) to pass on their genes to the next generation, and robustness and resilience on the other, in being able to deal effectively with any stresses that they are likely to encounter.

The study of how bacteria deal with stress can be very enlightening in this regard. Bacteria are simple compared to eukaryotes, with most bacteria having much smaller genomes than most eukaryotes. Bacterial genomes are dense with genes, and low in features like repetitive DNA. This is not because of their need to replicate quickly but because in the absence of selection, genes are easily lost by deletion (Mira et al. 2001). In looking at a bacterial genome we are looking at a fairly minimal set of genes for life, and genes that are involved in helping the organism resist stress will have been strongly selected for. Add to this the well-known experimental reasons for working with bacteria, and the fact that some of them cause infections, and it is evident that the study of bacterial stress responses will pay dividends in areas well beyond microbiology.

Simple principles must apply to any resilient system that is invoked only when the relevant stress is detected. First, the organism must be able to detect the stress and distinguish it from other stresses. Second, this detection must be linked in some way to the response, as it is no use being able to detect a stress unless the consequence is the mounting of a relevant response. Third, the response has to be appropriate to the particular stress. For example, there would be no point in responding to a dangerous down-shift in pH by turning on the genes that are required to deal with an osmotic shock, either through a failure of the detection system, the incorrect linkage to the response, or the faulty nature of the response itself. Fourth, the response must be switched off when the stress is no longer a problem, since not to do so would lead to a non-competitive reduction in efficiency for the organism overall. All of these features – detection of the stress, transduction of the signal, response to the stress, and termination of the response – are exemplified in the many bacterial stress responses that have been investigated, although the fourth point is less well studied that the other three.

1.2 Methods and General Principles

Much research on bacterial stress responses has focussed on describing the changes in gene and protein expression following stress, and evaluating the significance of these. In the pre-genomic era, the two major experimental approaches to this were

to identify proteins whose synthesis was induced or elevated following stress, and to find mutants that failed to cope with the stress, often by tagging followed by cloning of the genes involved, and investigate their regulation and the consequences of their absence. The focus has now shifted to more high throughput methods, principally proteomics and transcriptomics. Systems biology approaches that use 'omics data to build models of circuits and pathways are beginning to lead to a more holistic view of how stress responses are organised and integrated with other aspects of the biology of the organism, but we are still a long way from having organism-level models that enable us to directly link the overall stress response to a quantifiable measure of fitness. However, even having a list of the components and an idea of their individual properties is a good place to start. Consequently the armoury of molecular biology and biochemistry has been brought to bear on organisms and on purified proteins that are induced or up-regulated in response to stress.

The way that a bacterium responds to a stress depends on its growth medium, the level of the stress, the time over which the stress is applied, the precise nature of the strain used, and a myriad of other factors. One of the most important of these is probably which other stresses are also being applied at the same time, and investigations are now moving towards the recognition that at any time (and in particular in the bacterium's natural environments – which may be our gut, our blood stream, or our macrophages) a bacterium will be responding to multiple different stresses simultaneously. A successful bacterium like a pathogen must have evolved systems to simultaneously integrate the input of many different signals (temperature, nutrient availability, external pH, osmotic pressure, presence of external signalling molecules, oxygen tension, and many more) and respond to them in such a way as to maximise the bacterium's chance of survival. We are still only scratching the surface of understanding how bacteria do this. In many cases we do not understand exactly what damage these stressors can do to the cell, which means we often do not fully understand why some aspects of the responses that we see to these stressors are good ones for the organism to have.

Another key experimental variable is the choice of experimental organism. The largest amount of work on stress responses in bacteria has been done on various strains of *Escherichia coli* K12, the standard laboratory workhorse. Much that has been learned from *E. coli* can be applied to other bacteria, but this must be done with caution, as stress responses can show significant variation even between different strains of the same species. Although some responses, such as the heat shock response, are very well conserved, others are very species specific. *E. coli* for example is well adapted to survive low pH, and has several inducible systems that enable it to do this that are not present even in other enteric bacteria (Foster 2004). Also, the mechanism of regulation of the same response may vary significantly between different species, as is the case with the bacterial heat shock response (HSR). There is also abundant evidence that components of stress responses such as the HSR can, in different bacterial species, evolve to take on new cellular functions. This important point is discussed further below. Thus, while much of what is discussed below is based upon studies in laboratory strains of *E. coli*, it must be borne in mind that extrapolation is risky.

There are many different ways in which the proteins of a particular stress response enable the bacterium to deal with the stress. Bacteria may engage in damage-limitation, where they induce proteins that deal with the consequences of the stress, perhaps by repairing DNA damage, or (as will be discussed in detail below) refolding or degrading misfolded proteins. They may attempt to decrease the level of the stressor, for example by the induction of superoxide dismutates or catalases to deal with high levels of damaging superoxide or peroxide. They may alter their properties, for example by changing the proportion of saturated and unsaturated fatty acids in the membrane to make it more fluid in response to chilling. In the case of stress brought about by nutrient limitation, they are likely to improve their ability to scavenge for, or to import such nutrients, by the production of compounds such as siderophores for scavenging iron or the up-regulation of specific transporters, or they may switch on alternative metabolic pathways. Many stresses invoke more than one of these strategies. Behavioural responses are also common: bacteria can swim towards nutrients and away from stressors, and if all else fails they can simply sit the stress out, for example by forming spores or other highly robust forms.

Having made these general introductory points, I will now discuss the bacterial heat-shock response. It is an excellent example of a well-studied response where the components are understood and their properties have been studied in detail, the signals for inducing it are fairly clear (though probably not all documented), there are very interesting differences between different bacteria, and there is, of course, abundant evidence, discussed in the rest of this volume, that many of the proteins in this response have multiple moonlighting features.

1.3 The Heat-Shock Response

The heat shock response (HSR) is what it says on the tin: a response to heat. This does not mean that it is only induced in response to heat, but heat is a biologically relevant parameter which is easy to vary experimentally, and hence the HSR is probably one of the most studied of all stress responses. First described (Ritossa 1962) as a phenomenon that induced "puffing" in the polytene chromosomes of salivary glands of Drosophila that were moved from their normal growth temperature to 37 °C, (for more details see Chap. 2) it had been studied for nearly two decades before being looked at in any detail in *E. coli* or other bacteria (Neidhardt et al. 1984). The HSR occurs at temperatures a few degrees above the normal optimal growth temperature of the organism concerned, so organisms from the polar regions can show a HSR at 10 °C, whereas those that normally bask in hot springs require temperatures of nearly 90 °C to induce their HSR (Clark and Peck 2009; Trent et al. 1990). It consists of the induction or up-regulation of synthesis of a set of proteins, many of which are very highly conserved across kingdoms, and many of which have been shown to have key roles in cell growth, including in some cases at normal temperatures as well as under heat shock conditions. The predominant role of these proteins is in protein quality control – aiding the folding of some cellular proteins, and

degrading those that fail to fold correctly. The high degree of conservation of the response across all domains of life in terms of its components and the conditions that induce it, shows that this is a very ancient response indeed, likely having been present in some form in the last universal common ancestor.

Heat shock genes are induced by many treatments other than heat, including desiccation, osmotic stress, ethanol, and heavy metals. Even the original paper by Ritossa noted that the metabolic uncoupler DNP could induce heat shock. This shows that the response is not simply due to an increase in temperature, and any hypothesis concerning the mechanisms whereby the HSR is induced has to take account of this.

1.3.1 What Is the Role of the HSR?

The role of the HSR is best deduced from studies on mutants in which it does not take place. Such mutants exist in *E. coli*. In this organism, the HSR is induced by an expansion in the repertoire of promoters that are recognised by RNA polymerase, caused by the increased presence in the cell after heat shock of a particular RNA polymerase sub-unit, responsible for promoter recognition, called σ^{32} (Guisbert et al. 2008). A mutant that lacks the gene for this RNA polymerase sub-unit cannot mount a HSR. Such a mutant has two striking phenotypes. The first is that it is extremely temperature sensitive, failing to grow at temperatures above 20 °C, significantly lower than the optimum for *E. coli* of 37 °C (Zhou et al. 1988). The other is that a large number of its proteins are present as insoluble aggregates (Gragerov et al. 1991). From this it can be deduced that the HSR is something of a misnomer, since its expression is needed for growth even under non-heat shock conditions. It can also be deduced that a key role of the HSR must be to ensure that proteins do not aggregate, but either reach their correct soluble form, or be degraded if they fail to fold correctly.

1.3.2 Identification and Roles of the Key Components of the HSR

Although the members of the heat shock regulon can be identified by studies of the transcriptome or by over-production of σ^{32} (Richmond et al. 1999; Nonaka et al. 2006), such studies do not give any information about the roles or relative importance of these *members*. However, genetic experiments in *E. coli* have already provided this information. Because σ*rpoH* mutants of *E. coli* are highly temperatures sensitive, it is easy to select for mutants in these strains that have recovered the ability to grow at temperatures above 20 °C. Such mutants showed over-expression of a protein called GroEL, which was already known to be a heat shock protein (Kusukawa and Yura 1988). The temperature at which the mutants grew correlated well with the

extent to which the GroEL protein was over-expressed. No mutants were found when selections were done above 40 °C, but if a second round of selection was done on strains that already over-produced GroEL, such mutants could now be found. These all showed elevated expression of another heat shock protein called DnaK. Thus, the severe defect in growth caused by loss of the *rpoH* gene can be overcome by increased expression of GroEL and (at higher temperatures) DnaK, showing that of all the proteins of the HSR, these two are particularly important.

Over-expression of these two proteins is now known to be accompanied by the over-expression of other proteins encoded by the same operons. In *E. coli*, these are called GroES (co-expressed with GroEL) and DnaJ (co-expressed with DnaK). The central roles of these proteins was confirmed by further experiments done on strains containing *rpoH* mutations. The introduction of plasmids over-expressing either GroES and GroEL, or DnaK and DnaJ, largely prevented the protein aggregation seen in the cytoplasm of these strains (Gragerov et al. 1991, 1992). These studies showed that expression of both partner proteins was required for preventing aggregation: GroEL was ineffective without GroES, and DnaK was ineffective without DnaJ.

Thus there are two separate protein machines in the bacterial cell that are required for proteins to become fully soluble, even under normal growth conditions, and particularly after heat shock: the GroES/GroEL pair, and the DnaK/DnaJ pair. At the same time that these experiments were being done, related experiments in other organisms and using *in vitro* components was also studying the roles of homologues of these proteins, and the term "molecular chaperone" was coined for them in 1988 (Hemmingsen et al. 1988). Molecular chaperones were defined as proteins whose action was required for other proteins to attain their fully folded form. With these discoveries a new field of biology was born, and over the last 25 years an enormous amount of work has been done to document the cellular roles, mechanisms, and structures of many molecular chaperones (see also Chap. 2 for more discussion of protein folding and molecular chaperones). Some of the key findings in this field, which are relevant to the present volume, are summarised below. But first, the author will take a brief look at the way in which the HSR is regulated, since this may be relevant for the expression of these proteins in infecting bacteria.

1.3.3 The HSR Is Mostly an Unfolded Protein Response

Heat shock is generally studied simply by raising the incubation temperature of cultured cells, but as noted above, many other treatments can induce the HSR without any change in growth temperature. What is the signal (or signals) that cells detect in order to mount a HSR? The answer appears to be that a key signal is the presence of unfolded proteins in the cell. Several lines of evidence support this hypothesis. For example, the HSR can be induced in *E. coli* at 37 °C by the presence of amino-acid analogues (Goff and Goldberg 1985), by expression of proteins that fail to fold correctly (Parsell and Sauer 1989), and by the presence of precursors of

secretory proteins (Wild et al. 1993). This last point probably explains of the ability of ethanol to induce the HSR, since treatment of cells with ethanol inhibits protein secretion (Chaudhuri et al. 2006).

There are very plausible mechanisms that explain how accumulation of unfolded protein leads to the induction of the HSR in bacteria (reviewed in detail in Guisbert et al. 2008). The details of these vary between bacteria. In *E. coli*, accumulation of unfolded protein leads to stabilisation of the σ^{32} sub-unit of RNA polymerase. σ^{32} is normally very unstable, being degraded mostly by the FtsH protease, and the action of DnaK is needed for this degradation. However, DnaK also binds to unfolded proteins, so the model proposes that when unfolded proteins accumulate in the cell, DnaK is saturated by binding these proteins and hence is no longer available to bind σ^{32}. σ^{32} is thus stabilised, binds to RNA polymerase, and the promoters of the heat shock genes become activated. This model is well supported by experimental data, and a mathematical model of key elements in this system gives good agreement with experimentally determined events (El-Samad et al. 2005). In many other bacteria, the mechanism of regulation of the heat shock genes is different (repressor-mediated systems are more common that the alternative sigma factor system found in *E. coli*) but good evidence, including mathematical modelling, also supports an unfolded protein titration model in these cases as well (Narberhaus 1999; Inoue et al. 2012).

Thus, we have a mechanism for detection of the stressor, for transduction of this information, and we know the cellular role and the key components of the response. What do we know about the detailed roles of these key components?

1.4 The Cellular Networks That Assist Protein Folding in Bacteria

A concept that has gained increasing prominence in recent years is that of proteostasis (protein homeostasis) – the concept that cells have systems in place to maintain an overall balance of folded protein, that operate all the time but become particularly important under conditions that perturb protein folding (Balch et al. 2008). The key protein complexes identified by the genetic approaches above are integral parts of the proteostasis network in *E. coli* (and other bacteria), but they have somewhat different roles. In the next section, the roles of these two protein complexes, plus other components of the HSR network, will be discussed.

1.4.1 The Chaperonins

The GroEL protein is the *E. coli* representative of a class of proteins, the chaperonins, that have been shown to be essential in all domains of life (Fayet et al. 1989; Stoldt et al. 1996; Kapatai et al. 2006). The generic name for these proteins is Cpn60 proteins (for chaperonin 60, as the molecular weight of a monomer is approximately

60 kDa), and the small proteins that interacts with them are called Cpn10 proteins. Cpn60 and Cpn10 proteins are highly conserved in sequence and function; for example, the homologue from human mitochondria can complement for loss of the GroEL protein in *E. coli* (Nielsen et al. 1999).

Cpn60 and Cpn 10 proteins are abundant in bacteria, even in cells which have not been heat shocked. In a list of detectable proteins by relative abundance in *E. coli,* GroEL ranks in the top 3 %, or the top 1 % if the highly abundant ribosomal proteins are excluded; GroES is just outside the top 10 % (Ishihama et al. 2008). The Cpn60 proteins are usually found in a complex with a striking structure, normally consisting of two rings of sub-units, stacked in a "double doughnut" (Braig et al. 1994). In prokaryotic homologues, which are also found in chloroplasts and mitochondria, there are seven sub-units in each ring. Each ring of sub-units contains a central cavity where protein folding occurs. The past two decades have seen a very significant research effort into understanding the mechanism of these "protein folding machines", and although there is still controversy over some of the details, the basic reaction cycle has been thoroughly described (and is recently reviewed in Yébenes et al. 2011; Horwich and Fenton 2009; Horwich et al. 2007). The essence of the reaction cycle is that it involves binding of unfolded or partially folded client protein to the open end of one ring of the complex, followed by its displacement into the cavity inside the ring by the binding of Cpn10. Its subsequent ejection from the cavity requires the binding of another client protein and Cpn10 to the opposite ring. The cycling between binding at the two ends is mediated by the binding and hydrolysis of ATP.

There are several steps in the cycle that may aid protein folding. None of these involve Cpn60 providing any steric information to the folding client protein, indeed the role of Cpn60 proteins seems to largely be to block, or reverse, unfavourable pathways rather than to actively promote favourable ones. The first place at which it may assist is as the Cpn60 protein, with client protein bound, undergoes large movements to a conformation which can bind to Cpn10. As the client protein is bound to several positions on the Cpn60 protein complex, this may stretch the client protein and pull it out of any misfolded conformations that it might have entered. Subsequently, folding in the cavity prevents the client protein from interacting with any other folding proteins, which would be much more likely to occur in the crowded environment of the cytoplasm and which could lead to the formation of aggregates. It is also possible that confinement in the limited volume of the folding cavity may favour folding which minimises the volume of the folding protein.

Although there are some early reports of Cpn60 proteins associating with ribosomes, most evidence suggests that the action of Cpn60 proteins is on fully translated but unfolded or partially folded proteins. Some proteins bind Cpn60 but can also fold in its absence, others require it for folding under stress conditions, and others require it for folding at all times (Kerner et al. 2005). The most recent studies suggest that probably less than 60 proteins are in this last class, but that several of them are essential, which explains the essential nature of Cpn60 (Fujiwara et al. 2010). Numerous studies have attempted to define the rules that determine whether or not a given protein is likely to be a client of Cpn60, but a recent review of these approaches has concluded that to date they are not particularly successful, and that

we cannot yet predict from physico-chemical properties alone whether or not a given protein will be a Cpn60 client *in vivo* (Azia et al. 2012).

Much of the evidence on the function of the Cpn60 proteins as cytoplasmic chaperones is based on experiments done on *E. coli* and on the GroEL protein; as will be seen below, there are important observations from other organisms that show they have additional functions and locations which are of importance in consideration of their roles as moonlighting proteins.

1.4.2 The DnaK/DnaJ Chaperone Machine

The DnaK/DnaJ chaperone pair act upstream of the Cpn60/Cpn10 pair by a very different mechanism. They are non-essential for *E. coli* growth under normal conditions but are required for growth above 42 °C (Bukau and Walker 1998), but nonetheless many studies show they have a central role in protein folding, in particular in recovery from heat shock. Both proteins are abundant in the cell, and the levels of both are increased by the HSR. Like Cpn60 proteins, they are widespread through different domains, although they are absent from many archaea. The DnaK protein is the major bacterial Hsp70 homologue. The DnaJ protein has many homologues, although homology is often limited to a particular domain in the protein, called the J-domain, which is responsible for the interaction with DnaK homologues (Kelley 1998). Many species including *E. coli* contain several homologues of each protein, often with different functions. The discussion below solely concerns the DnaK/DnaJ pair, as these have the most central role in the cell.

Cpn60 proteins act by encapsulating their clients, whereas DnaK and DnaJ act by binding short amino-acid sequences. Analysis of these sequences shows that they are similar to sequences that will be mostly buried in proteins when they become folded (Rüdiger et al. 2001). One major role of the DnaK/DnaJ machine therefore appears to be to protect such sequences from forming incorrect associations with similar sequences on other proteins, which would lead to protein aggregation, until such time as the protein has folded and the sequences are no longer accessible. The ability to bind these sequences also means that the DnaK/J complex is implicated in disassembly of some oligomers, and in resolubilisation of protein aggregates. As with Cpn60 proteins, the DnaK/J complex acts in a cycle of binding and release, mediated by ATP hydrolysis (reviewed in Genevaux et al. 2007). Initially, unfolded proteins with exposed stretches of particular amino-acids bind to DnaJ, which presents them to DnaK which is in its ATP bound state. The interaction of DnaJ and client with DnaK stimulates ATP hydrolysis and leads to closure of the client binding site, protecting these regions on the protein, and to dissociation of the DnaJ protein. A single protein may, in its unfolded state, have several molecules of DnaK-ADP bound in this fashion. The cycle is completed by interaction with GrpE, a nucleotide-exchange factor, which catalyses the replacement of ADP with ATP and hence opening of the binding site and release of the bound protein from DnaK. Many, though not all, DnaK clients are subsequently captured by Cpn60 for further chaperoned protein folding.

A major role of DnaK in non-stressed cells is in transient protection of nascent chains of proteins which are emerging from the ribosome and which hence do not yet have all the information present that is needed for them to fold. The reason that deletion of *dnaK* is not lethal is that DnaK shares this role with another protein, trigger factor (Deuerling et al. 1999). Simultaneous deletion of both genes is lethal at temperatures above 30 °C, though this can be suppressed to an extent by over-expression of GroES and GroEL – demonstrating that chaperones can and do substitute for each other, despite their radically different mechanisms of action, under sufficient duress (Teter et al. 1999; Vorderwülbecke et al. 2005).

The central role of the DnaK/DnaJ machine in the overall protein folding network of the cell has been confirmed by a recent extensive proteomic study of the client proteins of both DnaK and TF (Calloni et al. 2012). This study shows that up to 25 % of cytoplasmic proteins interact with DnaK. Many of these are newly synthesised proteins, but there is evidence (as there is for GroEL) that some proteins repeatedly rebind to DnaK, suggesting that they are relatively unstable and that without regular chaperone maintenance, they would lose their function. DnaK clients are statistically more likely to form aggregation-prone intermediates as they fold, explaining why they need DnaK for efficient folding in the cell. This work also confirmed that many proteins that bind to DnaK go on to interact with GroEL, and reinforced the view that DnaK has a key central role in the chaperone network in the cell, although this role can to some extent be fulfilled by trigger factor if DnaK is absent. This redundancy may be important as DnaK has a wide range of other functions in the cell following stress. Interestingly, many DnaK clients are of relatively low abundance in the cell, which, together with data that shows that the tendency for proteins to aggregate is inversely correlated to their relative abundance (Tartaglia et al. 2007), implies that highly abundant proteins are under selection to evolve to be able to fold without the intercession of molecular chaperones.

The DnaK/DnaJ machine has many roles in survival of stress, often acting with other elements of the HSR in carrying out their function; thus, mutants in *dnaK*, although they are viable under normal conditions of growth, show generally reduced tolerance of stress. In these functions, the role of DnaK/DnaJ often appears to be one of reactivating proteins which have become damaged or unfolded in some way and have become transiently bound to "holders" – chaperones that bind unfolded proteins and prevent their degradation, but which cannot actively refold them. Examples include acting to refold proteins which have become bound to Hsp31 or to the small HSPs IbpA and IbpB after severe heat stress (Mujacic et al. 2004; Mogk et al. 2003); refolding proteins which have become bound to the redox-activated chaperone Hsp33 after oxidative stress (Hoffmann et al. 2004); and acting in concert with the prokaryotic Hsp90 homologue, HtpG, to reactivate proteins that have become aggregated (Genest et al. 2011). Many of these processes appear to be important only after fairly severe stress, which partly explains why neither DnaK, nor the proteins that it operates with, are essential under normal growth conditions. A key role that has been explored in some detail is its ability to refold proteins which have become trapped in protein aggregates, which it does in concert with the

heat shock induced AAA+-protease ClpB (reviewed in Doyle and Wickner 2009). Finally, DnaK and its partners are involved in directing some proteins to proteases, many of which are also HSPS, as is seen in the autoregulation of DnaK by the degradation of σ^{32} by FtsH, discussed above.

1.4.3 Further Examples of Components in the Cytoplasmic Proteostasis Network

In addition to the two major chaperone machines described above at the centre of the proteostasis network in *E. coli*, many other proteins that have a role in protecting, refolding, or degrading other proteins either under normal growth conditions or after exposure to different kinds of stresses. Some of these are discussed below, but the list is far from exhaustive. In particular, proteases are not discussed, but it is important to note that they have an essential role to play in removing damaged proteins from stressed cells (discussed in more detail in Chap. 24). The ways in which the activities of the various different chaperones and proteases are integrated in the overall proteostasis network (reviewed recently in Mogk et al. 2011) is not yet fully understood. However, a mathematical model (EcoFold) of the network (Powers et al. 2012) gives good agreement with experimental data, and it is likely that all the significant components have now been identified.

1.4.3.1 The Small Heat Shock Proteins

Small heat shock proteins are a large and very heterogenous group of proteins which are found in most organisms. In *E. coli*, they are represented by two proteins that are encoded by a single operon with two genes, *ibpA* and *ibpB*. They were first discovered in association with inclusion bodies (hence their name) and are strongly induced by heat shock, particularly at higher temperatures (Allen et al. 1992). However, they are not essential for the cell and their deletion leads to only moderate phenotypes even under heat shock conditions (Thomas and Baneyx 1998). Small heat shock proteins are examples of the class of proteins sometimes referred to generically as "holders". These are proteins whose role is thought to be mainly to protect unfolded proteins by holding them in a conformation where they are not degraded, until they can be refolded by "folders" – the more active, ATP-driven chaperones. Studies using purified proteins show that when both Ibp proteins are present during thermal denaturation of client proteins, those proteins can be more efficiently reactivated by the DnaK chaperone machine interacting with ClpB (Mogk et al. 2003; Matuszewska et al. 2005). *In vivo* data confirms that these proteins assist during extreme heat shock in keeping proteins in a state that can be re-activated (Kuczyńska-Wiśnik et al. 2002).

1.4.3.2 HtpG, the Bacterial Hsp90

Hsp90 proteins are important in eukaryotic cells, where they interact with a wide range of cochaperones and clients (described in Chap. 2) but in *E. coli* and other bacteria, their role is not so central. The sole representative in the *E. coli* genome is called HtpG, and, as is the case with the Ibp proteins described above, this protein is strongly induced upon heat shock but its absence does not lead to a severe phenotype (Bardwell and Craig 1988). It has been shown both *in vivo* and *in vitro* to interact with the DnaK chaperone machinery to assist in the refolding of proteins which have become denatured by heat, though its mechanism is not known (Thomas and Baneyx 1998, 2000; Genest et al. 2011). Its function thus appears to overlap to some extent with that of the small heat shock proteins, an interesting observation given that HtpG is an ATPase and the sHSPs are not.

1.4.4 Chaperones in the Periplasm

The second major semi-aqueous compartment in *E. coli* and other Gram negative bacteria, in addition to the cytoplasm, is the periplasm. This compartment represents between 20 and 40 % of the total cell volume (Stock et al. 1977), and differs in many important ways from the cytoplasm. It is less well separated from the external milieu, is oxidising, contains no ATP, and is an important physical location in its own right, but also for some of the proteins which are *en route* to the outer membrane or outside the cell altogether. Many proteins arrive in the periplasm in an unfolded state, having been kept that way in order to traverse the secretory machinery, and many need to be inserted into the outer membrane. All of these factors mean that it has its own repertoire of chaperones and folding catalysts as described below. These have been recently reviewed in Merdanovic et al. 2011, and are only discussed briefly here.

In the same way as the cell mounts a response to the presence of unfolded proteins in the cytoplasm, a separate response exists that regulates the levels of periplasmic proteins which have a role in protein folding. Indeed, identifying proteins whose levels are increased following the accumulation of unfolded proteins in the periplasm (such as outer membrane protein precursors) is one of the ways that their roles in protein folding were first discovered. Stresses in the periplasm are detected and communicated to the cell in at least two different ways (reviewed by Alba and Gross 2004; Vogt and Raivio 2012). Intriguingly, these systems are also involved in responding to events such as adhesion and so are also likely to play a role in mediating the changes in gene expression that take place during infection.

Many of the proteins with a role in periplasmic protein folding and insertion of outer membrane proteins are involved in covalent modification of the proteins; these are discussed in the next section. One protein which does appear to act as a chaperone by binding and holding proteins and hence reducing the likelihood of their aggregation is called Skp (also known as OmpH). Although this protein can bind to

several outer membrane proteins, deletion of its cognate gene is not lethal and does not cause a reduction in outer membrane protein insertion. However, simultaneous loss of SurA, another periplasmic protein with a protein folding role, is lethal, and proteomics analysis shows that Skp and SurA overlap in their client specificity such that at least one of them has to be present (Denoncin et al. 2012), a situation reminiscent of the trigger factor/DnaK pairing discussed above.

1.5 Covalent Modifiers of Protein Folding

In addition to molecular chaperones, which assist protein folding but do not alter the nature of the covalent bonds in proteins, there exist another set of proteins whose function is needed for efficient protein folding in bacteria. They are genuine enzymes with proteins as their substrates, unlike the chaperones. They consist of enzymes involved in formation and isomerisation of disulphide bonds, and enzymes which can catalyse *cis/trans* isomerisation of the peptide bond at X-Pro, where X is any amino-acid and Pro is proline. Disulphide bond formation and isomerisation take place almost exclusively in the periplasm, except in bacterial strains that have been deliberately engineered to have an oxidising cytoplasm, but proline *cis/trans* isomerisation may occur either in the cytoplasm or the periplasm. Indeed, two of the molecular chaperones discussed above (trigger factor and DnaJ) have this activity, although the extent to which it is important *in vivo* is not clear (reviewed in Wang and Tsou 1998; Hoffmann et al. 2010). The genes for these proteins are generally not essential, but knockout mutants show a variety of different phenotypes including slow growth in some conditions, and some double knock-outs show synthetic lethality.

Catalysis of disulphide bond formation is needed in the periplasm, despite the oxidising nature of that compartment, for efficient folding of proteins that contain disulphide bonds. The *E. coli* periplasm contains a network of proteins which connect the formation of disulphide bonds with electron transport. Disulphide bond formation is catalysed by the DsbA protein, which itself becomes reduced in this process (Bardwell et al. 1991). DsbA is then re-oxidised by the protein DsbB, and DsbB in turn is oxidised directly by interacting with the membrane quinone pool (Kobayashi et al. 1997). Loss of either of these two proteins results in defects such as a loss of motility (due to one of the flagellar proteins being unstable in its reduced form). Another protein, DsbC, can substitute for DsbA to a limited extent but is more active as a disulphide bond isomerase, rearranging disulphide bonds rather than introducing them (Missiakas et al. 1994; Shevchik et al. 1994).

Many genes are annotated as proline cis/trans isomerases in the *E. coli* genome and in the genomes of other bacteria, and the proteins they encode are found both in the cytoplasm and the periplasm. Although none are essential for growth, they have important roles in the cell, most notably in outer membrane biogenesis. Intriguingly, in some cases it has been shown that some aspects of their cellular action is not dependent on their PPIase activity, suggesting them to be proteins with a chaperone

role distinct from their catalytic role. For example, high levels of the periplasmic PPIase FkpA can suppress the formation of periplasmic inclusion bodies even if its PPIase activity is abolished by mutation (Arié et al. 2001).

In summary, there are many components to the bacterial HSR, which together with covalent modifiers of protein folding act together in a network to maintain cytoplasmic and periplasmic proteostasis in both normal and stressed cells, by a combination of protection of unfolded proteins from aggregation, refolding of nascent or pre-existing proteins, and protein degradation. The full workings and inter-relationships of the network remain to be completely described, but the two major chaperone machines of the cell, typified in *E. coli* by the GroEL/GroES and DnaK/DnaJ machines, are crucial to the functioning of the cytoplasmic network. The chaperonins are important because there are several essential proteins which cannot fold in their absence, and the DnaK/DnaJ machine is important in its interacting with numerous other components of the HSR, and other stress regulons, to promoter either protein refolding or protein degradation. A separate network exists in the periplasm, which is essential for outer membrane protein insertion and for the protection of periplasmic proteins from different stresses.

1.6 The Bacterial HSR, Proteostasis, and Infection

Given the significance of the HSR for viability of bacteria under different conditions, and given the obvious fact that bacteria which are causing infections are likely to be under considerable stress from host defences, it is not surprising that evidence has accumulated that shows induction of components of the HSR on infection, and some experiments have demonstrated that key proteins in the HSR are important in aiding bacteria during infection. What was perhaps more surprising was the realisation that stress proteins could and did have multiple roles in infection, which are not always necessarily related to their roles as understood from the *E. coli* paradigm. In this final section, I will review a selection of the evidence that shows that components of the HSR are also important in infection, and then briefly point to some of the evidence that additional roles have evolved for some stress proteins; this volume will of course deal with this topic in detail.

Evidence of induction of expression of chaperones on infection has been reported in numerous different bacteria, using both pre- and post-genomic methods. It was first reported for Salmonella infecting macrophages, where the homologues of GroEL and DnaK are among several proteins which are strongly induced (Buchmeier and Heffron 1990). Intriguingly, a later transcriptomic study of *S. enterica* bv. Typhimurium infecting macrophage showed a *decrease* of *groEL* transcription, although *dnaK* was up-regulated, and the genes for the small heat shock proteins IbpA and IbpB were very strongly up-regulated, showing again the danger of over-generalising from single studies on the HSR (Eriksson et al. 2003). Up-regulation of components of the HSR on infection, typically including Cpn60/10 proteins and DnaK/DnaJ homologues, has since been reported in many other bacteria, including *Staphylococcus aureus* (Qoronfleh et al. 1998), *Listeria monocytogenes* (Gahan

et al. 2001), *Mycobacterium tuberculosis* (Monahan et al. 2001), *Chlamydia trachomatis* (Gérard et al. 2004), *Rickettsia prowazekii* (Gaywee et al. 2002), *Neisseria gonorrhaeae* (Du et al. 2005), and others.

In isolation, up-regulation of gene expression or protein level does not provide conclusive evidence for a role of the proteins concerned. Such evidence relies on studies done on mutant bacteria where expression of the proteins in question is altered in some way, and interpretation of the data from such experiments is complicated by the very pleiotropic nature of mutants in some components of the HSR. Nevertheless, convincing evidence does exist. For example, it has been shown that if the normal heat shock regulation *dnaK* gene of *Brucella suis* is removed, the bacteria can still invade macrophages but cannot multiply in them, and are rapidly lost in a mouse model of infection (Köhler et al. 1996, 2002). Similarly, a DnaK-DnaJ depleted mutant of *S. enterica* bv. Typhimurium cannot survive in macrophages or cause systemic disease in mice (Takaya et al. 2004).

Experiments on the roles of the chaperonin genes in infection are more challenging because of the essential nature of these proteins. However, in many bacteria there are multiple copies of the chaperonin genes (Lund 2009), some of which are non-essential, and the role of one of these has been studied in Mycobacterial infection. It was shown that when the non-essential *cpn60* gene was deleted, *M. tuberculosis* could still grow as normal but it failed to form granulomas in experimental mice and guinea pigs (Hu et al. 2008). This is likely to be due to the fact that chaperonins are potent immunomodulators, a role which is probably independent of their role as chaperones. This unexpected but important aspect of their biology has been reviewed elsewhere (Maguire et al. 2002; Henderson et al. 2010).

1.7 Escaping from the *E. coli* Paradigm

This last observation brings up a central point which is that there is now excellent evidence, in particular from studies on pathogenic bacteria, and their interactions with their hosts, that we must think beyond the *E. coli* paradigm if we are to understand the role or roles of molecular chaperones in infection. Certainly they are important in protection against stress, as is shown in the studies above on their roles in aiding survival in macrophages. But they clearly have additional roles where their chaperone function is probably incidental.

A striking example of this is the finding that for many bacterial species, a Cpn60 protein acts externally to promote adherence to target cells. This has been shown for several very diverse bacterial species to date, including *M. tuberculosis* (where DnaK was also found on the cell surface), *Helicobacter pylori*, *Salmonella enterica* bv. Thyphimurium, *Clostridium difficile*, *Lactobacillus johnsonii*, *Brucella abortus*, *Legionella pneumophila*, and *Haemophilus influenzae* (see Hickey et al. 2009, 2010, and references therein – this is also described by Richard Stokes in Chap. 8). A recent paper even showed that some *E. coli* GroEL was found on the cell surface, and that if this amount was increased, macrophage clearing of *E. coli* cells was enhanced (Zhu et al. 2013).

On the face of it, this is a surprising finding. First, it is not at all clear how Cpn60 proteins get to the outside of the cell; they possess no recognisable signal sequence, and once assembled into large complexes would be expected to be impossible to transport across membranes. Second, Cpn60 proteins require ATP for their normal cellular function, and ATP is not expected to be present in high concentrations outside of the cell. Third, the complete chaperonin cycle also requires Cpn10, but it is hard to see how sufficient levels of Cpn10 could be maintained outside the cell. Cpn60 proteins are notoriously "sticky" and there has always been concern expressed that these results could be artefacts resulting from lysis of some cells followed by binding of liberated Cpn60 to the surface of other cells in the same culture, but careful controls in several of these studies make this interpretation very unlikely. Thus, it seems likely that Cpn60 proteins are genuinely operating to promote some aspect of attachment or adhesion, and they do so not as chaperones in the strict sense. They may act as relatively non-specific "sticky surfaces", but some studies have implicated particular receptors (for example, the LOX-1 receptor) as being involved in recognition of surface- exposed chaperonin proteins. The mechanism by which chaperonins reach the bacterial surface remains unknown, and this is an important area for future study.

1.8 Conclusions

This brief review has shown that bacteria contain highly adapted systems for detecting and surviving the many stresses and shocks to which they may be exposed, and we have a reasonable understanding of some of these. The HSR – more correctly called the unfolded protein response – involves the co-ordinated action of the Cpn60/Cpn10 and DnaK/DnaJ machines, which both also have central roles in normal cellular growth. Together with the other components of the HSR and other proteins which are not part of the heat shock regulon, these two machines assure that proteins are folded to their final native states or, if necessary, degraded. These systems also appear to have key roles to play in bacterial infection, not only by protecting the cells from the stresses that inevitably result, but by providing additional functions which are not necessarily related to their normal chaperone functions. One of these is that Cpn60, normally thought of as an ATP-dependent cytoplasmic chaperone, can also act on the cell surface as an adhesin, facilitating the attachment of bacterial cells to eukaryotic cells. This and other potential roles of these proteins will be discussed in more detail in the current volume.

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Chapter 2
A Brief Introduction to the Eukaryotic Cell Stress Proteins

Brian Henderson

Abstract The discovery of the heat shock response in *Drosophila* in the early 1960s led on to the elucidation of the cell stress response and the discovery of proteins of molecular mass of 10, 20, 40, 60, 70 and 90 kDa, amongst others, and which were termed the heat shock proteins. Beginning in the late 1970s, and continuing up to the present day, has been the identification of these heat shock/cell stress proteins and their mechanism of action, both as protein-folding proteins and as proteins with a range of other functions in various compartments of the cell and in the intercellular space. In addition to functioning as molecular chaperones, the heat shock/cell stress proteins can also function as cell surface receptors and as intercellular signalling molecules. This growing diversity of the biological functions of the cell stress proteins reveals that these proteins play roles in all aspects of cellular physiology and that these functions also contribute to whole body homeostatic control and to the dark side of human pathophysiology.

2.1 Introduction

The aim of this chapter is to introduce the reader to the growing number of eukaryotic proteins which are variously termed: heat shock proteins, cell stress proteins, molecular chaperones, chaperones and protein-folding catalysts (some of these proteins are shown in Table 2.1) in order to provide some background information for the other chapters. Already the reader can see problems. Why are there so many terms to describe a relatively small number (100–200) of eukaryotic proteins?

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Table 2.1 Some molecular chaperones and protein-folding catalysts in eukaryotes and prokaryotes

Family	Mammalian protein	Cellular location	Function	Bacterial homologue
Hsp10	Hsp10 or Cpn10[a]	Mitochondrion	Co-chaperone for HSP60	GroES (*E. coli*)
Thioredoxin	Thioredoxin(Trx), PDI[b]	Cytoplasm	Thiol:disulfide exchange	Thioredoxin, DsbA, DsbB
Hsp40	Sis1, auxilin	Cytoplasm	Stimulation of Hsp70 ATPase	DnaJ (*E. coli*)
Hsp60	Hsp60 or Cpn60	Mitochondrion	Protein folding within cavity	GroEL (*E. coli*)
Hsp70	Hsc70, BiP	Cytoplasm and ER	Prevention of aggregation	DnaK (*E. coli*)
Hsp90	grp94	Cytoplasm	Formation of specific proteins	HtpG

This table only shows a very small number of the known molecular chaperones
[a]Chaperonin
[b]PDI – protein disulphide isomerase

Indeed, a clear difficulty in the literature on cell stress proteins (CSPs) is trying to get to grips with the nomenclature, which is constantly enlarging and changing. The starting point for this chapter is a brief historic introduction to the eukaryotic cell stress response and the proteins that are involved in this process. This chapter will not deal with the homologues found in bacteria as this has been covered in Chap. 1.

2.2 A Brief History of the Eukaryotic Cell Stress Response

Stress as a physiological and psychological mechanism has been the subject of study since the mid to late nineteenth century, with Claude Bernard in France defining the internal environment of the body (*milieu interieur*) which remains constant in response to environmental changes (stresses) (see Bernard 1961 – a reprint of the original book). Walter Cannon in the USA identified the fight-or-flight response and coined the term homeostasis in 1926 to further define the concept of the dynamic equilibrium that had been hypothesised by Claude Bernard. Cannon was also the first to use the term *stress* in terms of the demands placed upon the body and its response to them (Cannon 1932). However, it was the Hungarian scientist, Hans Selye, who is most closely associated with the concept of stress and its role in health and disease and with the discovery of the circulating stress hormones (Selye 1956).

Selye's experiments on stressed animals did not address what was happening at the level of the individual cells. Clearly, stress has the ability to induce striking changes in specific cells such as those of the adrenal glands. The first 'experiment' to identify the effect of a specific stress, in this case heat, on an organism used the fruit fly, *Drosophila melanogaster.* This insect has been used by geneticists since its introduction by Thomas Hunt Morgan at the beginning of the twentieth century.

It has a number of advantages as an experimental subject including: rapid and prolific breeding potential, easily recognised characteristics (useful for spotting mutations) and giant (polytene) chromosomes. The latter are found in secretory cells such as in the salivary glands and in cells in the gut and contain multiple copies of the chromosomal DNA. These DNA strands are precisely aligned and form long linear structures visible at the resolution of the light microscope. Importantly, these chromosomes are transcriptionally active and sites of active transcription can be identified by the fact that the chromosome decondenses forming what is termed a 'puff'. It has been established, by radioisotope incorporation studies, that puffing and DNA synthesis are synonymous.

Our current understanding of the cell stress response can be traced back to the serendipitous finding of the geneticist (now sculptor) Ferruccio Ritossa, who was then working in Naples. One day Ritossa noticed an uncharacteristic pattern of puffs in the salivary gland polytene chromosomes that he was studying and on further investigation he discovered that a worker in his laboratory had inadvertently increased the temperature of an incubator and exposed *Drosophila* larvae, normally kept at an environmental temperature of 25 °C, to 30–32 °C. He calculated the correct conditions for the shift in temperature, repeated the exposure with appropriate controls and observed that new RNA synthesis (as indicated by changes in the pattern of puffs) could occur in just 2–3 min. With a 30 min period of heat shock, followed by a return to the normal environmental temperature of 25 °C, the new puffs disappeared in about an hour. If larvae were maintained at the elevated temperature, the puffs remained for up to 3 h before disappearing. With prolonged exposure to elevated temperature, puffs present in the chromosomes before the application of heat shock could regress or even disappear. These findings occurred with *Drosophila* larvae at different stages of development and in polytene chromosomes in sites other than the salivary gland suggesting that the heat shock effect was generic and not specific to this one gland system. Dissected salivary glands also demonstrated a heat shock puffing response (Ritossa 1962, 1996). Indeed, in 2013, at the time of writing, we have just celebrated the 50th anniversary of the discovery of the cell stress response (De Maio et al. 2012).

These salient findings were extended and expanded during the next decade and by the mid-to-late 1960s it was clear that exposure of cells containing polytene chromosomes to a variety of environmental stressors resulted in the transcription of novel genes and presumably in the synthesis of specific proteins. The 64,000 dollar question was – what were these proteins? This question was not answered until the 1970s when Alfred Tissières, University of Geneva and other investigators in this area (Tissières et al. 1974; Mirault et al. 1978) applied the new technique of sodium-dodecyl sulphate (SDS)-PAGE, a technique which denatures proteins and allows them to be separated according to their molecular mass. This revealed the appearance of seven new protein bands of distinct molecular masses in salivary glands after the application of heat shock. Furthermore, cellular levels of some proteins present before the application of elevated temperature either decreased or disappeared after treatment. Here was the first evidence for the existence of heat shock or cell stress proteins and it was at this time that the term was coined. It is from these

discontinuous PAGE gels, with their ability to provide accurate molecular masses, that the common names of the heat shock proteins (e.g. Hsp60, Hsp70) derived. Further work, using *in situ* hybridisation and *in vitro* protein synthesis with heat shock mRNA fractions was undertaken to conclusively demonstrate that the individual chromosomal puffs induced by stressors were related to the synthesis of the specific heat shock proteins.

During the 1960s and 1970s research on the heat shock response was undertaken solely with *Drosophila.* Thus the spectre that this response was specific to insects or even to *Drosophila* itself was always present. However, at the end of the 1970s/early 1980s reports of the existence of a heat shock response in chicken fibroblasts (Johnston et al. 1980), the gut bacterium, *Escherichia coli* (Tilly et al. 1983), yeast (Miller et al. 1982) and plants (Key et al. 1981) began to appear. Since this time it has become established that the heat shock/cell stress response is a universal phenomenon which occurs in all three of life's Kingdoms. By the late 1970s/early 1980s the *Drosophila* genes encoding heat shock proteins were being cloned and sequenced. This led on to the sequencing of many of these genes and the realisation of the evolutionary relationships between them (e.g. Bardwell and Craig 1984).

2.2.1 Identification of the Functions of Cell Stress Proteins

Larry Hightower, who has been a pioneer in the study of the physiological role of cell stress proteins, was the first to suggest that, as many of the cell stressors were protein chaotropes (agents able to denature proteins), then the most obvious function of the heat shock response was to cope with improperly folded proteins within the cell (Hightower 1980). A very simple experimental protocol was devised to test this hypothesis. Frog oocytes are large cells into which it is possible to directly inject proteins (a process termed microinjection). Oocytes were injected with native or denatured proteins and cells were assessed for the induction of the heat shock response. Only denatured proteins induced the heat shock response, thus establishing the link between protein unfolding within the cell and the induction of the heat shock/cell stress response (Ananthan et al. 1986).

2.3 Molecular Chaperones Make Their Appearance

Christian Anfinsen's group in the USA had conducted classic experiments in the 1960s/1970s to determine the mechanism of protein folding. Such experiments relied heavily on the small, stable, monomeric protein, ribonuclease. The key observation made by Anfinsen was that denaturation and reduction of ribonuclease (which has four disulphide bonds) resulted in unfolding of the protein, but that

removal of the denaturant and subsequent oxidation of the thiol (SH-) groups by atmospheric oxygen, resulted in the spontaneous reformation of the native state. From experiments like these came the paradigm that *all the information necessary for a polypeptide chain to fold into its native state is contained within its amino acid sequence*. This was a simple, logical and satisfying hypothesis/paradigm (Anfinsen 1973). However, the discovery of the heat shock response led to the partial overthrowing of this powerful paradigm.

The evolution of the term chaperone, or molecular chaperone, can be traced back to Ron Laskey and co-workers in Cambridge, England, who were studying the packaging of DNA into nucleosomes. These are oligomeric 'particles' containing 146bp of DNA wrapped around an octamer of the basic nuclear proteins known as histones. This is part of the evolved mechanism to package the enormously long DNA molecule into an exceedingly small volume. Nucleosome formation occurs rapidly in amphibian eggs once they become fertilised, with the basically charged histones binding to the negatively charged DNA. The nucleosomes can be dissociated with buffers containing high salt concentrations and it was therefore expected that DNA and histones should self-assemble into nucleosomes. Experimentally, this is not the case. Removal of the salt resulted in the formation of non-specific aggregates, but no nucleosomes. Laskey's group showed that homogenates of amphibian eggs (they used *Xenopus*) added to the histone/DNA mixture would promote nucleosome formation. They purified what turned out to be an abundant active component and identified it as an acidic nuclear protein that they called nucleoplasmin (Laskey et al. 1978). This protein alters the interaction between the histones and the DNA such that nucleosome formation is favoured over aggregate formation. Nucleoplasmin has two important properties, which shaped the subsequent development of the concept of molecular chaperones and chaperoning. The first is that the final product, the nucleosome, does not contain nucleoplasmin. The second is that if the conditions are right, nucleosomes can form in the absence of nucleoplasmin. Gentle dialysis of dissociated nucleosomes, which slowly lowers the salt concentration, allows them to reform naturally. This means that the nucleoplasmin does not provide steric information for the generation of the nucleosome, *but simply provides a means of enabling the interactions of the DNA and histones to be modified such that the natural self assembly is favoured over the formation of non-productive protein complexes*. Now, in prior centuries, and still in some parts of the world, the two sexes cannot meet alone and chaperones are required to prevent untoward interactions. Laskey used this analogy to describe nucleoplasmin as a 'molecular' chaperone which prevented 'unhealthy' interactions between the histones and the DNA (Dingwall and Laskey 1990).

Thus by 1978 the interaction of one protein with another 'protein' to aid interaction and form a properly folded complex had been identified and the term 'molecular chaperone' coined. Although nucleoplasmin was the first molecular chaperone to be described, the field of molecular chaperone biology can be said to have started with the discovery of another protein, chaperonin 60, and the analysis of its mechanism of action.

2.3.1 The Identification of Chaperonin 60 – The Prototypic Molecular Chaperone

The discovery of the prototypic molecular chaperone, chaperonin 60 (also known as Hsp60/HSPD1), can be traced back to two distinct areas of research: (i) the synthesis of the major chloroplast protein, Rubisco and (ii) the genetics of phage synthesis in *E. coli* (see also Chap. 1). In 1970, John Ellis, a chloroplast biologist, planned to use non-denaturing PAGE along with SDS-PAGE to study light-induced protein synthesis in chloroplasts. Intact chloroplasts were exposed to light and ^{35}S-methionine, and the proteins present in fractions of the chloroplast, and those newly synthesised, were determined by separating the proteins by PAGE and by SDS-PAGE and by subjecting the gels to autoradiography to identify bands containing radioactivity. The major labelled product was identified as the large subunit of the protein Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase). Rubisco is responsible for the fixation of CO_2 and around 15 % of the protein content of chloroplasts is Rubisco, making it the most abundant protein on the planet. When labelled/lysed chloroplasts were sampled at various times and the proteins were isolated by non-denaturing PAGE the radioactivity was initially seen to be part of a larger complex. With time, radioactive Rubisco large subunit was found to have dissociated from this complex. When this large molecular mass radioactive band was analysed by SDS-PAGE (which separates oligomeric proteins into their constituent subunits) a 60 kDa protein was identified. This was termed Rubisco binding protein (RsuBP) and was believed to be an oligomer of around 800 kDa with a subunit size of 60 kDa. Ellis's group also showed that it bound ATP (Ellis 1990). However, at the time of this finding, around 1980, its biological importance was totally unknown.

A decade earlier Costa Georgopoulos in Geneva, isolated mutants of *E. coli* which were temperature sensitive (i.e. would not grow at elevated temperatures) and in which bacteriophages such as phage λ or T4 would not grow (Georgopoulos and Hohn 1978 – see Chap. 1). It was later noted that the capsid protein of T4 aggregated at the cell membrane in these mutants and did not form the normal capsid. Genetic analysis identified that these mutations were associated with two genes which were termed *groEL* and *groES*. The product of the *groEL* gene was identified as a protein of 65 kDa subunit mass (termed GroEL) forming a tetradecameric complex visible in the electron microscope. The product of the *groES* gene (GroES) was also prepared and was shown to interact with GroEL forming a 1:1 complex in the presence of ATP.

So, by early to mid 1980 two groups are working on oligomeric proteins – one in chloroplasts, the other in *E. coli.* Each group realises the importance of their proteins, but have no idea of their function. It was the application of the nascent 'science' of Bioinformatics that wove these threads together. Sean Hemmingsen, in Ellis's group, cloned one of the genes encoding RsuBP and found that it had homology with a 65 kDa protein of *Mycobacterium leprae* called common antigen, as it is a powerful immunogen in bacterial infections generally. The key breakthrough was the discovery that the Rubisco binding protein sequence was homologous to

E. coli GroEL. This led to the publication in 1988 of a classic paper in Nature entitled 'homologous plant and bacterial proteins chaperone oligomeric protein assembly' (Hemmingsen et al. 1988). In this paper' the following statement was made: 'We have described a ubiquitous, conserved, abundant protein that is associated with the post-translational assembly of at least two structurally distinct oligomeric protein complexes. *(phage particles and Rubisco).* We conclude that the role of this protein [termed chaperonin 60] is to assist other polypeptides to maintain or assume conformations which permit their correct assembly into oligomeric structures'.

Ellis' 1988 publication, described above, only suggested that the newly-termed chaperonin 60 protein was involved in protein folding (Ellis and Hemmingsen 1989). However, it was George Lorimer's group, working at the time for the US pharmaceutical company, Dupont, that designed the key experiments to test the hypothesis that chaperonin 60 was involved in the folding of Rubisco into the correct configuration/conformation. Lorimer used Rubisco from the bacterium *Rhodospirillum rubrum.* This bacterial protein is simpler than that of the chloroplast protein, being composed of dimers of the large subunit. To determine if GroEL is involved in the folding of Rubisco, this protein has to be denatured. This is done with a chaotrope such as urea or guanidinium chloride. Using recombinant GroEL and the co-chaperone GroES (chaperonin 10 or Hsp10) and an equimolar concentration of denatured bacterial Rubisco (together with ATP and magnesium) it was found that the Rubisco folded into an enzymatically active form when the denaturant was removed. In the absence of the chaperonins, removal of the denaturant resulted in the formation of aggregated, enzymatically-inactive complexes. Thus the chaperonins were acting to aid the folding of denatured Rubisco (Goloubinoff et al. 1989; Lorimer 2001). This was the starting point for an intensive study of the mechanism of GroEL which has lasted from 1989 until the present day (Horwich 2011).

John Ellis, one of the pioneers of assisted protein folding has defined molecular chaperones as *'proteins that share the functional property of assisting the non-covalent assembly and/or disassembly of protein-containing structures in vivo, but are not permanent components of these structures when they are performing their normal biological functions'* (Ellis 1993).

To conclude, the current paradigm of protein folding encompasses both the self folding of small single domain proteins and the molecular chaperone-assisted folding of multi-domain and multi-subunit proteins. The last 20 years has seen enormous advances in our understanding of the mechanisms of individual molecular chaperones and has emphasised how complex the protein folding problem has been for evolution.

2.4 Control of the Eukaryotic Cell Stress Response

How is cellular stress, protein denaturation, protein misfolding and the overproduction of molecular chaperones and protein-folding catalysts (PFCs) controlled in the cell? The answer is as the result of the evolution of a highly conserved network of

interacting proteins and genes which generate specific patterns of transcription of the protein folding cell stress proteins. This depends upon a family of proteins called heat shock factors (HSFs). Mammals have four such proteins (HSF1-4) while *Drosophila* and yeast express only one (HSF1) (e.g. Björk and Sistonen 2010). The exact mechanisms of the cell stress response are still being elucidated and it is clear that the HSFs are involved in many aspects of cellular behaviour. A description of how bacterial cell stress proteins are regulated is provided in Chap. 1.

How do the HSFs function? In simple terms, the HSFs in the unstressed cell are monomers which interact with the cell stress proteins and form inactive complexes. Stress results in an elevation of unfolded proteins in specific cellular compartments, which competes with the HSF-molecular chaperone equilibrium and results in increased levels of free monomeric HSFs which can trimerise to form the active heat shock transcription complex. This complex can then translocate to the nucleus and induce the transcription of the genes for selected molecular chaperones and PFCs. As the levels of cell stress proteins increase in the appropriate cellular compartments, the concentrations of unfolded proteins declines, and more free molecular chaperones are available to bind to the HSFs and thus switch off the cell stress response (Sakurai and Enoki 2010).

The above description is a grossly simplified view of the cell stress response of the eukaryotic cells which, because of its numerous cellular compartments, can be thought of as having a range of intracellular cell stress responses (Hartl and Hayer-Hartl 2009; Hartl et al. 2011). Thus, there are different patterns of molecular chaperones and protein folding catalysts in the cell cytoplasm, mitochondrion, endoplasmic reticulum, lysosomes and nucleus. The terminology that is most often used to describe the cell stress responses in different cellular compartments is the unfolded protein response (UPR).

2.4.1 Cytosolic Stress Response

The eukaryotic cell cytosol is the compartment on which many of the early studies of the cell stress response were focused. The key proteins involved in protein homeostasis in the cytosol are Hsp70, Hsp90, Tric (or CCT), a cytosolic member of the chaperonin 60 family, and a number of co-chaperone families including Hsp40 which are thought to enhance the specificity of chaperone binding, and nucleotide exchange factors (NEFs) such as Hsp110, which are required to stimulate ADP release from Hsp70 and Hsp90 and promote binding of ATP. In addition to these proteins, the cytosol also contains a key piece of machinery for removing unfolded proteins. This is the protein ubiquitination system and the large complex proteolytic proteosome which removes unfolded proteins and subjects them to proteolysis (Buchberger et al. 2010; Hartl et al. 2011; Kubota 2009).

2.4.2 ER Stress Response

The endoplasmic reticulum (ER) harbours an even more complex network of interacting proteins to control protein homeostasis in this dynamic protein factory, which is the also the seat of protein decoration within the cell (Chakrabarti et al. 2011). The key proteins controlling folding in the ER include the Ig binding protein, BiP or glucose-regulated protein (Grp)78, Grp94/gp96, protein disulphide isomerase (PDI), calnexin and calreticulin (Walter and Ron 2011). The effect of stress on the ER has been termed the unfolded protein response (UPR) and variants of this term have been introduced for other cellular compartments (Walter and Ron 2011). Control of the ER UPR is the function of three transmembrane sensors: IRE-1 (inositol-requiring element 1), PERK (PKR-like ER kinase) and ATF6 (Activating transcription factor 6). In a manner reminiscent of the control of the HSFs, the molecular chaperone, BiP, binds to these proteins and retains them in an inactive state. However, with stress, the local levels of unfolded proteins starts to compete with the BiP for binding these three control proteins (Zhang and Kaufman 2006; Ron and Walter 2007). This results in their release and induction of the UPR. The activated PERK phosphorylates translation initiation factor elF2α thus decreasing protein synthesis and, indirectly, the levels of unfolded proteins. Activated ATF6 is processed to an active transcription factor which induces formation of BiP, PDI and GRP94/gp96 and IRE-1 enhances expression of genes involved in controlling proteostasis in the ER, including those proteins involved in the export and degradation of misfolded proteins (Chakrabarti et al. 2011; Walter and Ron 2011).

2.4.3 Mitochondrial Stress Response

If we take the mitochondrion, then the mitochondrial matrix contains a population of specific chaperones including chaperonin (Cpn) (Hsp10 – or in the new nomenclature – HSPE1), Cpn60 (Hsp60/HSPD1), mitochondrial (mt) Hsp70 (mortalin now HSPA9), mtGrpE and mtDnaJ. Alterations in the protein-folding environment within the mitochondrion induces the mitochondrial unfolded protein response (UPR^{mt}) which is the induction of expression of nuclear genes encoding mitochondrial cell stress proteins (e.g. Yoneda et al. 2004). This organelle-specific stress response utilises the transcription factors CHOP and C/EBPβ (Zhao et al. 2002).

2.4.4 Lysosomes and Cell Stress

Lysosomes are an intracellular vacuolar apparatus involved in proteolytic digestion. These organelles have long been recognised to be involved in cellular (protein) homeostasis through the process known as autophagy. In turn, autophagy, and

alterations in its activity is now being recognised to be involved in many homeostatic actions, for example, immunity (Levine et al. 2011) and also in a growing range of human diseases, for example, cancer (Mah and Ryan 2012). A growing number of molecular chaperones such as Hsp27, Hsp70 and Bag3 are being implicated in the control of autophagy and this is a rapidly growing area of molecular chaperone intracellular biology (e.g. Gamerdinger et al. 2011; Li et al. 2011).

It should be pointed out that one of the major areas of ignorance is how these various compartmentalised stress responses are integrated. Of particular interest is the role that invading microbes, such as viruses, bacteria and protozoa, play in the control of protein homeostasis. It should also be noted that the cell stress response and the cell stress proteins play a much wider role in cellular homeostasis than will be described in this chapter due to lack of space.

2.5 Current Understanding of the Intracellular Function of Selected Eukaryotic Molecular Chaperones

Studies carried out over the past 30 years have come to the conclusion that molecular chaperones and PFCs are essential intracellular proteins required to maintain the cellular proteome in a functional and folded conformation in its various compartments and this has major consequences in human health, human disease (Deture et al. 2010; Morimoto 2011; Xu et al. 2012) and the ageing process (Calderwood et al. 2009; Koga et al. 2011). The role of cell stress proteins in human disease has given rise to a new term – still to catch on – the chaperonopathies (e.g. Macario et al. 2007). There are probably 200 or more eukaryotic cell stress proteins and this chapter cannot cover them all. The proteins that will be discussed are only those that have been implicated in some aspect of microbial virulence or which are inherently interesting because of their moonlighting functions.

2.5.1 Hsp10/Chaperonin (Cpn)10/HSPE1

This volume deals largely with the non-folding aspects of molecular chaperones and PFCs and their moonlighting functions will be dealt with, in a microbial context, in the individual chapters. However Hsp10, a mitochondrial protein, is a fascinating example of a molecular chaperone first recognised as a secreted signalling protein. To be exact, the activity which was recognised was an immunosuppressive factor (termed early pregnancy factor (EPF)) secreted in the first trimester of pregnancy (Morton et al. 1977, 2000; Noonan et al. 1979) and this was eventually identified as Hsp10 in 1994 (Cavanagh and Morton 1994). It is to be wondered that if EPF had been molecularly identified in 1977, then the molecular chaperones that we currently recognise would now be moonlighting immunosuppressants.

However, this protein only comes into its own after the discovery that Cpn (Hsp)60 is a protein- folding molecular chaperone in 1988 (Hemmingsen et al.

1988) with Goloubinoff and co-workers revealing that the *E. coli* Cpn10 protein (GroES) is required for the folding of prokaryotic ribulose bisphosphate carboxylases (Goloubinoff et al. 1989). This same group then showed that the eukaryotic mitochondrion also contained a Hsp10 protein (Lubben et al. 1990). Note that the nomenclature to be used in this chapter is Cpn10/Cpn60 for the bacterial proteins and Hsp10/Hsp60 for eukaryotic proteins. It was then shown that Cpn10 could be chemically synthesised and the synthetic protein functioned as a co-chaperone (Mascagni et al. 1991). The role of Cpn10 in the working of the Cpn60 folding oligomer was first suggested by the finding that it enhanced ATP hydrolysis by Cpn60 (Gray and Fersht 1991). It was then shown that oligomeric Cpn10 bound to one end of the Cpn60 oligomer to aid in protein folding (Langer et al. 1992). However, it was only with the derivation of the crystal structure of Cpn10 (Hunt et al. 1996) and of the Cpn10/Cpn60 complex (Xu et al. 1997) that the interactions of these two proteins started to become elucidated.

Our understanding of the role of Cpn10 and Cpn60 in protein folding has come from the study of the *E. coli* proteins – GroES and GroEL respectively. The folding machine is composed of a 14 GroEL subunits composed into two heptameric rings each set back-to-back forming two central cavities which have an inner hydrophobic surface to which unfolded proteins can bind. The Cpn10 protein also forms a heptamer which is dome-shaped and functions as a cap or lid for the Cpn60 cavity (Fig. 2.1). The exact folding mechanism will be briefly described in the next section when we consider the Cpn (Hsp)60 protein oligomer.

The non-folding activities of Hsp10 are coming to the fore. The early studies that revealed that Hsp10 was immunosuppressive have been replicated in more modern studies. This has led the Australian biotech company CBio Ltd examining this protein for its therapeutic potential. This began with the discovery that human Hsp10 was a potent inhibitor of LPS-induced macrophage activation (Johnson et al. 2005). This led on to the clinical testing of human Hsp10 in several patient groups with nominal clinical effectiveness being seen in patients with rheumatoid arthritis (Vanags et al. 2006) and multiple sclerosis (Broadley et al. 2009). The authors group showed that Hsp10 is present in the circulation of healthy individuals and that these levels decrease in patients with periodontitis (gum disease). If provided with effective therapy, circulating levels of Hsp10 in these patents increases to normal levels (Shamaei-Tousi et al. 2007). With regard to human Hsp10, (XToll) it is unlikely that it will be further exploited, as the efficacy of the protein is low. This might simply be due to pharmacokinetic or pharmacodynamic considerations which may easily be overcome, or to some more serious limitation. The reader is referred to some recent reviews on the non-folding actions of Hsp10 (Corrao et al. 2010; Jia et al. 2011).

2.5.2 Hsp60/Chaperonin 60/HSPD1

As described, most of the studies of Cpn60 have been done on *E. coli* GroEL. This is a tetradecamer of two seven-membered rings stacked back-to-back to provide an

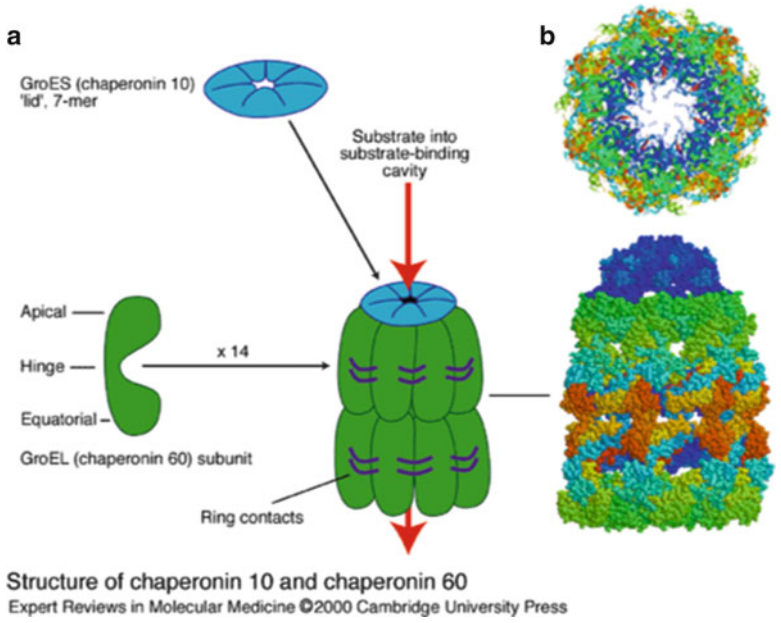

Fig. 2.1 Schematic diagram showing (**a**) the structure of chaperonin 10 and chaperonin 60 and providing some information on their interactions and (**b**) the crystal structures of *E. coli* GroES (*top*) and GroEL (*bottom*) (From Ranford et al. 2000 with permission)

ATP-dependent hydrophobic cavity to generate correct protein folding (Figs. 2.1 and 2.2). In *E. coli,* only a proportion of the cytoplasmic proteins require GroEL to fold, but amongst these are 13 proteins essential for bacterial survival (Kerner et al. 2005). Unfolded proteins are bound in the open cavity of what is termed a GroEL/GroES/ADP bullet complex through hydrophobic interactions. Next the complex binds GroES and ATP and this ATP state is capable of undergoing major conformational changes which change the internal structure of the cavity (Fig. 2.2). This results in the release of the enclosed protein into the cavity whose walls are now hydrophilic in nature. It is within this modified chamber that protein folding occurs. The key question that arose from these findings was whether folding within the GroES/GroEL chamber was an active or passive process. As reviewed by Horwich and Fenton (Horwich and Fenton 2009; Horwich et al. 2009), the GroEL/GroES cavity functions as a passive Anfinsen folding cage as was originally suggested by John Ellis (2003). In other words the segregation of the unfolded polypeptide within the GroEL/GroES cavity allows it time to follow the specific folding pathways leading to the correct conformational solution without interference with other unfolded proteins. This concept of molecular chaperones acting as 'molecular timers' and keeping client proteins separated from the bulk of the cellular proteins, will come up again in this chapter. An alternative hypothesis involving 'forced unfolding' (Lin et al. 2008) of proteins by GroEL is not favoured by Horwich's group (Horwich and Fenton 2009).

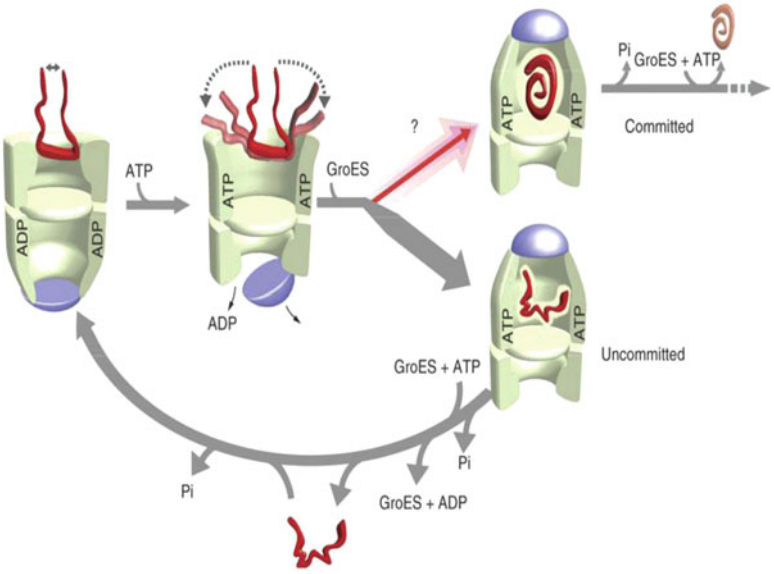

Fig. 2.2 Schematic diagram showing the interaction of a substrate protein with the GroEL folding cavity and the role of ATP binding and hydrolysis in the folding process (committed) In the GroEL folding cycle it is possible that complete folding is not obtained and the protein can undergo a second round of folding (uncommitted). The interaction of GroEL, GroES and ATP in the folding cycle are illustrated (Taken from Lin et al. 2008 with permission)

While, the GroEL/GroES mechanism is now reasonably well accepted, requiring the interactions of two systems of heptamers, there is evidence emerging from other bacteria that the described mechanism may be more complex. At the moment, this largely comes from the study of mycobacterial chaperonin 10/60 proteins. Thus the *Mycobacterium tuberculosis* Cpn60.2 protein both physico-chemically, and in terms of crystal structure, is, even at very high concentrations, a dimer, not a tetradecamer (Qamra et al. 2004; Qamra and Mande 2004; Shahar et al. 2011). Moreover the *M. tuberculosis* Cpn10 protein is not heptameric but is normally monomeric or dimeric (Fossati et al. 2004). It is unclear just how much the heptameric structure of GroEL/GroES can change and still generate a functional molecular chaperone (see Henderson et al. 2010).

In humans, the genes encoding Hsp10 and Hsp60 are side-by-side with a bidirectional promoter in between, resulting in co-regulation of these essential genes. The mitochondrial substrates for Hsp60 have not yet been identified. In mice, the inactivation of the HSPD1 gene is lethal (Christensen et al. 2010). It is beginning to be realised that mutations in the HSPD1 gene can result in human pathology. One of the most recently recognised such diseases has been termed MitCHAP-60 disease, a condition which can be differentiated from a more established human Cpn60-generated disease, spastic paraplegia 13 (SPG13 -another Hsp60-associated autosomal-dominant neurodegenerative disorder) by its autosomal-recessive

inheritance pattern, as well as by its early-onset, profound cerebral involvement and lethality (Magen et al. 2008). MitCHAP disease is proposed to be caused by a homozygous missense mutation G1512A → G in exon 2 at position 86 of the cDNA sequence, causing an aspartic acid → glycine exchange at amino acid 29 of the human Cpn60 protein sequence (D29G). This mutated protein is unable to fully complement an *E. coli* groEL mutant (Magen et al. 2008). Functional analysis of the expressed mutant protein revealed that the oligomer is unstable and dissembles more readily than the wild type and has decreased protein folding and ATP-ase activity (Parnas et al. 2009).

Chaperonin 60 is the master moonlighting protein. In a recently published review (Henderson et al. 2013) the author has reviewed the literature on Cpn60 moonlighting in bacteria, fungi, chloroplasts, protozoa, insects and mammals and has found around 40 incredibly diverse moonlighting functions for this protein. These include being able to produce pores in eukaryotic cells (Alder et al. 1990), acting as an insect neurotoxin (Yoshida et al. 2001) or insect gut toxin (Joshi et al. 2008). The human protein has a growing number of moonlighting functions which have been described (Henderson and Pockley 2010; Henderson and Martin 2011). The intracellular protein has a wide variety of functions including: having pro- and anti-apoptotic actions, modulating NF-kB, controlling cholesterol transport and controlling tumour suppression. As a secreted protein, human Hsp60 can bind to a variety of ligands including HIV glycoprotein, gp41 (Speth et al. 1999), high density lipoprotein (Bocharov et al. 2000) and Gram-negative bacterial LPS (Habich et al. 2005). Of potentially greater importance is the potent signalling activity human Hsp60 has with all leukocyte subsets including monocytes, macrophages, dendritic cells, B lymphocytes and T lymphocytes (Quintana and Cohen 2011; Henderson et al. 2013).

2.5.3 The Hsp70/HSPA1 Family

With the Hsp70/HSPA1 family we begin to explore the complexity of intracellular protein folding as the term Hsp70, which has been used for so long, actually refers to, in humans, at least 13 proteins with different and distinct protein folding functions and, one assumes from the limited literature, moonlighting functions (Kampinga et al. 2009). Thus we have Hsc70 (HSPA8) which is constitutively expressed and the stress-induced Hsp70 (HSPA1A) which can be induced by diverse factors such as heat shock, radiation, heavy metals and microbial infections (Noonan et al. 2007). These proteins are also found in distinct cellular compartments with the endoplasmic reticulum form of the protein known as Grp78/BiP (now HSPA5), the cytoplasmic Hsc70/HSPA8 and the mitochondrial Hsp70 protein known variously as mortalin/mtHSP70/GRP75/PBP74 (now HSPA9) (Hageman et al. 2011; Kampinga et al. 2009). Surprisingly, there is tissue-specific expression of some Hsp70 proteins. This HSPA1L and HSPA2 are cytotosolic proteins highly expressed in the testis (Kampinga et al. 2009).

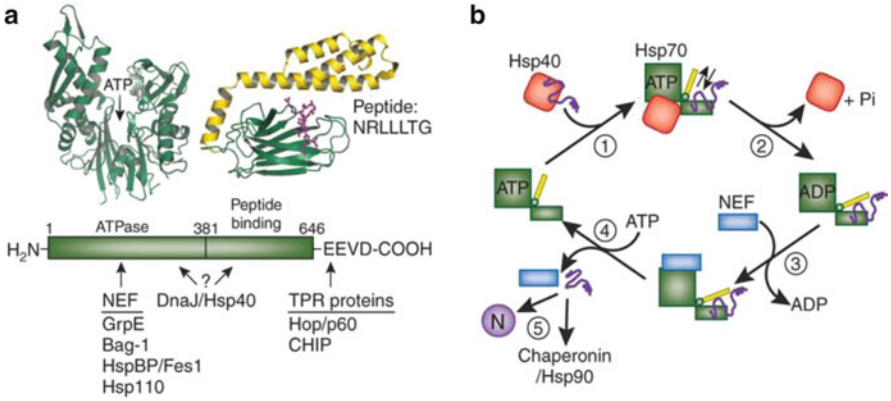

Fig. 2.3 The structure of and function of *E. coli* Hsp70. In (**a**) the structure of the ATP-binding and peptide-binding domains is shown as is the binding sites for various accessory proteins. In (**b**) is shown the so-called Hsp70 reaction cycle. This starts with Hsp40-mediated interaction of substrate protein with the ATP-bound Hsp70 (*1*). ATP hydrolysis to ADP which is enhanced by Hsp40, causes closing of the α-helical lid (in *yellow*) and thus in tight binding of the substrate protein, this is cotemporal with the dissociation of Hsp40 (*2*). Under the action of a nucleotide exchange factor (*NEF*) there is dissociation of ADP (*3*). Finally, binding of ATP results in the opening of the lid and the release of the substrate (*4*) which is either in a native state and does not rebind or is still unfolded and goes through another cycle (Reproduced from Hartl and Hayer-Hartl 2009)

The HSp70 proteins are highly conserved between orthologues and paralogues with a widespread population of protein clients whose interaction are both determined by the tissue compartment and the interactions with Hsp70 co-chaperones (Meimaridou et al. 2009). In this chapter only the stress-induced Hsp70/HSPA1A and the ER Grp78/BiP/HSPA8 will be briefly described. Hsp70 proteins are involved in multiple protein folding functions including: (i) the folding of newly synthesised proteins; (ii) refolding of misfolded and aggregated proteins; (iii) translocation of proteins through membranes and; (iv) control of regulatory proteins (kinases, cyclins, transcription factors, etc) (Mayer and Bukau 2005). For more details of the folding mechanism of bacterial Hsp70 (DnaK) refer to Chap. 1 (see also Fig. 2.3).

The function of Hsp70 family members is dependent on their ability to bind and release, so-called, client proteins through interaction with hydrophobic peptides of proteins in an ATP-dependent manner. Protein folding involves numerous cycles of client binding and release, coupled to conformational change of Hsp70s, driven by ATP hydrolysis and release. All Hsp70 proteins have two functional domains. These are the 40 kDa N-terminal nucleotide-binding domain (NBD) and a 25 kDa C-terminal substrate/peptide-binding domain (SBD) that recognizes exposed hydrophobic segments of clients (Fig. 2.3). These domains are connected by an inter-domain linker, which mediates their allosteric coupling. In the mammalian system, the SBD and NBD interact when Hsp70 is ADP bound (Smock et al. 2010). A simplistic description of the interaction of an Hsp70 protein with a client protein, perhaps a synthesising nascent peptide chain, would show the Hsp70-ATP complex recognising

the hydrophobic residues in the client through its SBD and interacting with them. This process is freely reversible. However, if sufficient binding affinity is generated, the ATPase activity of the Hsp70 is increased and the ATP is hydrolysed to ADP and the SBD undergoes conformational change increasing peptide binding affinity and trapping the peptide chain. The rate of ATP hydrolysis is normally also promoted by interaction of the Hsp70 with a large family of eukaryotic proteins known as J-domain co-chaperones, the best known being Hsp40 (now known as DNAJB1). These DNAJ family proteins significantly promote the ATPase activity of Hsp70 proteins when they bind peptides (Vos et al. 2008). The binding of Hsp70 to nascent protein chains prevent protein aggregation as the protein is formed. Protein release from Hsp70 binding requires that the original ATP-bound state of the Hsp70 is replicated. To achieve this, the Hsp70 must interact with so-called nucleotide exchange factors (NEFs – Rampelt et al. 2011) which are proteins (e.g. BAG-1 and Hsp110) that stimulate the release of ADP from Hsp70 and the subsequent binding of ATP to release the substrate binding pocket. Note that unlike Hsp60, whose client proteins must fit within the cavity, and therefore must have a size limit, there is no size limit to Hsp70 client proteins.

2.5.4 HSPA1A/Hsp70

Most studies of 'Hsp70', which is now recognised in humans to be a family of 13 proteins, have been done with HSPA1A, the so-called stress-inducible Hsp70 protein. Given the homology of these proteins it might be assumed that they are all virtually overlapping in protein folding function. However, it has recently been reported that there is marked variation in protein-folding activity in the Hsp70 family (Hageman et al. 2011). The variations in the intra- and extra-cellular functions of the Hsp70 proteins will be evaluated in the coming years. In addition to its protein-folding role, HSPA1A has attracted significant attention as a cell surface and secreted protein. Indeed, Hsp70 was the first molecular chaperone to be reported to be secreted by cells (Tytell et al. 1986; Hightower and Guidon 1989), and opened up a whole new field of molecular chaperone biology (Henderson and Pockley 2012). One early finding was that Hsp70 had a role in antigen presentation (Vanbuskirk et al. 1989). It is now appreciated that extracellular Hsp70 has prominent roles in immunity integrating the innate and adaptive systems (e.g. Joly et al. 2010; Wang et al. 2010) and also that this protein, which is present on the surface of tumour cells, is a promising vaccine target for immunotherapy (Stangl et al. 2011).

Cell surface Hsp70 is also part of a receptor complex for binding the Gram-negative pro-inflammatory component, lipopolysaccharide and this is explained in detail by Triantafilou and Triantafilou in Chap. 18. The other major role of extracellular Hsp70 is as an intercellular messenger with influence on human leukocytes. This was first identified by Asea and Calderwood (Asea et al. 2000) and has been confirmed by a number of workers (reviewed in Henderson and Pockley 2010).

It is therefore now well established that the inducible Hsp70 (HSPA1A) protein has a myriad of cellular and extracellular functions which are involved both in maintaining homeostasis and in the pathways of immunity that could relate to tissue pathology.

2.5.5 BiP/Grp78/HSPA5

A 78 kDa protein, known variously as glucose-regulated protein of 78 kDa (Grp78), binding immunoglobulin protein (BiP) and, in the new nomenclature, HSPA5, was initially identified as one of the major proteins upregulated in chicken fibroblasts when cells are grown in low glucose media or exposed to tunicamycin, a gycosylation inhibitor (Stone et al. 1974; Pouyssegur et al. 1977; Olden et al. 1979). It is now recognised that BiP is primarily a protein of the endoplasmic reticulum (ER) and a key player in the quality control of proteins processed in the ER and in the modulation of ER signalling in response to ER stress (Zhang and Zhang 2010) and the so-called unfolded protein response (UPR) (Ma and Hendershot 2004). This has been briefly touched on in an earlier section.

One of the more interesting and developing aspects of BiP is its role on the eukaryotic cell surface. This was first reported with hamster fibroblasts (Lee et al. 1984). It is now established that BiP can function as a signalling cell surface receptor for α2-macroglobulin (Misra et al. 2002, 2004). It also functions as a receptor for the binding of a variety of viruses which will be discussed in Honda and Tomonaga in Chap. 19.

Rheumatoid arthritis is a chronic inflammatory and destructive autoimmune disease in which the articular cartilage and subchondral bone of the joints are destroyed. While searching for putative autoantigens in articular cartilage one antigen was found – the ER chaperone – BiP. Attempts were made to see if BiP induced arthritis, which resulted in the finding that BiP was a potent anti-arthritic and anti-inflammatory molecule (Corrigall et al. 2001). Recombinant exogenous BiP induces cells to produce an anti-inflammatory cytokine network (Corrigall et al. 2008) which accounts for the fact that this protein is being used in a clinical trial that started in early 2012 to test its efficacy in rheumatoid arthritis (Panayi and Corrigall 2008).

2.5.6 Hsp90

First identified in the 1980s, it was the discovery that Hsp90 was inhibited by the antibiotics, such as geldanamycin (Whitesell et al. 1994), which has allowed the elucidation of the multiple functions of Hsp90. Like Hsp70, the term Hsp90 refers to a family of proteins found in the eukaryotic cytosol, ER and mitochondrion. Hsp90 requires a number of other proteins, particularly Hsp70, to promote correct protein folding and it appears that the different homologues in the eukaryote interact with different sets of client proteins (Johnson 2012). Most of these client proteins

are involved in intracellular signal transduction including steroid hormone receptors, transcription factors and kinases. A small number of non-signalling client proteins also have major cellular functions including telomerase and antigenic peptides that bind MHC proteins (Wayne et al. 2011). The importance of Hsp90 in controlling normal cellular homeostasis is one reason for the interest in this protein. The other major reason is that the actions of Hsp90 can be linked to cellular pathology, and in particular cancer (Travers et al. 2012). This link-up between Hsp90 and cancer was due to the discovery that this protein was involved in the activation of well known oncoproteins. There is now evidence emerging that transformed cells have an increased requirement for maintenance of intracellular protein homeostasis – again a process controlled by proteins like Hsp90 (Travers et al. 2012).

Our understanding of the protein folding mechanism of Hsp90 proteins is not as advanced as that with other chaperones (Fig. 2.4). Hsp90 proteins are dimers formed of three functional domains termed: the N-terminal binding domain (NBD), the middle domain (MD) which binds ATP, client proteins and co-chaperones and the C-terminal dimerisation domain (DD). Structural studies reveal that Hsp90 undergoes marked alterations in conformation during ATP hydrolysis and client binding cycles (Krukenberg et al. 2008). The client protein interaction cycle of Hsp70 has been described. A similar, but more complex set of interacting partners is required for Hsp90 protein folding. Studies of the folding of steroid hormone receptors has revealed an Hsp90 folding cycle as follows: (i) interaction of the receptors with Hsp70 and Hsp40; (ii) the co-chaperone, Hop binds to Hsp70 and Hsp90 stabilising the Hsp90-client protein early interaction; (iii) the co-chaperone p23 and a peptidylprolyl isomerase (e.g. FKBP52) displace the Hop and Hsp70 resulting in a mature Hsp90 complex. If the complex contains a kinase, then another protein, Cdc37, binds to the complex. The Hsp90-client protein complex has a very long half-life of 5 min and the client protein re-enters the folding cycle by again binding to Hsp70 and Hsp40 (see Picard 2006).

Like the other molecular chaperones described, Hsp90 is now known to be secreted by cells. Much less is known about the role this protein plays in, say, leukocyte activation. However, one of the least expected activities of this protein is its roles in tumour cell invasion, cancer metastasis and skin wound healing. Now in mammals there are two, or more, genes encoding Hsp90 – the major genes being Hsp90α and Hsp90β and these gene products form homodimers. The former (α) is an inducible protein while the latter (β) is constitutively formed (e.g. Hansen et al. 1991). The earliest indication of the role of Hsp90α in tumour growth was in a functional proteomic study of fibrosarcoma cells which identified this protein as a secreted component of invading cells (Eustace et al. 2004). Indeed, the release of Hsp90α in exosomes has been shown to be associated with cancer cell motility (McCready et al. 2010). The invasion of tumour cells *in vitro* can be blocked by cell impermeant anti-Hsp90α antibodies (e.g. Chen et al. 2010). Hsp90α induces tumour cell invasion via activation of cells through cell surface receptors CD91/LRP-1 and Neu and by upregulating NF-kB-induced integrin expression (Chen et al. 2010). A cell impermeable antibody to Hsp90α has also been shown to inhibit human lung cell metastases in SCID mice (Sidera et al. 2011).

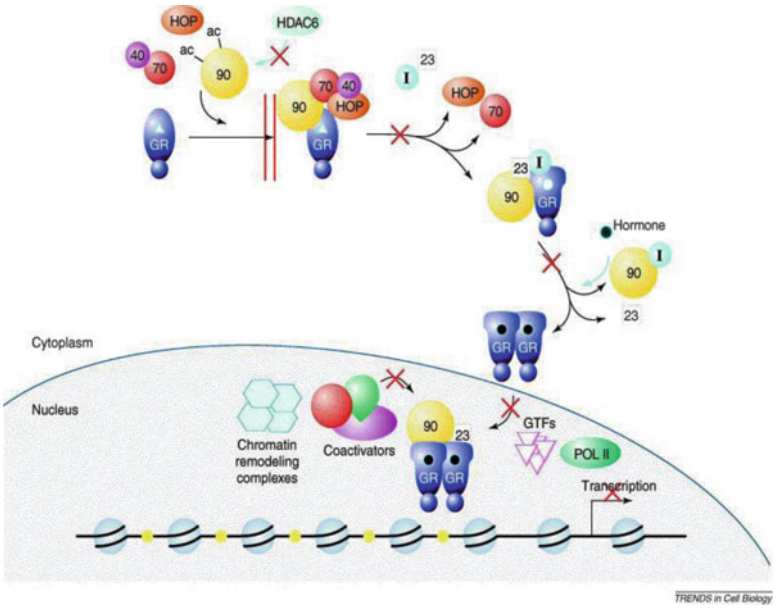

Fig. 2.4 The function of Hsp90 in interacting with the glucocorticoid receptor (*GR*). Hsp90 is responsible for folding the GR from a ligand-inaccessible form into a ligand-binding configuration. Deacetylation of Hsp90 by histone deacetylase 6 (*HDAC6*) allows the Hsp90–Hop–Hsp70–Hsp40 complex to interact with the nascent GR. Release of HOP and the association of p23 immunophilins (*I*) allow the conversion of nascent GR into a mature GR. Upon hormone binding, the GR dissociates from the chaperone complex, dimerizes and translocates into the nucleus, where it binds to the hormone response element in the promoter to induce transcription. The association of p23 and Hsp90 with the GR is thought to aid in the disassembly of the hormone receptor complex and release from the DNA. Hsp90 interactions with the GR and co-chaperones are dependent on HDAC6 deacetylation activity, and the inhibition of this deacetylation activity (*vertical red bars*) prevents Hsp90 from interacting with the GR. As this is the first step in the maturation process of GRs, inhibiting this initial step by inactivating HDAC6 prevents all subsequent steps leading up to the maturation of the GR into a ligand-binding form from occurring (*red crosses*). This ultimately leads to defects in GR-mediated gene activation (Reproduced from Aoyagi and Archer 2005)

Curiously, while acting as a pathological factor in cancer, it has recently come to light that secreted Hsp90α has major therapeutic potential in treating skin wounds, particularly the skin ulceration associated with chronic diabetes. This relies on the ability of this protein to enhance motility in the epithelial and fibroblast cell populations that are involved in promoting wound healing (reviewed by Li et al. 2012). Of therapeutic importance is the finding that a 115 residue fragment of the >700 residue Hsp90α contains the active site of the protein suggesting that a smaller peptide and peptide isosteres containing this beneficial activity can be developed (Cheng et al. 2011).

As can be seen, Hsp90 is a protein for all seasons and it is expected that many more biological roles for this protein, both within and without the cell will be found in the near future.

2.5.7 Gp96

The ER paralogue of the cytosolic HSP90 protein, known variously as gp96 (grp94, HSP90b1), is a molecular chaperone with a particular involvement with the toll-like receptors (TLRs) and integrins (McGettrick and O'Neill 2010). This protein was first identified as a protein regulated by glucose levels (Lee et al. 1983) and later as a tumour antigen (Srivastava and Old 1989). It quickly came to the attention of immunologists for its ability to bind peptides for antigen presentation (Nicchitta 1998). The focus on gp96 was its potential as a cancer vaccine as it bound antigenic peptides and also had the ability to link innate and acquired immune responses (Hilf et al. 2002). In this context the immune aspects of this protein seem to be outweighing its protein folding actions. However, like other molecular chaperones, gp96 transcription is upregulated by the accumulation of misfolded proteins (Kozutsumi et al. 1988). Gp96, like Hsp90, binds and hydrolyzes ATP (Li and Srivastava 1993) and functions as a chaperone for multiple protein including, like BiP, immunogloblulin chains (Melnick et al. 1994). A major advance in our understanding of gp96 occurred when a conditional gp96-deficient mouse restricted to granulocytes, monocytes and macrophages was generated (Yang et al. 2007). These KO animals developed normally but macrophages from them failed to respond to the normal ligands of both cell surface and intracellular toll-like receptors (TLRs – specifically TLR2/4/5/7/9). Mice were resistant to endotoxin shock and highly susceptible to the Gram-positive organism *Listeria monocytogenes*. The conclusion is that gp96 is a key chaperone for controlling TLR positioning and function in macrophages. More recently gp96 has been shown to be essential for correct platelet function (Staron et al. 2011). Thus this ER protein is being shown to have roles in homeostasis and in human disease that could not have been foreseen. The role of this protein in modulating bacteria-host interactions will be discussed in several chapters in this book.

2.5.8 Peptidylprolyl Isomerases (PPIs)

Like other protein structure-modulating proteins the peptidylprolyl isomerases have multiple functions including a variety of moonlighting actions. These very common proteins have protein folding actions as a result of their enzymic isomerisation of peptide bonds preceding prolines and can also function to bind peptides with immunosuppressive actions and confer activity on them (see also Chap. 1).

There are three families of PPIs which have been termed: cyclophilins (they bind to cyclosporine), FK506 (tacrolimus) binding proteins (FKBPs) and the parvulins (Theuerkorn et al. 2011). Each protein family has significant sequence identity, but between families there is limited sequence similarity and between the cyclophilins and FKBPs there is limited tertiary structural similarity. Initially most attention was focused on the immunophilins (cyclophilins and FKBPs) because of their roles in immunosuppression. However, such activity is not dependent on the prolyl isomerase

function of these proteins. Although, with some proteins prolyl isomerisation is the rate limiting step in folding, it was often found that inactivation of single or multiple PPI genes failed to produce a defined phenotype (Göthel and Marahiel 1999). A striking example of this is the inactivation of all 12 *Saccharomyces cerevisiae* immunophilin genes – which resulted in viable yeast (Dolinski et al. 1997). This suggests the immunophilins are not playing a major role in protein folding and possibly each protein has only a limited number of clients or has other distinct functions. The role played by PPIs in the cell is only slowly emerging (Edlich and Fischer 2006; Nagradova 2007). For example, there are 16 cyclophilins in *Homo sapiens* (Wang and Heitman 2005) and possibly the most interesting is cyclophilin D which plays a role in mitochondrial permeability by controlling the actions of the mitochondrial permeability transition pore (Giorgio et al. 2010). The major molecular chaperone, Hsp90, is now known to interact with a number of FKBPs including FKBP52 and FKBP51 in order to form appropriate protein folding complexes (Ebong et al. 2011). Unexpectedly, it has been found that inactivation of the gene encoding FKBP5 has major influences in the mouse on the control of the physiological stress response, neuroendocrine homeostasis and coping behaviour (Touma et al. 2011).

In more recent years attention has begun to focus on the third family of PPIs, the parvulins. There are only two parvulin genes in the human genome: Pin1 which interacts with phosphorylated Ser/Thr-Pro motifs and thus is involved in the quality control of phosphoproteins and parvulin 14 which is believed to be implicated in nuclear functions. Pin-1 was originally discovered as a protein essential for mitosis (Lu et al. 1996). In more recent years Pin1 has been shown to have roles in immunity (Tun-Kyi et al. 2011), cancer, ageing and neurodegenerative diseases (Lee et al. 2011).

Like the rest of the cell stress proteins described in this chapter, the cyclophilins, particularly cyclophilin A was early shown to be a secreted protein with pro-inflammatory properties. Thus it was secreted by LPS-stimulated mouse macrophages and had chemotactic activity (Sherry et al. 1992). There is now a substantial literature on cyclophilin A and it has been implicated in the pathology of chronic inflammatory diseases such as rheumatoid arthritis and atherosclerosis and is now clearly identified as a therapeutic target. Cyclophilin A binds to the cell surface receptor CD147 and this receptor is also considered to be an anti-inflammatory target (Satoh et al. 2010; Yurchenko et al. 2010).

2.6 Conclusions

This chapter has provided a short overview of the protein folding, intracellular function and extracellular actions of a range of the key molecular chaperones and protein folding catalysts that will be dealt with in the current volume or have major moonlighting activities. It is clear that these proteins play multifarious roles in cellular and organismal homeostasis and can participate in pathophysiological states. Their roles in microbial infection are only now starting to be revealed and rapid advances are being made in our understanding of their moonlighting roles in human infections.

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Chapter 3
New Ideas on Protein Moonlighting

Constance J. Jeffery

Abstract An increasing number and variety of proteins are being found to "moonlight" or have multiple, sometimes (apparently) unrelated functions. In this chapter, I describe moonlighting proteins in general – how moonlighting is defined, examples of some of the most common types of moonlighting proteins, how moonlighting functions evolved, and the fact that close homologues of moonlighting proteins might share all, some, or none of these functions. Moonlighting proteins include several taxon-specific crystallins, enzymes adopted for structural roles, enzymes that are also transcription factors, proteins with two different catalytic functions, and intracellular proteins with a second function outside the cell. The ability of so many proteins, over 200 identified to date, to moonlight also has an impact on genome annotation, selection of biomarkers, proteomics, and systems biology. The moonlighting functions of chaperones and heat shock proteins described in more detail elsewhere in this book are particularly important in disease, either by being secreted and affecting the activities of host cells, or by being displayed on the cell surface where they can play key roles in infection and virulence by pathogens.

3.1 Introduction

It is becoming increasingly common that a search for a protein that performs a specific biochemical function results in the identification of a protein that is already known to have a different function, sometimes a function that seems completely unrelated to the first function. It turns out that one method Nature uses to increase the number of functions that the estimated 24,000 genes in our genome can perform

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is to 'reuse' individual proteins for multiple purposes. There are many examples of these "moonlighting proteins" currently in the literature. A moonlighting protein is a single protein that has multiple functions that are not due to gene fusions, multiple RNA splice variants or multiple proteolytic fragments. Moonlighting proteins do not include families of homologous proteins, if the different functions are performed by different members of the protein family, or proteins that have multiple cellular roles that involve the same biochemical function in different locations. It has been known for many years that several soluble enzymes moonlight as structural proteins in the lens of the eye (crystallins) (Piatigorsky 1998) or bind to DNA or RNA as transcription factors or translational regulators (Hall et al. 2004; Zenke et al. 1996; Kennedy et al. 1992).

Over 200 other proteins have also been found to moonlight (for examples, see Table 3.1), and it is possible that many other proteins also have additional functions that have not yet been found. In addition to the examples described below, many other moonlighting proteins are described in more detail in other chapters in this book, in several review articles, and in a book on "gene sharing", an alternative name for protein moonlighting (Jeffery 1999, 2003, 2004, 2009; Piatigorsky 1998, 2007; Henderson and Martin 2011). Moonlighting proteins are widespread in many branches of life – from multicellular animals and plants to single celled yeast and bacteria. The known examples of moonlighting proteins exhibit many types of functions. They include cell surface receptors, enzymes, transcription factors, adhesins, scaffold proteins, and other proteins. Different combinations of biochemical functions are found, for example, an enzymatic function and a receptor-binding function are found in phosphoglucose isomerase/autocrine motility factor (PGI/AMF, Fig. 3.1). While some moonlighting proteins can perform multiple functions simultaneously, others perform one function at a time, and a variety of methods are used to trigger a switch between functions: binding a small molecule, joining a multiprotein complex, binding to DNA or RNA, and/or secretion from the cell.

3.2 Examples of Moonlighting Proteins

Many of the known moonlighting proteins fall in to a few functional categories – crystallins in the lens or cornea of the eye, a soluble enzyme adopted for a structural role, an enzyme that also binds DNA or RNA as a transcription factor or translation regulator, or as is the emphasis of this book, cytosolic proteins that are also found displayed on the cell surface or secreted to perform a second function. It's not clear why some of these categories of functions are observed repeatedly in moonlighting proteins. It might be due in part to the size or stability of the protein folds found in these proteins, physical characteristics that might enable evolution of a new ligand binding site or protein-protein interaction site without affecting the first function of the protein. Some moonlighting proteins are ubiquitous enzymes that appeared early in evolution, for example the enzymes of glycolysis, so that there have been billions of years for Nature to find a second use of them. In addition, some groups

Table 3.1 Examples of moonlighting proteins one function, another function, species

1. Crystallins

Crystallin (lambda), L- gulonate 3-dehydrogenase, rabbits and hares
Crystallin (zeta), NADPH:quinone oxidoreductase, camel, llama, guinea pig, Japanese tree frog
Crystallin (eta), retinaldehyde dehydrogenase, elephant shrews
Crystallin (nu), lactate dehydrogenase A, platypus
Crystallin (delta 2), arginosuccinate lyase (urea cycle) duck, ostrich
Crystallin (epsilon), lactate dehydrogenase B_4, hummingbirds, chimney swifts, duck, crocodiles
Crystallin (pi),glyceraldehyde 3-phosphate dehydrogenase, diurnal geckos
Crystallin (iota), cellular retinol binding protein type 1, diurnal geckos
Crystallin (tau), αlpha-enolase, turtle, lamprey, mola mola (ocean sunfish)

2. Soluble enzyme adopted for structural role

Citrate synthase, 14-nm cytoskeletal protein, tetrahymena
Delta-aminolevulinic acid dehydratase, proteasome inhibitory subunit, human
Ferredoxin-dependent glutamate synthase, subunit of SQD1E in sulfolipid biosynthesis, spinach
Carbinolamine dehydratase (4a), dimerization cofactor (DCoH), rat
Dihydroxyacetone kinase, binds to and affects the function of transcription activator DhaR, *E. coli*

3. Binding to RNA and/or DNA as translational or transcription regulator

Arg5,6 Reductase/kinase, DNA binding transcription regulator, *S. cerevisiae*
Galactokinase, transcriptional activator, *Kluyveromyces lactis*
Aconitase, iron-responsive protein, human cytoplasmic

4. Enzyme with two different catalytic functions

Glutamate racemase, DNA gyrase, *Mycobacterium tuberculosis*
Albaflavenone monooxygenase, terpene synthase, *Streptomyces coelicolor A3*
DegP (HtrA) chaperone, protease, *E. coli*
Ftsh chaperone, metalloprotease, *E. coli*

5. Cytoplasmic enzyme, chaperone, or other protein that is secreted or attached to the cell surface

Histone H1, thyroglobulin receptor, mouse
SMC3 (structural maintenance of chromosome 3, sister chromatin cohesion), bamacam (basement membrane), mouse
Phophosphoglycerate kinase, plasmin disulfide reductase, human
Phosphoglucose isomerase, neuroleukin, autocrine motility factor, differentiation and maturation mediator, mouse and human
Thymosin b4 (sequester actin), secreted chemotaxis ligand, bovine, human
Thymidine phosphorylase, platelet-derived endothelial cell growth factor, human
Ef-Tu, fibronectin binding, *Mycoplasma pneumoniae*
Beta-subunit pyruvate dehydrogenase, fibronectin binding, *Mycoplasma pneumoniae*
Aaa autolysin, fibronectin binding, *Staphylococcus aureus*
Aae autolysin, fibronectin binding, *Staphylococcus epidermis*
Atlc autolysin, fibronectin binding, *Stappylococcus caprae*
Enolase, fibronectin binding, *Lactobacillus plantarum*
Enolase, plasminogen receptor, *Streptococcus pneumonia*
Malate synthase, binds fibronectin and laminin, *Mycobacterium tuberculosis*
Superoxide dismutase, adhesin, *Mycobacterium tuberculosis*
Fructose-1,6-bisphosphate aldolase, adhesin, *Neisseria meningitidis*
Alcohol acetaldehyde dehydrogenase, adhesin, *Listeria monocytogenes*
Pyruvate-ferredoxin oxidoreductase (PFO), cell surface adherance protein, *Trichomonas vaginalis*
Hsp70/DnaK, plasminogen binding protein, *Neisseria meningitides* and *Mycobacterium tuberculosis*
Cpn60 chaperone, adhesin, *Helicobacter pylori*
Cpn60.1 chaperone, adhesin, *Chlamydiae pneumoniae*

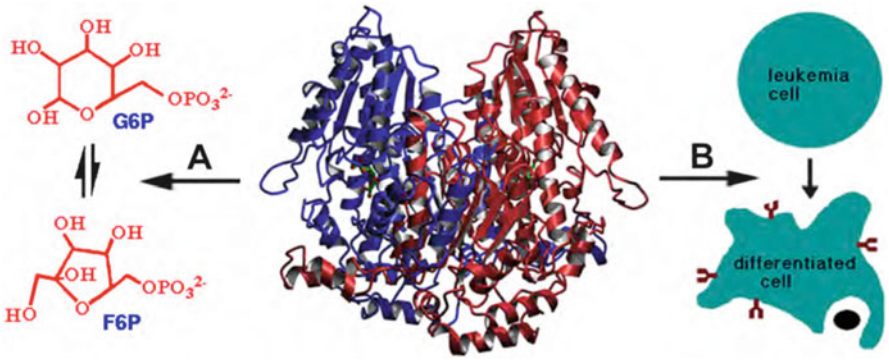

Fig. 3.1 Phosphoglucose isomerase is an example of a moonlighting protein. Phosphoglucose isomerase/autocrine motility factor is a ubiquitous cytosolic enzyme that catalyzes the second step of glycolysis, the conversion of glucose-6-phosphate to fructose-6-phosphate. When secreted by the cell it acts as a cytokine by binding to a cell surface receptor on target cells. Binding to its receptor causes pre-B cells to differentiate into antibody secreting cells and supports the survival of embryonic neurons

of proteins or types of functions have been the focus of more biochemical studies, so finding multiple examples of moonlighting proteins in those families might simply be due to that additional attention.

3.2.1 The Taxon Specific Crystallins

Crystallins are water soluble proteins that that are found in high concentrations in the lens of the eye. They give the lens an optimal refractive index so that clear images form on the photoreceptor cells in the retina. Several crystallins are identical to ubiquitous proteins found in lower concentrations in other cell types, where they are active as an enzyme (Table 3.1) (Piatigorsky and Wistow 1989; Piatigorsky 1998). For these proteins, the catalytic function was the original function of the protein, and these ubiquitous proteins were adopted to be lens crystallins in different species as eyes evolved many different times in different lineages.

Lambda crystallin, which is found in rabbits and hares, is the same protein as L- gulonate 3-dehydrogenase (Ishikura et al. 2005). Another rodent, the guinea pig, uses NADPH-quinone oxidoreductase as zeta crystallin (Huang et al. 1987), as do camels and llamas (Garland et al. 1991), both atriodactyla, as well as the Japanese tree frog (Fujii et al. 2001). Elephant shrews use retinaldehyde dehydrogenase, an aldehyde dehydrogenase class 1, as eta crystalline (Graham et al. 1996; Wistow and Kim 1991), and the platypus, a monotrene or egg-laying mammal, uses lactate dehydrogenase A as upsilon crystallin (van Rheede et al. 2003). Several birds use arginosuccinate lyase as delta 2 crystallin (ducks, ostriches) (Wistow and Piatigorsky

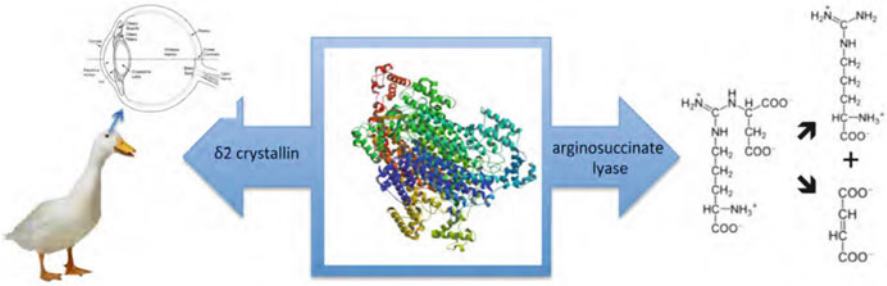

Fig. 3.2 Several taxon specific crystallins in the lens of the eye are catalytically active ubiquitous enzymes. The delta2 crystallin found in the lens of the eye in ducks is the same enzyme as arginosuccinate lyase in the urea cycle

1987; Barbosa et al. 1991; Chiou et al. 1991) (Fig. 3.2), and/or lactate dehydrogenase B_4 as epsilon crystallin (hummingbirds, chimney swifts, duck) (Wistow et al. 1987). The latter is also found in crocodiles (Wistow et al. 1987), which are reptiles that are more closely related to birds than are snakes and lizards. A lizard, the diurnal gecko, uses glyceraldehyde 3-phosphate dehydrogenase as pi crystallin (Jimenez-Asensio et al. 1995), and cellular retinol binding protein type 1 as iota crystallin (Röll et al. 1996). A third reptile group, the turtle, uses alpha-enolase as tau crystallin (Williams et al. 1985), as does the lamprey (jawless fish-like vertebrates) (Stapel and de Jong 1983) and the mola mola (the ocean sunfish, a bony fish) (Jaffe and Horwitz 1992).

3.2.2 Enzymes Adopted for Structural Roles

Other soluble enzymes were adopted for structural roles in protein multimers or large protein complexes in the cell. In *Tetrahymena*, citrate synthase functions as a soluble enzyme in the citric acid cycle but assembles to form the 14-nm cytoskeletal filament during cell mating (Numata 1996). In humans, delta-aminolevulinic acid dehydratase catalyzes a step in the biosynthesis of tetrapyrrole biosynthesis and also is an inhibitory subunit of the proteasome (Guo et al. 1994). This category also includes a moonlighting enzyme from plants; spinach ferredoxin-dependent glutamate synthase forms a complex with SQD1E in sulfolipid biosynthesis (Shimojima et al. 2005). Rat pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha plays a role in tetrahydrobiopterin biosynthesis as a homo-multimer in the cell cytosol, but in the nucleus it binds to and promotes the dimerization of hepatocyte nuclear factor 1 alpha to enhance HNF1A transcriptional activity (Citron et al. 1992). *E. coli* dihydroxyacetone kinase phosphorylates dihydroxyacetone and also binds to and affects the function of transcription activator DhaR (Bächler et al. 2005).

3.2.3 Enzymes That Bind to RNA and/or DNA to Regulate Translation or Transcription

Several other enzymes have evolved the ability to bind to DNA or RNA directly to regulate transcription or translation. Examples include *S. cerevisiae* Arg5,6 reductase/kinase in arginine biosynthesis (Hall et al. 2004) and *Kluyveromyces lactis* galactokinase in the galactose utilization pathway (Zenke et al. 1996), which are both transcriptional activators. Aconitase is an enzyme containing an iron-sulfur cluster in the citric acid cycle. Mammalian aconitase is also known as the iron-responsive protein, because when iron levels drop it loses its iron-sulfur cluster and binds to RNA to regulate translation of proteins involved in iron uptake (Kennedy et al. 1992).

3.2.4 Proteins with Intrinsically Disordered Domains

Still other moonlighting proteins have intrinsically disordered domains that fail to take on a folded three-dimensional structure until encountering a binding partner. This can enable some intrinsically disordered domains to have somewhat different folded structures when bound to different partners, and the interactions with different protein partners leads to different functional outcomes. This is observed for the Cdk-inhibitor p21Cip.1 (Kriwacki et al. 1996) and p53, in which one domain can interact with four different protein binding partners: sirtuin, cyclin A, CBP, and S100bb (Oldfield et al. 2005). In spite of their apparent binding promiscuity, many moonlighting proteins have specific functions under specific cellular conditions, they do not always bind with all their partners.

3.2.5 Two Active Sites: Enzymes with Two Catalytic Functions or Chaperones That Are Also Enzymes

Other enzymes combine two catalytic functions or a protease function with a chaperone function. *Mycobacterium tuberculosis* glutamate racemase is also a DNA gyrase (Sengupta et al. 2008), and *Streptomyces coelicolor* A3 albaflavenone monooxygenase contains a terpene synthase active site (Zhao et al. 2009). In *E. coli*, DegP (HtrA) and ftsh are examples of chaperones that are also proteases (Suzuki et al. 1997).

3.2.6 Intracellular Proteins with a Second Function When Secreted or Localized to the Cell Surface

By far one of the largest groups of known moonlighting proteins is comprised of intracellular proteins that have a second function outside the cell (Fig. 3.3). Dozens

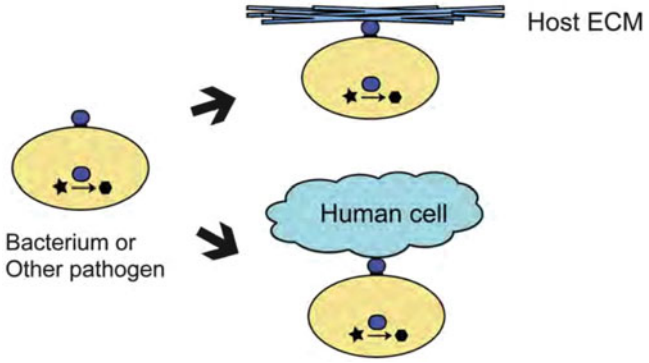

Fig. 3.3 An increasing number of cytoplasmic enzymes and chaperones are being found on the cell surface in bacteria and other pathogens. Some of these proteins moonlight as receptors for host cell surface proteins or for extracellular matrix (*ECM*) and play a role in infection and virulen

of cytosolic enzymes and chaperones, and even some nuclear proteins, are secreted to function extracellularly as soluble proteins or while attached to the cell surface, either the plasma membrane or the bacterial cell wall. For example, in mice, a core protein in the nucleosome, histone H1 is used as a cell surface receptor for a soluble protein, thyroglobulin (Brix et al. 1998), and another nuclear protein that is involved in sister chromatin cohesion, SMC3 (structural maintenance of chromosome 3), is the same protein as bamacam, a secreted protein that is a component of the basement membrane (Darwiche et al. 1999). Human phophosphoglycerate kinase is the same protein as plasmin disulfide reductase (Lay et al. 2000).

Other mammalian proteins serve as secreted cytokines, growth factors or chemotaxis ligands: human and mouse phosphoglucose isomerase is the same protein as neuroleukin, autocrine motility factor, and differentiation and maturation mediator (Gurney et al. 1986a, b; Watanabe et al. 1996; Xu et al. 1996). The actin sequestering protein thymosin b4 is a secreted chemotaxis ligand in cattle (Young et al. 1999). Thymidine phosphorylase is the same protein as the platelet-derived endothelial cell growth factor (Furukawa et al. 1992).

When some cytosolic enzymes or chaperones from bacterial pathogens are secreted or displayed on the cell surface, they can play key roles in infection and virulence. On the surface, the proteins enable attachment of the pathogen to host cells by serving as receptors or adhesins to host cell surface proteins or extracellular matrix. *Mycoplasma pneumoniae* translation elongation factor Tu (Ef-Tu) and the beta-subunit of pyruvate dehydrogenase both bind host fibronectin when they are displayed on the cell surface (Balasubramanian et al. 2008; Dallo et al. 2002). Fibronectin is also the target of *Staphylococcus aureus* Aaa autolysin, *S. epidermis* Aae autolysin, *S. caprae* Atlc autolysin, and *Lactobacillus plantarum* enolase (Heilmann et al. 2003, 2005; Allignet et al. 2002; Castaldo et al. 2009). Enolase, an enzyme in glycolysis, is also used as a cell surface receptor for a different host protein, plasminogen, by *Streptococcus pneumonia* (Kolberg et al. 2006). *Mycobacterium*

tuberculosis malate synthase has been shown to bind both fibronectin and laminin (Kinhikar et al. 2006). Other cytoplasmic enzymes that serve as adhesins when displayed on the cell surface include *Mycobacterium tuberculosis* superoxide dismutase (Reddy and Suleman 2004), *Neisseria meningitides* fructose-1,6-bisphosphate aldolase (Tunio et al. 2010), *Listeria monocytogenes* alcohol acetaldehyde dehydrogenase (Kim et al. 2006 – see Chap. 17), and *Trichomonas vaginalis* pyruvate-ferredoxin oxidoreductase (PFO) (Meza-Cervantez et al. 2011). Moonlighting proteins from two different species can also interact with each other; *Listeria monocytogenes* uses a moonlighting alcohol acetaldehyde dehydrogenase attached to its cell surface to bind to a moonlighting chaperonin (Cpn)60 on the surface of host cells (Kim et al. 2006 – described in detail in Chap. 17).

Numerous cytosolic chaperones and other heat shock proteins also serve a second function extracellularly. Just a few examples will be provided. Thus, *Neisseria meningitides* and *Mycobacterium tuberculosis* Hsp70/DnaK are plasminogen binding proteins (Knaust et al. 2007; Xolalpa et al. 2007). The *Helicobacter pylori* Cpn60 chaperone and *Chlamydiae pneumoniae* Cpn60.1 are adhesins (Kamiya et al. 1998; Wuppermann et al. 2008). In fact, many pathogens use Cpn60, its homologues and/or other chaperones and heat shock proteins, as adhesins or soluble signaling proteins. Other chapters in this book will focus on examples of Cpn60 homologues, other chaperones and other cell stress proteins used in this way.

While not exactly moonlighting in the initial intention of the term, there are also many proteins that are annotated in gene and protein sequence databases as having a second function in infection and virulence because on the cell surface they can be "adopted" for use by invading viruses, bacteria, or other pathogenic species as a means to attach to the host cell. These proteins are often annotated as "cell surface receptors" for the pathogen. Several examples of proteins used in this way will also be covered in later chapters in this book, including: Hsp90, Hsp70, Grp78(BiP) and ER-associated gp96.

3.3 How Do Proteins Become Moonlighting Proteins?

There are several potential reasons for a cell to reuse proteins, but perhaps the main reason proteins have evolved moonlighting functions is simply that Evolution uses whatever is available. Many of the proteins found to have moonlighting functions are involved in ubiquitous and ancient biochemical pathways, including most of the enzymes in glycolysis and several in the citric acid cycle. During three billion years since these enzymes first evolved, there has been ample time for these proteins to develop an additional binding site for participation in another biochemical pathway or a multi-protein complex. Changes in the regulation of protein expression also play a role in developing a second function. Several of the taxon specific crystallins appear to have been adopted for function in the lens of the eye without major changes in their structures. Instead, changes in protein expression patterns were a key factor in their being adopted for an additional function (Cvekl and Piatigorsky

1996). Once a protein has evolved a new function, the use of moonlighting proteins can provide benefits to the organism and so that second function can be retained through natural selection. The modern cell is complex but organized. In addition to providing the individual additional functions, moonlighting proteins can provide a means to coordinate cellular activities, provide feedback mechanisms, and aid in switching between different pathways in response to changing conditions.

For many of the proteins described above, and those discussed in the other chapters in this volume, the proteins have two roles inside and outside of the cell. It is not always clear how these cytosolic proteins end up outside the cell. Like most other cytosolic proteins, they lack a defined signal sequence, so they are not targeted to the classic ER-Golgi secretory pathway. They also don't share another clear consensus sequence or surface region that might label them to be secreted. How they get onto cell surface is not clear, but for many of the proteins it is apparently due to the use of unconventional secretion pathways, and this is currently an active area of research. Use of these alternative secretion methods might provide the cell with flexibility in where it localizes these proteins. The cell could have the option of keeping the protein in the cell or secreting some of it under different conditions or in response to specific stimuli.

3.4 Homologues of Moonlighting Proteins Are Not Always Moonlighting Proteins

It is important to note that finding that a protein moonlights does not mean that related proteins also moonlight. Even close amino acid sequence homologues of moonlighting proteins do not always have both functions, or in some cases have a different combination of functions (Fig. 3.4). Aconitase catalyzes the conversion of citrate to isocitrate in the citric acid cycle in many species, but it has different moonlighting functions in different organisms. The cytoplasmic enzyme in mammals and in *Mycobacterium tuberculosis* also function as the RNA binding iron-responsive protein to regulate translation of proteins involved in iron uptake (Kennedy et al. 1992; Zheng et al. 1992; Banerjee et al. 2007; Kaptain et al. 1991). In contrast, the mitochondrial aconitase in the yeast *S. cerevisiae* is not the iron-responsive protein, but it does have a second function in the maintenance of mitochondrial DNA (Chen et al. 2005, 2007). The delta 1 and delta 2 crystallins in the duck lens share 89 % amino acid sequence activity, but only the delta 2 isoform retains arginosuccinate lyase catalytic activity (Piatigorsky et al. 1988; Piatigorsky and Horwitz 1996). Other examples of homologous proteins with different moonlighting functions are described in more detail in other chapters in this book. In particular, several members of the Cpn60 protein family are receptors for proteins when displayed on the cell surface, but in different species they have been found to interact with different host proteins. In general it is not easy to predict if a protein has the same functions as a moonlighting homologue based on overall sequence homology alone. The presence

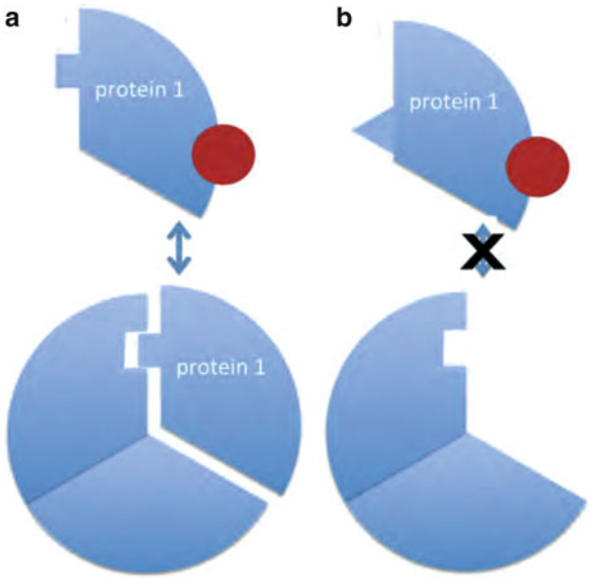

Fig. 3.4 Homologues of moonlighting proteins are not always moonlighting proteins. (**a**) A moonlighting protein, "protein 1", binds to a ligand (*red circle*) and also joins into a multi-protein complex to perform another function, but this second function is found only in the protein from a single species. (**b**) In another species, a homologue of protein 1 also binds the ligand, but differences in other regions of the solvent exposed surface of protein 1 prevent it from interacting with the multiprotein complex. Therefore, this homologue of protein 1 only has one function

of the key functional site amino acid residues can provide evidence of function, if those specific amino acids have been identified, but it is often necessary to test for each function by each protein individually.

3.5 Moonlighting Proteins Are Likely to Be Common

From the examples listed in this chapter, elsewhere in this book and also in many review articles, it is clear that moonlighting proteins comprise a diverse group of proteins that includes members of many protein families, and many different combinations of biochemical functions can be found within one protein. In addition, the mechanisms proteins can use to switch between functions, the proposed models for the evolution of moonlighting functions, and the potential benefits moonlighting proteins might provide to an organism through providing additional protein functions and through coordinating cellular activities, suggest that moonlighting proteins might be common. The previously published examples include over 200 known moonlighting proteins, but this probably still includes only a small percentage of proteins that moonlight. The number of known moonlighting proteins is growing each year, and it is currently not possible to predict how many proteins moonlight.

There is also currently no general straightforward method to identify which proteins encoded by a genome have multiple functions or for determining whether a protein of interest is a moonlighting protein. Characterization of a novel protein generally involves finding a function for a protein, but does not usually include a search for all possible additional functions of a protein. Many moonlighting functions have been found by serendipity. It is possible that many other proteins have additional functions that have yet to be found. In fact, moonlighting might be a common phenomenon among proteins. It is even possible that the majority of proteins might have some aspects of moonlighting functions.

The ability of so many proteins to perform multiple, sometimes apparently unrelated biochemical functions, affect several areas of current research, including interpreting and annotating genome sequences, proteomics, systems biology, selecting biomarkers for disease, and identifying the roles of proteins involved in infection and virulence. These will dealt with in turn:

3.5.1 Interpreting and Annotating Genome Sequences

Many protein functions annotated in sequence and structure databases are inferred from the known functions of amino acid sequence homologues. As illustrated above, the existence of moonlighting proteins complicates such interpretation because homologues can share all, none, one, or some moonlighting functions. Information describing the evidence that was used to annotate a protein function would be valuable to include in the annotation. How, when and where proteins moonlight and the locations of functional sites within the protein structure would also be valuable information to include.

3.5.2 Interpreting the Results of Proteomics Projects

The ability of a protein to have multiple biochemical functions and to be involved in different multi-protein complexes or pathways can complicate the interpretation of results from large scale proteomics projects that study protein expression, protein-protein interactions, protein localization, the effects of gene knockouts, and other protein characteristics. At the same time, because these high-throughput biochemical, genetic and computational screens provide valuable information on the functions of thousands of proteins simultaneously, without relying on a hypothesis of one or a few specific functions beforehand, they could be powerful methods for identifying potential moonlighting proteins. For example, a DNA binding function was found for an enzyme in the arginate biosynthetic pathway, Arg5,6, through a proteomics study using DNA oligonucleotide arrays (Hall et al. 2004).

3.5.3 Systems Biology

The modern cell is a sophisticated and highly organized network with many concurrent pathways and activities and a great deal of interaction between macromolecules. Moonlighting adds another level to our understanding of cellular complexity. For example, a moonlighting protein that has functions in both a biosynthetic pathway and a signaling pathway provides a connection between those two pathways. Moonlighting proteins can also provide a switch point between pathways, so that the cell can respond to changes in its environment. For example, in mammals, aconitase regulates the expression of proteins involved in iron uptake when iron levels drop, rendering the iron-dependent protein enzymatically inactive. Moonlighting might be a common mechanism of communication and coordination between the many pathways within a cell or between different cell types within an organism. The identification of moonlighting functions is important for understanding how the proteins in a cell and an organism work together.

3.5.4 Biomarkers and Disease

The roles of several moonlighting chaperones in modulating the activity of the immune system will be described in other chapters in this book. The roles of these and other moonlighting proteins in multiple biochemical pathways can result in the level of expression or localization of a moonlighting protein being affected by multiple, apparently unrelated, factors. This could result in changes in expression or localization (i.e. secretion into the blood) sometimes being related to the presence or progression of a disease and at other times being related to another function of the protein. This could complicate the choice of biomarkers for disease.

3.6 Summary

An increasing number and variety of proteins are being found to have multiple, seemingly unrelated functions. The most common known moonlighting proteins can be grouped as taxon specific crystallins, enzymes adopted for structural roles, enzymes that are also transcription factors, proteins with two different catalytic functions, and intracellular proteins with a second function outside the cell. In the last group, the chaperones and other heat shock proteins/cell stress proteins are well studied protein families that contribute many examples to the list of known moonlighting proteins. Their moonlighting functions are also particularly important because of their roles in disease. Several are required by pathogens for infection and virulence. Examples of moonlighting proteins from these families will be discussed in much more detail in other chapters of this book. Many of the topics described

above are important in studies for characterizing these proteins; questions about how they are secreted and/or become displayed on the surface of the cell, the potential use of proteomics or systems biology to identify additional family members that moonlight, how to annotate clearly sequence and structure databases when close homologues have different functions, which ones might serve as useful biomarkers, and how some of these moonlighting functions might be modified or otherwise exploited in disease treatment.

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Part II
Microbial Cell Stress Proteins Acting as Moonlighting Virulence Factors

Chapter 4
Chaperonin 10, a Pro- and Anti-inflammatory Host Modulator

Anthony Coates

Abstract Chaperonin(Cpn) 10 is a 10 kDa heat shock protein/cell stress protein which has an extraordinary range of biologically important activities. Human Cpn10 is found in the sera of pregnant women and pregnant animals. In this context it is known as Early Pregnancy Factor. It seems to be involved in immunosuppression which is associated with pregnancy and is clearly extracellular. Clinical trials with human Cpn10 have yielded contradictory results. Bacterial Cpn10 is involved in protein folding and in *E. coli* is known as GroES and is regarded as an intracellular protein. Bacterial Cpn10 is also highly immunogenic and stimulates powerful antibody and cellular immune responses. It seems to be a virulence factor, possibly leading to a number of diseases such as spinal bone resorption in tuberculosis (Pott's disease) and infertility in women (associated with *Chlamydia* spp infection). This chapter describes the characteristics of Cpn10 of both humans and bacteria.

4.1 Introduction

The discovery of chaperonin (Cpn) 10 was dominated by four ideas. The first idea in 1977 (Morton et al. 1977) was that Cpn10 was associated with pregnancy. It was found in the serum of pregnant women (Morton et al. 1977) and sheep (Morton et al. 1979). It was suggested that it might be involved in immunosuppression during pregnancy (Noonan et al. 1979). At that time, it was called early pregnancy factor (EPF) and was not identified as a member of the Cpn10 family until 1994 (Cavanagh and Morton 1994; Fletcher et al. 2001). The second idea was that it was involved in bacteriophage head morphogenesis and bacterial growth at

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nonpermissive temperatures (Tilly et al. 1981). See Chap. 1 for additional discussion on this point. This concept, which particularly focused on the role of Cpn10 in protein folding, was initially studied in detail in many laboratories (Goloubinoff et al. 1989; Laminet et al. 1990; Henderson 2010) without the knowledge that the human equivalent, Cpn10, was EPF. The gene which encodes Cpn10 is essential for bacterial growth (Fayet et al. 1989; Hu et al. 2008). X-ray crystallography studies (Hunt et al. 1996; Roberts et al. 1999, 2003) have revealed a heptameric structure with mobile loops which bind to Cpn60 or to itself (see Chaps. 1 and 2 for further discussion). The third idea was that it was a major antigen in bacteria, specifically *Mycobacterium tuberculosis* (Mt) (Baird et al. 1988,1989; Barnes et al. 1992). The fourth idea was that bacterial Cpn10 was a virulence factor (Henderson et al. 1996) and a member of the bacterial modulin class which induce cytokine synthesis and results in host tissue pathology. For example, MtCpn10 was shown to stimulate bone resorption (Meghji et al. 1997). It turns out that all four ideas are probably correct, and are discussed in more detail in this chapter. It is extraordinary that such a small protein can have so many, seemingly different characteristics. Even more puzzling is that Cpn10 can exhibit the opposite qualities of being both anti-inflammatory and pro-inflammatory. Cpn10 is not alone in holding such remarkable characteristics, because Cpn60 also has pro-and anti-inflammatory properties (Henderson et al. 2010b). Unsurprisingly, scientists have struggled to come to terms with the emerging data. The concept that Cpn10 is an intracellular molecule which is key to the correct folding of some proteins is widely accepted. However, the thought that Cpn10 is also found outside the cell, has been accepted more slowly. This is surprising because human Cpn10, which used to be called EPF, was first demonstrated to be present in human sera (Morton et al. 1977). Furthermore, *M. tuberculosis* Cpn10 has been shown to be secreted from Mt when this organism is residing inside macrophages (Fossati et al. 2003). This suggests that, like human Cpn10, MtCpn10 might have a role outside the bacterial cell during an infection. Contrarians have also suggested that Cpn10 does not actually have many different properties, rather some other molecule, such as a small peptide or lipopolysaccharide which is biologically active, is bound to Cpn10. For example, it has been suggested (Rosenkrands et al. 1999) that expression of Cpn10 in a foreign host such as *Escherichia coli* may lead to contaminants binding to the molecule, thus conferring additional properties. Mascagni and colleagues (Mascagni et al. 1991) synthesised the entire Mt Cpn10 protein, thus bypassing the need for expression in *E. coli,* and showed that this completely synthetic chaperone formed oligomeric structures which had the expected protein folding activity thus revealing that this protein did not require other chaperones for its activity. Interestingly comparison of this recombinant Mt Cpn10 with the native protein purified from cultured Mt found that the latter protein was able to induce γ-interferon (IFN) synthesis from peripheral blood mononuclear cells of healthy tuberculin reactors. In contrast, the recombinant protein was inactive in this respect (Rosenkrands et al. 1999). So, in the case of T cell reactivity, it is possible that an additional moiety is acquired from inside *M. tuberculosis* which enhances its activity. Bacterial lipopolysaccharide has been suggested as a possible

contaminant, but further work has shown that this is not the case (Henderson et al. 2010a). For example, Cpns are active in the absence of lipopolysaccharide, are active in the presence of a lipopolysaccharide blocking agent and lose activity in the presence of protease.

More surprises were in store. It was observed that some species, such as *M. tuberculosis,* have multiple chaperonin genes (Kong et al. 1993). A new nomenclature, which took this into account, was proposed (Coates et al. 1993) and suggests that the first Cpn which is discovered is allocated the number 1, the second, number 2 and so on.

Then, in 1994, it was revealed that EPF was a member of the Cpn10 family (Cavanagh and Morton 1994). This complicated matters. On the one hand, it was clear that bacterial Cpn10 was a major antigen (Barnes et al. 1992). On the other hand, human Cpn10 seemed to have immunosuppressive qualities (Morton et al. 1992). In addition, those studying protein folding within the cell, concentrated on intracellular Cpn10. In contrast, those who were interested in pro- and anti-inflammatory aspects of chaperones were assessing extracellular Cpn10 activity. To this day, where these fields overlap, there is still much misunderstanding.

What medical benefit might emerge from these ideas? The most obvious concept is that the pro-inflammatory properties might lead to a new vaccine for bacterial diseases (Ferrero et al. 1995) or better understanding of the pathogenesis of, for example *Chlamydia* spp. disease (Betsou et al. 2003a, b). Alternatively, the anti-inflammatory characteristic might lead to new immunosuppressive therapy for autoimmune diseases (Van Eden 2008). That Cpn10 may interact with the immune system in a unique way was proposed by Chan et al. (1995) because sequence and structural homologies were observed between bacterial Cpn10 and the MHC Class I/II peptide binding cleft. Further details of the pro-and anti-inflammatory properties are covered in the subsequent sections of this chapter.

4.2 Chaperonin 10 as an Anti-inflammatory Agent

When human chaperonin 10 was first discovered (Morton et al. 1977), it was called Early Pregnancy Factor (EPF). Since pregnancy involves the growth of non-genetically matched cells within the mother, it was thought that EPF could be implicated is some sort of immunosuppressive activity (Noonan et al. 1979). EPF was found in the serum within hours of fertilisation, binds to lymphocytes which release other factors which modulate the mother's immunity, in such a way that means that the baby is not immunologically rejected (Morton 1984). Details of these immunosuppressive properties included the observation (Cocchiara et al. 1986) that EPF suppresses the early expression of lymphocyte cell surface IgG in the presence of serum-free medium and Sepharose-Concanavalin (Con)A. EPF induced this suppressive effect at low picogram concentrations. Where does EPF come from? Interestingly, it seems that the embryo itself may produce EPF. It has been shown (Bose et al. 1989) that EPF is produced by preimplantation embryos and is found in the growth media of *in vitro* fertilised ova. EPF which is purified

from this growth media is immunosuppressive in a Con A-stimulated lymphocyte proliferation assay. The molecular mass of EPF in the early days was estimated to be 14 kDa (Bose et al. 1989). The sequence similarity between EPF and chaperonin 10 confirmed that they belonged to the same family of chaperonin 10. Cavenagh and colleagues showed that human Cpn10 reduced the inflammation of autoimmune encephalomyelitis in animals (Zhang et al. 2000, 2003; Harness et al. 2003). Interestingly, human Cpn10 reduced the infiltration of lymphocytes and macrophages into the spinal cord of rats during experimental autoimmune encephalomyelitis (Athanasas-Platsis et al. 2003). This is reminiscent of the inhibitory affect of MtCpn60.1 on leukocyte diapedesis in a mouse model of allergic lung inflammation (Riffo-Vasquez et al. 2012). Recombinant human Cpn10 also seems to have anti-graft rejection characteristics. Local treatment of skin grafts with Cpn10 has been shown to prolong skin graft survival time (Morton et al. 2000) in experimental models of transplantation. In another experimental model of autoimmune disease, Cpn10 prevents cutaneous lupus and suppresses nephritis in MRL-(Fas)lpr mice (Kulkarni et al. 2012).

It was also discovered (Ragno et al. 1996) that MtCpn10 has anti-inflammatory affects in an animal model of autoimmune disease, namely adjuvant arthritis. These results demonstrated that MtCpn10 delayed the onset and severity of arthritis. MtCpn10 also inhibits allergic inflammation in the lung (Riffo-Vasquez et al. 2004) by suppressing eosinophil recruitment and bronchial hyper-responsiveness in a mouse model of human asthma. It is unclear why an organism such as *M tuberculosis* produces MtCpn10 which mimics the anti-inflammatory properties of human Cpn10. It may be related to the underlying pathogenesis of the disease which is characterised by localised collections of cells, called granulomas. These are rich in macrophage-lineage cells. It is possible that *M. tuberculosis* Cpn10, like human Cpn10 (Athanasas-Platsis et al. 2003) and MtCpn60.1 (Riffo-Vasquez et al. 2012) inhibits the migration of cells. If this is the case, it could be expected that MtCpn10 and MtCpn60.1 are present in cells which contains Mt. These cells secrete cytokines as a result of being infected with Mt, and these attract other cells into the area. However, once these cells have arrived, they cannot leave because MtCpn10 and Cpn60.1 "freeze" them in place. The cells accumulate locally, and form a granuloma. Interestingly, mutant Mt which lacks the *mtcpn60.1* (Hu et al. 2008) does not form granulomas, although it does multiply in the tissues of the mouse and guinea pig. This suggests that MtCpn60.1 is the main molecule which inhibits migration of cells, and leads to granuloma formation. It was not possible to undertake the same experiment with Cpn10 mutants because the gene is essential and the mutant is not viable.

Human clinical trials have also been performed with Cpn10. Phase IIa clinical trials have been undertaken with Cpn10 treatment of patients with rheumatoid arthritis(Vanags et al. 2006), psoriasis(Williams et al. 2008) and multiple sclerosis (Broadley et al. 2009; Van Eden 2008). In a study with 23 rheumatoid arthritis patients, Cpn10 seemed to be well tolerated and efficacious in the treatment of symptoms (Vanags et al. 2006). However, in a subsequent trial Cpn10 did not show a significant difference between rheumatoid arthritis patients and those taking a placebo

(http://www.evaluatepharma.com/Universal/View.aspx?type=Story&id=251426. Accessed December 30th 2012). It is not known why contradictory results were obtained in these clinical trials.

4.3 Chaperonin 10 as a Pro-inflammatory Molecule

Monoclonal antibodies are useful tools to dissect out important antigens. The first set of monoclonals against *M. tuberculosis* was published in 1981 (Coates et al. 1981) and was used to identify Cpn60.2 (Young et al. 1985; Shinnick 1987), but missed Cpn10 and Cpn60.1 (Kong et al. 1993). A 10 kDa antigen, called BCG-a from *Mycobacterium bovis* BCG was purified in 1984 by Minden and colleagues(Minden et al. 1984) with the monoclonal SA12, and the 20 N-terminal amino acids were sequenced. They (Minden et al. 1984) showed that the 10 kDa antigen induced T-cell-mediated delayed type hypersensitivity in guinea-pigs which had been immunised with BCG, and also demonstrated a cross-reacting 10 kDa antigen in *M. tuberculosis*. This antigen is also recognised by sera from tuberculosis patients (Coates et al. 1989). It was not until 1989 (Baird et al. 1989) that the entire sequence of *M. tuberculosis* Cpn10 was determined and that its homology to other 10 kDa heat shock proteins (now called Cpn10) GroES of *E. coli* (Hemmingsen et al. 1988) and HtpA of *Coxiella burnetii* (Vodkin and Williams 1988) was discovered. At the time, it was suggested (Baird et al. 1989) the Cpn10 antigens are widely conserved amongst bacteria, are heat shock proteins (Vodkin and Williams 1988) and might protect against a range of bacterial infections, although possible associations with autoimmune disease should be borne in mind when considering heat shock proteins as vaccines.

However, surprises were in store. It seems that in leprosy and tuberculosis patients, IgG1 and IgG3 antibodies to specific *M. leprae* and *M. tuberculosis* epitopes are associated with disseminated disease and IgG 2 and IgG4 with disease limitation (Hussain et al. 2004). The mean intensity of the T cell and antibody responses also seems to be related to disease status. In the case of *Helicobacter pylori* infection of mice, Cpn10 was protective (Ferrero et al. 1995). But no vaccines for *H. pylori* are currently licensed. Curiously, MtCpn10 immunisation of guinea-pigs also protects against foot-and-mouth disease virus (Amadori et al. 1999) by a mechanism which may involve Cpn10 antibody which cross-reacts with histones released by virus-infected cells. These data suggest that bacterial Cpn10 may have protective properties, but the mechanism of protection may be complex.

Is bacterial Cpn10 a virulence factor? To complicate matters further, it appears that in certain circumstances, inflammation which is associated with bacterial Cpn10, may be harmful. For example, MtCpn10 may be involved in Pott's disease (Meghji et al. 1997), which is caused by severe bone resorption followed by spinal collapse in a small proportion of tuberculosis patients. Mtcpn10 induces bone resorption in bone explants cultures, leads to osteoclast recruitment and inhibits the proliferation of osteoblasts. This bone-resorbing activity resides within the flexible N-terminus of the molecule. This concept was derived from the observation that

MtCpn60.2 induces release of cytokines from human monocytic cells (Friedland et al. 1993) and that Cpn60 from the oral bacterium involved in periodontitis, *Actinobacillus actinomycetemcomitans,* resorbs bone (Kirby et al. 1995).

Another example of potentially harmful inflammation which is associated with bacterial Cpn10, is infertility which may be induced by *Chlamydia spp* infection of the female genital tract. The gene for Cpn10 of *Chlamydia trachomatis* (Ct) was sequenced in 1994 (Ho and Zhang 1994). In 1999 (Spandorfer et al. 1999) it was observed that antibodies to CtCpn10 were more prevalent in women with hydrosalpinx (in whom the fallopian tube is blocked with a serous exudate) than in women with male factor infertility. It was also found (Betsou et al. 1999) that antibodies to CtCpn10 were associated with chronic genital tract infection with *C. trachomatis*. Interestingly, antiCtCpn10 antibodies cross-react with human Cpn10 or EPF and infertility is associated with the presence of anti-CtCpn10 and anti-EPF antibodies (Betsou et al. 2003a). In mice (Athanasas-Platsis et al. 2000) anti-Cpn10 antibodies retard embryonic development at two key developmental stages- the one-two-cell stage and the peri-implantation stage. It seems that a significant proportion of women with tubal factor infertility make antibodies to CtCpn10 (LaVerda et al. 2000). Infertile women have greater CtCpn10 sero-reactivity than acutely infected women which suggests the greater the severity of the disease, the higher the degree of CtCpn10 recognition. In the general population there is also evidence of a correlation between antibodies to CtCpn10 and female subfertility (Karinen et al. 2004). This type of infertility may be related to the observation that cervical epithelial cells express higher levels of CtCpn10 protein in infertile than fertile women (Jha et al. 2009). Also, CtCpn10 induces apoptosis in endocervical epithelial cells (Jha et al. 2011). These data suggest that CtCpn10 may be involved in the pathogenesis of infertility.

Further clinical associations between bacterial Cpn10 and atherosclerosis (Borel et al. 2008), asthma (Betsou et al. 2003b), and macular degeneration (Kalayoglu et al. 2003) have been recorded. In addition, it has been suggested that Cpn10 may be an autoantigen in autoimmune pancreatitis and fulminant type 1 diabetes (Takizawa et al. 2009). There is insufficient evidence at this stage to say whether these observations have wider implications or not.

4.4 Conclusions

Cpn10 is an important component of all cellular life forms. When it is intracellular, it is involved in protein folding. When extracellular, it seems to act as a danger signal, with both pro- and anti-inflammatory characteristics. Whilst the intracellular properties of bacterial Cpn10 have been well characterised, the extracellular biology of this remarkable molecule have been less well explored. The reason for this, in my view, is that the scientific community has had difficulty in accepting that the extracellular properties of Cpn10 are real and are not artefacts. This view has been compounded by the diverse nature of these extracellular characteristics, which range

from anti-inflammatory to pro-inflammatory, essential for fertility to virulence factor for infertility, and from potential treatments for autoimmune disease to vaccines. Seemingly, Cpn10, appears to be important from the very beginning to the end of life (see Corrao et al. 2010, for further comments).

The confusion amongst scientists is understandable. On the one hand, there is the wealth of knowledge about the intracellular role of Cpn10 in the molecular mechanisms of protein folding. On the other hand there is the relative paucity of knowledge about the extracellular role of Cpn10, whose biological activities are claimed to be of potential importance in many different areas of medicine.

Is there a unifying hypothesis for all these different properties of Cpn10? Cpn10 is a molecule with an ancient lineage. It was almost certainly present in bacteria before eukaryotes existed. Srivastava and colleagues have suggested that a eukaryotic response to extracellular (released) heat shock protein could be an evolutionary remnant from primitive ancestral immunity (Srivastava et al. 1998). It has also been suggested that the immune system is somehow hard-wired to respond to 'danger' associated molecules of potential pathogens, or molecules which are released from cells during stress or tissue damage (Janeway 1992; Matzinger 1994). It is thought that antigen-presenting cells have pattern-recognition receptors which bind to and lead to upregulation of costimulatory molecules on other nearby antigen-presenting cells. Together with the interaction of antigenic peptides with major histocompatibility complex (MHC) an antigen-specific immune response is initiated. Cpn10 is upregulated (Gophna and Ron 2003), with many other heat-stress proteins, in response to stress. This could explain the powerful antigenicity of bacterial Cpn10. Why is human Cpn10 not treated as a danger molecule? A possible explanation could be that it is different to bacterial Cpn10s, and does not bind to the same receptors. However there does not seem to be a good data to explain this difference. In the case of Cpn60 (Davies et al. 2006), there is evidence that cell lines respond with a raised Hsp70 in the presence of bacterial Cpn60, but not in the presence of human Cpn60. It is possible that a similar situation exists for Cpn10 in this regard.

Why is bacterial Cpn10 a virulence factor in certain circumstances? It is likely that the pressure of evolution altered Cpn10 so that it could find a niche for itself inside a host, for example in the genital tract. In some way, it is quite possible that the enhanced inflammation which *C. trachomatis* Cpn10 induces in the fallopian tubes of infected women, is in some way advantageous to the microbe, perhaps providing additional nutrients. Whether it is an advantage to the organism to make the female subject sterile is difficult to say.

How could immunosuppression be explained in the context of the danger response? It is possible that high concentrations of Cpn10 are pro-inflammatory and very low concentrations are anti-inflammatory. There is no convincing evidence that this is the case for Cpn10, although human Cpn10 is immunosuppressive at very low concentrations (Cocchiara et al. 1986). However, there is a hypothesis which suggests that MtCpn60 is immunosuppressive at very low concentrations and immunostimulatory at high concentrations (Coates et al. 2007). Why should one molecule be both pro- and anti-inflammatory? Perhaps the reason is that the bacterium needs to control the immune system of a eukaryote. For

example, when the bacterium first enters the eukaryote, it is likely to do so in low numbers. It will be vulnerable to attack, but produce a heat-stress response which in turn leads to low concentration Cpn10 in the immediate micro-environment. This could suppress the immune response of the host, and allow the microbe to multiply and set up a colony in the host. However, large numbers of bacteria will produce higher concentrations of Cpn10 and eventually, the Cpn10 is recognised by the immune system which reacts by making antibody and a cellular response. In a healthy person, this immune response suppresses or eradicates the bacterial infection and cure ensues. In an immune-compromised subject, for example, the immune response may be inadequate and the infection could take hold. This hypothesis has been proposed previously (Coates et al. 2007) to help to explain why a mutant knockout of the MtCpn60.1 gene leads to a dramatic decrease of loose granulomas in mice (Hu et al. 2008) and was termed the granuloma activation-suppression cycle. Further work suggests that low doses of MtCpn60.1 inhibit the migration of mononuclear cells, thereby trapping them in the granuloma (Riffo-Vasquez et al. 2012). It remains to be seen whether this situation also applies to Cpn10, although it does seem to inhibit the migration of inflammatory cells into tissues *in vivo* (Athanasas-Platsis et al. 2003).

There is clearly much more to learn about Cpn10.

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Chapter 5
Helicobacter pylori Peptidyl Prolyl *cis, trans* Isomerase: A Modulator of the Host Immune Response

Manikuntala Kundu

Abstract *Helicobacter pylori* is an intriguing bacterium because of its ability to survive at low pH in the stomach and its relationship with gastric inflammation and cancer. Among its armamentarium of virulence factors is HP0175, a peptidyl-prolyl isomerase (PPIase), which is secreted and is a major antigen in patients with *H. pylori*-induced pathology. This review summarizes the moonlighting functions of HP0175, revealing that this secreted protein-folding catalyst regulates cell signalling in gastric epithelial cells and in monocytic cells to modulate the inflammatory response and apoptosis during *H. pylori* infection. In addition, it elicits a Th17 response thereby modulating the adaptive immune response. These activities reveal the importance of protein moonlighting in the generation of *H. pylori*-induced disease states.

5.1 Introduction

More than 50 % of the global human population harbours *Helicobacter pylori,* a spiral-shaped Gram-negative bacterium that is endowed with the ability to survive in the harsh, acidic environment of the stomach. Under circumstances where a robust immune response prevails, the infection remains clinically asymptomatic. However, a subset of individuals harbouring this organism develop duodenal and peptic ulcers, and 1 % of those that are infected develop adenocarcinoma or lymphoma of the stomach (Atherton 2006; Kusters et al. 2006; Nomura et al. 1994). According to the World Health Organization, *H. pylori* is classified as a carcinogen for distal gastric cancer. It is associated with stomach cancer and with gastric

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mucosa-associated lymphoid tissue (MALT) lymphoma (Eslick et al. 1999; Huang et al. 1998; Parsonnet et al. 1991; Uemura et al. 2001).

H. pylori is able to survive the acidic environment of the stomach by its ability to secrete an urease enzyme that neutralizes the acidic environment. It also shows extensive intrastrain and interstrain diversity, and exhibits a partially intracellular lifestyle. These traits confer a fitness advantage to the bacterium, accounting for its ability to persist. Establishment of infection by *H. pylori* is associated with secretion of proinflammatory cytokines as well as epithelial cell apoptosis. In order to colonize the gastric epithelium, it is likely that *H. pylori* needs to suppress a vigorous proinflammatory response (Blanchard et al. 2003; Blaser and Parsonnet 1994; Ismail et al. 2003). Therefore there is a dynamic interplay between the host immune response and the mechanisms employed by the bacterium to suppress inflammation. Injected toxins, secreted proteins and a host of surface-exposed molecules constitute the repertoire of molecules that modulate the immune response. *H. pylori* uses the Type IV secretion apparatus to inject toxins into host cells (Backert and Selbach 2008; Peek and Crabtree 2006). The best-studied pathogenicity island of *H. pylori*, the *cag* pathogenicity island, injects the cytotoxin-associated gene A (CagA) (Hatakeyama 2004), into host cells. CagA positive strains are associated with increased risk of gastric cancer (Rieder et al. 2005). The vacuolating cytotoxin, VacA is also associated with the pathology of the gastric epithelium (Palframan et al. 2012). It forms pores in the mitochondria. Apart from CagA and VacA, secreted proteins such as urease and γ-glutamyl transpeptidase also contribute to gastric inflammation and epithelial cell damage.

A host of secreted proteins of *H. pylori* have been identified by several groups (Atanassov et al. 2002; Bumann et al. 2002; Kim et al. 2002; McAtee et al. 1998). The focus of this review will be the secreted peptidyl prolyl *cis, trans* isomerase, HP0175. This protein belongs to the repertoire of antigens recognized by antibodies of patients with gastroduodenal ulcers. It is one of an increasing list of proteins endowed with moonlighting functions. HP0175 is characterized by a C-terminal peptidyl prolyl *cis*, *trans*-isomerase (PPIase) core identified on the basis of sequence similarity. While, on the one hand, it is a functional PPIase, on the other hand, it modulates immune responses in gastric epithelial cells as well as in cells of the monocytic lineage.

In this review, the author will discuss how HP0175: (i) triggers apoptosis while also being capable of eliciting a proangiogenic effect in gastric epithelial cells; (ii) elicits cytokine release from cells of the monocytic lineage, and; (iii) regulates differentiation of T cells.

5.2 Peptidyl Prolyl *cis, trans* Isomerase (PPIase)

Peptidyl prolyl *cis, trans* isomerases (PPIases) catalyze the cis/trans interconversion of prolyl imidic peptide bonds in proteins (Fanghänel and Fischer 2004 – see also Chap. 2). PPIases are ubiquitous. Three families of PPIases are recognized. These

are the cyclophilins, which are sensitive to the immunosuppressant cyclosporin (Fischer et al. 1989; Takahashi et al. 1989), the FK506 binding proteins (FKBPs) (Harding et al. 1989) and the parvulins (Hayano et al. 1991; Heikkinen et al. 2009; Rulten et al. 1999) which are insensitive to cyclosporine or FK506 (Rahfeld et al. 1994). Several PPIases exhibit moonlighting functions and are required for bacterial colonization. PPIases have been characterized as virulence factors. The Mip protein of *Legionella pneumophila* belongs to the cyclophilin family. The N terminus of the protein anchors the protein to the cell wall, while the C terminus exhibits PPIase activity (Hacker and Fischer 1993). It is an important virulence factor of *L. pneumophila* (Fischer et al. 1992) and a current account of the biology of this protein and its role in *L. pneumophila* virulence is presented in Chap. 6. The *Trypanosoma cruzi* Mip (Tc-Mip) is another virulence factor that is required for successful infection (Pereira et al. 2002). In addition, the streptococcal rotamase A (another term for peptidyl prolyl isomerase) is required for pneumococcal colonization (Hermans et al. 2006).

5.3 HP0175 and Gastric Epithelial Cell Signaling

5.3.1 Apoptosis

The gastric epithelium undergoes remodelling, underscored by defined histological changes initiated by the transition of the normal mucosa to chronic gastritis, followed by atrophic gastritis, intestinal metaplasia and finally adenocarcinoma (Correa and Miller 1998; Jones et al. 1999; Xia and Talley 2001). Apoptotic cells have been observed in chronic gastritis throughout the epithelial and lamina propria cells (Rudi et al. 1998). The CD95 pathway has been implicated in *H. pylori-induced* apoptosis (Rudi et al. 1998). One school of thought suggests that apoptosis of the gastric epithelium is intimately linked to the transition from gastritis to adenocarcinoma. An alternate point of view suggests that apoptosis is required to remodel the gastric epithelium and allow establishment of chronic *H. pylori* infection (Mimuro et al. 2007). Whatever the outcome, apoptosis is intimately linked to *H. pylori* infection.

Cell death may be the end point of several signaling pathways that are programmed to respond to cues or it may be the end point of an unprogrammed, destructive process associated with inflammation. Apoptosis is an orchestrated process involving shrinkage of the cell, condensation of the chromatin and disintegration of the cell into apoptotic bodies, designed to prevent inflammation (Bröker et al. 2005; Fuchs and Steller 2011). Apoptosis may involve signaling initiated by ligation of death receptors (the extrinsic pathway), their oligomerization and signal transduction mediated through the membrane proximal event of recruitment of procaspase 8 to the death-inducing signaling complex and its activation (Kischkel et al. 1995; Salvesen and Dixit 1999). Activated caspase 8 goes on to cleave

downstream effector caspases 3 and 7 (Muzio et al. 1997; Stennicke et al. 1998). The mitochondria play a central role in death via the intrinsic pathway (Estaquier et al. 2012; Oberst et al. 2008; Spierings et al. 2005; Wang and Youle 2009). Decrease in mitochondrial transmembrane potential involving formation of mitochondrial pores, leads to the release of cytochrome c (cyt c) (Liu et al. 1996), apoptosis-inducing factor (Susin et al. 1999), and Smac (DIABLO) (Okada et al. 2002) from the mitochondria. Cytosolic cyt c together with ATP/dATP induces a conformational change in apoptotic protease-activating factor-1, and formation of the apoptosome complex involving procaspase 9 (Acehan et al. 2002; Liu et al. 1996), leading to the proteolytic activation of procaspase 9 (Srinivasula et al. 1998). Activated caspase 9 then cleaves procaspase 3. At the crossover point between the extrinsic and intrinsic pathways is the cleavage of the Bcl-2 family member Bid by caspase 8 to form truncated Bid (t-Bid). By inserting into the mitochondrial membrane, t-Bid facilitates the pore-forming activity of the mitochondria (Li et al. 1998). *H. pylori* induces activation of caspase 8 and 3 and subsequent cleavage of PARP, leading to an increased level of apoptosis in gastric epithelial cells (Ashktorab et al. 2002).

The gastric epithelial apoptosis-inducing factors include VacA (Cover et al. 2001; Kuck et al. 2001); urease (Fan et al. 2000), γ-glutamyl transpeptidase (Shibayama et al. 2003) and the protein HP986 (Alvi et al. 2011). CagA functions as one of the major antiapoptotic factors (Mimuro et al. 2007). Since the outocome of the disease cannot be directly correlated to the genotype of the bacterium, particularly the *cag* pathogenicity island or the VacA-expressing genes, it is all the more important to understand the arsenal of factors used by the bacterium to modulate the inflammatory response.

One of the factors with apoptosis-inducing ability is the secreted PPIase HP0175 (Basak et al. 2005). Exogenous HP0175 is able to bind to TLR4 and to elicit signals leading to the activation of MAP kinases. Elevated expression of TLR4 has been reported in gastric biopsy samples associated with *H. pylori* infection compared with uninfected samples (Ishihara et al. 2004), suggesting a probable involvement of TLR4 in *H. pylori*-associated pathophysiology. The MAPK cascade is one of the evolutionarily conserved phosphorylation-regulated protein kinase cascades that influences cell survival or cell death. c-Jun N terminal kinase (JNK) and p38 (a MAP kinase (MAPK)) mediate various types of stress-induced apoptosis (Cargnello and Roux 2011). Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAPK kinase kinase family that plays a role in stress-induced apoptosis, principally through activation of the JNK or p38 MAPK signaling cascades (Ichijo 2004; Ichijo et al. 1997; Matsuzawa and Ichijo 2001). *H. pylori* is known to activate MAP kinases (Choi et al. 2003; Keates et al. 1999). Binding of HP0175 to TLR4 triggers activation of ASK1 upstream of the activation of p38 MAP kinase. This leads to the activation of caspase 8. The apoptotic signal is amplified by the generation of t-Bid, and engagement of the mitochondrial pathway leading to caspase 3 activation. The importance of HP0175 as an apoptosis-inducing agent has been confirmed using an *hp0175* knockout mutant (Basak et al. 2005).

5.3.2 *Epidermal Growth Factor Receptor (EGFR) Signalling and Vascular Endothelial Growth Factor (VEGF) Production*

CagA translocated into epithelial cells disrupts epithelial tight junctions by interaction with Zo-1 (Amieva et al. 2003). The phosphorylation of GPCR interactor 1 mediated by VacA (Fujikawa et al. 2003), likely facilitates binding of EGF ligands to the EGFR. Thus, there are likely to be multiple mechanisms of activation of EGFR and remodelling of the gastric epithelium during *H. pylori* infection. In order to gain a comprehensive understanding of the role of HP0175 on gastric epithelial cells, its ability to manipulate gene expression in AGS cells has been explored using global approaches. Our present knowledge arising from a microarray analysis of gene expression in AGS cells stimulated with HP0175 (unpublished), suggests that it modulates genes involved in tissue remodelling and angiogenesis, one of which is vascular endothelial growth factor (VEGF). Due to its role as an angiogenic factor, VEGF is suggested to play an important role in the pathology of *H. pylori* infection (Tuccillo et al. 2005). It is overexpressed in human gastric adenocarcinoma. It has a dual function in the reconstruction of the wounded mucosa during healing as well as in supporting tumor-associated angiogenesis. EGFR signalling is associated with VEGF production (Maity et al. 2000). Intriguingly, HP0175 elicits the production of VEGF in a TLR4-dependent manner (Basu et al. 2008). HP0175 triggers a novel signalling pathway in which TLR4 translocates to lipid rafts, recruits the tyrosine kinase lyn and transactivates EGFR. HP0175 therefore transactivates EGFR in an EGF- and ADAM-independent manner. HP0175-mediated remodelling of the gastric epithelium by apoptosis and VEGF production requires TLR4 signaling (Fig. 5.1). The effects of this PPIase with novel functions could well have a non-trivial role in *H. pylori* pathogenesis, particularly in *cag*A and *vacA*-negative strains.

5.4 HP0175 and Signaling in Immune Cells

HP0175 has been shown by *in vitro* experiments to be secreted by *H. pylori* (Kim et al. 2002). It could therefore be a potential candidate which could influence gene expression programs in mucosal macrophages once the epithelial cell junction is disrupted during the course of infection. TLRs play major roles in signalling in cells of the innate immune system. It could be argued that HP0175, shed during the process of infection, could have a bearing on modulating the cytokine milieu by virtue of its ability to engage and trigger TLR4 signaling in macrophages and monocytes. Using differentiated THP-1 cells, *in vitro* studies confirmed this hypothesis. HP0175 binds TLR4 and triggers MAP kinase signalling by activating ERK and p38 MAP kinases in a PI 3-kinase/Rac1-dependent manner leading to the activation of *IL-6* transcription (Pathak et al. 2006). This signalling culminates in the activation of (a) NF-κB and (b) the nuclear kinase MSK1, which

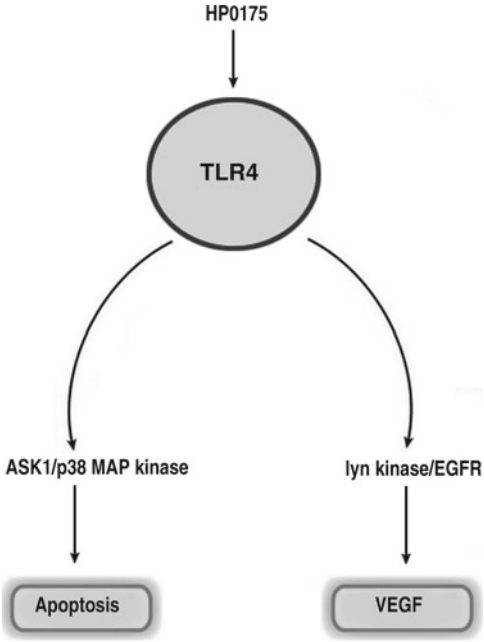

Fig. 5.1 HP0175 induces apoptosis and VEGF production in the gastric epithelial cell line AGS in a TLR4-dependent manner

phosphorylates histone H3 on serine 10 and the p65 subunit of NF-κB on serine 276. Phosphorylation of p65 on serine 276 facilitates its interaction with the transcriptional coactivators p300 and CBP (Zhong et al. 2002). Phosphorylation of H3 on serine 10 by MSK1 is documented to regulate transcription of a subset of genes (Thomson et al. 1999). Both these events are therefore likely linked to activation of transcription of *IL-6* (Pathak et al. 2006).

The cytokine milieu generated by antigen-presenting cells (APCs) dictates the differentiation of T cells into various lineages. Th1 cells are usually central to host defense against intracellular pathogens. *H. pylori* proteins, such as the neutrophil-activating protein, the cysteine-rich protein A, outer membrane protein 18 and *H. pylori* lipopolysacccharide are able to elicit gastric Th1 inflammation (D'Elios and Andersen 2009). The *H. pylori* flagellins are the principal TLR5 agonists (Wroblewski et al. 2010). However, the *H. pylori* flagellins and LPS are distinctly less proinflammatory than their counterparts from other organisms. Th2 cells confer resistance against helminthes. T_{Reg} cells are important in immune suppression and in peripheral self tolerance, whereas the newly recognized Th17 cells develop in response to IL-6, IL-25, TGF-β and IL-1β. IL-6 and IL-21 activate STAT3. STAT3 induces the expression of the transcription factors RORγt and RORα which promote IL-17A, IL-17F, IL-21 and IL-22 to stabilize T_{H17} cells. The Th_{17} subset of cells has recently been linked to *H. pylori* infection. Th_{17} cells produce IL-17 and IL-21 which drive an inflammatory response. Further, IL-21 production is associated with the production of matrix metalloproteases (MMPs). *H. pylori* infection is associated with the production of IL-17 and IL-21 (Caruso et al. 2007, 2008; Luzza et al. 2000;

Shi et al. 2010; Zhang et al. 2011). Interestingly, tumor-infiltrating lymphocytes (TILs) from *H. pylori*-infected patients with distal gastric adenocarcinoma produce IL-17 and IL-21 in response to HP0175 (Amedei et al. 2012). HP0175-specifc TILs also promote monocytic MMP-2, MMP9 and VEGF production. These findings suggest that HP0175 drives gastric Th17 responses. It is the first antigen of *H. pylori* to be identified as an inducer of the Th17 response. A long-lasting Th17-linked inflammatory response could lead to gastric cancer. It is therefore tempting to speculate that HP0175 could be a therapeutic target in the treatment of *H. pylori* pathology.

5.5 Conclusions

There is increasing evidence that bacterial moonlighting proteins play a role in bacterial virulence. Chaperones designed to handle cell stress in bacteria, and PPIases that are required for correct protein folding, are among the best studied classes of proteins with observed moonlighting functions. This review summarizes the moonlighting functions of HP0175 of *H. pylori*, a member of the PPIase family. HP0175 regulates cell signalling in gastric epithelial cells and in cells of the monocytic lineage to modulate the inflammatory response and apoptosis during *H. pylori* infection. In addition, it elicits a Th17 response thereby modulating the adaptive immune response. These attributes of HP0175 serve to highlight the importance of functional duality of the moonlighting proteins in microbial pathogenesis.

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Chapter 6
Macrophage Infectivity Potentiator Mip Exhibits Peptidyl-Prolyl-*cis/trans*-Isomerase Activity, Binds Collagen IV and Enables *Legionella pneumophila* to Transmigrate Across Tissue Barriers

Michael Steinert and Can Ünal

Abstract During human infection, *Legionella pneumophila* mainly replicates intracellularly within alveolar macrophages. In addition, the bacterial penetration of the alveolar epithelial barrier has a significant impact on the progress of Legionnaires´ disease. The macrophage infectivity potentiator (Mip) of *L. pneumophila* contributes to both the intracellular replication and the extracellular dissemination processes. Mip is exposed on the bacterial surface, exhibits peptidyl-prolyl-*cis*/*trans*-isomerase (PPIase) activity, and binds to the extracellular matrix (ECM) component collagen IV. Biochemical analyses revealed that the collagen-derived peptide P290 binds to a specific hydrophobic cavity of Mip, thus inhibiting bacterial epithelial transmigration *in vitro*. Based on NMR data and docking studies a model for the mode of interaction of P290 and Mip was developed. The present article focuses on Mip as a moonlighting protein with intra- and extracellular functions during *L. pneumophila* pathogenesis. Moreover, we discuss Mip-substrate based peptides as lead structures for anti-infective drug development.

6.1 *Legionella pneumophila*, an Environmental Human Pathogen

Legionella pneumophila is an environmental Gram-negative bacterium that naturally inhabits freshwaters, surviving on biofilms, or parasitizing, and surviving within, different protozoan species. This organism also thrives in man-made water

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systems including air conditioning systems and cooling towers. Infection of humans is by inhalation of *L. pneumophila*-contaminated aerosols, which can lead to a severe and life-threatening pneumonia called Legionnaires´ disease (Gaia et al. 2005; Steinert et al. 2002). Upon transmission, the bacteria invade and replicate within alveolar macrophages and epithelial cells. Chest radiographs of infected patients typically demonstrate patchy, peripheral, non-segmental consolidations. Attributes of infection are fibrinolysis, cellular infiltrations of neutrophils and macrophages, and alveolar damage followed by dispersal of *L. pneumophila* to other organs (Winn and Myerowitz 1981). The penetration of the lung epithelial barrier, formed by epithelial cells and the basement membrane, seems to be an essential step in the pathogenesis of Legionnaires´ disease. The basement membrane is composed of extracellular matrix (ECM), in which type IV collagen is the most prominent component (Dunsmore and Rannels 1996). In later stages of infection, *L. pneumophila* disseminates to the patient's spleen, kidneys, bone marrow and lymph nodes (Watts et al. 1980). Cellular infection assays, guinea pig infections, Mip-binding assays with collagen IV, bacterial transmigration experiments across an artificial lung epithelium barrier, collagen IV peptide arrays and NMR spectroscopy assisted modelling revealed that the *L. pneumophila* virulence factor Mip (macrophage infectivity potentiator) contributes to both, the intracellular replication of the pathogen and to bacterial dissemination within the lung (Conlan et al. 1986; Wintermeier et al. 1995; Wagner et al. 2007; Ünal et al. 2011). In the present article we discuss Mip as a moonlighting virulence protein with intra- and extracellular functions during *L. pneumophila* pathogenesis.

6.2 Macrophage Infectivity Potentiator (Mip): A Surface-Exposed Peptidyl-Prolyl-*cis/trans*- Isomerase, Contributes to Intracellular Pathogenicity of *L. pneumophila*

Soon after its first description, Mip was shown to be a FKBP-Type PPIase, which was a novel finding within this protein family (Fischer et al. 1992). Mip is differentially expressed under the control of LetA, which is a global regulator of virulence associated *L. pneumophila* genes (Wieland et al. 2002; Shi et al. 2006). Biochemical and molecular studies have shown that Mip is a basic 24 kDa surface protein (pI 9.8) that possesses a N-terminal signal sequence, which is cleaved off when the protein is transported through the cytoplasmic membrane (Köhler et al. 2003). The protein consists of two domains that are connected via a very long α-helix (Riboldi-Tunnicliffe et al. 2001). The fold of the C-terminal domain (residues 100–213) is closely related to the human FK506-binding protein (FKBP12), and like FKBP12, Mip exhibits peptidyl-prolyl-*cis/trans*- isomerase (PPIase) activity. The α-helical N-terminal domain is responsible for the formation of very stable Mip homodimers (Köhler et al. 2003). In a nuclear magnetic resonance (NMR) investigation, we were able to solve the solution structure of free $Mip^{77\text{-}213}$ and the $Mip^{77\text{-}213}$/rapamycin complex (Ceymann et al. 2008). The C-terminal domain forms an extremely stable complex with rapamycin, which

identifies Mip as a typical member of the FK506-binding protein (FKBP) type PPIases. Mediated by a hinge in the long α-helix, both FKBP domains of Mip are subject to large fluctuating movements allowing for flexible cooperative binding of potential target structures (Horstmann et al. 2006).

The macrophage infectivity potentiator (Mip) protein was the first virulence factor of *L. pneumophila* shown to be relevant for intracellular infection. Infectivity defects of Mip-negative *L. pneumophila* mutants show that Mip is needed for optimal replication of *L. pneumophila* in macrophage cell lines, alveolar macrophages, blood monocytes, lung epithelial cells and protozoan hosts (Cianciotto et al. 1995; Cianciotto and Fields 1992; Wintermeier et al. 1995). The Mip-negative mutants were approximately 50- to 100-fold less infective compared with their isogenic Mip-positive parental strains. However, no differences in intracellular bacterial numbers were observed between the wild-type isolates and the *Legionella* strains expressing Mip variants with strongly reduced or no PPIase activity. These data indicated that the enzymatic activity of Mip does not contribute to the intracellular virulence of *L. pneumophila.* Apart from *L. pneumophila*, FKBP homologues of Mip-like proteins are also present in numerous other microorganisms including various pathogenic bacteria (Horne et al. 1997; Bas et al. 2008; Maeda et al. 2010; Norville et al. 2011). Interestingly, the supposed functions of these Mip-like proteins are very diverse and their natural substrates remain to be identified. Until now, this also held true for the intracellular function of the *L. pneumophila* Mip protein.

6.3 Collagen Binding Protein Mip Enables *L. pneumophila* to Transmigrate Through *In Vitro* Lung Epithelial Barrier

Guinea pig infections with Mip-positive *L. pneumophila* strains and Mip-negative mutants revealed that Mip contributes to the bacterial colonization of the lung and to the subsequent dessimination into extrathoracic organs (Wagner et al. 2007). Since the bacterial transmigration across the alveolar septum requires penetration of the basal membrane, the interaction of Mip and the extracellular matrix (ECM) was analysed. In order to test whether Mip interacts with components of the lung epithelial barrier, Mip was tagged with horseradish peroxidase (Mip-HRP) or with the fluorescent dye fluorescin isothiocyanate (Mip-FITC). Its binding to NCI-H292 lung epithelial cells, to ECM of these cells and to purified immobilized ECM proteins revealed that Mip is a collagen IV binding protein. Transwell assays with *L. pneumophila* and recombinant *E. coli* HB101 strains demonstrated that Mip also enables these bacteria to transmigrate across a barrier of NCI-H292 lung epithelial cells and ECM (NCI-H292/ECM barrier). Degradation assays with ^{35}S-labelled ECM proteins later supported the hypothesis of a concerted action of Mip and proteolytic enzymes (Wagner et al. 2007; Juli et al. 2011). In this regard, it is interesting to note that Mip enhances the levels of a phospholipase C-like activity in the supernatants of cultured *L. pneumophila* (Debroy et al. 2006).

6.4 Collagen IV-Derived Peptide P290 Binds the Hydrophobic Cavity of *L. pneumophila* Mip and Interferes with Bacterial Epithelial Transmigration

After identifying human collagen IV as the major extracellular binding target of the Mip protein, we determined the binding sequence of collagen IV by using a peptide array. Probing this array with recombinant Mip protein identified the surface-exposed Mip-binding peptide sequence P290 (IPPCPSGWSSLWI). The localization of P290 within the native protein was assessed with the help of the published crystal structure (pdb 1LI1) of collagen IV in the NC1 domain of human collagen IVα1. In co-precipitation assays, pre-incubation of Mip with P290 or the PPIase inhibitor FK506 interfered with the binding of Mip to immobilized P290. In competitive binding studies, P290 reduced the binding of Mip to collagen IV in a dose-dependent manner. In accordance with these findings, the bacterial transmigration of *L. pneumophila* across the transwell-based lung epithelial cell barrier (NCI-H292/ECM barrier) was significantly inhibited by the addition of P290 to Mip-positive bacteria. Remarkably, this treatment of wild-type *L. pneumophila* with P290 resulted in transmigration rates comparable to Mip deficient bacteria or wild-type bacteria treated with the PPIase inhibitor rapamycin. Taken together, this data demonstrated that the collagen IV-derived peptide P290 specifically binds Mip, competitively interferes with the binding of Mip to collagen IV and inhibits *L. pneumophila* transmigration across an *in vitro* lung epithelial barrier model (Ünal et al. 2011).

6.5 NMR Spectroscopy-Assisted Modelling of the Mip^{77-213}/P290 Complex and Development of Improved P290 Variants

To attain insight into the solution structure of the Mip/P290 complex, and to better understand details regarding interactions within this complex, NMR and docking were used (Fig. 6.1). A similar approach for Mip alone, or in complex with its inhibitor rapamycin, had previously given a detailed picture of molecular dynamics and interactions (Ceymann et al. 2008). In the solution structure of the Mip/rapamycin complex, amino acid residues D142 and Y185 were shown to form hydrogen bonds to rapamycin, stabilizing the complex. Since binding of rapamycin efficiently inhibits PPIase activity, the hydrophobic cavity of the protein most certainly is the active site of the enzyme. The NMR experiment identified D142 as part of the interaction surface of Mip and P290. In addition Y185 is influenced by the presence of P290, but not to the same extent. In the docking structure of Mip^{77-213} and P290, both residues form hydrogen bonds to P290 (Y185(Mip) – P129(P290) and D142(Mip) – S135(P290)). By utilizing structural data of human PPIase FKBP12, the most closely related human orthologue of Mip, it was

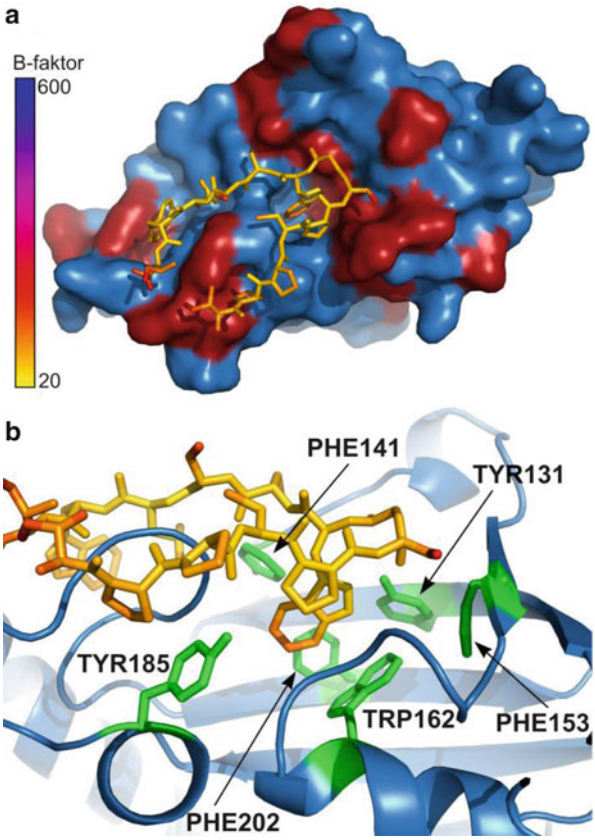

Fig. 6.1 Average structural model of the $^{Mip77-213}$/P290 complex. (**a**) Mip is shown as *blue* surface, residues that were classified as being part of the interaction surface are coloured in *red*. P290 is shown as stick model coloured according to the calculated B-factors. (**b**) Enlarged cutaway view of the Mip binding pocket. P290 is shown as *yellow* sticks. Mip is shown in *blue* cartoon representation and residues forming the hydrophobic cavity are highlighted in *green*

possible to optimize the inhibitory characteristics of P290. The similarity between Mip and FKBP12 is less distinct outside the binding pocket and therefore the terminal residues of P290 cannot attach equally well to the surface of FKBP12. Accordingly, the overall FKBP12/P290 complex is less stable. A peptide microarray containing P290 variants with single amino acid substitutions revealed variants, which exhibit ameliorated binding to Mip. Furthermore, cyclization of P290 without amino acid substitutions resulted in an improved Mip binding. The use of D-amino acids for the synthesis of protease-resistant P290 showed that Mip is able to distinguish between L- and D-peptide forms of P290, since D-P290 lost its capacity to bind Mip (Ünal et al. 2011).

To determine if changes in the primary and secondary structure of P290 predicted by B-factor calculations and protein microchip experiments improve the inhibitory effects on bacterial transmigration in the transwell system, two of the most promising candidates (cP290 and cP290_1) and P290 at a concentration of 15 μM were compared, respectively. In the chemically synthesized cyclic variant cP290 (DPPCPSGWSSLW-Dpa), the terminal Ile-residues were replaced by Asp and diaminopimelic acid (Dpa) residues, respectively. In addition to cyclization,

cP290_1 (DPPC**L**SG**F**SSLW-Dpa) contains the amino acid substitutions P131L and W134F. Both peptides improved inhibition of bacterial transmigration as compared to P290 and represent interesting lead structures for anti-infective drug developments (Ünal et al. 2011).

6.6 Conclusions

The collagen binding protein Mip of *L. pneumophila* contributes to the intracellular infection of phagocytic cells and to the bacterial dissemination within infected lungs of guinea pigs. Considering that both processes are critical for *Legionella* pathogenicity, Mip may serve as an interesting anti-infective drug target. The high homology between Mip and human PPIases like FKBP12 contraindicates the use of FK506 and rapamycin during *L. pneumophila* infection since these PPIase inhibitors are immunosuppressants. However, variants of the collagen IV derived peptide P290 could represent promising candidates for competitive inhibition of Mip-collagen IV binding. As future direction, and to better understand how Mip and degradative enzymes interact and finally perforate tissue barriers, four hypotheses should be tested. Firstly, Mip binds to collagen IV and contributes to adhesion and intimate interaction of *L. pneumophila* within the lung tissue. Secondly, Mip activates degradative enzymes of *L. pneumophila* or the host tissue. Thirdly, the PPIase activity of Mip interconverts proteolytically insensitive *cis* prolyl bonds of host proteins into their proteolytically sensitive *trans* prolyl bond congeners. This could increase host protein degradation (e.g. of the basal lamina) by enzymes of *L. pneumophila* or the host tissue. Fourthly, Mip influences the bacterial secretion process towards a more degradative phenotype. Very recent results revealed that Mip significantly influences the secretome of *L. pneumophila* (Steinert et al. unpublished).

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Chapter 7
Evolution of Bacterial Chaperonin 60 Paralogues and Moonlighting Activity

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Abstract Around two thirds of genome sequenced bacteria encode one chaperonin 60 protein with the other third encoding between two and eight chaperonin 60 paralogues. A surprising finding is that these bacterial proteins have a wide, and growing, range of additional functions both within the bacterium, but principally when the Cpn60 protein exits the cell and exists on the bacterial cell wall or in the bacterium's external milieu. These findings have occurred at the same time that it has been realised that bacterial Cpn60 proteins can assume lower oligomeric forms than that of the prototypic tetradecameric *E. coli* GroEL. It is possible that lower oligomeric forms of Cpn60 may more readily be secreted and interact with biopolymers in a distinct manner to that of the tetradecameric homologues and paralogues. How the Cpn60 moonlighting functions evolved is a key question to be addressed. To address this question we postulate that the chaperonin genes have been subject to different selective constraints over evolutionary time. Gene duplication, followed by sequence divergence, resulted in the evolution of paralogous Cpn60 proteins that have distinct moonlighting activities. Moreover, these functional variations might be acquired by incorporating chemically dissimilar substitutions at functionally important residues.

7.1 Introduction

The central dogma of molecular biology outlines three steps in the transfer of information present in the genetic material to the synthesis a polypeptide chain. These polypeptides perform a predestined function. However, for the proteins to be functional, they need to be folded in an appropriate conformation. Cells have

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evolved a family of proteins, aptly known as molecular chaperones, to assist the folding of other proteins. The chaperone proteins are ubiquitous, highly conserved and have been demonstrated to bind naïve and unfolded substrate proteins via exposed hydrophobic patches, which otherwise are buried in the native folded conformation (Hartl 1996; Hartl and Hayer-Hartl 2003). Molecular chaperones were initially identified as a group of proteins that are abundantly expressed during heat stress. Therefore, these proteins are also known as Heat Shock Proteins (HSPs) and consequently classified according to their molecular masses such as Hsp100, Hsp90, Hsp70, Hsp60, small Hsp families. See Chaps. 1 and 2 for more detailed discussions of the molecular chaperones of prokaryotes and eukaryotes.

One of the best characterized families of HSPs is the Hsp60 family, which is commonly referred to as the chaperonin (GroEL or Cpn60) family. Chaperonins form a characteristic cylindrical assembly whereby they assist misfolded or unfolded substrate proteins to reach their native state upon encapsulation in the cylindrical cavity in ATP dependent cycles of binding and release (Hartl and Martin 1995). Chaperonins are classified into two classes based on their systematic occurrence, cellular localization and co-chaperonin dependence. The group I class includes members from the cytosol of prokaryotes and endo-symbiotically related membrane bound eukaryotic organelles such as mitochondria and chloroplasts. These chaperones require assistance of the co-chaperonin, Hsp10 as a lid for encapsulating the substrates. On the other hand, group II chaperonins include members from the eukaryotic cytosol and from archaea. These chaperonins possess a protrusion in their apical domain, which acts like a built-in lid for substrate encapsulation and therefore are independent of the co-chaperonin (Fig. 7.1). Examples of group I chaperonins include GroEL from *Escherichia coli* and several eubacteria (Horwich et al. 2007), while CCT chaperonins and the well-studied thermosome from the archaeal branch of life are members of group II chaperonins. Whereas the group I chaperonins form homo-tetradecameric complexes, some members of the group II family form hetero oligomers (Fig. 7.1a). Moreover, the two groups differ in their subunit movements; while the subunit movement in group I chaperonins is sequential, for group II it is concerted. This review focuses on the structure, function and evolutionary aspects of the group I chaperonins.

7.2 Structure and Function of the Chaperonins

Understanding of the biology of chaperonin function is dominated by studies on *E. coli* GroEL and its co-chaperonin GroES. Since the discovery of chaperonin function, genetic, biochemical and structural studies on *E. coli* GroEL-GroES have led to systematic knowledge on various aspects of its function (Hartl 1996; Hartl and Hayer-Hartl 2003; Hartl and Martin 1995; Horwich et al. 2007). From these studies GroEL-GroES have emerged as the panacea in preventing all the undesirable consequences of intracellular protein misfolding.

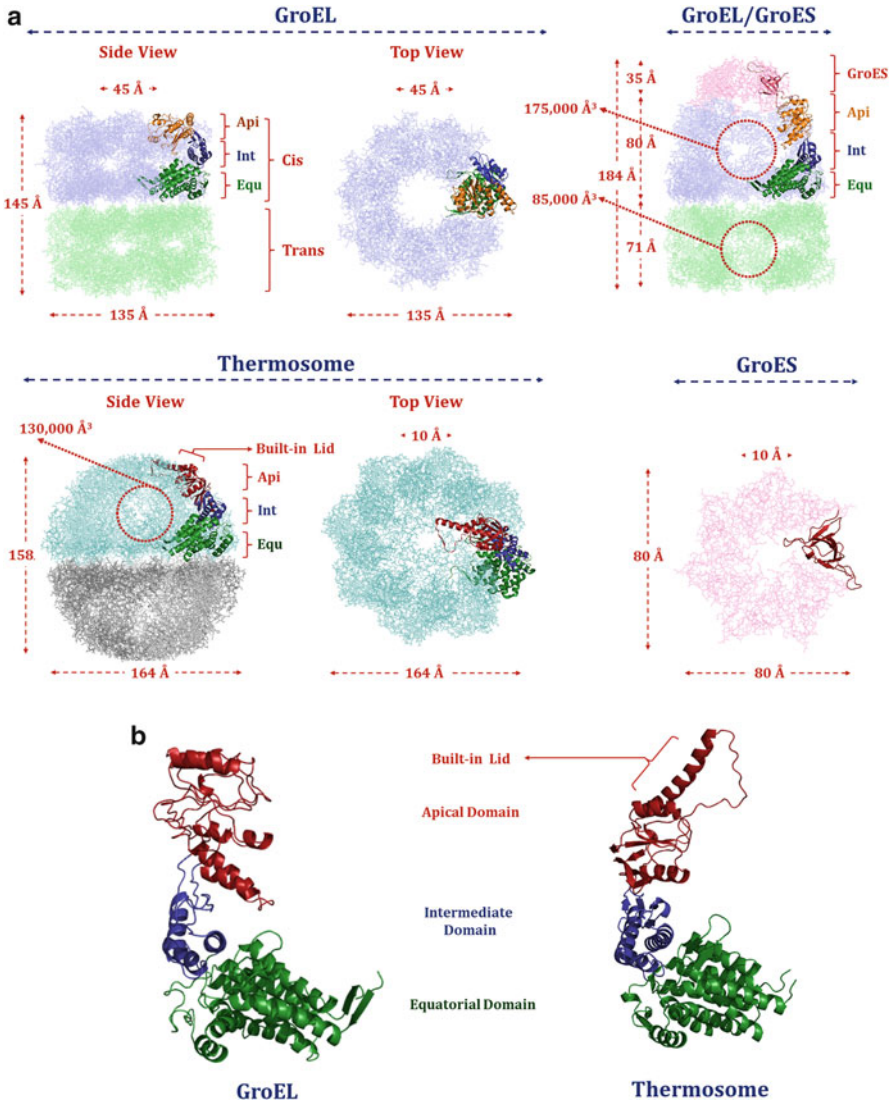

Fig. 7.1 Architecture of group I and II chaperonins. (**a**) Crystallographic models of *E. coli* GroEL, GroES and GroEL-GroES representing the Group I chaperonins and the thermosome from *T. acidophilum*, representing the Group II chaperonins, are presented. Individual domains in one subunit of GroEL and thermosome are indicated. *Api* apical domain, *Int* intermediate domain, *Equ* equatorial domain. GroES acting as a lid binds to GroEL asymmetrically, at the cis GroEL ring, wherein the general substrate polypeptides are encapsulated. Other open ring is termed the trans ring. Thermosome forms a symmetric complex, showing a "closed" cavity. Illustrations are generated using Pymol 1.3 (DeLano Scientific LLC, USA). Co-ordinates for the molecules GroEL (1OEL), GroEL-GroES and GroES (1AON) and thermosome (1A6E) were obtained from PDB. (**b**) Crystallographic models of GroEL and thermosome monomers as indicated depicting colour coded individual domains. The built-in lid in thermosome is shown that replaces GroES as cap

Structurally, GroEL possesses a three-domain architecture. The central region of the polypeptide, spanning amino acid residues 191–376, constitutes the apical domain. This domain is rich in hydrophobic residues and thus binds substrates and GroES. The equatorial domain spans two extremities of the GroEL polypeptide, that is, residues 1–133 and 409–523. This domain is responsible for the ATPase activity and bulk of the inter-subunit and inter-ring interactions. The hinge forming intermediate domain spans two regions on the polypeptide namely, residues 134–190 and 377–408, and transmits signals between the equatorial domain to the apical domain owing to nucleotide and substrate binding, respectively (Hayer-Hartl et al. 1995; Kumar et al. 2009; Mayhew et al. 1996; Xu et al. 1997) (Fig. 7.1b).

GroEL's chaperoning ability is dictated by the formation of a tetradecamer constituting two isologous heptameric rings, each enclosing a cavity for substrate proteins to bind (Fig. 7.1 see also Chap. 2). The substrate-bound *cis* cavity is expanded upon capping by GroES and thus forms a sequestered environment of ~175,000 $Å^3$ to enable the substrate polypeptides to fold. The *trans* cavity, on the other hand, remains constrained with a capacity of ~85,000 $Å^3$ (Fig. 7.1). The volume of the cavity in group II chaperonins is ~130,000 $Å^3$, which is significantly smaller than the *cis* cavity of GroEL but larger than the *trans* cavity (Lars et al. 1998). GroEL's wide substrate repertoire constitutes about 10–15 % of the cellular proteins in *E. coli*. Thus, the major intracellular function of GroEL is understood to be as an essential folding machine (Horwich et al. 2007).

7.3 Multiple Copies of GroELs/Chaperonin 60 in Bacteria

The ubiquitous occurrence of GroEL, or more correctly chaperonin (Cpn)60 (as GroEL is the Cpn60 protein of *E. coli*), across species might be explained by its essential role in the protein folding process. High architectural conservation among Cpn60s from different species indicates that the mechanism of GroEL is universally conserved. Moreover, several Cpn60 homologues from other bacteria have been shown to function in *E. coli*, suggesting that the substrate spectrum of the respective Cpn60s must overlap considerably (Goyal et al. 2006; Lund 2009). Since Cpn60 needs to interact with a wide range of substrate proteins, sequence analysis of the substrate-binding apical domain has revealed significant plasticity in its sequence and structure (Dekker et al. 2000; Goyal et al. 2006) (Fig. 7.3). On the other hand, the equatorial domain required for oligomerization through inter-subunit interactions exhibits higher conservation.

The availability of complete genome sequences of various bacteria has revealed the presence of multiple copies of *groEL* genes; with one (or more) of the multiple genes in operonic arrangement with the cognate co-chaperonin, *groES*. Examples of bacteria hosting multiple *groEL* genes include actinobacteria, α-proteobacteria, cyanobacteria and chlamydiae (Lund 2009). The discovery of multiple copies of Cpn60s poses interesting questions about the function of these extra copies. First,

whether all the copies of Cpn60 retain their function as protein chaperones? Second, do they distribute the substrate pool either temporally or based on factors such as function and/or composition of the substrates? Such a distribution has been demonstrated in the case of the rhizobial Cpn60s, wherein different Cpn60 copies play different roles in nitrogen fixation, probably by encountering different substrates (see Lund 2009). Third, do the extra copies of chaperonins acquire additional functions, owing to their substrate promiscuity?

Multiple chaperonin genes in different bacteria and subcellular organelles might have arisen through either gene duplication in different lineages or by horizontal gene transfer (Goyal et al. 2006; Lund 2009). Invariably such evolutionary events lead to functional divergence, and it is interesting to address if paralogous Cpn60s too have acquired new functions. It is well known that apart from the role as a protein-folding chaperone, several Cpn60 proteins exhibit non-chaperonin activities, such as binding to other biopolymers, eliciting immune responses and functioning as insecticides as explained in the following sections (Joshi et al. 2008) and in other chapters of this volume. The presence of multiple copies of chaperonins in different bacterial lineages are therefore believed to have introduced new functional roles for the different copies of the chaperonins (Piatigorsky and Vistow 1989). Exerting multiple roles by a single protein is known as moonlighting and has been widely observed in Nature and thus understanding the molecular basis of moonlighting is important to appreciate its implications for the function of Cpn60 proteins.

7.4 Moonlighting in Proteins

Moonlighting, initially known as gene-sharing (Piatigorsky and Vistow 1989), is defined as the ability of a single polypeptide to perform multiple unrelated functions (Jeffery 2004a). The definition, however, excludes the polypeptides that have resulted from gene-fusion, alternative splicing or inherently promiscuous enzymes. One of the earliest moonlighting functions was discovered by identification of carbohydrate metabolic functions in certain vertebrate eye lens proteins (Piatigorsky and Vistow 1989). Since then, it is estimated that several hundred moonlighting proteins have been identified with around two dozen of these proteins performing multiple unrelated biological functions. Accumulation of the number of moonlighting proteins identified has added another dimension to the complexity of cellular networks. Although, moonlighting in eukaryotic proteins is well documented, evidences for the prokaryotic proteins have only been described recently (Muro-Pastor et al. 1997; Ostrovsky de Spicer and Maloy 1993; Walden et al. 2006). Different mechanisms have been proposed for moonlighting, including secretion into extracellular space, interactions with nucleic acids, changes in physico-chemical parameters such as temperature or redox condition of the cell, changes in oligomeric status or changes in the cellular concentration of ligands, substrates, co-factors or products (Jeffery 2009; Kumar and Mande 2011).

The evolution of moonlighting might be a result of two fundamental cellular phenomena. One, moonlighting is typically exhibited by proteins which occur ubiquitously (Jeffery 1999). For example, many of the enzymes involved in carbohydrate metabolism are ubiquitous and hence during evolution an extra function might have been incorporated into these proteins. Examples include several glycolytic enzymes (Fig. 7.2a). Two, moonlighting has been proposed for the proteins that are constitutively overproduced in the cell, such as crystallins (Piatigorsky 1998; Jeffery 2004b) (Fig. 7.2b). Chaperonin 60 turns out to be a good candidate for exhibiting moonlighting activity since it fits both of these features well: high sequence conservation and elevated cellular expression levels.

In addition, two models of the evolution of moonlighting activity have been proposed which are based on the fundamental structural aspects of the proteins: (i) the **allostery model** and (ii) the **adaptability model**. The allostery model arises out of the common observation in proteins, namely the larger-than-required size of many of enzymes. Since the majority of proteins exhibit a larger structure than is necessary for performing their specific function, the apparent unused large surface areas exposed on proteins might have evolved new pockets and active sites for performing novel function (Jeffery 1999). Examples for the allosteric model include the glycolytic enzymes (Huberts and van der Klei 2010; Jeffery 2004b). On the other hand, the adaptability model is based on the location of a protein in different cell types. Proteins expressed in different cell types might exhibit different activities owing to the local necessity and the binding partners encountered Piatigorsky 1998). The examples for this model include the classical lens proteins and several molecular chaperones such as Hsp60, Hsp70 etc. Therefore, one protein performing several functions would be advantageous for the cell in terms of energy conservation (Jeffery 2004b). We discuss here two examples of moonlighting proteins: crystallins and phosphoglucoisomerase (PGI). Complete discussion on the biology of moonlighting with more examples is provided in Chap. 3, written by Connie Jeffery.

7.4.1 Crystallins

Crystallins were the first proteins to be discovered to show moonlighting function (Piatigorsky and Vistow 1989). Crystallins are the principal structural proteins in the lens of the vertebrate eye, constituting about 90 % of the lens protein content. Crystallins are principally represented by α, β and γ variants, while other variants assist assembly of the principal crystallins in certain vertebrates. However, certain crystallins also have been demonstrated to display additional functions in other locations of the body. These examples include the δ-crystallin from ducks, which is the enzyme, argininosuccinate lyase, while the ε and η-crystallins have been shown to exhibit lactate dehydrogenase activity (Bateman et al. 2003; Hendriks et al. 1988) (Fig. 7.2b). Moreover, turtle τ-crystallin shows α-enolase activity (Wistow et al. 1998). Due to their high concentrations in the eye lens, however, such

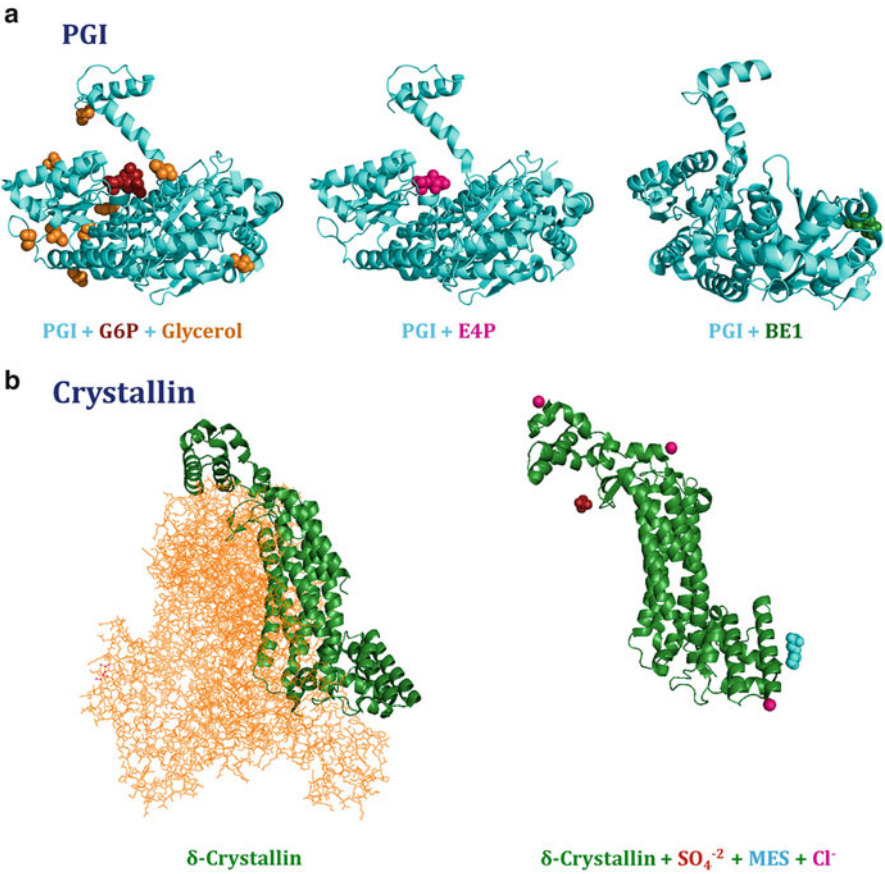

Fig. 7.2 Examples of moonlighting. (**a**) Phosphoglucoisomerase (*PGI*) is glycolytic enzyme involved in the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate. PGI assumes several extracellular cytokine functions. Presented is PGI in complex with its natural substrate, glucose-6-phosphate (1U0F), the cytokine activity inhibitor, erythrose-4-phosphate isocitrate (1IRI) and n-bromoacetyl-aminoethyl phosphate (1C7Q). The ligands glucose-6-phosphate (G6P), glycerol, inhibitors Erythrose-4-phosphate isocitrate (E4P) and n-bromoacetyl-aminoethyl phosphate (BE1) are presented as space filled in red/blue, gold, pink and green, respectively. (**b**) Moonlighting activity of crystallins. Cartoon representations of duck δ-crystalline monomers are presented in the apo (1U15) and holo (1U16) conformations. Apo form exists as a tetramer and is involved in the lens function, while the holo form is involved in the argino succinate lyase activity. Ligands 2-(N-morpholino)-ethanesulfonic acid (MES), chloride ions and sulphate ion are presented as space filled in cyan, pink and maroon, respectively

metabolic role is unlikely and thus they might have only the structural function in the lens (Wistow and Piatigorsky 1988). Therefore, the crystallins expressed in different cell types might have evolved additional functions in accordance with the cell type requirement.

7.4.2 *Phosphoglucoisomerase*

Phosphoglucoisomerase (PGI) is a glycolytic enzyme which catalyses the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate (Read et al. 2001). Moonlighting activity in PGI has been widely documented (Cao et al. 2000; Chaput et al. 1988; Gurney et al. 1986; Hansen et al. 2005; Schulz and Bahr 2003; Tanaka et al. 2002; Watanabe et al. 1996; Xu et al. 1996). Apart from its original glycolytic function in the cytosol, PGI has been demonstrated to perform several unrelated cytokine-like extracellular functions: (a) When secreted from T-cells, PGI promotes the survival of nerve cells, and thus is known as a neuroleukin (Chaput et al. 1988; Gurney et al. 1986); (b) as the well-known autocrine motility factor (AMF), which promotes cell migrations, and is believed to be involved in cancer metastasis (Watanabe et al. 1996; Tanaka et al. 2002) (Fig. 7.2a); (c) as a maturation factor (MF), promotes myeloid cell differentiation and may play some role in leukaemia (Xu et al. 1996); (d) as a serine protease inhibitor when bound to myofibrils (Cao et al. 2000) and; (e) as an implantation factor activity in the ferret (Schulz and Bahr 2003, 2004). PGI, therefore, appears to have acquired several diverse biological functions depending on its location in different cell types.

With the examples such as those described above, proteins are believed to evolve moonlighting functions either due to the development of additional binding sites, other than their original active site (allostery model), or alteration of functions based on the requirements of the expressing cell type (adaptability model).

7.5 Moonlighting in Chaperonin 60 Proteins

Chaperonin 60, as described above, is a 900 kD cylindrical assembly of 14 monomers in two rings, which encloses cavities for the non-native protein substrates to bind (Fig. 7.1). The non-specific binding is a consequence of recognition of substrates by the hydrophobic surfaces of Cpn60 presented by the apical domains. Therefore Cpn60 has been attributed the protein folding function of the bacterial cytosol and intracellular organelles. However, recent discoveries on the Cpn60 molecules from other bacteria and eukaryotic organelle have been expanding the substrate repertoire and imparting new functions to Cpn60 (Basu et al. 2009; Kumar et al. 2009; Kumar and Mande 2011). Yeast mitochondrial Hsp60 has been shown to act as a protein chaperone *in vitro* and *in vivo*. However, this same chaperone has been demonstrated to bind single stranded DNA *in vitro* and play a role in stability and transmission of nucleoids *in vivo* (Kaufman et al. 2003). Moreover, Cpn60 homologues from several insect symbionts such as *Enterobacter aerogenes* (Yoshida et al. 2001) and *Xenorhabdus nematophila* (Joshi et al. 2008) have been shown to exhibit insect toxicity. The toxicity of Cpn60 from *X. nematophila* has been demonstrated to be alleviated upon its interaction with α-chitin. Mutational analysis, followed by biochemical characterizations of these Cpn60s showed that the amino-acid residues

critical for toxicity are distinct from those essential for chaperone activity, suggesting that the two functions are independently operated. Furthermore, several pathogen-borne Cpn60s, such as the surface-associated Cpn60 from the proteobacterial pathogen, *Legionella pneumophila* (Garduño et al. 1998 – see Chap. 9) and several mollicute pathogens (Clark and Tillier 2010), have been implicated in host cell invasion, owing to their association with the cell surface. A more detailed analysis of the Cpn60 of *L. pneumophila* is given in Chap. 9. Moreover, Cpn60 from pathogenic *E. coli* (Reddi et al. 1998) and *from Aggregatibacter actinomycetemcomitans* (Kirby et al. 1995) have been implicated in bone resorption. Taken together, these observations suggest that Cpn60s from different organisms have evolved moonlighting functions, probably to support the organism either at different stages of growth or for the purposes of virulence.

The substrate repertoire of Cpn60 has been found to be expanded from polypeptides to various biopolymers. Since binding to different biopolymers is independent of each other, and that Cpn60 might encounter different macromolecules in the cell, this protein might have evolved the capability to distinguish between the protein and non-protein substrates. The ability to differentiate between polypeptides and other biopolymers might be conferred upon Cpn60 by its specific conformational features such as the hydrophobic surfaces of the apical and equatorial domains. Moreover, employing mass spectrometry and NMR coupled with hydrogen-exchange techniques, it has been shown that *E. coli* GroEL is inefficient in binding to extended polypeptides but is able to bind the collapsed, molten globule-like folding intermediates of the substrates effectively (Gervasoni et al. 1996; Goldberg et al. 1997; Robinson et al. 1995; Zahn et al. 1994). Furthermore, Cpn60's preference to interact with α/β proteins without any sequence similarity suggests that the discrimination among the substrates by this protein might be by the formation of central cavities enclosed in the heptameric rings and thus a lower oligomeric form might not differentiate different biopolymers (Kumar and Mande 2011). The moonlighting Cpn60s from different prokaryotic species might indeed exhibit different oligomeric species and thereby be involved in distinct biochemical functions, as observed in *Helicobacter pylori*, *Mycobacterium tuberculosis* and several intracellular pathogenic bacteria (Basu et al. 2009; Kumar et al. 2009; Lin et al. 2009). The moonlighting functions of Cpn60 homologuess might arise due to subtle alterations in substrate specificity as a consequence of differences in the oligomeric states of these proteins. Such an ability to differentiate between polypeptides and non-protein substrates by modulating their oligomeric properties appears promising to us from our recent studies on the paralogous Cpn60s of *M. tuberculosis* (Basu et al. 2009; Kumar et al. 2009; Qamra and Mande 2004; Qamra et al. 2004).

7.5.1 Moonlighting by M. tuberculosis *Cpn60 Proteins*

Mycobacterium tuberculosis, the causative pathogen of tuberculosis, encodes two chaperonin homologs; GroEL1 (Cpn60.1 or Hsp60) and GroEL2 (Cpn60.2 or

Hsp65). Sequence identity between the two GroELs is about 60 % (Kong et al. 1993). GroEL2, the first to be discovered (Henderson and Martin 2011), is essential (Stewart et al. 2002) and has been implicated in inducing host cytokine responses (Friedland et al. 1993; Lewthwaite et al. 2001). On the other hand, Cpn60.1 is dispensable (Stewart et al. 2002). Deletion of the *cpn60.1* gene in mycobacteria shows similar growth patterns as the wild type, *in vitro* and *in vivo* (Hu et al. 2008; Ojha et al. 2005). A Cpn60.1 deletion mutant in *Mycobacterium smegmatis* is deficient in formation of biofilms (Ojha et al. 2005). In contrast, deletion of the same gene in *M. tuberculosis* fails to have much effect on biofilm formation but the mutant fails to be able to induce granuloma formation in mice or guinea pigs (Hu et al. 2008). In *M. smegmatis*, biofilm formation is implicated for its chaperone function and thus requires the interaction of Cpn60.1 with KasA, an enzyme involved in membrane lipid metabolism (Ojha et al. 2005). Moreover, both mycobacterial Cpn60 proteins are secreted and consequently are involved in eliciting several host cell immune responses via macrophage and monocyte stimulation (Friedland et al. 1993; Khan et al. 2008; Lewthwaite et al. 2001; Riffo-Vasquez et al. 2012). For more information on the extracellular roles of *M. tuberculosis* Cpn60.2 the reader should consult Chap. 8 by Richard Stokes.

Biochemical and biophysical studies showed that the recombinant *M. tuberculosis* Cpn60s exist as dimers unlike *E. coli* GroEL and consequently, are ineffective as chaperones (Kumar et al. 2009; Qamra and Mande 2004; Qamra et al. 2004). Moreover, recombinant Cpn60.1, the dimeric form, was demonstrated to co-localize with the nucleoids isolated from *M. tuberculosis* extracts (Basu et al. 2009). In addition, structural studies on the apical domains of *E. coli* GroEL (Xu et al. 1997), *M. tuberculosis* GroEL1 (Sielaff et al. 2011) and GroEL2 (Qamra and Mande 2004) showed identical substrate binding cleft (Fig. 7.3). Furthermore, using peptide arrays, GroEL1 has been shown to bind polypeptides derived from GroEL1's native substrate, KasA, indicating that GroEL1 is a *bona fide* chaperonin (Ojha et al. 2005).

Chaperonin 60.1 has been demonstrated to exist in different oligomeric forms, as a dimer, heptamer and tetradecamer and the conversion between the heptamer and the tetradecamer is mediated by a phosphorylation switch (Kumar et al. 2009). Considering that the tetradecameric form of Cpn60.1 might be an active chaperonin, it has been proposed that the phosphorylation event might act as an energy (ATP pool) conservation mechanism in slow growing *M. tuberculosis* (Kumar et al. 2009; Kumar and Mande 2011). Furthermore, such multiple oligomeric forms of chaperonins were observed in the chloroplast (Dickson et al. 2000) and mitochondrial chaperonins (Levy-Rimler et al. 2001), wherein they existed in the monomeric, single ring heptameric and double ring tetradecameric forms and the conversion from single ring to the double ring form is concentration and GroES dependent. Moreover, as described earlier, yeast mitochondrial Cpn60 proteins were demonstrated to associate with the stability and transmission of the nucleoid DNA (Kaufman et al. 2003). Taken together, these observations tend to suggest that the Cpn60 protein might switch between different functional forms by modulation of its oligomeric status.

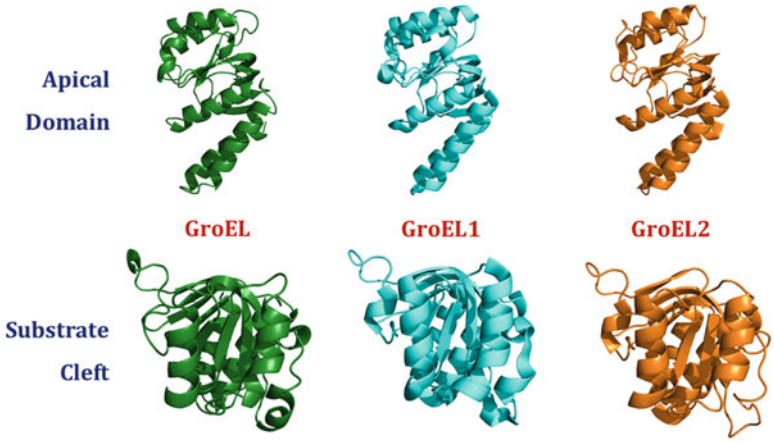

Fig. 7.3 Conserved substrate binding clefts in GroELs. Apical domains and the substrate binding clefts of the indicated GroEL homologues are presented. Co-ordinates for *E. coli* GroEL (1AON), *M. tuberculosis* GroEL1 (3M6C) and GroEL2 (1SJP) were obtained from PDB

7.5.2 *Functional Dichotomy in* M. tuberculosis *Cpn60 Proteins*

Structural features which confer substrate dichotomy on Cpn60 proteins would be interesting to study. The equatorial domain in GroEL is responsible for its oligomerization (Xu et al. 1997). While the equatorial domain is principally buried in the tetradecameric form, it gets increasingly exposed in the lower oligomeric forms. However, the substrate interacting face of the apical domain remains exposed independent of the oligomeric status. Therefore, it might be possible that different functions of *M. tuberculosis* Cpn60.1 arise out of different oligomeric forms; dimer interacting with DNA via its nucleotide binding equatorial domain and the tetradecamer being involved in the chaperone function. Chaperonin 60.1 therefore presents a unique way of moonlighting – distributing its distinct functions into different oligomeric forms. The evolutionary significance of the functional divergence is therefore essential to understand the basis such a behaviour.

7.6 The Evolution of the Chaperonin 60 Protein

As stated before, the chaperonins are distributed into two different groups based on their phylogenetic distribution. While the group I belongs to prokaryotes and eukaryotic organelles, the group II belongs to archaea and eukaryotes. Although the evolution of group II chaperonins from eukaryotes (Archibald et al. 2000, 2001) and archaea (Archibald et al. 1999; Archibald and Roger 2002) is well documented, that

of group I chaperonins is still in its infancy (Dekker et al. 2011; Goyal et al. 2006; Hughes 1993; Levy-Rimler et al. 2001; Techtmann and Robb 2010). We have attempted to address evolutionary aspects of GroELs in our laboratory based on sequence analysis, functional studies and biochemical experiments (Goyal et al. 2006; Kumar et al. 2009; Kumar and Mande 2011).

Two experimental studies on the directed evolution of GroEL have delineated the functional significance of individual domains of GroEL. In one study, employing random mutagenesis to derive GFP-folding GroEL variants resulted in mutants with diminished ability to recognize its natural substrates and, consequently deficient in functioning as a general chaperone (Wang et al. 2002). In another study based on random mutagenesis of GroELs, apical domain has been shown to be capable of absorbing large insertions or deletions, unlike the highly conserved equatorial domain (Kumar et al. 2009). Therefore, GroEL, even as it encounters a wide range of substrates by maintaining plasticity in the apical domain, it requires conserved architecture anchored by the equatorial domain to be functional as a chaperone.

Conserved residues among group I chaperonins map onto ATP binding pseudo-Walker motif and the substrate binding sites on *E. coli* GroEL structure (Brocchieri and Karlin 2000). Highly conserved charge clusters line the central cavity and the intra subunit interfaces, and thus are presumed to play a role in interacting with the substrate and constituting the quaternary structure. Interestingly, the less conserved segments were mapped outside wall of the cylinder (Brocchieri and Karlin 2000). Furthermore, the residues mapped to the functionally important regions on GroEL have been predicted to be selected by positive selection (Fares et al. 2002a, 2005).

Phylogenetic studies on the two groups of chaperonins predicted the divergence of the two groups at a Last Universal Common Ancestor (LUCA), which further diverged in terms of type and number of subunits in a ring and requirement of the co-chaperonin (Dekker et al. 2011). Co-occurrence of paralogous chlorophyll and nucleomorph chaperonins in the same eukaryotic cells has been implicated in the divergent evolution of the organellar chaperonins (Wast et al. 1999). Similarly, several mycobacterial species host multiple paralogues of GroEL (Kong et al. 1993; Qamra and Mande 2004). Phylogenetic studies on chaperonins predicted single gene duplication event in the common ancestor of *M. tuberculosis*, *M. leprae*, and *Streptomyces albus* that might have duplicated the chaperonin genes. In addition to evolving novel functions, Cpn60 has also been implicated to play role in evolution of several proteins by buffering mutations and elevated temperatures (Fares et al. 2002b; Rudolph et al. 2010).

To understand the basis for the divergence of multiple copies of chaperonins in bacteria we have performed divergence analysis on GroEL homologues from completely sequenced bacterial genomes (Table 7.1). Homologues of *M. tuberculosis* Cpn60.1 were identified using BLAST against 1,129 bacterial genomes (Cummings et al. 2002), aligned using ClustalW and an unrooted phylogenetic tree was generated from the 1859 Cpn60 homologues using MEGA 5.0 (Tamura et al. 2011). The tree depicts Cpn60 proteins from bacteria belonging to eight phyla, wherein multiple copies of Cpn60 proteins are identified for actinobacteria, cyanobacteria and chlamydia.

Table 7.1 Number and distribution of *M. tuberculosis* GroEL1 homologues in Bacteria

Phylum	Number of completely sequenced genomes	Number of sequences with expect value less than e^{-10}
Actinobacteria	138	326
Bacteriodetes/Chlorobi	81	98
Chlamydiae	12	83
Cyanobacteria	28	62
Firmicutes	212	415
Mollicutes	32	10
Proteobacteria	500	1,011
Spirochaetales	35	36
Total number of sequences with Expect Value less than e^{-10}		2,041

***Phylum Proteobacteria*:** The phylum proteobacteria is divided into five classes: α-, β-, γ-, δ- and ε- proteobacteria. Three of the five classes of proteobacteria; γ-, δ- and ε- proteobacteria show the presence of a single copy of the *groEL* gene, while α- and β-proteobacteria possess two or more copies of this gene. Interestingly, the conserved C terminal $(GGM)_4M$ repeat sequence has been observed in all the Cpn60 proteins in this phylum (Farr et al. 2007; Suzuki et al. 2008; Tang et al. 2006). Examples for this phylum include rhizobiaceae family, members of which have GroEL copies ranging from one to seven (Lund 2009). Interestingly, *Bradyrhizobium japonicum* hosts five *groESL* operons. Among these, *groESL2*, *groESL4* are constitutive and the other stress induced (Fischer et al. 1993). Moreover, *Buchnera aphidicola*, a member of γ-proteobacteria, hosts a single copy of *groEL* in operonic arrangement with *groES*, typically overexpressed at elevated temperatures (Baumann et al. 1996) (85).

***Phylum Actinobacteria*:** Actinobacteria clade represents another Gram positive bacterial phylum hosting multiple copies of *groEL* gene (Goyal et al. 2006). The comparison of the protein sequences across different lineages showed that the duplicated copies of chaperonin 60s, Cpn60.1 and Cpn60.2, are distributed to different phylogenetic branches (Fig. 7.4), suggesting that the duplication event might have occurred in the common ancestor of actinobacteria. Interestingly, unlike the rhizobiaceae family, only one of the actinobacterial *groEL* genes is in operonic arrangement with *groES* (Goyal et al. 2006). The absence of a *cpn10* copy with the other *cpn60*(s) could be either due to a duplication (or multiplication) of *cpn60* alone or loss of a *cpn10* copy after duplication of the operon. For example, *M. smegmatis* hosts three copies of *cpn60*, namely *cpn60.1*, *cpn60.*2 and *cpn60.3*, but only a single copy of *cpn10* that is associated with *cpn60.1* (Ojha et al. 2005; Rao and Lund 2010). *M. smegmatis* Cpn60.2 exhibits greater sequence identity (93 %) with *M. tuberculosis* Cpn60.2 than with other copies. Some of the actinobacteria host single *cpn60* genes, such as in *Bifidobacterium longum* and *Tropheryma whipplei*, which is homologus to the actinobacterial Cpn60.2 and the gene is located away from *cpn10* on the chromosome. Moreover, *Gordonia bronchialis DSM 43247* hosts three Cpn60 copies, which are distributed to three different clades of phylogenetic tree. *G. bronchialis* Cpn60.1

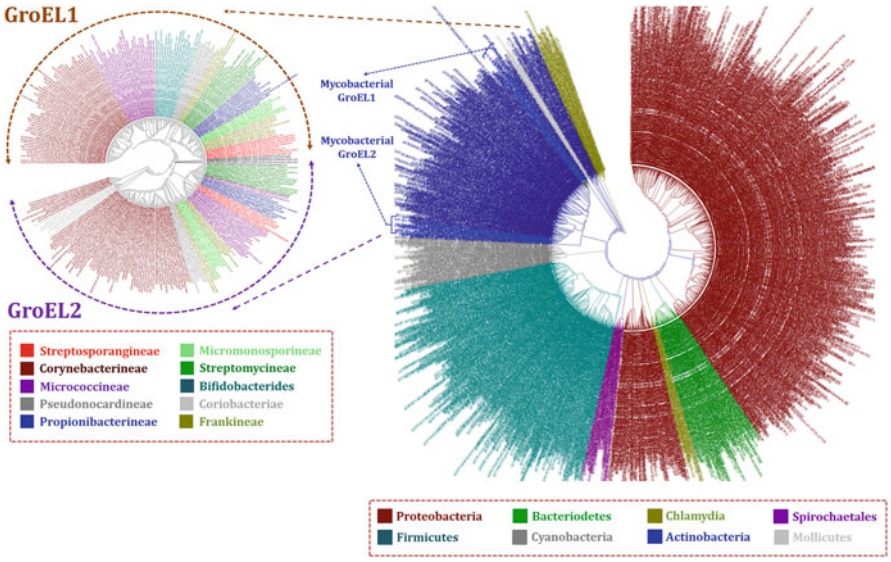

Fig. 7.4 Unrooted phylogenetic tree of bacterial chaperonins. A phylogenetic tree was generated using the 1,129 complete bacterial genomes. Protein sequences that are homologus to *M. tuberculosis* GroEL1 were aligned using the ClustalW program and an unrooted tree was constructed using MEGA 5.0. The bacterial phyla are colour coded as indicated. Regions corresponding to mycobacterial GroEL1 and GroEL2 are indicated in blue. Inset shows the actinobacterial branch expanded with individual bacterial families color-coded. Branches of the tree corresponding to GroEL1 and GroEL2 sequences are indicated in orange and purple, respectively

and Cpn60.2 are homologous to the mycobacterial Cpn60.1 and Cpn60.2, respectively, while Cpn60.3 is phylogenetically distant from these two copies.

***Phylum Cyanobacteria*:** Members of cyanobacteria phylum are characterized by multiple copies of *cpn60*s and a single copy of *cpn10* (Huq et al. 2010). *Nostoc punctiforme* PCC 73102 has three copies of *cpn60* with only one *cpn10* gene (Ran et al. 2007). Analogous to actinobacterial Cpn60s, these copies are similar in sequence to the corresponding homologues across the species, but are distant from the other copies within the species. Likewise, bacteria belonging to synechocystis species host two copies of *cpn60*, encoding Cpn60.1 and Cpn60.2, and one copy of *cpn10* (Lehel et al. 1993). Cpn60.1, but not Cpn60.2, has been demonstrated to complement the loss of GroEL in an *E. coli* mutant (Tanaka et al. 1997), while Cpn60.2 is essential under stress conditions (Tanaka et al. 1997).

7.7 Inference on Cpn60 Evolution

The size of the bacterial genomes ranges between 1.5 and 13 Mb (Cummings et al. 2002; McCutcheon et al. 2009; Schneiker et al. 2007). Owing to the absence of introns, the size of a bacterial genome can be logically correlated with the

number of cistrons. Since the number of protein families is limited, it is reasonable to imagine that the genome size variation probably correlates with the number of paralogous genes. While several bacteria with average genome size host one *cpn60* gene, bacteria with large genome sizes have been shown to encode multiple copies of the *cpn60* gene; up to five copies in *B. japonicum* harbouring a 9.2 Mb genome (Fischer et al. 1993).

The process of evolution of multiple genes might be due to either horizontal gene transfer (xenologues) or gene duplication (paralogues). To understand the basis of the evolution of Cpn60 paralogues we need to understand the process of gene duplication. Two models have been proposed on the consequences of gene duplication: (i) neofunctionalization model and (ii) subfunctionalization model. The neofunctionalization model, as the name suggests, assumes that the duplicated gene acquires a new function upon acquiring adaptive mutations. The model is supposed to be free of the selection pressure since the ancestral gene continues to function normally (Ohno 1970). Contrariwise, the subfunctionalization model assumes that the duplicated gene acquires neutral mutations and consequently retains one of the ancestral functions. Therefore, each of the ancestral functions (sub-function) is acquired by the duplicated genes (Force et al. 1999; He and Zhang 2005; Lynch and Force 2000). Although, examples for both the models have been identified, the subfunctionalization model has been widely observed (He and Zhang 2005). The phylogenetic distribution of the Cpn60 sequences has been observed in agreement with the 16S rRNA tree. The Cpn60 sequences were clustered according to the host bacteria in the phylogenetic tree, suggesting duplication and rapid evolution of the genes (Fig. 7.4). Thus, the duplication event of *cpn60* genes seem to have occurred in ancestors of certain clades, rather than being horizontally transferred across different species.

7.8 Domain Conservation in Cpn60

In the duplicated Cpn60 proteins of clades such as mycobacteriacea, the two paralogous classes of the Cpn60s: Cpn60.1 and Cpn60.2, are distinguished by the characteristic C-terminal sequence. While the proteobacterial and cyanobacterial Cpn60s display a hydrophobic GGM tripeptide repeat motif, the actinobacterial Cpn60.1 and Cpn60.2 display histidine rich and GGM repeat motifs, respectively.

The apical domain, which spans the central part of the Cpn60 primary structure, is responsible for binding a wide range of substrate molecules (Kumar and Mande 2011). Owing to its wide range of substrate interactions, this domain appears less conserved. Only six residues in proteobacterial Cpn60s and 12 residues in actinobacterial Cpn60s are conserved (Table 7.2). Interestingly, the apical domains of cyanobacterial Cpn60s displayed 40 conserved residues. Moreover, the equatorial domain, which spans two extremes of the Cpn60 polypeptide, and is responsible for the inter-subunit interactions, the formation of essential Anfinsen cage and ATPase activity, exhibits higher conservation. Five point residues and three peptide stretches in proteobacterial Cpn60s and 15 residues and one peptide stretch in actinobacterial

Table 7.2 Conserved residues among GroEL1 homologues

Phylum	GroEL domains	Conserved residues
Proteobacteria	Apical domain	Ser200, Pro276, Gly279, Asp288, Gly315, Arg365
	Equatorial domain	Gly52, Gly436, Asn454, Gly489, Val496, Ser506, Gly85-Asp-Gly-Thr-Thr-Thr90, Gly411-Gly-Gly413 Asp492-Pro493
	Intermediate domain	Gly172, Gly191, Gly377, Gly407, Ala402, Ala403
Actinobacteria	Apical domain	Gly197, Gly281, Gly317; Leu220, Leu258; Pro278; Arg284, Arg344, Arg367 and Asp290
	Equatorial domain	Gly69, Ala77, Gly85-Asp-Gly-Thr-Thr-Thr90, Gly102, Gly109, Gly118, Gly409, Gly413, Gly414, Pro448, Asn455, Gly457, Gly475, Asp493, Val497, Ser507
	Intermediate domain	Gly158, Gly381, Thr384, Asp397
Cyanobacteria	Apical domain	Gly196, Ser199-Pro-Tyr201, Arg208, Leu219, Lys224, Leu231, Leu235-Glu236, Ala256-Leu257, Thr259-Leu-Val-261, Asn263, Gly267, Ala276-Pro277, Phe279-Gly280, Arg283, Asp289, Leu293-Thr294, Glu301, Gly316, Lys325, Arg343, Ser356, Lys362, Arg366, Ala368, Leu370, Gly372-Gly373, Ala375
	Equatorial domain	Arg12, Leu15-Glu-Gly17, Thr29, Gly31-Pro32, Ala56, Asn67, Gly69, Leu72, Thr80, Gly85-Asp-Thr-Thr-Thr-Ala91, Gly102, Asn105, Gly109, Gly118, Gly412-Gly-Gly414, Pro450, Asn457, Gly459, Gly462, Gly477, Gly492, Asp495, Val499, Ser509, Thr516-Thr-Glu518
	Intermediate domain	Asp153, Gly157, Ala163, Val167, Gly171-Val-Ile173, Glu176-Glu177, Ser180, Thr183, Glu189-Gly-Met191, Lys378-Val-Gly-Ala381, Thr383-Glu-Thr-Glu386, Lys388, Lys391-Leu392, Glu395-Asp396, Asn399, Thr401, Ala403-Ala404, Glu407-Gly408

Cpn60s are conserved. Moreover, 48 residues are conserved in equatorial domains of cyanobacterial Cpn60s. The intermediate domain, which connects the apical and equatorial domains in the primary and tertiary structure, shows moderate conservation. Since the domain is responsible for the *en bloc* movement of the substrate recognition domain, a few conserved residues were identified in Cpn60s from three phyla. Eight residues in the proteobacterial Cpn60s and four residues in actinobacterial Cpn60s were conserved. As expected, cyanobacterial Cpn60s displayed 33 conserved residues in intermediate domains (Table 7.2).

In a nut shell, these observations imply that the apical domain, owing to its promiscuity in substrate interactions, is less conserved. Moreover, displaying structural similarity, this domain has been attributed the promiscuous peroxiredoxin origin (Dekker et al. 2011). The equatorial domain, on the other hand, is greatly conserved owing to the inter-subunit interactions, thereby, the formation of the essential Anfinsen cage

and the ATP binding for its activity. The intermediate domain is fairly conserved, since it needs to regulate the *en bloc* movement of the apical domain in response to the presence of nucleotide in the equatorial domain. Therefore the conservation profile for the Cpn60 monomer is the highest for equatorial domain, followed by the intermediate domain, while the apical domain shows the least conservation.

7.9 Conclusions

Chaperonin 60 functions as the constitutional protein chaperone in several bacteria. Recent studies have discovered additional novel functions for paralogous Cpn60 proteins in certain pathogenic bacteria. Moreover, these studies have expanded the substrate catalogue for Cpn60s from the polypeptides to other biopolymers and therefore, the resulting functional divergence has been attributed to different oligomeric status and cellular localization. Lower oligomeric forms are implicated in binding and transport of extended polymers such as DNA, while the higher oligomeric forms might be involved in the protein folding function. Considering the dimensions, it is reasonable to assume that the lower oligomeric form might be secretory and involved in eliciting the immunological responses and transport of biopolymers, while the higher oligomeric forms might be confined to the cytoplasm and protein folding actions.

In addition, we propose that chaperonin genes have been subjected to different selective constraints during evolution. Gene duplication followed by sequence divergence resulted in paralogous Cpn60s that can perform different functions. Moreover, these functional variations might be acquired by incorporating chemically dissimilar substitutions at functionally important residue positions.

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Chapter 8
Mycobacterium tuberculosis Chaperonin 60 Paralogues Contribute to Virulence in Tuberculosis

Richard W. Stokes

Abstract With the human population reaching seven billion it is estimated that one third of this population are infected with the causative agent of tuberculosis, *Mycobacterium tuberculosis.* Curiously, this bacterium has evolved to survive within the macrophage, a key cell population involved in cell-mediated immunity to infectious bacteria. Such survival in this natural bacterial-killing cell population means that *M. tuberculosis* has evolved strategies to combat the killing machinery of the macrophage. Amongst the growing number of *M. tuberculosis* virulence factors are two paralogues of chaperonin (Cpn)60 or Hsp60 termed Cpn60.1 and Cpn60.2. The c*pn60.2* gene is essential and involved in the maintenance of cell viability through "normal" chaperoning activities. In contrast, *cpn60.1* is non-essential and appears to have minimal chaperone activity. However, both Cpn60.1 and Cpn60.2 have various moonlighting functions including: acting as secreted signaling molecules, modulators of host immunity, surface located bacterial ligands and bacterial cell wall components. How these proteins leave the cytosol to function at the surface of the bacterial cell wall, or even in the extracellular milieu is still not clear. That they can act as bacterial virulence factors is becoming clear, although recognition of Cpn60.2 by the host may instead mediate a host defence mechanism. These various activities will be described.

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8.1 Introduction

The vast majority of the genus, *Mycobacterium,* are saprophytic species that, like other Actinomycetes, are found in soil and water. Unfortunately, some species are the causative agents of severe disease in *Homo sapiens.* For example, the *M. avium-intracellulare* complex, *M. kansasii* and *M. fortuitum* are opportunistic pathogens of man, that predominantly cause disease in immunocompromised individuals. Others can infect and cause severe disease in the immunocompetent population. These include *Mycobacterium tuberculosis, M. leprae* and *M. ulcerans* of which the best known and most important is *M. tuberculosis* (*M.tb*), the causative agent of tuberculosis (TB). TB causes more death in adults than does any other single bacterium and it is estimated that in 2010 there were approximately ten million new cases of TB (Dye and Williams 2010). Ominously, it is also estimated that TB is the current cause of around two million deaths worldwide each year (Anonymous 2009). The relatively recent emergence of multidrug resistant and extensively drug resistant TB is now a serious problem and threatens to make the disease incurable (Jain and Mondal 2008; Mitnick et al. 2008). To add to our woes, the recent AIDS pandemic has compounded the infection rates and morbidity of TB, due to the diminished CD4 T-cell mediated immunity of the AIDS patient (Getahun et al. 2010). Despite the recent successes in the global treatment of TB, it remains a devastating disease of mankind that continues to infect increasingly higher numbers of people (Dye and Williams 2010).

Mycobacterium tuberculosis is an evolved intracellular pathogen generally found within macrophages (MΦs) in the lung. MΦs are phagocytic cells which form part of the effector arm of the host cell-mediated immune system. They kill invading microbes, although not as efficiently as neutrophils, by a variety of mechanisms such as generation of reactive oxygen intermediates (ROI), cationic peptides, LRG-47 and by undergoing apoptosis. Clearly, given the prevalence of tuberculosis, *M.tb* can survive the initial move to the lung and the subsequent intracellular environment. Here it starts to replicate with its bacterial antigens being processed and thus resulting in the development of adaptive immunity with the production of interferon-γ (IFNγ) by T-cells (Orme 2004; Cooper 2009; Torrado et al. 2011). IFNγ is a major activator of MΦs, enhancing their ability to control bacterial replication through a process discovered in the 1960s and termed classical macrophage activation. MΦ activation results in the elevation of the killing mechanisms seen in resting MΦs (such as ROI and apoptosis) and in the induction of new killing mechanisms mediated by RNIs or phagosomal maturation to lysosomes. This exposure to IFNγ-activated MΦs, following the development of adaptive immunity, marks a change in the pathogenesis of TB. Bacterial growth slows down to the point where there is little bacterial replication and what little there is, is countered by host immunity (Gill et al. 2009; Ehlers 2009). This coincides with a change in the physiology of *M.tb* which assumes a state known as dormancy or the latent/chronic phase. In this condition the metabolism of *M.tb* changes to the use of lipids instead of carbohydrates as a carbon source and to the use of the glyoxalate shunt for energy

production. However, although the host manages to control the replication of the *M.tb* bacilli, the obvious danger is that the bacteria still survive and can remain viable for the remaining lifespan of the TB infected patient. The host response to these persisting *M.tb* is to form an organised collection of cells that surround and wall off the bacteria in a hypoxic environment where they are commonly found within lipid rich foamy MΦs, surrounded by lymphocytes, giant cells and fibroblasts. This structure is known as a granuloma (or a tubercle in clinical terminology) and functions to contain the TB bacilli, presumably preventing their spread and replication. It is assumed that granuloma formation is a host-protective process. However, *M.tb* within a granuloma can remain in a dormant physiological state for decades until some external factor results in diminished immune regulation of the granuloma that then facilitates reactivation of bacterial replication, a breakdown of the granuloma's integrity and breakthrough into the airways of the lung. At this point the patient is said to be productive and will cough up these bacteria which, if inhaled by a new host, complete the infectious cycle.

Mycobacterium tuberculosis has clearly evolved strategies for evading and subverting the antimicrobial effector mechanisms of the host immune response. The prevalence of TB demonstrates the success of these evolved strategies. That said, only 10 % of immunocompetent individuals infected with *M.tb* develop clinical disease over their lifetime. Within the other 90 % of infected individuals, the bacterium survives in a dormant, yet viable state for decades. These data indicate that there is an ongoing conflict between pathogen and host with the balance being tipped in the favour of fulminating bacterial disease in some cases, whereas usually the host can keep the pathogen in check. Which way the balance tips in any given individual is greatly affected by environmental factors (Lienhardt 2001; van der Eijk et al. 2007), but also by genetic variation in host susceptibility (Newport and Levin 1999; Doffinger et al. 2006; Stein 2011) and the virulence of the infecting *M.tb* isolate (Collins and Smith 1969; North and Izzo 1993; Orme 1999; Sassetti and Rubin 2003). It has long been known that the virulence (defined as the ability to produce a progressive infection) of separate strains or isolates of *M.tb* can vary in animal models of tuberculosis (Steenken et al. 1934; Alsaadi and Smith 1973; Orme 1999) Recently, members of the so called Beijing family of *M.tb* strains have been shown to have greater infectivity and virulence in man and have rapidly spread around the world (Bifani et al. 2002; Lasunskaia et al. 2010). It is therefore logical to propose that specific bacterial genes are critical for the survival and virulence of *M.tb* within the host and that identification of these virulence genes and their products will facilitate the design of novel vaccines and therapies to treat tuberculosis by providing novel targets for pharmacological research. This has resulted in a concerted effort to identify *M.tb* virulence factors using a variety of methodologies (Braunstein et al. 2002; Smith 2003; Sharma and Tyagi 2007). Surveying a transposon mutant library for survival in MΦ (Rengarajan et al. 2005) or *in vivo* (Sassetti and Rubin 2003) has identified gene sets that appear to be required for survival under these experimental conditions which could therefore be considered the essential genes for virulence. However, alternate methodologies have identified other gene sets that appear to be virulence

factors for the survival of *M.tb* in MΦ (Schnappinger et al. 2003; Li et al. 2008, 2010) and *in vivo* (Talaat et al. 2004; Lamichhane et al. 2005). A direct comparison of the gene sets identified to be virulence factors in three separate MΦ infection studies showed only limited overlap (Li et al. 2010). Undoubtedly, differences in methodologies account for some of this variation but different interpretations of what defines a virulence factor should also be taken into account. Whether it is defined as a genomic difference between a virulent and an avirulent strain of *M.tb* or as an expression difference between broth grown and intracellular bacteria or between intracellular strains of varying virulence, the identification of a gene product as a virulence factor is open to interpretation.

Although an understanding of the molecular details controlling the pathogenesis of TB is by no means complete, recent research has begun to determine the microbiology and immunology of this phenomenon (Glickman and Jacobs 2001; Smith 2003; de Chastellier 2009; Barry et al. 2009; Stokes and Waddell 2009). *Mycobacterium tuberculosis* within its host encounters numerous stresses including residence within an intracellular environment, exposure to MΦ killing mechanisms, exposure to the effector mechanisms of the host's adaptive immune response and changes to oxygen and nutrient availability (Ehrt and Schnappinger 2009). The pathogen responds to these stresses in several ways (Ehrt and Schnappinger 2009; Stokes and Waddell 2009) including the induction of stress proteins of which the chaperonin (Cpn)60 homologue is perhaps the best known.

8.2 The Chaperonin 60 (Hsp60/Hsp65/Cpn60) Proteins of *Mycobacterium tuberculosis*

The mycobacterial Cpn60 homologue (often called Hsp65 in mycobacteria) was first identified as a member of the "common antigen" family (Thole et al. 1988a) and an immunodominant antigen in TB patients and in experimental animal infections (Young et al. 1988; Young 1990). That the Cpn60 of *M.tb* is a strong immunogen is not surprising, as members of the Cpn60 family are major antigens in several pathogens and strong antibody responses to Cpn60 are to be found following bacterial, protozoan and helminth infections (Young 1990). Early studies on the mycobacterial Cpn60 protein included the identification of B cell and T cell epitopes, identifying numerous epitopes of varying degrees of species specificity and species cross-reactivity (Anderson et al. 1988; Thole et al. 1988b). It was shown that *M.tb* Cpn60 could stimulate T-cell responses in human subjects, irrespective of whether they were infected with *M.tb* (Lamb et al. 1986; Thole et al. 1988a). This raised the possibility that recognition of conserved epitopes in the Cpn60 family could lead to autoimmunity (Lamb et al. 1989; Dubaniewicz 2010). The interested reader may like to refer to Chap. 4 which discusses the co-chaperone of Cpn60, namely Cpn10, another immunomodulatory mycobacterial protein which is likely to also play a role in the pathogenesis of TB.

Studies have shown that experimental adjuvant arthritis in rats and mice (a model of rheumatoid arthritis (RA) in humans) can be induced by the injection of intact mycobacteria or complete Freund's adjuvant (a mixture of *M.tb* components within a mineral oil vehicle) (McLean et al. 1990; Cohen 1991). In contrast, pre-immunization with recombinant Cpn60 or virally expressed Cpn60 leads to suppression of and/or protection from adjuvant arthritis (Billingham et al. 1990; Yang et al. 1990; López-Guerrero et al. 1994; Haque et al. 1996). An explanation for how *M.tb* Cpn60 induces autoimmunity has not been determined but it appears that repeated exposure to bacteria, especially pathogens containing proteins with a high similarity to host mammalian antigens, affects the host's ability to discriminate between self and non-self antigens (Moudgil and Sercarz 1994). As mycobacterial and mammalian Hsp60 homologues share 60 % homology (Jindal et al. 1989) and mycobacterial infections are commonly chronic and may remain with the patient all their lives, it can be seen why mycobacterial cell stress proteins are generally implicated in autoimmune diseases. The autoimmunity induced by mycobacterial Cpn60 could merely be an unavoidable consequence of the homology seen between Cpns of all species and is of no advantage to host or pathogen. However, the possibility that the autoimmunity is an occasional "by product" of an *M.tb* virulence strategy should not be discounted. As will be shown below, the Cpn60 of *M.tb* appears to be actively transported to the outer layers of the bacterial cell wall and beyond where it interacts with the host. The data showing that the extracellular Cpn60 is acting as an immunomodulator, a bacterial ligand mediating attachment to MΦs and a cell signaling molecule indicates that this molecular chaperone of *M.tb* has a role in the pathogenesis of TB.

8.2.1 Mycobacterial Paralogues of Cpn60

Most bacteria contain a single Cpn60 gene but it is becoming clear that in approximately 30 % of the bacteria that have been currently sequenced, multiple copies of Hsp60 exist (Lund 2009). The role of Cpn60 paralogues in the evolution of the moonlighting actions of this protein family has been discussed in Chap. 7. This is true for *M. leprae* (Rinke de Wit et al. 1992), *M.tb* (Kong et al. 1993), *M. bovis* (Wang et al. 2011) and *M. avium paratuberculosis* (Goyal et al. 2006) which all have two copies of Cpn60, whereas *M. smegmatis* has three copies (Rao and Lund 2010). Phylogenetic analysis suggests that the two paralogues resulted from a single gene duplication event followed by varied rates of evolutionary change (Hughes 1993) with the third homologue in *M. smegmatis* appearing to have been acquired by horizontal gene transfer (Rao and Lund 2010). The two paralogues of Cpn60 in pathogenic mycobacteria are designated Cpn60.1 (GroEL1, *M.tb* genome accession number Rv3417c) and Cpn60.2 (GroEL2, Hsp65, *M.tb* genome accession number Rv0440). The Cpn60.1 and Cpn60.2 proteins from *M. tuberculosis* only share 61 % sequence identity (Kong et al. 1993) while there is 95 % identity between Cpn60.2 of *M.tb* and *M. leprae* (Shinnick et al. 1987). This implies that

Cpn60.1 and Cpn60.2 would have divergent functions (Qamra et al. 2005 – see also Chap. 7 for further discussion of this point). Comparable to the GroEL function in *E. coli*, Cpn60.2 shows hydrophobicity-based protein folding activity and acts as a "normal" chaperonin. However, this function seems to result from the formation of a Cpn60.2 homodimer that is less ATP-dependent than is the GroEL of *E. coli* (Qamra et al. 2004; Shahar et al. 2011 see Chap. 7). Both Cpn60.1 and Cpn60.2 behave as dimers *in vivo* and *in vitro* which is unlike other bacterial Cpn60s that exist as tetradecamers (Qamra et al. 2004; Shahar et al. 2011 – see Chap. 7). As it appears that *M.tb* Cpn60.2 is acting as a GroEL equivalent, it was surprising to find that *cpn60.1* appears to be arranged in a putative operon with *cpn10* (GroES, *M.tb* genome accession number Rv3418c) (Kong et al. 1993), while *cpn60.2* is found elsewhere on the chromosome. However, recent studies show that the apical domains of *M.tb* Cpn60.1 and Cpn60.2 have conserved their three-dimensional structure and appear to be like the *E. coli* GroEL. Thus, it seems that while Cpn60.2 functions as the general housekeeping chaperonin, Cpn60.1, like Cpn60.2, can also act as a chaperonin (Sielaff et al. 2010), although, at the moment, this is only based on structural homology.

Further support for the divergent functions of Cpn60.1 and Cpn60.2 came from the attempts to delete these genes in mycobacteria. It was found that a knockout mutant can be obtained for *cpn60.1* in *M. smegmatis* (Ojha et al. 2005), *M.tb* (Hu et al. 2008) and *M. bovis* BCG (Wang et al. 2011). In contrast, *cpn60.2* can not be deleted and has been shown to be an essential gene required for the survival of *M.tb* (Hu et al. 2008). The fact that *cpn60.2* is essential, lends support to the idea that it acts as the main housekeeping chaperone for *M.tb* in much the same way as GroEL does in *E. coli*. The role of Cpn60.1 is less clear. While it can possibly act as a chaperonin (Sielaff et al. 2010), deletion of the gene in *M.tb* did not result in a dramatic phenotype (Hu et al. 2008) suggesting that any chaperonin activity is not essential for bacterial survival. Growth of the mutant in broth and in MΦs was found to be equal to that of the wild-type parent (Hu et al. 2008). However, the mutant failed to grow in mice as rapidly as did the wild type, attaining comparable bacterial load in the lung and spleen only at later time points. This was associated with differences in the granulomatous inflammation in both mice and guinea pigs, with the mutant infected lungs showing only minimal inflammation in mice at 15 weeks post-infection, even though bacterial numbers were similar to that of the wild type (Hu et al. 2008). This suggested that Cpn60.1 is essential for the induction of normal granuloma formation during *M.tb* infection. The finding that levels of TNFα, IFNγ, IL-6 and IL-12 in the lungs of mice infected with the mutant were significantly lower than that seen in mice infected with wild-type bacteria up to 15 weeks post-infection (Hu et al. 2008) suggests that inflammation is affected throughout the course of the infection and indicates that Cpn60.1 is important in the induction of this inflammation. It may seem counter-intuitive that a putative *M.tb* virulence factor would induce inflammation, a host response usually associated with defence against bacterial infection. However, it is important to note that *M.tb* has a level of resistance to the effector arm of cell-mediated immunity and, in fact, resides within the very cells that are part of this response. It should also be

noted that the mutant lacking Cpn60.1 was unable to induce a response in a human blood granuloma assay (it failed to stimulate multinucleate giant cell formation) showing that the relationship of this protein to granuloma formation is not simply an animal artifact (Cehovin et al. 2010).

The growth of a *M. bovis* BCG Cpn60.1 mutant in broth was equal to that of the wild type although more protein was secreted into the supernatant by the mutant (Wang et al. 2011). Cell wall lipids were altered in the mutant and it was more susceptible to hydrogen peroxide (Wang et al. 2011). When growth in mice was investigated, the mutant was slightly less persistent in the lungs and spleen but retained its ability to protect vaccinated mice against a challenge with *M.tb* (Wang et al. 2011). Thus, like *M.tb,* the growth of a *M. bovis* BCG Cpn60.1 mutant is not greatly affected. However, it was shown for BCG that Cpn60.1 was necessary for bacterial cell wall integrity and resistance to hydrogen peroxide, but is not essential for the vaccine potential of BCG.

8.3 Secretion of Chaperonin 60 Proteins by *M.tb*

It can thus be seen that the two Cpn60 paralogues of *M.tb* differ in their essentiality for bacterial survival and also in their function. That these stress proteins have other roles besides that of acting as a protein chaperone is becoming clear, with increasing evidence that they are secreted signaling molecules, modulators of host immunity, surface located bacterial ligands and bacterial cell wall components. A useful term for these additional roles of *M.tb* Hsp60 has been suggested by Henderson and his colleagues (Cehovin et al. 2010; Henderson et al. 2010) who called them "moonlighting" functions – a term initially introduced by Connie Jeffery (1999). A full description of protein moonlighting is provided by Jeffery in Chap. 3. Some resistance to this idea that stress proteins may have other functions, besides acting as chaperones or protein-folding catalysts has been forthcoming and seems, at least in part, to be connected to the dogma that *M.tb* Cpn60s acts only as chaperonins and are therefore located intracellularly where they can function to mediate protein folding and do not transfer across the plasma membrane. In fact, it is commonly believed that detection of Cpn60 in a culture supernatant is indicative of cell lysis (Sonnenberg and Belisle 1997). It is therefore worthwhile examining the evidence that both Cpn60.1 and Cpn60.2 are normally to be found both within the cytosol and on the outer layers of the cell wall where they can be shed or actively secreted into the extracellular environment.

With the demonstration that mycobacteria have multiple copies of Cpn60 and the increasing demonstrations that Cpn60 has "moonlighting" functions, (see Henderson et al. 2013) it becomes easier to accept that Cpn60 may have functional roles that involve its location other than in the cytosol. Indeed, Cpn60 has been shown to be secreted and to be located within the outer layers of the cell wall of *M.tb*. The identification of MΦ receptors that mediate binding of intact mycobacteria via Cpn60.2 (Hickey et al. 2009, 2010) necessitates that the Cpn60.2 must be located at the cell

surface of the mycobacteria. In addition to this evidence, it has been demonstrated that mycobacteria do contain several cell stress proteins, including Cpn60, within their outer cell wall by using various methodologies such as electron microscopy (Esaguy and Aguas 1997), antibody binding (Gillis et al. 1985; Esaguy and Aguas 1997; Hickey et al. 2009) and proteomics (Stokes, unpublished observations and (Rosenkrands et al. 2000; Wolfe et al. 2010)). In fact, using isobaric tags for relative and absolute quantitation (iTRAQ), Cpn60.1 and Cpn60.2, along with Hsp70, Hsp10 and Hsp16 have been shown to be among the most prevalent of proteins within the outer cell wall capsular layer of *M.tb* (Stokes unpublished observations). This is further supported by studies analyzing proteins in the cell wall of *M.tb* (Wolfe et al. 2010), by demonstrating the presence of Hsp16 in the cell wall (Cunningham and Spreadbury 1998) and by protein gel analysis of *M.tb* capsule (Hickey et al. 2009). Furthermore, Cpn60.1 has been shown to be secreted by *M.tb* into the supernatant of broth cultures (Cehovin et al. 2010). Interestingly, at the same time point that Cpn60.1 first appears in culture filtrates (6 days), no Cpn60.2 can be found (Hickey et al. 2009; Cehovin et al. 2010) even though it is on the surface of the bacteria (Hickey et al. 2009). This would imply that Cpn60.1 is actively secreted, perhaps to facilitate its actions on host cells. However, it is worth noting that Cpn60.2 secretion (or release) can be induced by the removal of zinc from the culture medium (De Bruyn et al. 1989). A more recent study on the cell surface proteins of *M. avium subsp. hominissuis* using surface biotinylation and mass spectrometric protein identification, identified the presence of the molecular chaperones: Cpn10, Cpn60.1, Cpn60.2, DnaK and ATP-dependent Cpl protease. In addition, the classic cell surface glycolytic proteins, GAPDH and enolase, were also present (McNamara et al. 2012).

To date no mechanism for the active secretion of Cpn60.1 has been identified, nor has a mechanism for how Cpn60.2 and the other cell stress proteins access their outer cell wall location been discovered. The means by which these, and, for that matter, the many other cell wall-located and secreted proteins that exit the mycobacterial cytosol are poorly understood. Although significant progress has been made in identifying the protein secretion systems of mycobacteria (Abdallah et al. 2007; Digiuseppe Champion and Cox 2007), none of the systems identified appear (at least, as yet) to be involved in the transport of Cpn60 proteins across the plasma membrane. However, possible mechanisms for the egress of Cpn60 and other cell stress proteins can be postulated. For instance, secretion may be due to their hydrophobic surfaces allowing them to interact with membrane phospholipids and other lipidic molecules within the largely hydrophobic milieu of the lipid rich mycobacterial cell wall, as suggested for other bacteria (Hennequin et al. 2001). Indeed, one report has shown that GroEL, human Hsp70, Cpn60.2 and DnaK all have the capacity to induce the formation of pores in lipid bilayers (Alder et al. 1990). Additionally, GroEL can promote lipid bilayer stability during protein folding activity (Török et al. 1997), indicating its ability to traverse the plasma membrane. Alternatively, Cpn60 may engage more specific export mechanisms such as 'hitch-hiker'-based export via the recently described mycobacterial Twin-Arginine Translocation (Tat) pathway (McDonough et al. 2005; Lee et al. 2006). Proof-of-principal for the

secretion of cell stress proteins exists, even though they have not been specifically applied to the *M.tb* chaperonins. For example, *M.tb* Cpn10 protein appears to be secreted from the bacterium, and shares some structural elements common to the N-terminal region of Cpn60 (Hughes 1993). See Chap. 4 for further discussion of *M.tb* Cpn10. In addition, the active secretion of mammalian Hsp60 (Merendino et al. 2010) and Hsp70 (Mambula et al. 2007) demonstrate that Cpn60 could be secreted to the mycobacterial cell surface and beyond.

8.4 The Moonlighting Functions of Bacterial Cpn60 Proteins

Once Cpn60 has traversed the plasma membrane and lipidic layers of the mycobacterial cell wall, what functions does it exert? The growing literature on this topic would suggest that it has several functions. The immunomodulatory function of Cpn60 in autoimmunity has already been covered above, but other cell-cell signaling mechanisms for Cpn60 have also been discovered. The Cpn60 of the oral bacterium *Aggregatibacter actinomycetemcomitans* stimulates the activity of the bone-resorbing osteoclast population resulting in the breakdown of murine calvarial bone (Kirby et al. 1995; Henderson et al. 2003). Interestingly, the Cpn60 proteins from both humans and some other bacteria (e.g. *E. coli*) also have this function (Reddi et al. 1998; Meghji et al. 2003). Curiously, the Cpn60 proteins of *M.tb* not only do not share this biological action but the Cpn60.2 protein has absolutely no influence on osteoclasts (Meghji et al. 1997) while the Cpn60.1 protein actually functions as an inhibitor of osteoclast generation (Winrow et al. 2008). Given the fact that the osteoclast is a multinucleate cell similar, at least in appearance to the Langhans giant cell of the tuberculoid granuloma, it is curious that the *M.tb* Cpn60.1 protein should promote giant cell formation (Cehovin et al. 2010) but block osteoclast formation (Winrow et al. 2008). Thus this Cpn60.1 protein is a useful probe to define the differences between the genesis of giant cells and osteoclasts.

More obviously connected to the virulence of *M.tb* is the effect of Cpn60 on the induction of host cell MΦ production of cytokines, ROI and RNI. The early studies did not differentiate the two *M.tb* Cpn60 paralogues but still showed that the Cpn60 of *M.tb* (actually the Hsp65 or Cpn60.2 protein) induced the production of TNFα, IL-6 and IL-8 by the human macrophage-like cell line, THP-1 (Friedland et al. 1993) and TNFα and IL-6 by murine peritoneal MΦs (Peetermans et al. 1995). Interestingly, murine MΦs also produced RNI in response to Cpn60 which was TNFα-dependent and inhibited intracellular replication of the protozoan pathogen, *Toxoplasma gondii* (Peetermans et al. 1995). However, whether RNIs play any role in human MΦs is still a topic of some controversy (Fang 2004). Chaperonin 60 treatment of human monocyte-derived MΦs induced the pro-inflammatory cytokines TNFα and IL-1β and increased the expression of the surface complement receptor 3, but did not result in increased induction of ROI or MHCII expression, indicating a lack of classical MΦ activation (Peetermans et al. 1994). As it is classical IFNγ-mediated activation that is able to control *M.tb* intracellular replication

(Cooper and Flynn 1995; Doffinger et al. 2006), it would not be advantageous to *M.tb* to induce this response, while an increase in complement receptor 3 expression may aid the uptake of the bacteria in an advantageous manner (Stokes et al. 1993; Velasco-Velázquez et al. 2003).

Following the discovery of the two *M.tb* Cpn60 paralogues, it was possible to compare the ability of *M.tb* Cpn60.1 and Cpn60.2 to induce cytokine production by MΦs. While both Cpn60.1 and Cpn60.2 stimulate human MΦ to produce IL-1, IL-6, IL-8, IL-10, IL-12, TNFα and GM-CSF but not IL-4 or IFNγ, 100 fold less Cpn60.1 was required to stimulate comparable amounts of these cytokines (Lewthwaite et al. 2001). Furthermore, Cpn60.1, but not Cpn60.2, signalling was shown to involve CD14 (Lewthwaite et al. 2001). Both Cpn60.1 and Cpn60.2 have only a partial requirement for MyD88 to induce MΦ cytokine production. Additionally, both have a requirement for Toll-like Receptor (TLR)-4, with Cpn60.2 having an additional requirement for TLR2 (Cehovin et al. 2010). Additional studies showed that both Cpn60.1 and Cpn60.2 utilize the ERK/1 and MAPK signaling pathways to induce cytokine production by MΦ (Lewthwaite et al. 2007). When whole blood leucocyte populations are stimulated with Cpn60.1 and Cpn60.2, only IL-1β and IL-6 and not IL-8, IL-10, IL-12 or IFNγ were produced by the mixed cell population. In this model, Cpn60.2 was a more potent stimulator than was Cpn60.1 and was the only one that induced TNFα production (Cehovin et al. 2010). The contrasting results with those previously reported for MΦ (Lewthwaite et al. 2001) indicated that the interaction of Cpn60.1 and Cpn60.2 with whole blood is very different from that seen with purified MΦ, due, presumably to interactions between these leukocyte populations. Mande's group have also shown that *M.tb* Cpn60.1 induces TLR2 expression on macrophages and can then use this receptor to inhibit PPD-induced induction of IL-12p40 synthesis (Khan et al. 2008).

8.5 Binding of Cpn60 to Immune Effector Cells

The demonstration that TLRs, CD14 and MyD88 are necessary for appropriate signaling to take place in response to Cpn60 does not mean that they are necessarily the receptors for Cpn60. In another model studying the interaction of lipopolysaccharide (LPS) with MΦ, it was shown that CD14, TLR4, MD2 and other cell surface moieties form an intricate complex that mediates binding and cell signaling in response to LPS (Triantafilou and Triantafilou 2005 – described in more detail in Chap. 18). Perhaps a similar complex of MΦ surface receptors is needed to interact with mycobacterial cell wall glycolipids and proteins. Nevertheless, the search for MΦ receptors that bind Cpn60 has indicated a number of cell surface proteins that may bind to Cpn60.1 (Henderson and Mesher 2007). Binding of Hsp70 and Cpn60.1 from *M. bovis* BCG to DC-SIGN has also been reported (Carroll et al. 2010). In contrast, it appears that, in the absence of serum (a situation that would be found within the alveolar space where *M.tb* first encounters MΦs), Cpn60.2 binds strongly to the MΦ surface receptor, CD43 (sialophorin, leukosialin) and that this receptor/

ligand interaction accounts for 30–40 % of all binding of *M.tb* bacilli to MΦs (Hickey et al. 2009, 2010). Whether this binding can be considered a true receptor/ligand interaction and not just an interaction of "sticky" chaperonins with a host glycoprotein is not unequivocally determined. However, two observations strongly suggest that this interaction is a specific binding of the two moieties: (i) although both Cpn60.1 and Cpn60.2 are present in large amounts in the outer cell wall capsule of *M.tb* (Stokes, unpublished data), only Cpn60.2 binds to isolated CD43 (Hickey et al. 2009) and (ii) Hsp70 was also shown to bind to isolated CD43 but does not mediate binding of whole bacteria to CD43 on MΦs (Hickey et al. 2009, 2010). It is interesting to note that Cpn60.1 was found to bind to approximately 90 % of circulating human monocytes compared to <50 % binding with Cpn60.2 (Cehovin et al. 2010). This may reflect the very different surface receptors found on monocytes and MΦ, although CD43 is expressed on both.

The finding that Cpn60.2 can interact with purified CD43 (Hickey et al. 2009, 2010), does not necessarily mean that they interact with MΦ surface CD43 in isolation. It is possible that CD43 interacts with mycobacteria within the context of a group of MΦ surface molecules, as was described above for LPS (Triantafilou and Triantafilou 2005 – see Chap. 18 for more details). In this model, CD43 would co-operate with other surface MΦ receptors to facilitate efficient bacterial binding and/or signal transduction via interaction with one or more bacterial surface molecules. The demonstration that soluble CD43 can overcome the deficiency of mycobacterial binding to MΦ from CD43 knockout mice (Fratazzi et al. 2000) suggests that, although *M.tb* can bind CD43 directly, it may also interact with other MΦ receptors. In addition to Cpn60.2, numerous other mycobacterial cell wall constituents have been identified as ligands that mediate binding to MΦ and several MΦ receptors have been shown to be involved in this binding (El-Etr and Cirillo 2001; Schäfer et al. 2009; Mishra et al. 2011). Alternatively, it may be that Cpn60.2 and CD43 do interact in isolation and that this interaction anchors the *M.tb*, thereby facilitating subsequent ligand-receptor interactions to effectively take place such as binding by the phagocytic CR3 receptor (Melo et al. 2000; Rooyakkers and Stokes 2005), or signaling via TLRs (Means et al. 1999; Thoma-Uszynski et al. 2001; Reiling et al. 2008). It is noteworthy here that CD43 often plays the role of an intercellular binding modulator, allowing some receptor-ligand interactions to take place more readily, while limiting other interactions (Ostberg et al. 1998).

Whether this interaction of CD43 with *M.tb* Cpn60.2 is to the advantage of the bacteria or the host is not clear yet. It is known that absence of CD43 results in more rapid bacterial growth in MΦs and a more severe pathology resulting from *M.tb* infection *in vivo* (Randhawa et al. 2005). Increased growth of *M.tb* in CD43 null MΦs is due to a reduction in TNFα-mediated apoptosis of these MΦs that then allows for greater bacterial replication (Randhawa et al. 2008). This would suggest that recognition of Cpn60.2 by CD43 is a host defence mechanism and not a bacterial virulence strategy. Recognition of an essential *M.tb* protein that results in induction of a mechanism to control the intracellular replication of the pathogen would be a good defence strategy for the host. As *M.tb* can not survive without Cpn60.2, it has little opportunity to avoid this immune defence mechanism. However, whether the

induction of TNFα-mediated apoptosis via CD43 is facilitated by Cpn60.2 or some other *M.tb* surface moiety binding to the CD43 is not yet unequivocally determined. Another intriguing possibility is that the role of secreted Cpn60.1 may be to counter the host defence mechanisms initiated by recognition of Cpn60.2, thus providing one possible explanation for the evolution of two Cpn60 paralogues in *M.tb*.

Genetic evolution analyses provide evidence that an ancient mycobacterial ancestor gained an additional Cpn60 copy at some point and since that time Cpn60.1 has undergone a more rapid level of nonsynonymous mutation, apparently leading to a form that no longer functions in protein folding, while Cpn60.2 has evolved to facilitate protein folding without the need of Cpn10 (Hughes 1993; Qamra et al. 2004). This is described in more detail in Chap. 7. That GroE (GroEL + GroES) is necessary for the formation and maintenance of the *E. coli* cell wall suggests that these chaperonins may have originally located to the cell wall to facilitate cell maintenance (McLennan and Masters 1998). In addition, Cpn60.1 from *M. smegmatis* has been implicated in the formation of mycolic acids, again suggesting a functional role within the mycobacterial cell wall (Ojha et al. 2005). These observations suggest that at least one reason that bacterial molecular chaperones leave the cytosolic space is to facilitate their role in maintenance of the cell wall. Thus, the additional "moonlighting" roles that molecular chaperones have may have evolved as a byproduct of this extracellular localization. An additional means by which the Cpn60 proteins may have attained additional functions relates to the fact that the mycobacteria contain multiple copies of these proteins. The finding that only Cpn60.2 is necessary for viability suggests that Cpn60.1 and Cpn60.2 have unique roles within the bacterium (Hu et al. 2008) and that Hsps can evolve to have additional functions if another functional copy is retained for housekeeping functions related to cellular viability (Hu et al. 2008).

8.6 Conclusions

Protein moonlighting appears to be a relatively rare phenomenon and yet *M.tb* has at least 11 moonlighting proteins, including four molecular chaperones, involved in the virulence of this organism (Henderson et al. 2010). This suggests either that this bacterium has followed a curious evolutionary trajectory or that many more bacterial proteins will be found to have moonlighting virulence attributes. This curious use of moonlighting proteins to survive within the host is amplified by the fact that highly conserved proteins, such as the chaperonins, have evolved multiple functions. These moonlighting activities of the Cpn60 proteins seem biased toward enhancing interactions with myeloid cells. The finding that Cpn60.2 can promote macrophage cytokine synthesis without inducing the characteristic changes of the classically activated macrophage suggests this protein is an alternative macrophage activator. It is not clear if this state of macrophage activation has anything to do with interaction of Cpn60.2 with CD43. The other curious interaction is the finding that the Cpn60.1 protein seems to be involved with the

generation of pathological multinucleate giant cells and yet this same protein inhibits the generation and activation of the naturally-occurring multinucleate osteoclasts. The *M.tb* Cpn60.2 protein has no influence on osteoclasts while the *E. coli*, *A. actinomycetemcomitans* and the human Cpn60 proteins are potent inducers and activators of osteoclasts. Much more work is need to understand the molecular and cellular interactions between the *M.tb* chaperonins and myeloid cells and the pathogenesis of tuberculosis.

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Chapter 9
The *Legionella pneumophila* Chaperonin 60 and the Art of Keeping Several Moonlighting Jobs

Rafael A. Garduño and Audrey Chong

Abstract The title of this chapter intends to emphasize the fact that, as the essential chaperonin 60 of the bacterial pathogen *Legionella pneumophila*, HtpB has a main day job in protein folding, in addition to several alternate night jobs, depending on where it is located. The alternate virulence-related jobs that we have described for HtpB include the interaction with host cell surface receptors (which results in signaling and a variety of host cell responses), attraction of host cell mitochondria, modification of the host cell actin cytoskeleton, induction of bacterial filamentation, and interaction with specific host cell proteins, i.e. S-adenosyl methionine decarboxylase (which plays a role in the synthesis of host cell polyamines). These alternate HtpB jobs were primarily discovered as strong phenotypes after expression of recombinant HtpB in bacteria, yeast and mammalian cells. It is fascinating that HtpB possesses the extraordinary ability to functionally adapt to the disparate cellular environments of prokaryotic and eukaryotic cells. Although we have not yet been able to decipher the molecular basis for this adaptation, HtpB stands out as an artful moonlighting chaperonin capable of serving several night jobs.

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9.1 Introduction

Since this book is centered on stress proteins and protein moonlighting, it would be redundant (and thus unnecessary) to introduce in this chapter the concept of moonlighting (as applied to protein biochemistry – described in Chap. 3), or the chaperonins as a family of essential stress proteins involved in protein folding (described in Chaps. 1 and 2). Instead, we will introduce here *Legionella pneumophila* (the pathogen) and the roles that its chaperonin 60, subsequently referred here as HtpB (for high temperature protein B), seems to play in pathogenesis. The multifunctional nature of HtpB has been recently discussed in a historical context (Garduño et al. 2011), but in this chapter, emphasis will be placed on those HtpB functions potentially relevant to the establishment of *L. pneumophila* in its host cells.

Legionella pneumophila is an intracellular, Gram-negative bacterial parasite of freshwater amoebae, and an accidental human pathogen able to infect alveolar macrophages and colonize the human lungs (reviewed by Newton et al. 2010). Human infection is often initiated by the inhalation of contaminated aerosol, which, in susceptible individuals, results in an atypical pneumonia known as Legionnaires' disease (Winn 1988).

The intracellular events ensuing the initial entry of *L. pneumophila* into host cells (i.e. amoebae or human macrophages) have been well studied at the cellular and molecular levels. These events include modifications to the normal organelle and vesicular trafficking of the host cell and the establishment of the *Legionella-containing* vacuole (LCV), a membrane-bound compartment suited for the intracellular replication of *L. pneumophila* (reviewed by Isberg et al. 2009; Newton et al. 2010). Prominent among these modifications are the recruitment of mitochondria and rough endoplasmic reticulum by the LCV, which has been hypothesized to be a mechanism of nutrient acquisition by *L. pneumophila*. The morphological sequence of LCV establishment shows a remarkable conservation in all host cells capable of supporting the intracellular growth of *L. pneumophila*, suggesting that the mechanisms of pathogenesis developed by *L. pneumophila* to infect freshwater amoebae, are applicable to the highly evolved human macrophage (reviewed by Al-Quadan et al. 2012). In this respect, a *Legionella* type IV secretion system (T4SS), known as the Dot/Icm system, remains as the primary virulence factor needed for LCV establishment in amoebae and macrophages (reviewed by Hubber and Roy 2010; Isberg et al. 2009; Newton et al. 2010). The Icm/Dot T4SS exhibits the most numerous and complex array of redundant secreted effectors in relation to any other T4SS described to date (reviewed by Ensminger and Isberg 2009; Luo 2012; Ninio and Roy 2007). The redundancy of the Dot/Icm secretion substrates explains why single genetic deletions of secreted virulence effectors are not associated with distinct phenotypes, or only result in partial virulence defects. This is in sharp contrast with mutations within the structural components of the Icm/Dot T4S apparatus, which usually result in a complete inability to grow intracellularly in amoeba, macrophages, and all other potential host cells that support the intracellular replication of *L. pneumophila*. It has been thus postulated that *L. pneumophila* uses its Dot/Icm

T4SS to target basic cellular processes common to most eukaryotic cells. In fact, the molecular mechanisms associated with some Icm/Dot effectors target basic vesicular and organelle trafficking processes that are highly conserved in eukaryotes (Al-Quadan and Abu Kwaik 2011; Franco et al. 2012; Haenssler and Isberg 2011; Neunuebel et al. 2012).

In addition to the Dot/Icm system, other virulence factors have been described, primarily adhesins and invasins that mediate binding to host cell surface receptors and the internalization of *L. pneumophila* by host cells. Among these, is the *Legionella* chaperonin HtpB, which we demonstrated binds to specific host cell surface receptors and is capable of mediating the internalization of HtpB-coated inert polystyrene microbeads by HeLa cells, which are epithelial cells regarded as non-phagocytic (Garduño et al. 1998b). Because antibiotic-treated *L. pneumophila* (incapable of *de novo* protein synthesis) can bind to and invade host cells (Horwitz and Silverstein 1983), and the conditioning of the LCV can begin within minutes after internalization (Roy et al. 1998), it has been proposed that the virulence factors involved in adherence and invasion are both pre-formed in infectious *L. pneumophila* cells and involved in the early establishment of the LCV. Based on this proposal, we have hypothesized that, as an invasion factor, HtpB indeed participates in the early establishment of the LCV. But for HtpB to play alternative roles in modifying host cell processes, it has to deviate from a conventional cytoplasmic location, and be strategically located outside of the bacterial cell cytoplasm.

Therefore, in the remaining sections of this chapter we will describe the experimental data that supports the localization of HtpB in extracytoplasmic bacterial compartments, as well as in several compartments of the host cell. Then, the experimental evidence that supports the functional diversity of HtpB in these various locations will be presented, speculating on the potential mechanisms by which HtpB could achieve its variety of functions as a bacterial extracytoplasmic moonlighting chaperonin.

9.2 The *Legionella* Chaperonin 60 Can Be Found in Extracytoplasmic Bacterial Locations and in Host Intracellular Compartments

Several independent studies provide evidence that HtpB can be found in extracytoplasmic bacterial locations and released into the LCV. Hoffman et al. (1990) reported that virulent legionellae suspended in Dulbecco-modified Eagle's medium (DMEM, a nutrient-rich defined medium commonly used for the culture of mammalian cells) had the ability to display HtpB on their cell surface, as determined by fluorescence microscopy using a primary HtpB-specific rabbit polyclonal antibody and a fluorescein-labeled anti-rabbit secondary antibody. In this same study, legionellae-infected HeLa cells showed an intense, diffuse labeling of LCVs by fluorescence microscopy using the same antibodies. Since labeling of bacterial cells

within LCVs would have produced a punctuate pattern of fluorescence, the diffuse labeling pattern observed suggested that HtpB was free in the LCV. The suggestion that HtpB accumulates in phagosomes (or LCVs) was also formulated by Blander and Horwitz (1993), based on unpublished immunogold labeling experiments. Garduño et al. (1998a) reported a series of detailed immunogold labeling experiments where ~58 % of the cell associated HtpB was found in association with the *L. pneumophila* cytoplasmic cell membrane, periplasm and outer membrane. These labeling results were compatible with previous biochemical fractionation studies by Gabay and Horwitz (1985), which led these investigators to consider HtpB as the major cytoplasmic membrane protein of *L. pneumophila*. Fractionation studies by Garduño et al. (2002) confirmed the association of HtpB with the outer membrane of *L. pneumophila* grown intracellularly in HeLa cells. The presence of HtpB in the *L. pneumophila* periplasm was also confirmed by the results of Galka et al. (2008) who found HtpB among the proteins present in outer membrane vesicles (OMVs). Virtually all Gram-negative bacteria have the ability to produce OMVs as exvaginations of their outer membranes, whose cargo is defined by periplasmic proteins (reviewed by Kulp and Kuehn 2010). Finally, the immunogold labeling results of Garduño et al. (1998a) clearly showed the presence of HtpB free in the lumen of LCVs, in legionellae-infected HeLa cells.

The mechanism by which HtpB is mobilized to bacterial extracytoplasmic locations, or released from the bacterial cell, is unknown. However, the fact that mutants with defects in the Dot/Icm T4SS accumulate HtpB in the periplasm, and have no surface-exposed HtpB (Chong et al. 2006), strongly suggest that T4S is responsible for translocating HtpB from the periplasm to the bacterial cell surface. The mechanism that places HtpB in the periplasm of *L. pneumophila* remains, nonetheless, unaccounted for.

To determine whether HtpB remains confined to the LCV's lumen, or whether it is capable of crossing the LCV membrane, we tagged HtpB with a C-terminal fusion with CyaA (adenylate cyclase from *Bordetella pertussis*). Because the adenylate cyclase activity of CyaA depends on the presence of calmodulin, and calmodulin is only found in the cytoplasm of mammalian cells, a statistically significant increase in cAMP levels in cells infected with *L. pneumophila* carrying the HtpB: CyaA fusion, indicated that HtpB reaches the cytoplasm of legionellae-infected cells (Nasrallah et al. 2011b). This finding defined a previously unknown compartment for HtpB, and opened up new possibilities for alternate HtpB functions. Translocated effectors from intracellular bacterial pathogens that replicate in a membrane-bound compartment (usually referred to as the pathogen-containing vacuole or inclusion) can be free in the cytoplasm of host cells, from where they can interact with soluble host cell molecules. In addition, translocated effectors could remain associated to the membrane of the pathogen-containing vacuole or inclusion, from where they can play a different role, e.g. recruiting host cell molecules (Cox et al. 2012; Weber et al. 2006). Immunogold labeling of legionellae-infected macrophages (Fernandez et al. 1996) had previously suggested (in agreement with the cAMP assay) that HtpB can be free in the host cell cytoplasm, but we deemed it important to determine whether HtpB could also be found in

association with the LCV membrane. For this, we purified phagosomes/LCVs from legionellae-infected cells and immunogold labeled them as intact, non-permeabilized structures, previous to being fixed and processed for conventional transmission electron microscopy. The method used to purify and label early *L. pneumophila* phagosomes is summarized in Fig. 9.1a. The experimental results that support the association of HtpB with the cytoplasmic face of the phagosomal/LCV membrane are shown in Fig. 9.1b-g.

In summary, HtpB has been localized to the *L. pneumophila*'s cytoplasm and cell envelope, and has been found free in the medium surrounding the bacterial cell (which, in legionellae-infected cells, becomes the lumen of the LCV), in the cytoplasmic side of the LCV membrane, and free in the cytoplasm of legionellae-infected cells.

9.3 Moonlighting Jobs of HtpB in Its Different Locations

The main role (regular day job) of chaperonins is in helping other proteins to fold properly in an ATP-dependent manner (as described in Chaps. 1 and 2). Protein folding is considered to define the essential nature of chaperonins, mainly because many of the substrates folded by chaperonins are proteins with essential metabolic or structural functions (Houry et al. 1999; Kerner et al. 2005). Although the protein folding ability of HtpB has not been as yet experimentally proven, we have shown that its encoding gene, *htpB*, is essential and cannot be deleted from the *L. pneumophila* genome (Chong et al. 2009; Nasrallah et al. 2011a). In addition, the *L. pneumophila* genome carries only one chaperonin gene copy, *htpB* (Nasrallah et al. 2011a). Therefore, it is reasonable to surmise that as the only *L. pneumophila* chaperonin 60 protein, HtpB has a regular day job as an essential folder of *Legionella* proteins. To perform this essential job HtpB must form a tetradecameric barrel structure that associates with an heptameric ring of co-chaperonin (the HtpA protein) that, in turn, acts as a lid to the barrel (reviewed by England et al. 2008; Horwich et al. 2007 and described in Chaps. 1 and 2). In addition, this multimeric complex should be located in the cytoplasm of *L. pneumophila*, where ATP and the protein substrates to be folded would be readily available. Besides its regular day job, we have found that HtpB is capable of keeping several alternate (moonlighting) jobs, which will be now discussed according to the locations where HtpB could be present.

9.3.1 HtpB in the Cytoplasm of L. pneumophila

The overexpression of *htpB* (alone) in *Escherichia coli* induces filamentation. Because in *E. coli* the recombinant HtpB (rHtpB) remains in the cytoplasm, filamentation must be an effect of cytoplasmic rHtpB, produced in the absence of the co-chaperonin HtpA, and therefore represent a protein folding-independent function of

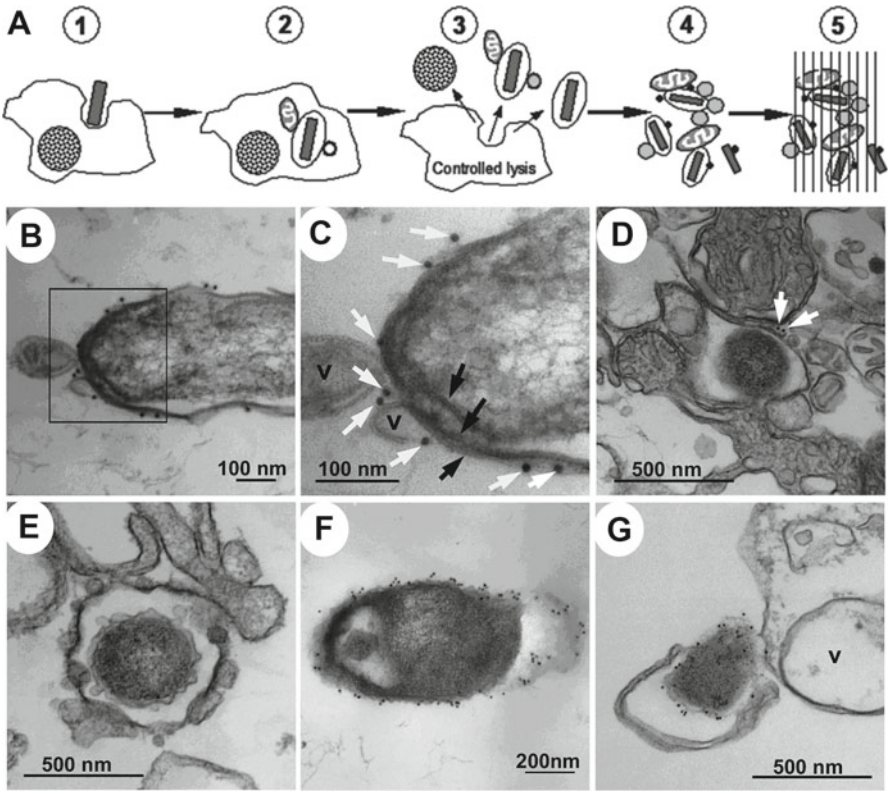

Fig. 9.1 HtpB is localized to the cytoplasmic side of LCVs. (**a**) Graphical depiction of the steps followed to isolate phagosomes, after the method of Chakraborty et al. (1994), and prepare them for immunogold labeling and transmission electron microscopy: *1* CHO cells or U937-derived human macrophages were incubated with *L. pneumophila* strain Lp02 at a bacteria-to-cell ratio of ~300. *2* Inoculated cells were incubated for 3 h to allow bacterial internalization and intracellular establishment. *3* Cells were then scraped, lysed by 12 passages through a 27-gauge needle, and the lysate was centrifuged at low speed to remove nuclei and unbroken cells. *4* Phagosomes were pelleted by centrifugation, washed and incubated overnight at 4 °C, with a rabbit anti HtpB polyclonal antibody (anti-HtpB Pab). A 'no primary antibody' control and a control labeled with a rabbit anti-*L. pneumophila* major outer membrane protein (MOMP) antibody (anti-MOMP) were included. Samples were then secondarily labeled with a goat anti-rabbit antibody conjugated with 10 nm gold spheres. *5* Labeled phagosomes were fixed in glutaraldehyde, and then processed for electron microscopy. Ultrathin sections were observed on a JEOL JEM 1230 transmission electron microscope, and images captured digitally. (**b**) Phagosome isolated from CHO cells labelled with anti-HtpB PAb, also shown in (**c**) at a higher magnification. Phagosomes isolated from U937 cells labelled with (**d**) anti-HtpB PAb or (**e**) anti-MOMP. Lp02 cells not fully contained in a phagosome (or free) and labeled with (**f**) anti-HtpB PAb or (**g**) anti-MOMP. *Black arrows* indicate phagosome or bacterial membranes; *white arrows* indicate immunogold staining on the cytoplasmic side of the phagosome. V, host vesicle

HtpB. Filamentation by overexpression of rHtpB has also been observed in *Bordetella pertussis* and in *L. pneumophila* (Allan 2002). It is important to note here that overexpression of the *B. pertussis* chaperonin (Cpn60) in *E. coli*, or the expression of the *E. coli* chaperonin (GroEL) in *B. pertussis*, *L. pneumophila*, and *E. coli* does not induce filamentation, suggesting that HtpB is somewhat unique in this respect. Heat shock of *L. pneumophila* (42 °C in a shaken broth culture) produces a high proportion of filamentous legionellae (C.C. Sze, Nanyang Technological University, Singapore, personal communication), but the same conditions (known to induce high levels of expression of a number of heat shock proteins, including chaperonins) do not result in a rod length increase in *B. pertussis* or *E. coli*.

The mechanism by which cytoplasmic HtpB induces bacterial filamentation is currently under investigation. However, we have hypothesized that this is an indirect effect accomplished through the interaction of HtpB with proteins involved in cell division and septum formation. Regardless of the mechanism involved, HtpB remains the first (and only) identified *L. pneumophila* protein implicated in filamentation, a process that seems to be important for both the survival of *L. pneumophila* in the environment (Piao et al. 2006) and the invasion of lung epithelial cells during human infections (Prashar et al. 2012).

9.3.2 *HtpB Associated with the Cytoplasmic Membrane of* L. pneumophila

As previously stated, HtpB was characterized as the major cytoplasmic membrane protein of *L. pneumophila* by Gabay and Horwitz (1985). However, from their studies, it is impossible to determine whether HtpB occurs as an integral membrane protein, or as a tightly associated (yet superficial) protein. If HtpB would insert itself into the cytoplasmic side of the inner membrane, it could act as a lipochaperonin, an activity originally described for GroEL oligomers in *E. coli* (Török et al. 1997). In addition, insertion of HtpB into the cytoplasmic membrane could, at least in part, explain how HtpB gets into the *L. pneumophila* periplasm. That is, HtpB could cross the inner bacterial membrane in a fashion similar to that described for cell-penetrating peptides (reviewed by Zorko and Langel 2005). Therefore, it is possible that the HtpB fraction associated with the cytoplasmic membrane of *L. pneumophila*, estimated to be 16 % of the immunogold-labeled epitopes by Garduño et al. (1998a), is in transit to the periplasm and eventually to the bacterial cell surface.

9.3.3 *HtpB in the Periplasm of* L. pneumophila

We have estimated that approximately 1 % of the cell associated HtpB in *L. pneumophila* is free in the periplasm and able to be extruded into the extracellular environment (and be detected by immunoblotting) upon osmotic shock (unpublished

results submitted for publication). We have hypothesized that the periplasmic HtpB fraction is in transit to the bacterial cell surface, or to be packaged into OMVs. However, either of these two outcomes would have functional implications for HtpB in different compartments. Thus, we have not ascribed to date any particular moonlighting job to the periplasmic fraction of HtpB.

9.3.4 *HtpB in the Outer Membrane of* L. pneumophila *and on the Bacterial Cell Surface*

Fractionation studies suggest that HtpB can be tightly associated with the *L. pneumophila* outer membrane (Garduño et al. 2002), but as discussed for the HtpB fraction associated with the cytoplasmic membrane, it is currently not known whether HtpB is an integral or just a superficial outer membrane protein.

Regardless of the nature of HtpB's association with the outer membrane, we have described a distinct role for the surface-exposed HtpB as a bacterial invasin that mediates the binding of *L. pneumophila* to, and its internalization by, HeLa cells (Garduño et al. 1998b). Because HeLa cells are epithelial, non-phagocytic cells, the process of invasion must be actively triggered by the interaction of HtpB with cell surface receptors. Experimental evidence for the involvement of HeLa cell surface receptors was directly provided by receptor down-modulation experiments with purified HtpB, and competition experiments between *L. pneumophila* and HtpB coated beads (Garduño et al. 1998b). However, we have now provided additional experimental evidence for the interaction of HtpB with a 70–76 kDa HeLa cell membrane protein. Figure 9.2 shows the various methods used to identify this HeLa cell membrane protein, and their corresponding results. However, the identity of this 70–76 kDa protein(s) remains to be determined.

Integrins (Long et al. 2003), Toll-like receptors (Nussbaum et al. 2006; Ohashi et al. 2000; Vabulas et al. 2001), and the cellular prion protein (Watarai et al. 2003) have been reported to act as receptors for different chaperonins. Moreover, several chaperonins (discussed in other chapters of this book) have been documented to interact with mammalian cell surface receptors and act as immuno-modulatory signalling molecules (Bethke et al. 2002, and reviewed by Henderson 2010). However, it is not known at this time whether or not HtpB can interact with the aforementioned chaperonin receptors. What is known is that the HtpB exposed on the surface of *L. pneumophila* (or bound to the surface of inert high-density polystyrene microbeads) is capable of eliciting immuno-modulatory host cell responses. For instance, HtpB interacts with putative macrophage surface receptors and triggers a signalling cascade that involves the activation of protein kinase C, and results in the production of interleukin 1 beta (IL-1β) (Retzlaff et al. 1996). This IL-1β response was not due to contaminating LPS, because the response was greatly reduced by heat inactivation, a treatment that would affect HtpB function, but not LPS-induced effects (Retzlaff et al. 1996).

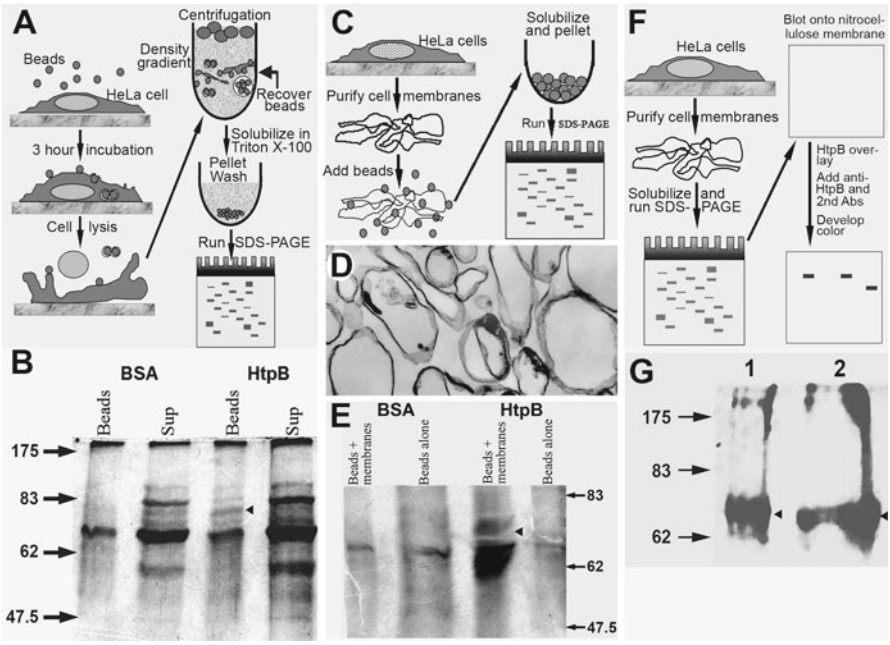

Fig. 9.2 HtpB interacts with a HeLa cell membrane protein of 70–76 kDa. (**a**) In the first experiment, cells were allowed to interact with HtpB- or BSA-coated beads for 3 h, and then were lysed in deionized water containing 0.01 % Triton X-100. The lysate was separated in a Percoll continuous density gradient by centrifugation, and the band containing beads was recovered. Beads were then treated with 0.1 % Triton X-100, and subsequently pelleted to separate the beads from the Triton X-100 supernatant. Beads were washed to eliminate peripherally associated proteins not tightly bound to the protein coated beads. Both beads and supernatants were then prepared for SDS-PAGE. (**b**) Silver stained gel showing the results from the first experiment in *panel A*. *Arrows and numbers* on the *left* indicate the position of the corresponding molecular mass standards given in kDa, and each lane is labeled according to the sample contained. A unique protein band of ~76 kDa (marked with the *black triangle*) was identified in association with the HtpB-coated beads. (**c**) In a second experiment, plasma cell membranes were purified from lysed HeLa cells following the method of Atkinson and Summers (1971). The purified membranes were then allowed to interact with HtpB- or BSA-coated beads for 1 h. Membranes were solubilized in 0.1 % Triton X-100, and beads were then pelleted and prepared for SDS-PAGE. (**d**) Electron micrograph of an ultrathin section of the purified membranes, showing that the preparation was very clean. (**e**) Silver stained gel showing the results from the second experiment in *panel C*. Gel is labeled as per indicated in (**b**). A single protein band of ~73 kDa (marked with the *black triangle*) was identified in association with the HtpB-coated beads that interacted with the purified membranes. (**f**) In a third experiment, a preparation of HeLa cell membranes was solubilized in 0.1 % Triton X-100 and separated by SDS-PAGE. The separated proteins were then blotted onto a nitrocellulose membrane and overlaid with purified HtpB. The overlay was then washed and developed with an anti-HtpB polyclonal antibody and a secondary antibody conjugated with alkaline phosphatase. (**g**) Immunoblot showing two different membrane preparations (*1* and *2*) overlaid with HtpB. The *black triangles* indicate single protein bands of ~70 kDa to which the overlaid HtpB was bound

Finally, the engagement of host cell surface receptors by the HtpB exposed on the surface of *L. pneumophila*, can initiate other signalling cascades across the host plasma cell membrane, or across the early phagosome and LCV membranes. We have hypothesized that this type of signalling is involved in the HtpB-mediated modification of the host cell actin cytoskeleton, as it will be discussed below (Sects. 9.3.5 and 9.3.6, where we present the experimental evidence that supports this HtpB role).

9.3.5 HtpB in the Lumen of LCVs

L. pneumophila abundantly releases HtpB into the lumen of LCVs. It is not clear why this is so, mainly because the released chaperonin cannot protect *L. pneumophila* from the stress imposed by host cell defensive responses. We have hypothesized that the HtpB found inside LCVs is meant to interact with the luminal side of the LCV membrane, where it can signal through the interaction with membrane integral proteins. The HtpB that is still associated with the bacterial cell surface could also signal in this manner. In addition, it is reasonable to surmise that it is from this location (the LCV lumen) that HtpB gets translocated across the LCV membrane to reach the cytoplasmic side of the membrane, or be released into the host cell cytoplasm. This would be particularly true if *L. pneumophila* produces OMVs in the LCV, which could then fuse with the LCV membrane and expose their cargo on the cytoplasmic side of the membrane.

Evidence for the proposed signalling across the LCV membrane has been primarily provided by our experiments with mammalian cells exposed to HtpB-coated microbeads, which would obviously be secretion incompetent and unable to produce OMVs. These microbeads are internalised into tight phagosomes in HeLa cells (Garduño et al. 1998b), CHO cells, and U937-derived human macrophages (Chong et al. 2009). While still bound to the host cell plasma membrane, or contained in early phagosomes, HtpB-coated beads attract mitochondria and induce changes in the actin cytoskeleton (Chong et al. 2009).

After staining with the fluorescent stain Mitotracker™, CHO cells show a nice array of well-defined individual mitochondria distributed as a cloud around the cell cytoplasm. In spite of mitochondria occupying space all throughout the cell, we were able to quantify a clear difference in the ability of HtpB-coated beads to associate with mitochondria, in relation to a number of control beads including GroEL-coated beads (Chong et al. 2009). Transmission electron microscopy confirmed that phagosomes containing HtpB-coated beads tightly interact with mitochondria, to the point of causing the mitochondrial envelope to deform (Chong et al. 2009). Occasionally, it is possible to see an electron dense material bridging the phagosomal membrane and the mitochondrial outer membrane, as shown in Fig. 9.3. In fact, phagosomes containing *L. pneumophila* or HtpB-coated beads are often purified with tightly attached mitochondria (e.g. Fig. 9.1d, and Chong et al. 2009), suggesting that the bridging between the phagosomal and mitochondrial membranes is

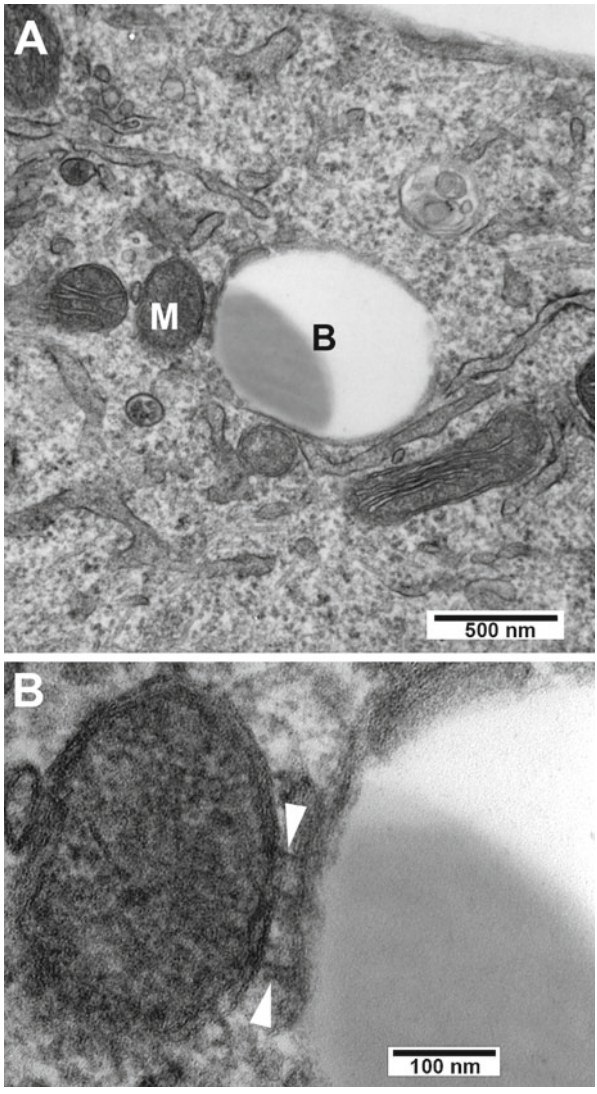

Fig. 9.3 Electron micrographs (**a** and **b**) showing the presence of an electron dense material (*arrowheads*) bridging the membrane of a phagosome containing an HtpB-coated bead (marked with a *B*) and the mitochondrion marked with an *M*. (**b**) shows a high magnification close-up of the area of interest between the phagosome and mitochondrion

strong enough to resist the purification process. An alternative explanation to the signalling hypothesis leading to mitochondrial recruitment is that some HtpB molecules accrued onto the bead's surface, but not covalently bound to the bead's surface, could integrate into the phagosomal membrane and pass on to the cytoplasmic side of the membrane. From this location, which will be further discussed below (Sect. 9.3.6), HtpB could directly interact with mitochondria.

The same possibilities discussed above for mitochondria recruitment could also apply to the HtpB-mediated effect on the host cell actin cytoskeleton, which involves a rearrangement of polymerised actin. This rearrangement causes stress fibers to

disappear, and peripheral bundles of actin fibers to appear, an effect we have named 'framing' (Chong et al. 2009). In this case, however, we have shown that exactly the same framing effect is observed when HtpB is expressed as a recombinant protein (alone – in the absence of HtpA) in CHO cells (Chong et al. 2009). That is, HtpB elicits the same effect on the mammalian actin cytoskeleton when presented from without (the lumen of LCV) or from within (the host cell cytoplasm). We have hypothesized that there must be a common signalling intermediate that can be encountered from either side of the phagosomal membrane and that HtpB and this putative signalling intermediate interact right at the phagosomal membrane as integral proteins.

9.3.6 HtpB on the Cytoplasmic Surface of the LCV Membrane

As a protein associated with the cytoplasmic surface of the LCV membrane, HtpB would be in a position to effectively interact with and recruit host cytoplasmic molecules. In legionellae-infected cells, LCVs would contain live bacteria capable of translocating Icm/Dot effectors into the host cell cytoplasm. We have thus proposed that in legionellae-infected cells the translocation of HtpB across the LCV membrane is a consequence of T4S, without HtpB necessarily being a T4SS substrate. That is, we have speculated that in legionellae-infected cells the permeability of the LCV membrane is somewhat compromised as a result of the T4S activity of *L. pneumophila*, and that the HtpB in the LCV lumen would have an opportunity to interact with the compromised membrane and pass onto the cytoplasmic side of the LCV membrane, or further into the cytoplasm of the host cell (Chong et al. 2009). Of course, this mechanism would not apply to internalised HtpB-coated beads.

We believe that the HtpB that remains associated with the cytoplasmic side of the LCV membrane is involved in the recruitment of mitochondria to the LCV and in delaying phagosome-lysosome fusion (Chong et al. 2009). Finally, in this location, HtpB could recruit other, as yet unidentified, host factors necessary for the conditioning of the LCV, or for nutrient acquisition by the contained *L. pneumophila* cells.

9.3.7 HtpB as a Free Soluble Factor in the Host Cell Cytoplasm

To address the potential effects that HtpB could have as a free soluble factor in the host cell cytoplasm, we expressed it as a recombinant protein in the cytoplasm of the yeast *Saccharomyces cerevisiae*, which constitutes an excellent, genetically tractable, model eukaryote. In yeast, HtpB was capable of inducing pseudohyphal growth (Nasrallah et al. 2011b), for which it required a functional Ras2-mediated signaling cascade. That is, yeast mutants lacking Ras2, or downstream effectors of

the Ras2 signaling cascade were unable to form pseudohyphae upon expression of rHtpB (Riveroll 2005). After conducting a series of yeast two-hybrid protein-protein interaction experiments we identified a specific interaction between HtpB and S-adenosyl methionine decarboxylase (SAMDC), an enzyme involved in the synthesis of spermidine and spermine in eukaryotic cells. Overexpression of SAMDC, resulting from an increase in gene copy number, also induced pseudohyphal growth in *S. cerevisiae*, strongly suggesting that the mechanism by which HtpB induces pseudohyphal growth in yeast involves an interaction with SAMDC and modulation of polyamine levels. In fact, polyamines have been reported to regulate the dimorphic (yeast-to-hyphae) transition in a number of fungi species (Herrero et al. 1999; Jin et al. 2002).

The interaction of HtpB with mammalian and amoebal SAMDC has been confirmed in a series of dot-blot and overlaying assays with purified HtpB (Nasrallah et al. 2011b). Based on the fact that the intracellular growth of *L. pneumophila* is enhanced by the addition of exogenous polyamines and inhibited by the pharmacological inhibition of SAMDC activity (Nasrallah et al. 2011b), we have speculated that one of the HtpB contributions to the intracellular establishment of *L. pneumophila* is to induce the host cell to produce increased levels of spermidine and spermine through the interaction with SAMDC. In addition, it seems reasonable to surmise that increased polyamine levels could have a secondary effect on intracellular signaling, and thus we hypothesize that a molecular link exists between HtpB, polyamine levels and Ras signaling.

Other potential host proteins that interact with HtpB, found through our yeast two-hybrid screens, are the mitochondrial co-chaperonin (mtHsp10), and a mammalian Merlin-associated protein. The interaction between HtpB and mtHsp10 is not surprising, and could in fact be speculated to be meaningful to the recruitment of mitochondria to the LCV. That is, HtpB associated with the cytoplasmic side of the LCV membrane could interact with mtHsp10 associated with the mitochondrial surface. It should be remembered here that mtHsp10 is one of the many mitochondrial proteins synthesized in the eukaryotic cell cytoplasm that are subsequently imported into mitochondria. Therefore, mtHsp10 must interact with the protein translocation complex of the mitochondrial outer membrane, which displays protein-recognizing receptors on the mitochondrial surface (reviewed by Becker et al. 2012; Schmidt et al. 2010). It should be thus possible to find mtHsp10 attached to the mitochondrial surface, which would be available to interact with HtpB.

Finally, Merlin is a tumor suppressor factor that belongs to the Ezrin-Radixin-Moesin family of proteins, which interact with the actin cytoskeleton (McClatchey and Fehon 2009; Xu and Gutmann 1998). Merlin, itself, interacts with the tubulin and actin cytoskeletons and associates with a number of partner proteins, and HtpB seems to interact with one of these partners. It remains to be experimentally determined whether the HtpB fraction that is free in the host cell cytoplasm, or associated with the phagosomal membrane, needs to interact with this Merlin-associated protein to cause framing. We have also recently determined that HtpB interacts with actin (Fig. 9.4). However, the *E. coli* GroEL chaperonin showed the same ability as HtpB to interact with actin (Fig. 9.4). Since GroEL-coated beads and recombinant

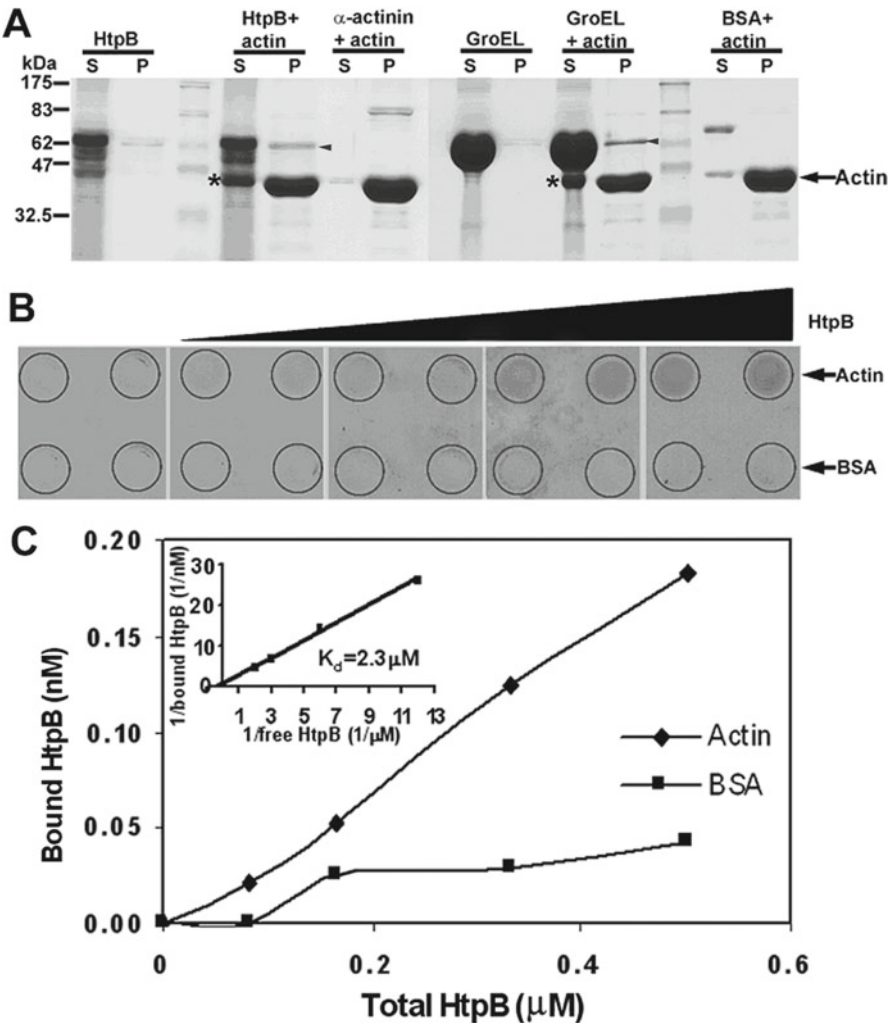

Fig. 9.4 HtpB and GroEL bind to soluble and polymerized actin. (**a**) Results from a co-sedimentation assay conducted according to the standard protocol provided by Cytoskeleton Inc. F-actin (18 μM) was incubated with HtpB (17 μM), α-actinin (1 μM), GroEL (22 μM), or BSA (1 μM). Supernatants (S) and pellets (P) resulting from a 100,000 × *g* ultracentrifugation of the samples were analyzed by SDS-PAGE and stained by Coomassie Blue. Molecular weight markers are indicated on the *left*. Soluble actin (G-actin) polymerizes spontaneously and can be sedimented by ultracentrifugation. Proteins that interact with polymerized actin (F-actin) will co-sediment and appear in the pellet fraction. *Arrowheads* indicate the presence of HtpB or GroEL in the pellet fractions. If spontaneous actin polymerization is inhibited (likely through binding to G-actin), actin will remain in the supernatant fraction. *Asterisks* indicate actin present in supernatant fractions; *arrow* indicates the expected position of the actin band. (**b**) HtpB binds to F-actin in an overlay assay. F-actin (2 μg per dot) and BSA (2 μg per dot) were immobilized onto a nitrocellulose membrane (*circular outlines*). Pieces of the dot-blotted membrane were floated in PBS containing different concentrations of HtpB. HtpB binding was detected by immunoblotting with a *L. pneumophila* HtpB-specific monoclonal antibody (Helsel et al. 1988). (**c**) Densitometric quantitation of HtpB binding to immobilized F-actin and BSA as per (**b**). The dissociation constant (K_d) of the HtpB:F-actin interaction was extrapolated from the Lineweaver-Burke plot (*inset*). Actin, α-actinin (positive control for interaction with polymerized actin) and BSA (negative control for interaction with actin) were from Cytoskeleton Inc., GroEL was from Stressgen Biotechnologies Corp., and HtpB was purified in-house (Chong et al. 2009)

GroEL expressed in the cytoplasm of CHO cells was not competent at inducing framing (Chong et al. 2009), we concluded that the mechanism by which HtpB induces framing does not involve a direct interaction with actin.

9.4 Conclusion

HtpB in the bacterial cytoplasm seems to meet the essential protein folding needs of *L. pneumophila*, defining the conventional day job for HtpB. However, it is the several moonlighting night jobs of HtpB that make this chaperonin so unique. We hope to have convincingly presented the experimental evidence that clearly points at HtpB as a moonlighting chaperonin that plays distinct roles according to its location in the bacterial cell, or in the *L. pneumophila*-infected cell. These alternate roles are played by HtpB in the absence of its cognate co-chaperonin HtpA, and might not require the conventional oligomerization of HtpB into a tetradecameric barrel structure. The road to discovery of potentially new moonlighting HtpB jobs and the elucidation of the molecular mechanisms behind those already established, is now wide open. Our immediate plan is to exploit the functional differences between HtpB and GroEL to define (through a detailed analysis of amino acid sequences) motifs and domains responsible for the moonlighting nature of HtpB. In this respect, we would like to invite investigators within the field of stress proteins in general, and chaperonins in particular, to take advantage of our experimental models in an attempt to advance our understanding of both the biology of chaperonins and their contributions as ancient virulence factors.

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Chapter 10
Chaperonin 60.1 of the *Chlamydiae* (cHSP60) as a Major Virulence Determinant

Aruna Mittal and Rajneesh Jha

Abstract *Chlamydiae* are Gram-negative intracellular obligate bacterial pathogens causing major health problems. *Chlamydia trachomatis* is the most common agent of bacterial urogenital infections responsible for cervicitis, ectopic pregnancy, tubal infertility, and pelvic inflammatory disease (PID) in women. Other than genital complications, *C. trachomatis* is a major cause of trachoma, the leading cause of preventable blindness in humans. *Chlamydia pneumoniae* causes respiratory disease and has been associated with asthma and atherosclerosis. The persistent infection of *Chlamydia* modulates the immune system of the host for its own survival, which remains a problem, and often leads to immunopathogenesis. The chronic infections not only exhibit highly unusual forms of intracellular *Chlamydiae*, but also display induction of Chlamydial Heat Shock Protein (cHSP) 60 and reduction in the expression of other chlamydial antigens. cHSP60 has been identified as strong immunodominant antigen and target of both humoral and cell-mediated immunity (CMI) responses. The cHSP60 released from the *Chlamydia* infected cells is capable of eliciting potent localized proinflammatory immune response resulting in the production of antibodies against cHSP60 and other inflammatory mediators which makes them one of the major virulence determinants of *Chlamydiae.* It is established that Chlamydia has three genes encoding chaperonin 60 paralogues, however all of our information on the chaperonin 60 protein of Chlamydia comes from chaperonin 60.1. This chapter is focused on the moonlighting action of the chlamydial chaperonin 60 protein, cHSP60.1.

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10.1 Introduction

Chlamydiae are Gram-negative, eubacteria and obligate intracellular parasite in eukaryotic cells, which thrive in a host derived membrane bound vacuole termed an "inclusion" (Abdelrahman and Belland 2005). The family *Chlamydiaceae* comprises the known human chlamydial pathogens; mainly *C. trachomatis and C. pneumoniae. C. trachomatis* infection causes, most commonly, bacterial sexually transmitted disease (known as chlamydia) and infection of the eyes (trachoma) (Gerbase et al. 1998). *C. pneumoniae* is one of the most studied chronic pathogens which has been associated with community-acquired pneumonia, bronchitis and progression of cardiovascular disease (CVD) (Watson and Alp 2008). The genome size of *C. trachomatis* encodes for an estimated 900 proteins while that of *C. pneumoniae* has around 1,100 open reading frames (ORFs) (Subtil and Dautry-Varsat 2004). The persistent infection of both of these chlamydial species exhibit a highly unusual form of intracellular *Chlamydiae*, which also display induction of cHSP60 and reduction in the expression of other chlamydial antigens, like major outer membrane protein (MOMP) and lipopolysaccharide (LPS) (Beatty et al. 1994). In *Chlamydiae*, the 60-kDa cHSP60, or GroEL (Ct110), and the 10-kDa cHSP10, or GroES (Ct111), are genetically linked as they are encoded by genes arranged on the bicistronic groESL operon (Morrison et al. 1990). These two proteins bind to each other and prevent incorrect folding and denaturation, thus *Chlamydiae are* able to survive and persist in host cells and this may be maximized by higher expression of cHSP60 and cHSP10 (Karunakaran et al. 2003). Among a panel of recombinant *C. trachomatis* proteins, cHSP60 has been identified as a major virulence determinant, being a strong target of both humoral and CMI responses (Follmann et al. 2008). Also, cHSP60 is the second most abundant protein in cell lysates of this organism and is loosely associated with the cell surface.

Chlamydia reproduction involves two separate developmental stages. In the extracellular environment the bacteria exist in a transit form called an elementary body (EB). Within the host cell the organism exists in a reproductive form called the reticulate body (RB). The presence of cHSP60 in both the EB and RB phases of the bi-phasic life-cycle of *Chlamydiae* makes it more dispensable to release into the extracellular milieu during the persistence phase of this organism (Linhares and Witkin 2010). The cHSP60 released from the *Chlamydia* infected cells is capable of eliciting potent localized pro-inflammatory immune responses. Chronic or intermittent release of cHSP60 into the extracellular milieu results in the production of antibodies against cHSP60, and the generation of other inflammatory mediators often remains a problem which leads to immunopathogenesis (Kimani et al. 1996). The consequence of *Chlamydial* infection depends on an intricate balance between secreted cytokines by the lymphocytes and its interaction with host cells. It is well known that *C. trachomatis* modulates the immune system of the host for its own survival and induces persistent infections (Golden et al. 2000; Joyner et al. 2002). The single acute infection does not cause severe pathology associated with chlamydial disease but the recurrent infections are

responsible for persistent inflammation (Paavonen and Lehtinen 1996). Typically, in *Chlamydiae* infections, the T-helper (Th) 1 cell product, interferon (IFN)-γ, is the most important factor in host defense while the Th-2 cell activation product, interleukin (IL)-10 has been linked with disease susceptibility (Beatty et al. 1993). Therefore, induction of Th-1 or Th-2 type cell responses due to persistent insult of virulence factor like cHSP60 is an important determinant of chlamydial disease pathogenesis.

10.2 Structure and Homology of cHSP60

The sequence of cHSP60 from the *Chlamydia* genome database is highly homologous and conserved throughout evolution. It belongs to a group of chaperone proteins ranging in molecular weight from 15 to 110 kDa which are produced in response to infection, stress, inflammation and other cellular insults (Engel et al. 1990 – see also Chaps. 1 and 2 for further discussion of molecular chaperones). cHSP60 exhibit greater than 80 % homology between *Chlamydia* spp., 60 % identity with other bacteria and 50 % homology with human HSP60 (hHSP60) and also exhibits immuno-regulatory properties primarily by inducing pro-inflammatory responses (Zugel and Kaufmann 1999). Interestingly, *cHSP60* was duplicated at the origin of the *Chlamydiae* lineage presenting three distinct molecular chaperones, namely the original protein cHSP60-1 (Ct110), and its paralogous proteins cHSP60-2 (Ct604) and cHSP60-3 (Ct755) (Karunakaran et al. 2003), but cHSP60-1 or cHSP60 was found to be more abundant and more immunodominant than the other two proteins (McNally and Fares 2007). Further information on the evolution of the chaperonin 60 protein is detailed in Chap. 7. The transcription of the cHSP60 gene in *C. trachomatis* has been shown to be immediately induced in infected cells exposed to high temperatures (45 °C), and the expression levels of cHSP60 and its co-chaperone cHSP10 increase under heat-stress conditions but only the cHSP60 complements its function in thermo-sensitive mutation in HeLa cells under heat-stress conditions (Karunakaran et al. 2003).

Since, cHSPs are phylogenetically conserved, and have high sequence homology, the immune response against epitopes within the region of cHSP60 may elicit an autoimmune response due to cross-reactive epitopes of the hHSP60. Also, the homology between bacterial and hHSP60 and the fact that hHSP60 may be increased locally at the site of infection, may indicate a role for HSP60 autoimmunity in the immunopathology of chlamydial infections (Jones et al. 1993). The pair-wise amino-acid sequence alignment of hHSP60 and cHSP60 showed four epitopes with 100 % identity and other 13 epitopes with identities varying from 33 to 75 % (Campanella et al. 2009). The comparison between the three-dimensional structure of cHSP60 and hHSP60 shows the position of the four epitopes with 100 % homology (Fig. 10.1). Also, 7 of 13 B-cell peptide epitopes recognized by human anti-sera exhibited cross-reactive antibody binding to homologous peptide sequences in hHSP60. Such self-reactive B-cell immunity to HSP60 may

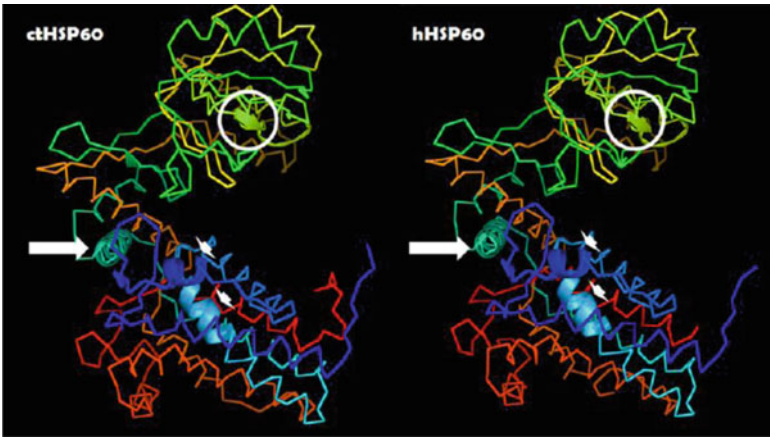

Fig. 10.1 Computed structures of the human and chlamydial HSP60 proteins showing four epitopes in which the sequence in the chlamydial protein is identical to that in the human protein (Adapted from Cappello et al. 2009)

contribute to chlamydial disease pathogenesis (Yi et al. 1993). The sensitization to hHSP60 is associated with humoral immune response to conserved epitope of the cHSP60 in patients with PID and there is also serologic evidence of exposure to *Chlamydiae*. This suggests that an autoimmune response to hHSP60 may arise as a consequence of a *C. trachomatis* upper genital tract infection in those women who develop sensitivity to cHSP60 epitopes that cross-react with epitopes of the hHSP60 (Domeika et al. 1998).

10.3 *C. trachomatis* Heat Shock Protein 60 and Female Reproductive Immunopathology

Several studies in women have identified a relationship between titres of serum immunoglobulin G (IgG) against cHSP60 with chronic infection, salpingeal fibrosis, ectopic pregnancy, PID and tubal infertility (Domeika et al. 1998; Toye et al. 1993; Ondondo et al. 2009). It is reported that because of the molecular mimicry and chronic antigenic stimulation, antibodies to cHSP60 in women with chlamydial-related pathology also react with hHSP60 (Yi et al. 1993; Sziller et al. 1998). This eventually leads to loss of tolerance and the generation of immunity to conserved amino acid sequences that are also present in the homologous hHSP60. Moreover, high levels of anti-cHSP60 antibodies play a pivotal role in *C. trachomatis-associated* immune mediated inflammation, leading to PID, fallopian tube obstruction and ectopic pregnancy (Brunham and Peeling 1994; Paavonen 1996). Also, a significant association has been found between anti-chlamydial antibodies in serum and antibodies to hHSP60 in follicular fluid

of infertile women (Cortinas et al. 2004). The persistent inflammation of the upper genital tract leads to low implantation rate as hHSP60 is expressed in early stage of embryogenesis and a cross-reacting antibody may induce destruction of the embryo (Jakus et al. 2008). However, one of the studies confirms the association between tubal factor infertility (TFI) and antibodies to cHSP60, whereas no connection was observed between TFI and antibodies to hHSP60 (Hjelholt et al. 2011). Thus, detection of IgG antibodies to cHSP60 indicates persistence and severity of *C. trachomatis* in the upper genital tract resulting from chronic inflammatory sequelae like PID and infertility (Dutta et al. 2007, 2008).

The availability of immunogenic peptide sequences and recombinant cHSP60 has facilitated detailed serological studies among *C. trachomatis*-infected women. Studies of antibodies to the cHSP60 antigen among women with different sequelae of chlamydial infection showed reliable results in seroprevalence to the cHSP60 antibodies (Brunham et al. 1992; Toye et al. 1993). Furthermore, among women with laparoscopically visualized PID, those with high antibody titers to cHSP60 have had significantly more severe inflammatory manifestations (Brunham et al. 1985). The women with antibody responses to cHSP60, among those with the severe forms of *C. trachomatis* infection, may induce tissue damaging immune responses. The distinctive immunodominance of cHSP60 as a serologic antigen is observed in seroepidemiologic studies of individuals with complicated chlamydial infection (Brunham and Peeling 1994). However, immune sensitization to HSPs probably requires prolonged exposure at elevated concentrations; confirming women with recurrent infections have significant humoral and CMI responses towards cHSP60 (Witkin et al. 1998). It is clear that low level infections can produce relatively low quantities of cHSP60 and that women who suffer from infertility and ectopic pregnancy often have high levels of antibody to cHSP60 (Wagar et al. 1990; Toye et al. 1993). This is also true with the transcript level of cHSP60 which is significantly higher in *Chlamydia*-infected infertile than fertile women (Jha et al. 2009).

The treatment of *Chlamydia* infected cells with IFN-γ inhibits chlamydial growth, while permitting expression of cHSP60 and down regulation of MOMP expression. This can be correlated in chronically or acutely infected individuals; continued cHSP60 expression secondary to the action of IFN-γ produced by CMI might ultimately drive chronic inflammatory responses associated with fibrosis and scarring. Furthermore, IFN-γ response to cHSP60 are significantly associated with reduced risk of infection (Cohen et al. 2005), whereas repeated chlamydial infection or PID are associated with cHSP60-specific IFN-γ response (Debattista et al. 2002). Moreover, systemic and mucosal IFN-γ responses are correlated with preferential systemic targeting of $CD4^+$ T-cells and cHSP60 response is mainly $CD4^+$ T-cell mediated which follows distinct Th1 and Th2 pathways (Ondondo et al. 2009). Studies have shown that although antibodies help in clearance of infection, their major role is in the augmentation of Th1 activation. The occurrence of IgG and IgA antibodies to MOMP is reported to be significantly higher in patients with primary chlamydial infections while the prevalence of antibodies against cHSP60 is higher in those with recurrent infections. This trend is similar as the proliferative response

of cervical lymphocytes induced by MOMP is significantly higher in patients with primary infections; however proliferative responses to cHSP60 was found more commonly in patients with recurrent infections (Agrawal et al. 2007). In addition, cHSP60 affects mucosal immune response by secreting IFN-γ, TNF-α and IL-10 from mononuclear cells which contributes to immunopathogenesis in women leading to fertility-related disorders (Srivastava et al. 2008).

10.4 *C. pneumoniae* Heat Shock Protein 60 and Its Contribution in Atherosclerosis

Chlamydia pneumoniae HSP60 (cHSP60) has been detected in atherosclerotic plaques and serum antibodies to cHSP60 strongly correlated with the ability of *C. pneumoniae* to be detected in plaques (Kol et al. 1998). cHSP60 produced by *C. pneumoniae* can induce an autoimmune response due to high antigenic homology and cross-reactivity of antibodies with hHSP60 (Huittinen et al. 2001). Also, hHSP60 antibody levels correlated with the presence of *C. pneumoniae* antigen in atheromas (Fong et al. 2002). However, Esposito and coworkers. contradicted this finding and suggested that the hHSP60 antibody response is not related to *C. pneumoniae* infection and subsequent CVD (Esposito et al. 2011). Of course, there are studies suggesting that the host immune response to cHSP60 may lead to autoimmunity to hHSP60 which triggers hypersensitivity and immunopathology, consequently, to the development of atherosclerosis (Milioti et al. 2008).

Several studies have suggested that cHSP60 may act as a possible mediator of target cell activation (Krull et al. 2006; Bulut et al. 2002). Additionally, the mitogenic effect of endogenous hHSP60 in *C. pneumoniae* infection also contributes to vascular smooth muscle cell (VSMC) proliferation during the stages of atherogenesis and lesion formation (Hirono et al. 2003). P44/p42 mitogen-activated protein kinase (MAPK) activation is a crucial event in *C. pneumonia*- and cHSP60–induced human VSMC proliferation through the involvement of toll-like receptor (TLR) 4 which does not necessarily require an active infection to induce proliferation (Sasu et al. 2001). Furthermore, it can contribute to low-density lipoprotein (LDL) oxidation; another central pathological mechanism in atherogenesis by mononuclear cells exposed to *C. pneumoniae* (Kalayoglu et al. 1999a). Lipid-loaded macrophages (foam cells) are a major cellular component of atherosclerotic lesions and chronic infection of foam cells with *C. pneumoniae* could exacerbate the inflammatory response which is associated with the initiation and progression of atherosclerotic lesions (Blessing et al. 2002). Exposure of macrophages to cHSP60 followed by LDL-oxidation causes a marked increase in the number of foam cells and accumulation of cholesterol esters (Kalayoglu et al. 1999b). The oxidized LDL promotes the mitogenic actions of *C. pneumoniae* in the vasculature, accompanied by activation of ERK1/2 and an upregulation of HSP60 that generates atherogenesis (Chahine et al. 2011).

cHSP60 is a highly expressed chlamydial protein and is a known stimulator of macrophages and vascular endothelial cells which induces the expression of TNF-α and metalloproteases further activating adhesion molecules in atherosclerotic plaque macrophages, indicating *Chlamydia*-dependent pro-atherogenic processes (Kol et al. 1998). This event preferentially polarizes a Th1 inflammatory response through maturation of monocyte-derived dendritic cells which contribute to T-cell derived immunopathology of atherosclerosis (Ausiello et al. 2006). This has been implicated in macrophage-rich areas of human atherosclerotic plaques and maintains innate immune responses in vascular endothelial cells by activating nuclear factor (NF)-kB via TLR4 in a myeloid differentiation factor 88 (MyD88)-dependent pathway (Bulut et al. 2002). Further, cHSP60 positive atheromatous tissue exhibited higher expression of TLR2/4, IL-8, monocyte chemoattractant protein-1 (MCP-1), cell adhesion molecules (ICAM1 & VCAM1), extracellular signal-regulated protein kinases (ERK1/2) and NF-kB in comparison to cHSP60 negative coronary artery disease (CAD) patients. This suggests that cHSP60 activates NF-kB via TLRs and the resulting expression of MCP-1 and other adhesion molecules by *C. pneumoniae* in human endothelial cells may participate in monocyte/macrophage recruitment in atheromatous plaque in CAD patients during the early stages of atherogenesis (Jha et al. 2011a).

10.5 Chlamydial Heat Shock Protein 60 and Host Cell Survival

The survival or persistence of *Chlamydia* in infected cells is regulated by many factors including responses generated by the host. The cHSP60 is known to be associated with the outer membrane and can be exposed to the extracellular milieu to induce immune activation at the site of infection and this is relevant to immune responses generated by *Chlamydia*-induced cell death. This may have impact on innate immune responses wherein pro-inflammatory mediators from dying cells are released which may promote long-term tissue damage. Further, cHSP60 induces apoptosis in primary trophoblasts and the JEG3 cell line through a caspase dependent pathway. This further suggests a contribution of cHSP60 in the pathogenesis for poor fertility and pregnancy outcome in women with persistent *C. trachomatis* infection (Equils et al. 2006).

Chlamydia spp., produces large quantities of cHSP60, which is reported to be localized in the cytoplasm and in the outer membrane of the host cell during persistent infection (Bavoil et al. 1990). Also, cHSP60 has an amino acidic sequence similar to its human counterpart, with the exclusion of the mitochondrial localization signal. It may be possible to correlate some cellular responses during *Chlamydia* spp. infections with the accumulation of cHSP60 in the cytoplasm of the host cell and its possible association with caspase-3 activation. In fact, cHSP60 has the ability to induce apoptosis of cervical epithelial cells with an upregulation of caspase-1,

3, 8 and 9. Binding of cHSP60 to TLR mediates, through an adapter molecule, MyD88, via a pathway involving Fas-associated death domain (FAAD) protein and caspase-8, the initiator caspase associated with Fas-mediated apoptosis and further activation of NF-kB. Indeed, cHSP60 signals apoptosis through TLR which in turn induces the synthesis of the precursor of the pro-inflammatory cytokine; IL-1β. Interestingly, binding of cHSPs to TLR also activates caspase-1, resulting in proteolysis and secretion of mature IL-1β (Aliprantis et al. 2000). Caspase-1 contains an active site cysteine necessary for cleavage of cytokine precursor pro-IL-1β and pro-IL-18 (Cerretti et al. 1994; Lu et al. 2000). This mechanism suggests that cHSP60 is involved in activation of caspase-1 which is correlated with the release of mature IL-18 from epithelial cells and to IL-1β, which is involved in apoptosis and chlamydial pathology (Jha et al. 2011c).

In *C. pneumoniae* also, apoptosis is a highly regulated cellular process that consists of diverse upstream pathways for transducing extracellular death signals into intracellular events and a common downstream effectors pathway for amplification of caspases (Fan et al. 1998). The live and inactivated forms of *C. pneumoniae* induce a necrotic form of cell death which boosts the apoptotic cell death induced by the accumulation of oxidized LDL by macrophages (Yaraei et al. 2005). Also, *C. pneumoniae* infection activates apoptosis related proteins which can either induce or inhibit apoptosis (Fischer et al. 2001). In human atherosclerotic lesions, enhanced expression of cHSP60 has been detected (Mosorin et al. 2000) and despite the inhibitory effect that *Chlamydia* infection on apoptosis induced by various agents, some findings suggest a putative role for caspase-dependent apoptosis in spreading infection (Dean and Powers 2001). In a study with human atheromatous plaques, levels of Bax, caspase- 3, 8 and 9 were higher whereas levels of anti-apoptotic molecules were lower in cHSP60 positive CAD patients (Jha et al. 2011b). Moreover, regulatory caspase-8 is directly activated by death receptors, whereas caspase-9 activation follows mitochondrial stress (Herold et al. 2002) and both pathways merge by activating executioner caspase-3 (Chandler et al. 1998). Therefore, the signaling of cHSP60 appears to be mediated through apoptotic rather than necrotic and also both caspase-dependent and independent pathways may have a role in *C. pneumoniae* associated cardiovascular complication (Jha et al. 2011b).

10.6 Conclusions

The role of cHSP60 in the immunopathogenesis of infertility in *C. trachomatis* infected women is well established. The high titer of antibodies to cHSP60 is a very useful marker for predicting disease severity. The exposure of cHSP60 at the local site of infection is responsible for the production of a variety of cytokines by immune cells which, in turn are responsible in prolonging infection by exerting immuno-stimulatory effects that contribute to the immunopathological conditions associated with infertility. The cHSP60 antibodies are also an associated risk predictor for CAD in *C. pneumonia*e infection. The cHSP60 can be considered as an

important inflammatory mediator which may stimulate VSMC proliferation and contribute to lesion formation in the artery. Overall, exposure of cHSP60 could significantly affect mucosal and vascular immune functions by modifying the release of cytokines and chemokines leading to severe immunopathological conditions such as infertility and atherosclerosis.

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Chapter 11
Insect Symbiotic Bacterial GroEL (Chaperonin 60) and Plant Virus Transmission

Rena Gorovits and Henryk Czosnek

Abstract GroEL is a multifunctional protein belonging to the conspicuous family of chaperones active in prokaryotic and eukaryotic cells. GroEL of *Escherichia coli* is a heat shock-like protein (Hsp60). It is involved in the correct folding of newly synthesized proteins, and participates in protein aggregation and in repair of damaged proteins. GroEL is essential for the morphogenesis and the capsid assembly of a number of *E. coli* bacteriophages. In eukaryotic cells, HSPs were shown to promote virus replication and survival. GroEL homologues are produced not only by free living bacteria but also by bacteria living in total symbiosis with insects and located in specialized eukaryotic cells called bacteriocytes. Symbiosis, which occurred some 200 million years (MY) ago, has led to a reduction of the bacterial genome by two third, accompanied by the adaptation of the endosymbiotic bacteria to novel functions such as providing the host with essential amino acids and other nutrients. It seems that circulative plant viruses have taken advantage of the high production of GroEL by the endosymbionts to devise a protective mechanism allowing viral particles to safely cross the haemolymph-filled body cavity of the insect, while translocating from the digestive tract to the salivary system. It seems that the relationship between chaperones and plant viruses, and the insects that vector them, have lasted for geological times (Fig. 11.1). Here we analyze the relationship of endosymbiotic GroEL with viruses in a number of insect-circulative virus systems. Moreover, we show how we can exploit this relationship to devise diagnosis tests for a number of viruses and generate virus-resistant plants by expressing insect endosymbiotic GroEL proteins.

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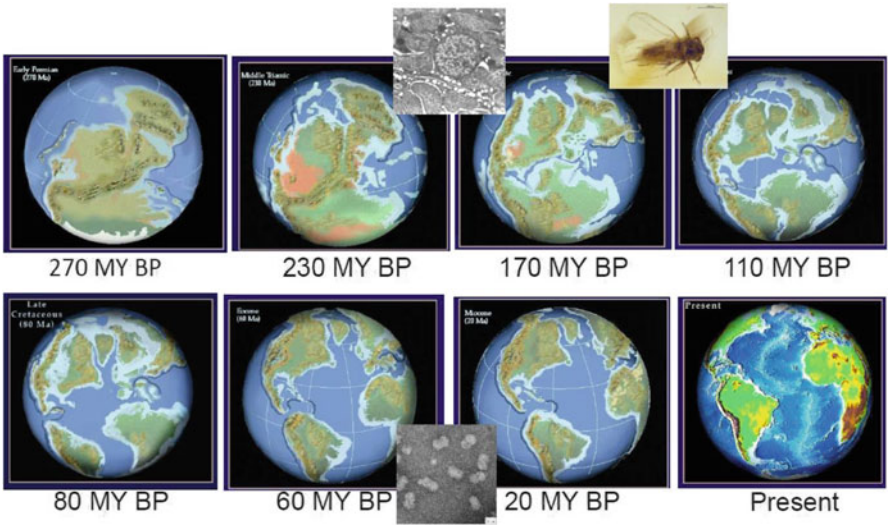

Fig. 11.1 Long-term association (and interactions?) between whiteflies, endosymbionts and geminiviruses. The map of the continent drift is from http://vishnu.glg.nau/edu/globa/text.html. *MY BP* million years before present. Evidence for the presence of endosymbionts (~200 MY BP), whiteflies (~120 MY BP) and begomoviruses (~30 MY BP) is indicated

11.1 Introduction

11.1.1 Free Bacterial and Eukaryotic GroEL

GroEL (chaperonin 60) is a molecular chaperone found in bacteria. GroEL of *Escherichia coli* is a heat shock protein (Hsp60) with 60-kDa subunits. It has a barrel shape formed by a homo-oligomer of 14 subunits, which are arranged in two rings consisting of seven subunits each stacked on each other . GroEL has extensive sequence similarity with other members of the chaperonin-60 family. To function properly, GroEL requires the co-chaperone protein GroES, a single heptameric ring of 10-kDa subunits. GroEL is involved in folding and assembly of proteins in an ATP-dependent manner regulated by GroES (Ellis and van der Vies 1991; Hartl 1996). Each GroEL subunit of 547 amino acid residues consists of three structural domains: the equatorial (residues 5–132 and 408–522), apical (residues 190–375) and intermediate (residues 133–189 and 376–407) domains (Braig et al. 1994). Unfolded proteins are recognized by the apical domain of the protein and are encapsulated in one of the cavities, where they are refolded (Roseman et al. 1996). In eukaryotic cells the proteins Hsp60 and Hsp10 are structurally and functionally nearly identical to GroEL and GroES, respectively**.** Hsp60s are common to prokaryotes, mitochondria, and chloroplasts (Gupta 1995). Molecular chaperones are essential for the morphogenesis and the capsid assembly of a number of *E. coli*

bacteriophages, including λ, T4, and T5 (Zeilstra-Ryalls et al. 1991). In eukaryotic cells, HSPs are thought to promote virus replication and survival (Escaler et al. 2000; Mayer 2005). Further details of the sequence, structure and function of both the chaperonin 10 and chaperonin 60 proteins of prokaryotes and eukaryotes is to be found in Chaps. 1, 2 and 7.

11.1.2 GroEL Encoded by Insect Bacterial Symbionts

11.1.2.1 Primary and Secondary Endosymbionts

About 20 % of all insects bear endosymbiotic bacteria living in symbiosis with their animal hosts. Most of these are γ-proteobacteria closely related to *E. coli* (Buchner 1965). Insect pests to agriculture such as aphids, leafhoppers, mealybugs, psyllids, and whiteflies, bear such bacteria. Insect endosymbionts are located in specialized host cells called bacteriocytes usually located in the abdomen of the insect (Baumann 2005). The bacteria cannot be cultured outside the host. The insect benefits from the symbionts by their provision of essential nutrients (Douglas 1998; Moran and Baumann 2000). Bacteriocyte-associated endosymbionts are transmitted from the mother to the offspring, during the development of the egg in the ovarioles (Baumann 2005). Comparisons of endosymbiont rRNA sequences indicated that the symbiotic association began about 250–300 MY ago (Moran and Telang 1998).

Insect bacterial symbionts are divided into two general types. Primary endosymbionts (P-endosymbionts) are present in bacteriocytes, are transovarially transmitted to offspring and are essential for the survival of the host (Baumann 2005). The primary endosymbionts are *Buchnera aphidicola* (Munson et al. 1991) in aphids and *Portiera aleyrodidarum* (Thao and Baumann 2004) in whiteflies. P-endosymbionts have reduced genomes (van Ham et al. 2003; Sloan and Moran 2012), which resulted from early large deletions and chromosomal rearrangements (Silva et al. 2001). Insects house other morphologically diverse bacteria that do not appear to perform essential functions and which are facultative. These are designated as secondary endosymbionts. These bacteria have probably been acquired more recently than primary endosymbionts (Baumann 2005).

11.1.2.2 Whitefly Bacterial Symbionts

Bemisia tabaci (Hemiptera: Alerodidae) is considered a species complex comprised of 11 well-defined groups as determined by sequence comparisons of the mitochondrial cytochrome oxidase I gene (Frohlich et al. 1999; De Barro et al. 2010), which mostly overlap geographic location, but also point to extensive migrations. *B. tabaci* contains the primary symbiont *Porteria aleyrodidarum* as well as six secondary symbionts belonging to the genera *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Rickettsia*, and *Wolbachia*. Whereas most symbionts are localized in bacteriocytes,

Rickettsia can be within these cells or can occupy the entire body cavity of the insect (Brumin et al. 2011). While *Porteria* was found so-far in all *B. tabaci* populations, not all the secondary symbionts are found in all individuals in a population, or in all populations. For example in Israel, *Hamiltonella* was detected only in populations of the B (or MEAM1) biotype, while *Wolbachia* and *Arsenophonus* were found only in the Q (or MED1) biotype. *Rickettsia* was abundant in both biotypes. *Cardinium* and *Fritschea* were not found in any of the populations (Chiel et al. 2007). The primary endosymbionts of whiteflies, like aphids, are vertically transmitted through bacteriocyte inclusions into the oocyte (Costa et al. 1996). While some secondary symbionts are transmitted through the egg from mother to offspring, others are transmitted horizontally between species (Russell et al. 2003). *Wolbachia* was found to alter the host reproduction (Zchori-Fein and Brown 2002).

11.2 Insect Endosymbiotic GroEL and Circular Transmission of Viruses

11.2.1 Circular Transmission of Plant Viruses in Their Insect Vector and the Role of GroEL in the Safe Translocation of the Virus

The mode of transmission of plant viruses by their insect vector can be divided into four classes (Hogenhout et al. 2008): (1) non-persistent stylet-borne (virus restricted to the stylets, transmission takes seconds to minutes, maintained in the vector for a few hours, not present in the haemolymph); (2) semi-persistent foregut-borne (moves up to the foregut after ingurgitation, transmission takes minutes to hours, maintained for hours, not present in the haemolymph); (3) persistent circulative (circulates in the insect digestive system from where it moves to the haemolymph and salivary glands until transmitted to plants – hours to days latent period, retained for days to the entire lifespan, does not multiply in vector); (4) persistent propagative (similar to persistent circulative, with the exception that the virus multiplies in the vector and is often transmitted to the progeny through the egg).

Arthropods such as aphids, leafhoppers and whiteflies transmit many viruses infecting plants in a circulative manner (Raccah and Fereres 2009). During feeding on an infected leaf, the flexible insect stylets find their way between cells until they reach the phloem sap, a process that may take a few minutes. While feeding, the insects ingest virus particles which rapidly enter the oesophagus and the filter chamber. Virions are subsequently transported through the gut wall into the haemolymph and from there they reach the salivary glands. Then, the virus translocates into the salivary duct and is finally excreted with the saliva during feeding, infecting the plant the insect feeds on. It takes from 8 to 36 h from the time the virus is ingested until it is effectively transmitted to other plants (latent period). It is believed that the haemolymph acts as a reservoir in which the virus is retained in an infective form for many days, sometimes during the entire lifespan of the insect.

To overcome the proteolytic environment of the haemolymph, it was suggested that plant viruses take advantage of a GroEL homologue synthesized by the insect endosymbionts. First shown for the luteovirus *Potato leafroll virus* (PLRV), it has been demonstrated by *in vitro* binding assays and *in vivo* depletion using antibiotics that a 60 kDa GroEL homologue produced by the primary endosymbiotic bacterium (a *Buchnera* sp.) of *Myzus persicae* and released in the haemolymph is a key protein in the transmission of the virus, preventing or retarding breakdown of virus particles (van den Heuvel et al. 1994). The general role of endosymbiotic GroEL in virus transmission has been confirmed with the whitefly transmitted geminivirus *Tomato yellow leaf curl virus* TYLCV (Morin et al. 1999). The localization of GroEL in aphid haemolymph and salivary glands indicated a compartmentalization paralleling that of circulative viruses (Filichkin et al. 1997).

11.2.2 Luteoviruses and Aphids

11.2.2.1 *Potato Leafroll Virus* (PLRV)

The role of endosymbiotic GroEL in plant virus circular transmission by insects was first discovered by studying transmission of PLRV by *M. persicae* (van den Heuvel et al. 1994). Using virus overlay assays, it was found that PLRV binds to a 63 kDa GroEL homolog produced by the primary symbiont of the insect, a *Buchnera* sp. This GroEL, one of the most abundant proteins in aphids, had 92 % sequence similarity with *E. coli* GroEL and other members of the chaperonin-60 family. Feeding aphids on artificial diet containing tetracycline eliminated the GroEL protein from the insect haemolymph and reduced the efficiency of PLRV transmission by more than 70 %. The PLRV CP was undetectable in the treated insects.

11.2.2.2 *Barley Yellow Dwarf* (BYDV)

BYDV-PAV is transmitted by the aphid species *Rhopalosiphum padi* and *Sitobion avenae*. The highly specialized BYDV-vector relationships suggest that there are specific interactions between BYDV virions and aphid cellular components. Two endosymbiotic genes were cloned and expressed from the two insect vectors; the products presented characteristics of the chaperonin-60 (cpn60) and of GroEL from *M. persicae* and *E. coli*. These proteins bound BYDV particles *in vitro* suggesting a potential involvement of endosymbiotic chaperonins in interactions with virions during their trafficking through the aphid (Filichkin et al. 1997). The role of GroEL in luteovirus transmission by aphids was recently questioned (Bouvaine et al. 2011). It was argued that since *Buchnera aphidicola*, the Gram-negative symbiotic bacterium of aphids, GroEL from two aphid species *R. padi* and *A. pisum* could not be detected in insect bacteriocyte-free haemolymph, the chaperon is unlikely to interact with BYDV. The alternative scenario proposed was that the virus interacts with GroEL derived not from *Buchnera* but from aphid secondary symbionts (Oliver et al. 2010).

11.2.3 Geminiviruses and Whiteflies

11.2.3.1 *Tomato Yellow Leaf Curl Virus* (TYLCV)

Similarly to PLRV in *M. persicae*, a GroEL homologue produced by endosymbiotic bacteria housed in bacteriocytes of the whitefly *Bemisia tabaci*, seems to have a cardinal role in safeguarding begomoviruses in the haemolymph. We have studied the Interaction between the whitefly endosymbiotic GroEL and the begomovirus TYLCV. GroEL proteins present in whitefly and aphid share high homology in their amino acid sequence. GroEL is present in the haemolymph of *B. tabaci* as a native 14-mer unit, each subunit having a mass of 63 kDa (Morin et al. 1999). TYLCV particles displayed affinity for the *B. tabaci* endosymbiont GroEL homologue in a virus overlay assay. Moreover, the TYLCV coat protein (CP) and *B. tabaci* GroEL interacted physically in the yeast two-hybrid system (Morin et al. 2000). Disturbing the GroEL-TYLCV association *in vivo* by feeding insects with an antibody raised against Buchnera GroEL leads to the degradation of the virus and to a markedly decrease in transmission efficiency of the virus (Morin et al. 1999). Interestingly, *B. tabaci* GroEL interacted as well with the CP of the non-transmissible *Abutilon mosaic begomovirus* (AbMV), indicating that the amino acid residues at position 124, 149 and 174, which prevented AbMV from crossing into the insect haemolymph (Höhnle et al. 2001), did not prevent binding to GroEL.

Contrary to *M. persicae*, where the PLRV-binding GroEL is encoded by the genome of the primary endosymbiont, in *B. tabaci*, the TYLCV-binding GroEL is encoded by a secondary symbiont. GroEL was cloned from all the endosymbionts present in the good TYLCV transmitter B biotype (containing the primary symbiont *Portiera* and the facultative *Rickettsia*) and the poor TYLCV transmitter Q biotype (containing the primary symbiont *Portiera* and the facultative *Wolbachia* and *Arsenophonus*) from Israel (Chiel et al. 2007). In yeast two hybrid system and protein pull-down assays, only GroEL produced by the facultative secondary endosymbiont *Hamiltonella* was able to bind TYLCV CP. GroEL from the other symbionts were unable to bind TYLCV CP. Taken together, the GroEL protein produced by *Hamiltonella* (present in the B biotype, but absent in the Q biotype) facilitates TYLCV transmission. The other symbionts from both biotypes do not seem to be involved in transmission of this virus (Gottlieb et al. 2010). It is interesting to note that a Q biotype population from Spain, which harbors *Hamiltonella*, was able to transmit efficiently TYLCV (Gottlieb et al. 2010). In addition to GroEL, whitefly HSP70 interacts with begomoviruses in the insect (Götz et al. 2012). The expression of the insect Hsp70 gene (mRNA and protein) was upregulated by the presence of TYLCV; fluorescence *in-situ* hybridization (FISH) showed that TYLCV and another begomovirus, *Squash leaf curl virus* SLCV, co-localized within midgut epithelial cells. Feeding whiteflies with antibodies raised against HSP70 increased virus transmission, suggesting a protective role of HSP70 by inhibiting virus mobility in the insect (Götz et al. 2012).

11.2.3.2 *Cotton Leaf Curl Virus* (CLCuV)

CLCuV transmission is another case where GroEL produced by a secondary endosymbiont binds to a begomovirus. CLCuV infects cotton plants in the Indian subcontinent. the virus is exclusively transmitted by *B. tabaci*. Following the finding that *Hamiltonella* GroEL interacts with TYLCV, a study was conducted to find out whether endosymbionts of *B. tabaci* are similarly involved in CLCuV transmission in Rajasthan, India. The local *B. tabaci* population belongs to AsiaII genetic group and harbors the primary endosymbiont *Portiera* and the secondary endosymbiont *Arsenophonus*. Purified GroEL proteins from *Portiera* and *Arsenophonus* were purified; pull-down, co-immuno-precipitation and yeast two hybrid assays showed that only the GroEL protein of *Arsenophonus* could interact with the CLCuV CP (Rana et al. 2012).

11.2.4 Grapevine Leafroll-Associated Virus 3 and Mealybugs

Mealybugs, like aphids and whiteflies, bear primary endosymbionts (coined *Candidatus* Tremblaya princeps) whose major function appears to be the synthesis of amino acids lacking in plant sap. As in aphids and whiteflies, they are acquired through the egg and are stored within bacteriocytes. Mealybugs are unusual in having beta-proteobacterial endosymbionts; while the P-endosymbionts of aphids and whiteflies are all γ-proteobacteria. Mealybugs also harbor secondary endosymbiotic bacteria (related to *Sodalis glossinidius)* of γ-proteobacteria (von Dohlen et al. 2001). Mealybugs are the principal vectors of *Grapevine leafroll-associated virus 3* (GLRaV-3), an ampelovirus that causes grapevine leafroll disease (Cabaleiro and Segura 1997)**.** It has been proposed that GroEL homologs produced by the mealybugs primary symbionts are involved in virus transmission, but virus-GroEL binding assays have not been performed (Gatehouse et al. 2012).

11.3 Use of Whitefly Endosymbiotic GroEL Expressed in Plants to Trap Viruses and Confer Resistance to Viruses

11.3.1 GroEL Structure and Sites Involved in the Binding of Viruses

The mechanism by which the 14 × 14 nm barrel-shape GroEL (Sigler et al. 1998) interacts with the 25–30 nm virus particles (Fig. 11.2) presumably differs from its chaperone function (Miller and Rasochová 1997) since virions are too large to enter the 4.5 nm diameter GroEL cavity (Braig et al. 1994). The apical domain of GroEL

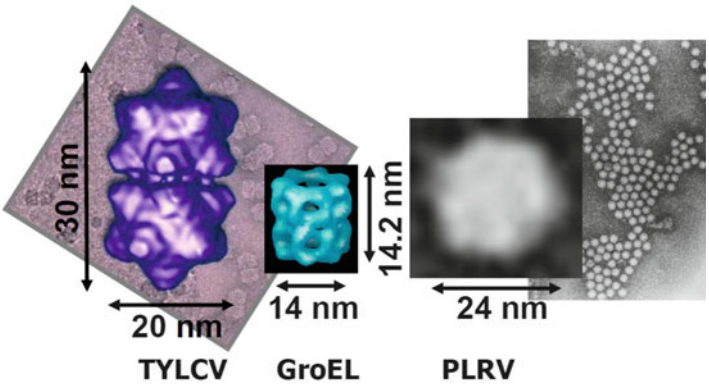

Fig. 11.2 Relative sizes of the *barrel-shaped* 14 mer-GroEL and of the begomovirus TYLCV and the luteovirus PLRV. The two viruses bind to GroEL *in vivo* and *in vitro*

has been implicated in polypeptide binding (Fenton et al. 1994), a process which may require ATP hydrolysis. The ATPase activity of GroEL is regulated by GroES, a single heptameric ring of 10-kDa subunits (Martin et al. 1991).

Mutational analysis of the gene encoding the *Buchnera* GroEL revealed that the PLRV-binding site was located in the equatorial domain and not in the apical domain which is generally involved in polypeptide binding and folding. Both the N- (amino acid residues 9–19) and C-terminal (residues 427–457) regions of the equatorial domain were implicated in virus binding (Hogenhout et al. 2000). On the other hand, it was found that the N-terminal region of the read-through domain of BYDV and other luteoviruses has a high affinity for *Buchnera* GroEL and is essential for virus persistence in the aphid vector (van den Heuvel et al. 1994). The stoichiometry of GroEL-virus binding is not known.

11.3.2 GroEL from B. tabaci *Endosymbionts is Able to Trap Viruses* In Vitro

The ability of whitefly endosymbiotic GroEL to bind TYLCV CP was exploited to devise a simple procedure for trapping and identifying plant viruses *in vitro* (Akad et al. 2004). GroEL was purified from *B. tabaci* secondary *endosymbiont Hamiltonella* following cloning, sequencing (GenBank accession number AF130421), and expression in *E. coli*. The test was first developed for diagnosis of TYLCV. PCR tubes coated with GroEL were incubated with sap from infected tomato plants. A PCR mixture containing TYLCV-specific primers was added and TYLCV DNA was amplified demonstrating that this virus was efficiently trapped by GroEL. The test was able to detect TYLCV in a single whitefly. The same procedure has been successfully used to detect several other whitefly-transmitted geminiviruses, such as AbMV and *African*

cassava mosaic virus (ACMV). The spectrum of plant viruses tested was extended to circulative and non-circulative viruses. Only those viruses possessing a globular (or geminate) shape, a CP with a high positive charge, a high percentage of arginine, and a high isoelectric point were able to bind GroEL. Members of the genera Begomoviruses, Cucumoviruses, Luteoviruses and Tospoviruses complied to these criteria and were able to bind GroEL *in vitro*. On the other hand Closteroviruses, Nepoviruses, Potyviruses and Tobamoviruses, which do not comply with these criteria were unable to bind GroEL *in vitro* (Akad et al. 2004).

11.3.3 Tomato and Nicotiana benthamiana *Plants Expressing GroEL from* B. tabaci *Endosymbionts Are Resistant to Viruses*

Since whitefly GroEL binds to viruses from different taxonomic families, this GroEL property was exploited to generate transgenic tomato plants expressing the chaperone under the control of a phloem-specific promoter. The rationale was that once inoculated by their vector, phloem-limited circulative viruses would be trapped by GroEL in the plant phloem. As a result, the virus movement and replication could be inhibited to a point where plants would be able to resist the virus. This approach was first attempted by infecting GroEL-expressing transgenic tomato plants with TYLCV (Akad et al. 2007). Upon infection, the plants showed no or mild symptoms and yielded fruit, while the non-transgenic control plants were stunted and did not yield. GroEL-TYLCV complexes were detected in the sap of infected transgenic tomato plants. Non-transgenic susceptible tomato plants grafted on resistant GroEL-transgenic scions remained susceptible, although GroEL translocated into the grafted plant and GroEL-TYLCV complexes were detected in the grafted tissues (Akad et al. 2007).

This concept was extended to generate transgenic GroEL-expressing *N. benthamiana* plants, which were infected with two viruses binding GroEL *in vitro* (TYLCV and *Cucumber mosaic virus* [CMV]) and two viruses that do not bind GroEL (*Tobacco mosaic virus* [TMV] and *Grapevine virus A* [GVA]) *in vitro* (Akad et al. 2004). As predicted, the plants were tolerant to TYLCV and CMV but not to TMV and GVA. Virus-GroEL complexes were detected in TYLCV- and CMV-inoculated transgenic *N. benthamiana*, but not in plants inoculated with TMV- and GVA (Edelbaum et al. 2009).

11.3.4 GroEL is Associated with TYLCV Aggregates in Infected GroEL-Expressing Transgenic Plants

In insects such as whiteflies and aphids, endosymbiotic GroEL contributes to the safeguard of circulative viruses by binding viral particles. We have proposed that by sequestering virions, GroEL prevents the invasion of vital organs and cells by

potentially deleterious viruses (Czosnek et al. 2001). Hence we may postulate that in transgenic plants expressing GroEL, the chaperone will protect the host plant in the phloem in a way similar to that it does in the insect haemolymph. Indeed GroEL-TYLCV complexes were detected in the sap of GroEL-expressing tomato and *N. benthamiana* (Akad et al. 2007; Edelbaum et al. 2009). Analysis of TYLCV CP aggregation in the phloem of infected tomato plants may provide clues as to the manner GroEL interacts with the CP. We have shown that upon TYLCV infection of susceptible tomato plants, TYLCV CP rapidly forms aggregates of increasing size containing infectious particles and viral replicative dsDNA molecules, reminiscent of viral factories. These aggregates are first seen by FISH in the cytoplasm, then in the nuclei of phloem-associated cells. In resistant tomato plants, the formation of large aggregates is somewhat inhibited (Gorovits et al. 2012). The size of the aggregates was estimated by ultracentrifugation in sucrose gradients. In non-transgenic *N. benthamiana* plants, as in susceptible tomato, the TYLCV CP was associated with large aggregates or inclusion bodies (Fig. 11.3). By comparison, in virus tolerant GroEL-expressing *N. benthamiana*, most of the CP was confined in mid-sized aggregates; GroEL was associated with the CP-containing fractions. Consequently, we propose that as part of the mechanism conferring tolerance to transgenic plants, GroEL does not allow the development of large virus aggregates, which may facilitate virus multiplication.

11.4 Conclusions

The role of bacterial GroEL chaperonins in mediating correct folding of newly synthesized proteins has been identified in the early 1990s (Horwich et al. 1993). The functions of bacterial GroEL have been extended to stress response and bacteriophage assembly. GroEL is not only produced by free bacteria but also by bacteria living in symbiosis with their animal hosts, especially insects, where the chaperone plays a cardinal role in transmission of viruses to plants.

The relationship between chaperones and plant viruses, and the insects that vector them, presents multiple facets, as fits for moonlighting proteins. As a rule, viruses do not encode HSP70s, although many induce the host chaperones to promote virus multiplication (Aranda et al. 1996; Aparicio et al. 2005). For example, HSP70 and HSP90 are required for *Red clover necrotic mosaic virus* (RCNMV) RNA replication; they interact with p27, a virus-encoded component of the 480-kDa replicase complex, on the endoplasmic reticulum membrane (Mine et al. 2012). Plant closteroviruses are a unique virus family that encode HSP70 homologues (Zhu et al. 1998). It has been postulated that this gene was acquired by recombination with a host HSP70 mRNA. *Beet yellows virus*-encoded HSP70 was shown to facilitate virus translocation from cell to cell, but has no effect on virus multiplication and encapsidation (Peremyslov et al. 1999).

We have studied the role of GroEL produced by endosymbionts of the whitefly B. tabaci in the circulative transmission of the begomovirus TYLCV by the insect.

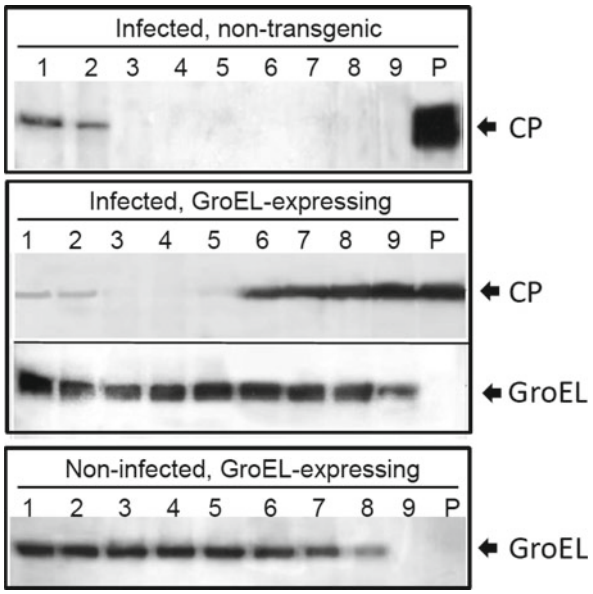

Fig. 11.3 Redistribution of TYLCV CP aggregates in virus-resistant GroEL-expressing *N. benthamiana* plants, as analyzed following sedimentation on linear 10–50 % sucrose gradients. Gradients were divided into 10 fractions, 1 (*top*) to 9 (*bottom*), and pellets (*P*). Aliquots were subjected to SDS-PAGE and western blotted. *CP* and *GroEL* were detected using specific antibodies. Note that most of the CP in GroEL-expressing plants at 10 dpi shifted from large (fraction P) to mid-size aggregates (fractions 6–9)

Begomoviruses, whiteflies and endosymbionts have interacted for geological times begomoviruses: (1) fossils anatomically similar to modern whiteflies have been found in ~120 MY old amber (Schlee 1970); (2) multiple head-to-tail repeats of regions of the modern begomovirus *Tomato golden mosaic virus* have been found in *Nicotiana* species suggesting that about 25 MY ago viral sequences have integrated the genome of tobacco ancestors during *Nicotiana* speciation (Bejarano et al. 1996) and; (3) the GroEL-producing endosymbiotic bacteria have been associated with whiteflies for the last 200 MY (Baumann et al. 1993). Such a long-lasting relationship implies that the partners have developed co-evolutionary mechanisms that insure on one hand the efficient transmission of the virus, and on the other hand prevents the virus from invading insect tissues, causing irreparable damages (Rubinstein and Czosnek 1997; Pan et al. 2012). Indeed the CP of TYLCV (and of other) begomoviruses possess a nuclear localization signal allowing to penetrate (experimentally) the nuclei of *Drosophila* eggs (Kunik et al. 1998). In this regard, it is possible that the insect has taken advantage of the endosymbiotic proteins, especially a GroEL homologue, to facilitate the transit of the virion until it is expelled during feeding, instead of attempting to neutralize and destroy it. The same strategy of survival based on virus interaction with endosymbiont GroEL has been adopted by aphids for the transmission of luteoviruses (van den Heuvel et al. 1994). It is tempting to assume that non-circulative viruses are in fact unable to circulate because their shape makes them incapable to bind to endosymbiotic GroEL (Akad et al. 2004). *Tomato chlorosis virus* (ToCV) and *Lettuce infectious yellows virus* (LIYV) may constitute good examples supporting this theory. These viruses are phloem-restricted RNA viruses members of the genus *Crinivirus*

(family *Closteroviridae*). They appear as long flexuous particles of ~850 nm in length encapsidating two genomic RNAs. ToCV and LIYV are transmitted by *B. tabaci* in a non-circulative, semi-persistent manner (Wintermantel and Wisler 2006; Chen et al. 2011). These viruses reach the whitefly anterior foregut where they may be retained for 1–2 days, and are egested during feeding. Interaction of LIYV with its vector is mediated by the viral minor CP, a protein necessary for virus inoculation and retention by *B. tabaci* (Chen et al. 2011). ToCV and LIYV do not reach the midgut and do not cross into the haemolymph where GroEL is conspicuous. Hence viruses that do not interact with endosymbiotic GroEL have found other ways to survive in the insect host, either by remaining in the insect stylets for a short time binding to cuticular proteins (e.g. aphid-transmitted CMV and PVY, Raccah et al. 1985) or as in the case of Tospoviruses and their thrips vector, by invading cells of the larval midgut and multiplying in the new host, decreasing its fitness on the way (Whitfield et al. 2005).

We have used the capacity of GroEL to bind viruses of the Begomovirus family to devise diagnostic tests and to renter virus-susceptible tomato and *N. benthamiana* resistant by expressing endosymbiotic GroEL (Akad et al. 2007). This strategy twisted around the primary biological function of the chaperone, which in our eyes is to ensure infection by binding the viral particle and protecting it from destruction. In the resistant GroEL-expressing plants, GroEL circulates in the plant phloem where it binds incoming viral particles transmitted by whiteflies during feeding, thanks to their endosymbiotic GroEL. In the transgenic plants, GroEL by binding virions (with an unknown stoichiometry) likely decreases the amount of free particles, therefore preventing or slowing down the formation of large viral aggregates, a feature of infection of susceptible plants (Gorovits et al. 2012).

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Chapter 12
Histoplasma capsulatum Chaperonin 60: A Novel Adhesin and Vaccine Candidate

Joshua Daniel Nosanchuk and Allan Jefferson Guimarães

Abstract HSP60 has a key role in immunoregulation and the abundance of HSP60 proteins in mammalian and microbial cells impacts diverse biological functions in both. Therefore, it may be essential for innate immune cells to distinguish HSP60 proteins by their endogenous or infectious origin. *Histoplasma capsulatum* (Hc) is able to express an Hsp60 on its surface that facilitates engagement with macrophages through complement 3 receptors (CR3) and leads to phagocytosis of the fungus where the pathogen survives within phagosomes. HcHsp60 plays important metabolic roles related to the fungal adaptation to temperature and oxidative stress conditions. Hc is also able to secrete this chaperone, which could play important roles in modulation of the antifungal immunological responses. Vaccination with recombinant HcHsp60 has conferred protection in mouse models against intravenous and pulmonary infections by Hc. Passive immunization using monoclonal antibodies to HcHsp60 has provided excellent results against Hc in mouse models, mainly by activating the antifungal functions of macrophages and inducing a Th-1 type immunoresponse. As Hsp60 is a common fungal antigen, passive immunization with Hsp60 mAbs has been evaluated against distinct fungal pathogens, including coupling the antibody with radionuclides (radioimmunotherapy) in order to increase the antifungal potential of these mAbs. In this chapter, we detail the research progresses on Hsp60 of Hc with particular focus on its characterization as an adhesin and utilization in vaccination and passive immunization.

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12.1 Introduction

Histoplasmosis is a cosmopolitan mycosis caused by the fungus, *Histoplasma capsulatum* (Hc). Hc is a dimorphic pathogen and the morphological presentation, filamentous or yeast, depends mainly on temperature and nutritional conditions (Maresca and Kobayashi 1989). It can be found as a saprophytic mould in soils enriched with organic nitrogen sources, such as areas contaminated with bird or bat droppings (Disalvo et al. 1970; Emmons 1950, 1956a, b; Emmons et al. 1966; Alteras 1966; Smith 1971a, b; Zeidberg et al. 1952). After disturbances in the environment, the microconidia or hyphal fragments become airborne and can be inhaled by a susceptible host (Guimaraes et al. 2006), undergoing conversion to the pathogenic yeast form after reaching the terminal bronchioles of the lung and deposition in the alveoli (Allendoerfer et al. 1997; Couto et al. 1994). As a facultative intracellular parasite, the interaction of Hc with phagocytic cells is a critical component of the host response to infection (Newman 2005) and is a complex and obscure phenomenon. Within the phagocytic cells, yeast may travel to hilar and mediastinal lymph nodes where they gain access to the blood circulation for dissemination to various organs, such as liver and spleen (Wheat and Kauffman 2003).

Environmental exposure to Hc is exceedingly common for persons living within areas of high endemicity (Wheat and Kauffman 2003), where optimal environmental conditions are a moderate climate with a relatively constant humidity level (Maresca and Kobayashi 1989). Fifty million people are infected in the United States of America and it is estimated that half a million new human infections occur annually in this country (Cano and Hajjeh 2001). Common endemic areas are located in Midwestern and Southeastern parts of the USA, with approximately 80 % of individuals displaying reactivity to histoplasmin skin test (Ajello 1971; Goodwin and Des Prez 1978; Wheat 1997). High endemicity areas can also be found in Latin America, particularly within Brazil, Venezuela, Ecuador, Paraguay, Uruguay and Argentina (Borelli 1970; Wheat 1997, 2001). In some regions, such as the Midwestern and Southeastern portions of Brazil, the prevalence can reach values as high as 63.1 and 93.2 %, respectively (Londero and Ramos 1978; Zancope-Oliveira et al. 2005; Guimaraes et al. 2006).

The clinical manifestations of histoplasmosis are governed by environmental and genetic factors, such as the magnitude of exposure (i.e. the number of fungal particles inhaled), the immunological status of the host, and the virulence of the infective strain (Goodwin et al. 1981; Kauffman 2007). The vast majority of infected persons in endemic areas (~90 %)(Pfaller and Diekema 2010), have either no symptoms or very mild illnesses that were never recognized as being histoplasmosis (Wheat et al. 2007). The most common presentation is pneumonia, though the disease can also manifest as a fulminant life-threatening disseminated sepsis that may involve virtually any tissue (Bradsher 1996; Csillag and Wermer 1956; Goodwin and Des Prez 1978; Meloan 1952; Wheat 1994). Individuals in the setting of immunosuppression, such

as those chronically receiving steroids, patients on chemotherapy (Kauffman 2007) or with advanced HIV disease are also at significant risk for severe infection due to reactivation of latent lesions or primary disease, with a rate of 95 % of disseminated form in individuals with AIDS who become infected (Wheat 1996). Fortunately, therapy with potent anti-retrovirals has reduced the numbers of HIV-associated histoplasmosis cases in the USA.

The *in vivo* mycelium-to-yeast transition is a central biological phenomenon, since it results from morphological adaptation to a temperature increase and this mechanism is correlated with the capability of Hc to cause disease (Maresca and Kobayashi 1989). The majority of Hc strains easily convert to the yeast phase and destroy macrophage monolayers *in vitro*, which correlates with virulence in mouse models of infection (Eissenberg et al. 1991). Because only the yeast phase is parasitic, factors which affect morphogenesis have been of interest for understanding and controlling pathogenicity. This temperature adaptation is accompanied by a heat shock response (Lambowitz et al. 1983), in which cellular ultrastructural modifications occur, such as reduction in the number and organization of the mitochondrial cristae (Borgia et al. 1990). This heat-induced phenomenon is also accompanied by changes in several different biochemical and metabolic processes including respiration and cysteine metabolism (Maresca and Kobayashi 1989, 1993). The complete transition occurs over several days, and triggers temperature-dependent activation and expression of yeast specific genes (Maresca 1995). Respiration is the main catabolic process in the yeast phase, with the highest mitochondrial ATPase activity and electron transport efficiency occurring at 37 °C. It results in a rapid decline in intracellular ATP levels which are followed by uncoupling of oxidative phosphorylation.

12.2 Chaperonin 60 Localization and Anti-stress Chaperone Function in *H. capsulatum*

Heat-shock proteins have been identified as molecular chaperones that are highly conserved among several eukaryotic and prokaryotic species and grouped by their molecular mass and degree of sequence homology (Burnie et al. 2006). Heat shock proteins are an essential part of the heat shock regulon that helps maintain cellular homeostasis by playing crucial protective roles regarding protein renaturation and stabilization, such as facilitating folding/unfolding, directing assembly and disassembly of protein complexes, preventing aggregation of nascent polypeptides and toxicity, coordinating translocation/sorting of newly synthesized proteins into correct cellular compartments, degradation of aged/damaged proteins via the proteasome, regulating cell cycle and signaling, and also protecting cells against stress/apoptosis (Saibil 2008; Li and Srivastava 2004). For more information on the cell

stress response and on the functions of prokaryotic and eukaryotic molecular chaperones the reader is referred to Chaps. 1 and 2.

Hc increases the synthesis of some constitutively expressed heat shock proteins when undergoing transition from mycelium-to-yeast forms (Kamei et al. 1992), or when stressed by heat, low pH or H_2O_2, which might relate to resistance against a hostile environment and pathogenicity (Kamei et al. 1992). Overall, these proteins display an expression peak between 34 and 37 °C (Shearer et al. 1987; Caruso et al. 1987; Minchiotti et al. 1992). In general, heat shock expression is part of a developmental process and temperature adaptation; strains expressing higher levels of Hsps are temperature resistant and possess a higher virulence level (Caruso et al. 1987; Minchiotti et al. 1992; Maresca 1995). Changes in membrane fluidity, such as with the addition of saturated fatty acids, induce a heat shock response, which results in a mitochondria-retained ATPase activity coupled to electron transport, a thermotolerant state, and shortens the time required for mycelium-to-yeast phase transition (Maresca and Kobayashi 1993). This phenomenon is correlated with the induction of a strong increase in molecular chaperone mRNA transcription, whereas treatment with an unsaturated fatty acid reduces or eliminates the transcription level of these genes at 37 °C (Carratu et al. 1996).

Hsps are abundant and widely distributed within the cell, but they are mainly concentrated in specific cell compartments such as the mitochondria, chloroplasts, endoplasmic reticulum and nucleus (Habich and Burkart 2007). Their physiological function depends on subcellular localization, but the appearance of these stress glycoproteins and modification on the intracellular distribution and function may occur by several mechanisms of heat shock responses (Jethmalani et al. 1997; Soltys and Gupta 1997). The control of the cell stress response, or unfolded protein response (UPR) is detailed in Chaps. 1 and 2. A unique feature of the Hsp60 of the fungus Hc is that it is also found as clusters on the cell wall, suggesting its localization within vesicles, as well as being free within the cell (Long et al. 2003); however its functions in this subcellular location are poorly understood. These proteins are also released to the extracellular milieu by stressed cells, pointing to a potential role of these proteins as intercellular signaling molecules (Barreto et al. 2003; Davies et al. 2006; Hunter-Lavin et al. 2004).

Our group has shown that Hsp60 levels increase in response to temperature stress in both cytoplasm and cell wall subcellular fractions (Guimaraes et al. 2011d). However, the change of Hsp60 cell wall levels was not significantly different during heat shock, suggesting that, in the conditions tested, Hsp60 had a constitutive and regulatory function in the cell, orchestrating traffic of proteins to the cell surface where it is present at levels close to saturation, independent of the overall expression within the cell. This fact, in association with the presence of distinct Hsps in the cell wall, strongly suggests that these proteins could be recruited to re-organize the fungal surface under stress conditions.

The capacity of Hsp60 to interact and work as a carrier molecule suggests wide-ranging regulatory functions of this protein as a chaperone, as they possess all the features of such protein categories (Fig. 12.1). Differential interactions of Hc Hsp60 have been dissected in both cytoplasmic and cell wall fractions, and

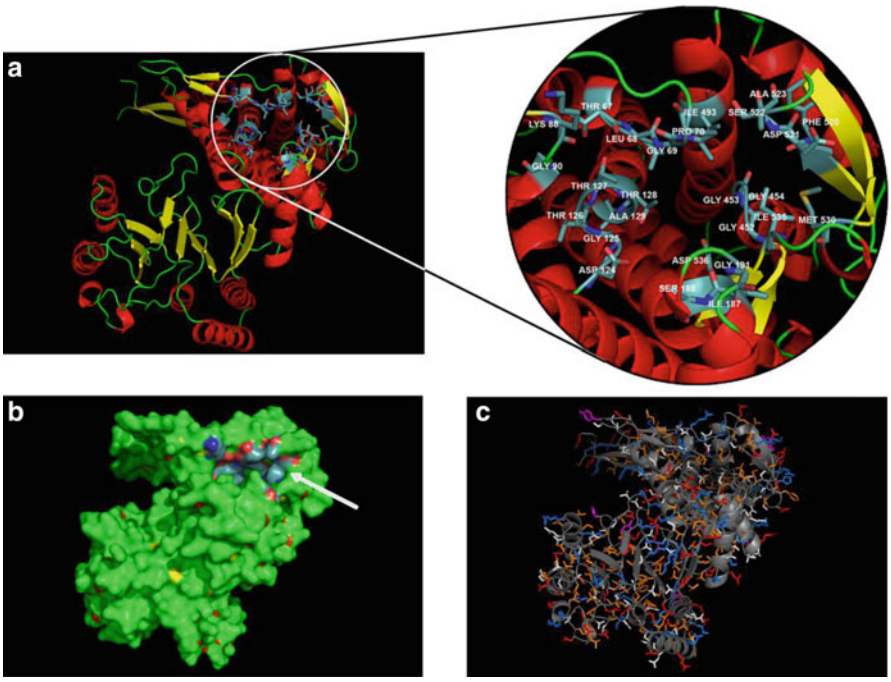

Fig. 12.1 Hc Hsp60 molecularly displays chaperonin properties. (**a**) ATP binding sites were mapped to a pocket within the Hc Hsp60 structure. (**b**) Surface accessibility displays the localization of the ATP binding site within a cleft (indicated by a *white arrow*). *Gray*, *red* and *blue* and *green* are used to represent hydrophobic, negatively charged, positively charged respectively. (**c**) The cleft is mostly composed of hydrophobic residues (side chains labeled in *orange*)

we identified common and unique interactions within each subcellular compartment (Guimaraes et al. 2011d). The interactome reveals that Hc Hsp60 engages nuclear chaperones, small chaperones and Hsp90 families. Temperature increases interactions between Hsp60 and Hsp70 in the cell wall. Furthermore, cell wall Hsp60 more broadly interacts with enzymes related to carbohydrate metabolism, suggesting a trafficking function of the Hsp60 related to enhanced energy acquisition under stress conditions. Additionally, Hsp60 apparently contributes to cell wall changes that allow the pathogen to survive under stress conditions (Shaner et al. 2008). Hence, this protein participates as a key regulator of diverse cellular processes, including cell signaling, replication, oxidative stress responses, expression of virulence associated proteins, and amino acid, protein, lipid, and carbohydrate metabolism (Guimaraes et al. 2011d). Interestingly, the binding of HcHsp60 by antibody may dysregulate the chaperone functions of the protein. Disruption of this interactome, especially during stress response conditions, could impact the capacity of the fungus to cause disease and emerge as a new avenue for therapeutic interventions.

12.3 Chaperonin 60 Adhesin Function and Non-lytic Phagocytosis

As a facultative intracellular parasite, the interaction of Hc with phagocytic cells is a critical component of the host response to infection (Newman 2005). Hc yeast must survive and/or subvert the hostile antimicrobial environmental within phagocytes (Allendoerfer et al. 1997), which includes fungicidal mechanisms dependent on hydrogen peroxide and nitrogenous species (NOS pathway) (Eissenberg and Goldman 1987). Interestingly, HcHsp60 is the major ligand that engages the integrin CR3 (CD11b/CD18) expressed on the surface of macrophage/monocytes (Long et al. 2003; Habich et al. 2006). Engagement of CR3 is followed by Hc internalization, which, interestingly, is associated with the inhibition of the respiratory burst (Eissenberg and Goldman 1987; Guimaraes et al. 2008; Wolf 1987). Notably, yeast cells actively alkalinize phagosomal pH to 6.5 and effectively inhibit phagolysosomal fusion, thereby preventing exposure to the acidic hydrolytic lysosomal enzymes, prohibiting accumulation of vacuolar ATPase, which is important for proton accumulation in phagosomes (Strasser et al. 1999). These processes facilitate the capacity of the pathogen to survive and replicate within host cells leading to subsequent dissemination (Eissenberg and Goldman 1987; Wolf et al. 1987; Wheat and Kauffman 2003).

A study characterizating Hsp60 species revealed that all proteins, from microbes to mammals, bound the CR3 receptor and elicited inflammatory responses of mouse macrophages; however, each used a different binding moiety, which implies the existence of distinct receptor structures for Hsp60 binding and activation of specific cell types depending on the engaged receptors (Habich et al. 2003). Human and HcHsp60 share similar domains for binding to primary macrophages and J774.1 6 macrophage-like cells (Fig. 12.2). In addition, the interaction of Hsp60 with immune cells exhibits immunoregulatory properties, such as Hsp60-induced release of inflammatory mediators and regulation of innate and adaptive immune responses (Habich and Burkart 2007). Besides the interaction of HcHsp60 with the CR3 receptor complex on the cell surface of macrophages (Long et al. 2003), this protein could be interacting with other critical macrophages surface proteins, regulating the effector functions of these cells or even exerting other important chaperonin-like functions that modify the pathogenesis of Hc (Guimaraes et al. 2011d).

12.4 Immunotherapy Against the *H. capsulatum* Chaperonin 60

12.4.1 H. capsulatum *Chaperonin 60 as Vaccination Target*

Macrophages have been considered the most important cells in resistance against Hc. Interferon gamma (IFN-γ) has been reported as a key cytokine to activate anti-Hc functions of these cells, mainly by the induction of nitric oxide (NO),

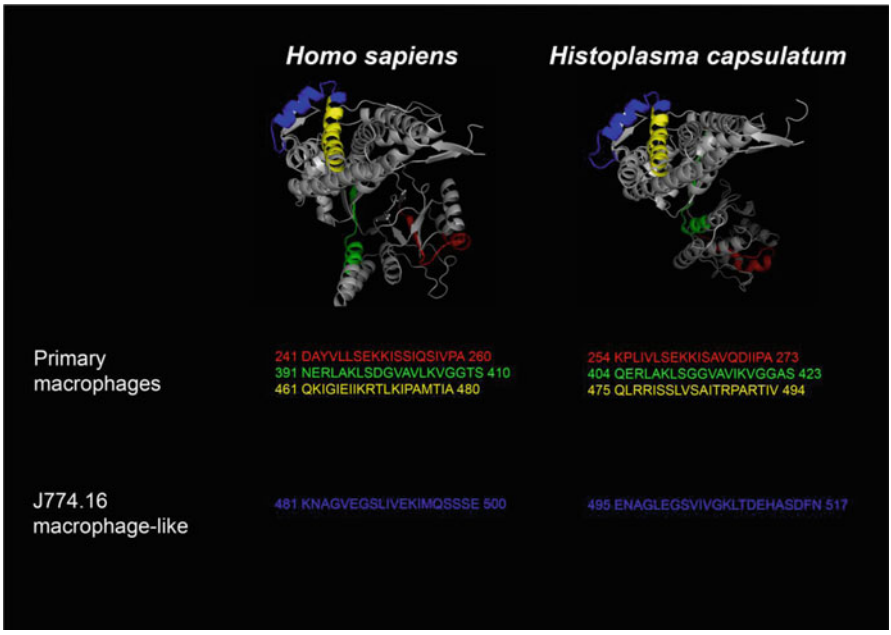

Fig. 12.2 Similarities between Hc and human Hsp60 regarding interaction moieties used by these two proteins to anchor to primary mouse macrophages or macrophage like J774 cells

which purportedly deprives Hc of iron (Lane et al. 1994). The interaction of human monocyte-derived macrophages with Hc reveals some interesting contrasts to that of murine macrophages. Human macrophages mount a vigorous oxidative burst in response to ingestion of Hc, yet the organism resists the harmful effects of these molecules and Hc is able to replicate within these cells (Wolf 1987). On the other hand, in murine macrophages, this fungus fails to trigger an oxidative burst and can actively inhibit the generation of toxic oxygen molecules (Eissenberg and Goldman 1987; Wolf et al. 1987). Indeed, recent evidence suggests that DCs can restrict the differentiation of conidia into yeast (Newman 2005; Newman et al. 2011) and may be the key antigen-presenting cells that initiate cell-mediated immunity (Deepe et al. 2008).

Host protective immunity involves interaction of infected monocytes/macrophages or DCs with T-cells (Wu-Hsieh and Howard 1984; Allendorfer et al. 1999; Zhou et al. 1995). Both $CD4^+$ and $CD8^+$ T cells contribute to host resistance in primary infection. Mice that lack T cells either genetically or by depletion with mAb to T cell receptors (TCR) α/β, display higher susceptibility to Hc infection (Allendoerfer et al. 1997; Zhou and Seder 1998). Selective reduction of $CD4^+$ T cells produces fatal histoplasmosis in naive mice but clearance of Hc from organs is impaired in mice that lack $CD8^+$ T cells (Zhou et al. 1998). Adoptive transfer of Hc-reactive $CD4^+$ T cells confers protection. Thus, in primary infection, $CD4^+$ T cells are critically needed for survival, whereas $CD8^+$ T cells are necessary for optimal clearance.

Activation of T-cells can be achieved by immunization with several protein-containing antigens and extracts from Hc (Gomez et al. 1991; Sa-Nunes et al. 2005). In particular, Hsp60 is an immunodominant antigen and a potent inducer of protective cellular immunity. A cutaneous delayed-type hypersensitivity response to the antigen was apparent in the C57BL/6, BALB/c, and CBA/J mouse strains injected with Hsp60 and immunization prolonged survival of each strain of mouse lethally infected with Hc by intravenous or intranasal routes (Deepe and Gibbons 2002a; Gomez et al. 1991, 1995). The protective domain of Hsp60 was mapped to the fragment 3, between aa 172 and 443 (Deepe et al. 1996), which caused the most vigorous responses by cells from yeast-immunized mice *in vitro* and conferred protection against sublethal and lethal challenges in mouse models.

Regarding the cellular requirements, the depletion of CD4(+) T cells during the inductive phase of vaccination completely abolished the protective effect (Deepe and Gibbons 2002a; Gomez et al. 1995). Protection was strictly dependent on the presence, activation and proliferation of a specific Vβ8.1/8.2+ subset of T CD4+ lymphocytes (Deepe and Gibbons 2002b; Scheckelhoff and Deepe 2002). These cells are responsible for the production and increase in levels of certain Th1 inflammatory cytokines, such as IL-12, IFN-γ, and TNF-α (Lazar-Molnar et al. 2008; Cain and Deepe 1998; Zhou et al. 1995). However, studies in the expressive phase of vaccination show that the elimination of CD4 (+) or CD8 (+) T cells does not significantly modify fungal recovery from organs of infected animals or survival from a lethal challenge (Deepe and Gibbons 2002a).

12.4.2 Targeting Heat Shock Protein 60 with Antibodies

As Hc Hsp60 is an immunogenic molecule, it has been described as a potential target for antibody therapy (Guimaraes et al. 2009, 2011b). MAbs were generated against recombinant Hsp60 and their protective efficacy characterized in mouse models (Guimaraes et al. 2009). Interestingly, IgG2a and IgG1 mAbs to Hsp60 were protective whereas an IgG2b was disease enhancing. Protection was correlated to a reduction of fungal burden, decreased tissue damage, and prolongation in survival (Guimaraes et al. 2009, 2011c). Cytokine analyses revealed that the protective IgG2a mAbs induced a strong Th1-type host response. Notably, the IgG2b recognized the same region on the protein to which a protective IgG1 also bound in a competitive manner, suggesting that protection was strictly regulated by isotype. This finding was supported by data from mice treated with methamphetamine that developed significant increases in their IgG2b levels and also had accelerated and exacerbated disease (Martinez et al. 2009).

The protective mAbs to Hsp60 modified the intracellular fate of the yeast. IgG1 and IgG2a mAbs to surface Hsp60 activated the antifungal properties of macrophages in a dose dependent manner similar to what has been described in other pathogen-antibody models, including antibody interactions with other fungi (Mukherjee et al. 1996; Guimaraes et al. 2009) and for antibodies to heat shock proteins in other pathogens (Zugel and Kaufmann 1999; Macura et al. 2007). Interestingly, increased rates of

Hc yeast cell phagocytosis by the IgG1 subclass mAbs was primarily via Fc receptors whereas the IgG2a mAbs utilized both Fc and CR3 receptors to augment phagocytosis (Guimaraes et al. 2009). Phagosomal maturation was significantly increased in the presence of the protective mAbs and correlated with a reduction in intracellular yeast survival. In contrast, yeast cultured with the disease enhancing IgG2b mAb replicated at an enhanced rate within macrophages.

Interestingly, the protective mAbs were noted to induce aggregation of Hc yeast cells (Guimaraes et al. 2011b). Despite the negative charge on the Hc surface due to the presence of α-1,3-glucans on the cell wall, which increases the electrostatic potential surrounding the cells leading to repulsion, agglutination occurred only when cells were brought together due to a result of Brownian movement during which cellular collision permitted interaction. However, Hc yeast aggregation was an effect of concentration, but the magnitude of aggregation efficiency was dependent on the dissociation constant and subclass of each mAb characterized (Guimaraes et al. 2011a). Additionally, we used an optical tweezer to measure real-time interactions between single cells in the presence of opsonins and found a correlation of time for aggregation and binding constant, with the protective mAb being more effective than the non-protective mAb. This study also shows a cooperative function of Fc and CR3 receptor for the phagocytosis of large particles while small aggregates can be phagocytosed mainly through Fc receptors. Overall, it is unclear what the impact of agglutination potential of the antibodies is during infection. However, the antibodies may keep replicating cells agglutinated, which can reduce the dissemination of the fungus, and these clusters of cells may be more effectively targeted by host responses.

Recently, our group has extended the application of this panel of mAbs by coupling radionuclides to the Hsp60 mAbs and tested them against other human pathogenic fungi such as *Cryptococcus neoformans* and *Candida albicans* (Bryan et al. 2012) , which are the most common causes of fungal infections in immunocompromised patients. This approach, known as radioimmunotherapy (RIT), uses the exquisite ability of antibodies to bind antigens to deliver microbicidal radiation. To create RIT reagents which would be efficacious against all major medically important fungi, we have selected monoclonal antibodies (mAbs) to common surface fungal antigens, such as heat shock protein 60 (HSP60) and assessed cytotoxicity of these compounds after exposure of yeast to either radiolabeled mAbs or controls. Bi-188-mAbs to Hsp60 reduced the viability of both fungi by 80–100 % (Bryan et al. 2012). Our results suggest that it is feasible to develop RIT against fungal pathogens by targeting common antigens and such an approach could be developed against fungal diseases for which existing therapy is unsatisfactory.

12.5 Conclusions

Hsp60 proteins display high homology across all kingdoms. Hc Hsp60 is a key regulator of several cellular processes, including cell signaling, replication, expression of virulence associated proteins, and amino acid, protein, lipid, and carbohydrate

metabolism (Guimaraes et al. 2011d). Hsp60 apparently contributes to the regulation of cell wall modifications that allow the pathogen to survive under stress conditions.

As Hc is able to secrete Hsp60 and due to the promiscuity and broad interaction capacity of this protein, and, in its secreted form, Hsp60 could also act as a host proteins scavenger. These interactions could potentially modify the way that host cells recognize the pathogen and possibly modulate the host immune responses. This possibility opens numerous interesting avenues for future study, including drug targeting and immunotherapeutic approaches for combating life-threatening fungal infections in addition to histoplasmosis.

Most existing treatments for invasive fungal infections are chemotherapeutic drugs that interfere with certain biological processes in fungal cells and rely on the collaboration of patients' immune system. These are inherent drawbacks of antifungal drugs, especially as fungal cells have alternative pathways to the ones targeted by the drugs, which increases the potential for resistance. Current drugs often fail to eradicate invasive fungal infections and hence the challenge is to create novel treatments.

Although vaccine development for Hc is an exciting area of research, vaccination may not be effective in immunocompromised individuals, since they cannot necessarily orchestrate an immunoresponse. Passive immunization strategies could be of valuable use in this set of individuals, since MAbs to pathogen cell surface antigens are able to modify the complex dynamic host-pathogen interplay. Besides, MAbs to Hsp60, in combination with other mAbs to cell surface antigens or antifungal drugs could act synergistically, providing a more effective in protective efficacy. These new strategies could improve the treatment of patients with histoplasmosis or even administered prophylactically in outbreaks high-risk individuals, such as HIV-infected.

Radioimmunotherapy have emerged as a promising alternative for antifungal drugs. The technology of attaching radionuclides to the mAbs is mature and can be easily translated from the cancer field into infectious diseases. Using mAbs against promiscuous antigens, such Hsp60, would eliminate the need for specific mycological diagnosis, minimize toxicity of exposure to ineffective antifungals and potentially shorten treatment durations. Moreover, the activity of radiolabeled mAbs is independent of the host immune condition as RIT is directly lethal to the targeted cells.

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Chapter 13
The Role of Stress-Induced Activation of HSP70 in Dendritic Cells, CD4$^+$ T Cell, Memory and Adjuvanticity

Thomas Lehner and Yufei Wang

Abstract Inducible HSP70 is the hallmark of cell stress and it may mediate CD4$^+$ T cell memory and adjuvanticity. *In vitro* interaction between human DC exposed to thermal or oxidative stress and CD4$^+$ T cells elicits homeostatic memory. The mechanism involves activation of the NF-κB signalling pathway that leads to expression of membrane-associated IL-15 molecules. These interact with the IL-15 receptor complex on CD4$^+$ T cells, activating the Jak3 and STAT5 phosphorylation signalling pathway to induce CD40 ligand expression, T-cell proliferation and IFN-γ production. CD40 ligand on CD4$^+$ T cells in turn re-activates CD40 molecules on DC. The proliferating CD4$^+$ T cells were characterized as CD45RA$^-$CD62L$^+$ central memory cells. Importantly, the circuit is independent of antigen and MHC class-II interaction with TCR. This was confirmed and extended *in vivo* studies in BALB/c mice by SC immunization with ovalbumin (OVA) mixed with alum. Furthermore, inflammasomes were elicited, with significant activation of caspase 1, production of IL-1β, and adjuvanticity, demonstrated by enhancing OVA-specific serum IgG antibodies and CD4$^+$ T cell proliferation. The novel finding that alum, the most commonly used adjuvant in vaccines induces HSP70 suggests that stress is involved in the mechanism of adjuvanticity. This was confirmed by inhibition studies with the HSP70 inhibitor PES (phenylethynesulfonamide), which inhibited both inflammasomes and the adjuvant function. Parallel studies were then pursued with oxidative, K$^+$ releasing and a metal ionophore agents. All three stress agents induced

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HSP70, inflammasomes, interactions between splenic CD11c DC and $CD4^+$ T cells and the adjuvant function. The results suggest that the three stress agents elicit HSP70, inflammasomes, homeostatic memory and adjuvanticity, commensurate with those of alum. These findings extend our understanding of the mechanism of adjuvanticity and may provide an alternative strategy in developing novel adjuvants.

13.1 Prolegomenon (Editor)

Adjuvants are essential for the protection of human health, but it is curious that even in twenty-first century we do not understand their workings. The best known adjuvant is Freund's complete adjuvant (FCA) which is a suspension of sonicated *Mycobacterium tuberculosis* in oil (Freund et al. 1947). The work reported in this chapter on the potential adjuvant actions of host Hsp70 started from initial studies that demonstrated that the *M. tuberculosis* Hsp70 protein (DnaK) signalled to leukocytes, in this case primate CD8 lymphocytes, causing the release of the CC chemokines, CCL3-5 (Lehner et al. 2000). Studies of the effects of *M. tuberculosis* Hsp70 on myeloid cells found that this protein stimulates monocytes to secrete CCL3-5 through a mechanism involving the transmembrane receptor of the TNFα gene superfamily, CD40 (Wang et al. 2001). Most reports of Hsp70 binding to monocytes support the hypothesis that signalling is through TLR4, although a number of other receptors have been implicated (Henderson and Pockley 2010). There is another study of the human Hsp70 protein that confirms that this protein does bind to CD40. However the receptor binding site in the human Hsp70 protein is within the N-terminal ATP-binding domain (Becker et al. 2002), which is distinct from the identified receptor binding site in the *M. tuberculosis* Hsp70 protein (Wang et al. 2002) which is in the C-terminus of this protein. This suggests that the leukocyte-activating moonlighting activity of these two Hsp70 proteins evolved independently. *Mycobacterium tuberculosis* Hsp70 stimulated monocytes to generate IL-12, TNF-alpha, NO, and C-C chemokines and is of relevance to adjuvanticity. Dendritic cells exposed to *M. tuberculosis* Hsp70 exhibited significant increases in cell surface expression of CD80, CD83, CD86, CCR7 and HLA class II. Both CD80 and CD86 are the co-stimulatory signals required for T cell activation and are induced by adjuvants. Truncation mutagenesis and peptide mapping identified the receptor binding site of *M. tuberculosis* Hsp70 within a 20-mer peptide sequence of the C-terminus (Wang et al. 2002, 2005). Later studies from the same and another group revealed that *M. tuberculosis* Hsp70 also bound the HIV co-receptor, CCR5 (Whittall et al. 2006; Floto et al. 2006).

Since these studies were conducted it has been shown that *M. tuberculosis* releases large amounts of Hsp70 (Hickey et al. 2009 – see Chap. 8). Indeed, an earlier paper had reported that *Mycobacterium bovis* released two ATPases which could inhibit ATP-induced monocyte apoptosis. One of these two enzymes turned out to be the Hsp70 protein of this bacterium (Zaborina et al. 1999). In conclusion, the *M. tuberculosis* Hsp70 protein has multiple actions on leukocytes. The studies

presented in this chapter, which have evolved from studies of *M. tuberculosis* DnaK, reveal that the stress induced Hsp70 protein in human cells (HSPA1A) also has marked effects on leukocytes which suggest that this protein may function in adjuvant activity and that stress is a clear factor in adjuvant action.

13.2 Introduction

Immunological memory to pathogens following natural infection, or vaccination prevents infection on re-exposure to the pathogen. Dendritic cells in mucosal tissues or skin take up the pathogen, process it and migrate to lymph nodes where they interact with T cells and elicit effector and memory T cells. The $CD4^+$ subset of T cells help production of both B cell antibodies and $CD8^+$ T cytotoxic cells. Immunological memory can last a lifetime, as has been demonstrated for up to 75 years with smallpox infection (Hammarlund et al. 2003). Most, if not all vaccines, need an adjuvant to enhance immune functions and they have long been largely recognized as critical components of vaccines. Alum (aluminium salt) has been the sole clinically approved adjuvant, and only recently has it been subjected to critical investigation.

Maintenance of immunological T cell memory may involve low level antigen-independent T cell division in lymphoid tissue, referred to as basal homeostatic proliferation of memory T cells (Tough and Sprent 1994; Murali-Krishna et al. 1999; Intlekofer et al. 2006). There is considerable evidence that memory T cells do not require TCR-MHC interaction to survive, as reported with a variety of microorganisms (Goldrath and Bevan 1999; Swain et al. 1999). Virus specific $CD4^+$ T cells persist after viral clearance and the $CD4^+$ Th1 and Th2 memory T cells are capable of rapid proliferation in response to encounter with LCMV (Varga and Welsh 1998). Homeostatic proliferation is necessary to maintain a stable $CD4^+$ memory T cell population, as has been demonstrated with smallpox vaccination (Hammarlund et al. 2003). However, the mechanism responsible for the maintenance of T cell memory is not fully understood.

Stress induces a series of important structural and functional changes in cells, including activation of genes coding for heat shock proteins, alteration of protein translation and changes of a number of kinases. Heat has a significant effect on innate and adaptive immunity, such as lymphocytes migration (Wang et al. 1998), T cell proliferation, activation and modulation of DC function (Hatzfeld-Charbonnier et al. 2007), and overall enhances host defense mechanisms against microbial infection. Host derived HSP70 is an important endogenous danger signal that stimulates innate immunity by activating DC (Matzinger 2002). Oxidative stress also activates inflammasomes and triggers maturation of pro-inflammatory cytokines such as IL-1β, a key mediator of inflammation. Inflammasomes consist of intracellular receptors called nucleotide-binding oligomerization domain-like receptors (NLSs), along with Cardinal and apoptosis associated speck-like protein containing a caspase recruitment domain (ASC) and these form a caspase-1 activating complex.

This acts as an intracellular sensor for microbial infection and can be also activated by endogenous danger signals, such as stress (Martinon et al. 2009). Intracellular stress sensors involve NLR which sense endogenous danger signals (Mariathasan and Monack 2007). Commonly, NLRP3 with Cardinal and ASC initiates formation of the inflammasome protein complexes, which induce caspase-1 leading to activation of IL-1β pro-inflammatory cytokine (Martinon et al. 2009).

The objectives of this review are to discuss: (a) the reciprocal interactions between DC and $CD4^+$ human T cells, induced by thermal, oxidative, K releasing and ionophore stress agents, engaging the IL-15/IL-15 receptor complex, followed by CD40L-CD40 activation, resulting in an antigen-TCR independent circuit; (b) to demonstrate that a number of stress agents induce the homeostatic pathway, which is mediated by inducible HSP70; (c) a comparative study of alum and stress agents to show that both induce iHSP70 and elicit inflammasomes and; (d) to demonstrate that the stress agents may act as potent adjuvants comparable with that of alum.

13.3 Stress-Induced Upregulation of Endogenous iHSP70 in DC Derived from Human PBMC

Monocyte-derived DC were studied by exposing DC to thermal stress at 41.5 °C for 90 min. Cell surface and intracellular HSP70 were examined by flow cytometry and Western-blots following overnight incubation at 37 °C. An increase in cell-surface HSP70 expression of DC was observed from 1.7 ± 0.3 to 4.3 ± 0.8 % ($p = 0.04$; Fig. 13.1a) and intracellular HSP70 MFI from 141 ± 15 to 210 ± 29.6 ($p = 0.047$) (Fig. 13.1b). Western blot analysis demonstrated that the inducible HSP70 was upregulated by heat treatment of DC (Fig. 13.1c). A dose-dependent increase in HSP70 was also found when DC were exposed to oxidative stress with NA arsenite (Fig. 13.1c). To confirm the specificity of heat-induced HSP70, DC were treated with the inducible HSP70 inhibitor KNK437 (Koishi et al. 2001), which showed significant inhibition of HSP70, both the cell-surface and intracellular HSP70 ($p = 0.032$ and $p = 0.039$ respectively, Fig. 13.1a, b). Representative flow cytometry profiles are presented in Fig. 13.1e.

13.4 Upregulation of maIL-15 on DC by Stress

Thermal exposure of DC also showed a significant increase in membrane associated (ma)IL-15 from 16.8 ± 3.8 to 33.7 ± 5.1 % ($p = 0.008$), which was inhibited on treatment with KNK437 to 17.4 ± 5.5 % ($p = 0.029$), suggesting that the inducible HSP70 may be responsible for IL-15 induction (Fig. 13.1d, e). Similarly, treatment of DC with the oxidative stress agent induced a dose-dependent increase in maIL-15 (Fig. 13.1f). These results suggest that human

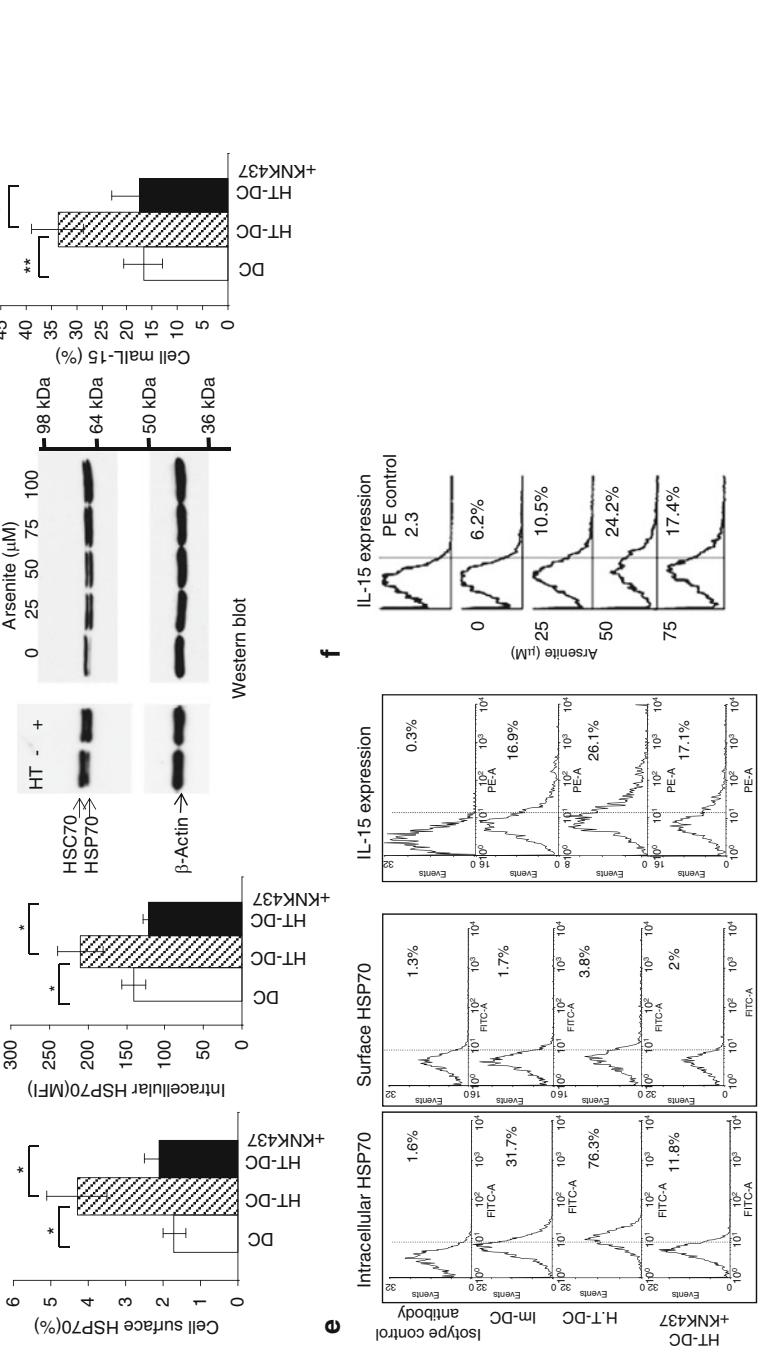

Fig. 13.1 Increase in DC expression of HSP70 and IL-15 induced by stress. The effect of heat treatment (HT) and the HSP inhibitor KNK437 on (**a**) cell surface and (**b**) intracellular HSP70 expression; (**c**) HSP70 induced by HT and oxidising stress by sodium arsenite treatment of DC demonstrated by Western-blots of DC lysate; (**d**) induction of IL-15 expression by H-T of DC and inhibition by KNK437; (**e**) flow cytometry profile of HSP70 and IL-15 expression of HT-DC and (**f**) dose-dependent induction of IL-15 expression by sodium arsenite treatment of DC. Three or more experiments were carried out in **a**–**f**. *$p < 0.05$, **$p < 0.01$ indicate significant differences between the bracketed columns by the paired Student's t test

monocyte-derived DC respond to thermal and oxidative stress by expressing intracellular and cell surface HSP70. Furthermore, they elicit maIL-15, which is critical in the development of homeostatic memory.

13.5 Stimulation of CD4⁺ T Cell Proliferation and CD40L Expression by Stressed DC

In order to examine the function of DC treated by stress agents we used a co-culture model by incubating DC with autologous CD4⁺ T cells at a ratio of 1:10. DC were exposed to thermal stress at 41.5 °C for 90 min, co-cultured with autologous CD4⁺ T cells and after 7 days T cell proliferation was studied by the CFSE dilution method. Heat-treated DC significantly enhanced CD4⁺ T cell proliferation from 14.1 ± 2.7 % with untreated to 20.3 ± 2.9 % with heat treated DC ($p = 0.003$, Fig. 13.2a). The proliferating cells stimulated by heat treated DC were largely $CD4^+CD45RA^-$ ($p = 0.0011$), $CD45RA^-CD62L^+$ central ($p = 0.001$) and not $CD62L^-$ effector memory T cells (Fig. 13.2b, c). This is consistent with the findings that isolated central memory CD4⁺ T cells showed significantly greater proliferation than effector memory CD4⁺ T cells stimulated by heat treated DC. Activation of CD4⁺ T cells by DC exposed to thermal stress was also demonstrated by an increase in CD40L expression on CD4⁺ T cells with increasing proportion of heat treated DC ($p = 0.036$, Fig. 13.2d). A similar increase was observed in the production of IFN-γ ($p = 0.039$, Fig. 13.2e). Thermal stressed DC also upregulate the secondary response to tetanus toxoid (TT) in primed subjects. A significant increase in the proliferative response was seen when the heat treated DC were co-cultured with CD4⁺ T cells and stimulated with TT (36.6 ± 2.4 %), compared with DC kept at 37 °C (27.3 ± 1.9 %) (Fig. 13.2f, g). Thus, thermal stress of DC increases significantly CD4⁺ T cell proliferation, both in antigen independent and TT dependent co-cultures. The proliferating cells are CD4⁺ $CD45RA^-$ and predominantly central memory T cells. An increased proportion of the CD4⁺ T cells express IFN-γ.

13.6 TCR-Independent Stimulation of CD4⁺ T Cell Proliferation by Stressed DC

In an attempt to define the mechanisms of DC-stimulated antigen-independent CD4⁺ T cell proliferation, the effect of heat treated-DC was compared with the DC culture supernatant. Indeed, CD4⁺ T cell proliferation was cell-cell contact- dependent as the DC culture supernatant failed to elicit CD4⁺ T cell proliferation. The cytokines IL-2, IL-4, IL-7 and IL-15 were not detected in the DC culture supernatants (data not shown). The potential dependence of CD4⁺ T cell proliferation on maIL-15 was examined by antibody blocking assays. T cell proliferation by heat treated DC was shown to be dependent on maIL-15 expression as significant

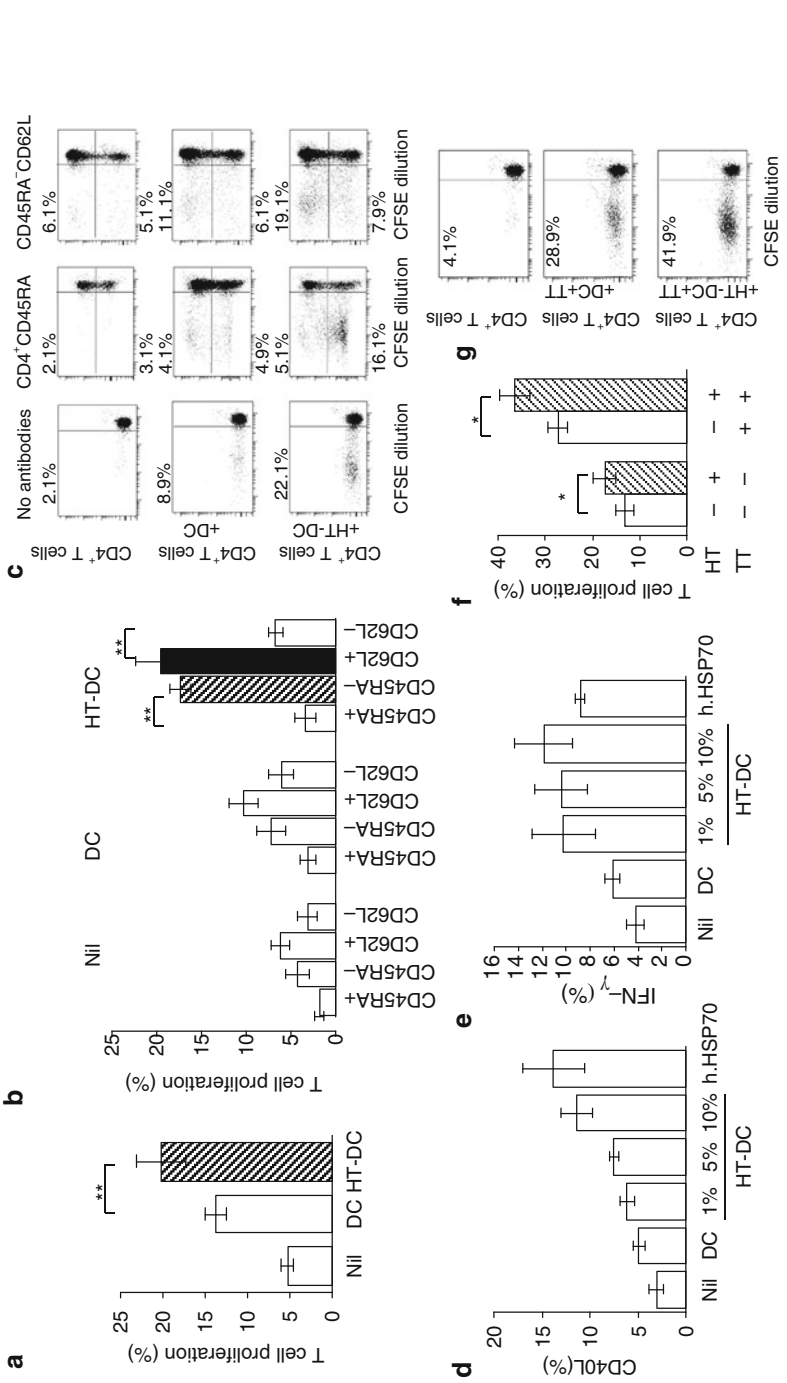

Fig. 13.2 (**a**) Stimulation of CD4+ T cell proliferation by heat treated (HT)-DC assayed by the CFSE method, (**b**) HT-DC stimulating proliferation of CD4+ central memory T cell subsets and (**c**) flow cytometry illustrations of CD4+, CD45RA− memory and CD45RA−CD62L+ central memory T cell proliferation. (**d**) The effect of increasing the proportion of HT-DC cocultured with CD4+ T cells on CD40L expression, (**e**) IFN-γ production, (f) the effect of HT-DC on TT stimulated T cell proliferation and (**g**) flow cytometry illustrations of TT stimulated CD4+ T cell proliferation; three or more independent experiments were carried out in (**a–e**) and the results are presented as mean ± sem. *h* human, *p<0.05, ** p<0.01 indicate significant differences between the bracketed columns calculated by the paired Student's t test

inhibition was found with anti-IL-15 antibodies, compared with the isotype control ($p = 0.043$; Fig. 13.3a). On the other hand, the independence of heat-induced $CD4^+$ T cell proliferation from the HLA-class II-TCR interaction is evident from the lack of inhibition of proliferation when the $CD4^+$ T cells were treated with HLA-class-II antibodies or the ZAP70 inhibitor, compared with that of IL-15 antibody ($p < 0.01$; Fig. 13.3b). The TCR-HLA class II dependence is clearly seen by inhibition of TT stimulated proliferation with antibodies to HLA-class II (Fig. 13.3c), unlike that by heat treatment (Fig. 13.3c).

The interaction between DC maIL-15 and $CD4^+$ T cells was then focused on the IL-15R complex, which consists of high affinity IL-15Rα chain subunit along with the IL-2/15Rβ (CD122) and common γ chains (or IL-2, CD132). Steady state $CD4^+$ T cells express low levels of IL-15R α, β and γ chains, which are significantly upregulated after stimulation with recombinant or maIL-15. To find out which of the 3 IL-15 receptor chains is involved in stimulating $CD4^+$ T cell proliferation by DC maIL-15, we co-cultured HSP70-activated DC with autologous $CD4^+$ T cells and used anti-IL-15Rα, β and γ chain antibodies. The results suggest that only antibodies to the γ chain (γc) significantly inhibited $CD4^+$ T cell proliferation ($p = 0.038$, Fig. 13.3d). This was confirmed by γc siRNA knock down of $CD4^+$ T cell proliferation, compared with unrelated siRNA (Fig. 13.3e, f). These results suggest that an antigen-independent memory circuit between DC and $CD4^+$ memory T cells can be stimulated by stress-induced DC through an IL-15 dependent mechanism.

13.7 The Signalling Pathways Mediating Transcription of IL-15 in DC and CD40L Expression in $CD4^+$ T Cells

The signalling pathway engaged in transcription of IL-15 in DC involves IKKα and IKKβ proteins, which phosphorylate IKB leading to its ubiquitination and proteolysis in the cytoplasm (Ghosh et al. 1998), allowing NFκB p50/p65 localization and NF-κB responsive gene transcription. Stimulation of DC maIL-15 expression by thermal, oxidative stress or microbial HSP70 was inhibited with IKKα, IKKβ and IKB kinase inhibitors (Fig. 13.4a); the flow cytometry profiles are shown in Fig. 13.4b. NF-κB nuclear localization in DC was significantly increased when the cells were exposed to thermal ($p = 0.009$) and oxidizing ($p = 0.012$) stress (Fig. 13.4c).

The signalling pathway between γc chain engagement and CD40L expression, expected to involve Jak3 and STAT5 proteins was then examined. Addition of Jak3 and STAT5 inhibitors to co-cultures of heat-treated DC and $CD4^+$ T cells inhibited upregulation of CD40L ($p < 0.0001$), whereas a control agent for Jak3 inhibitor had no effect (Fig. 13.4d). The flow cytometry profiles are shown in Fig. 13.4e. These results suggest that the signaling pathway in $CD4^+$ T cells engage the Jak3/STAT5 phosphorylation pathway leading to NF-κB nuclear localization.

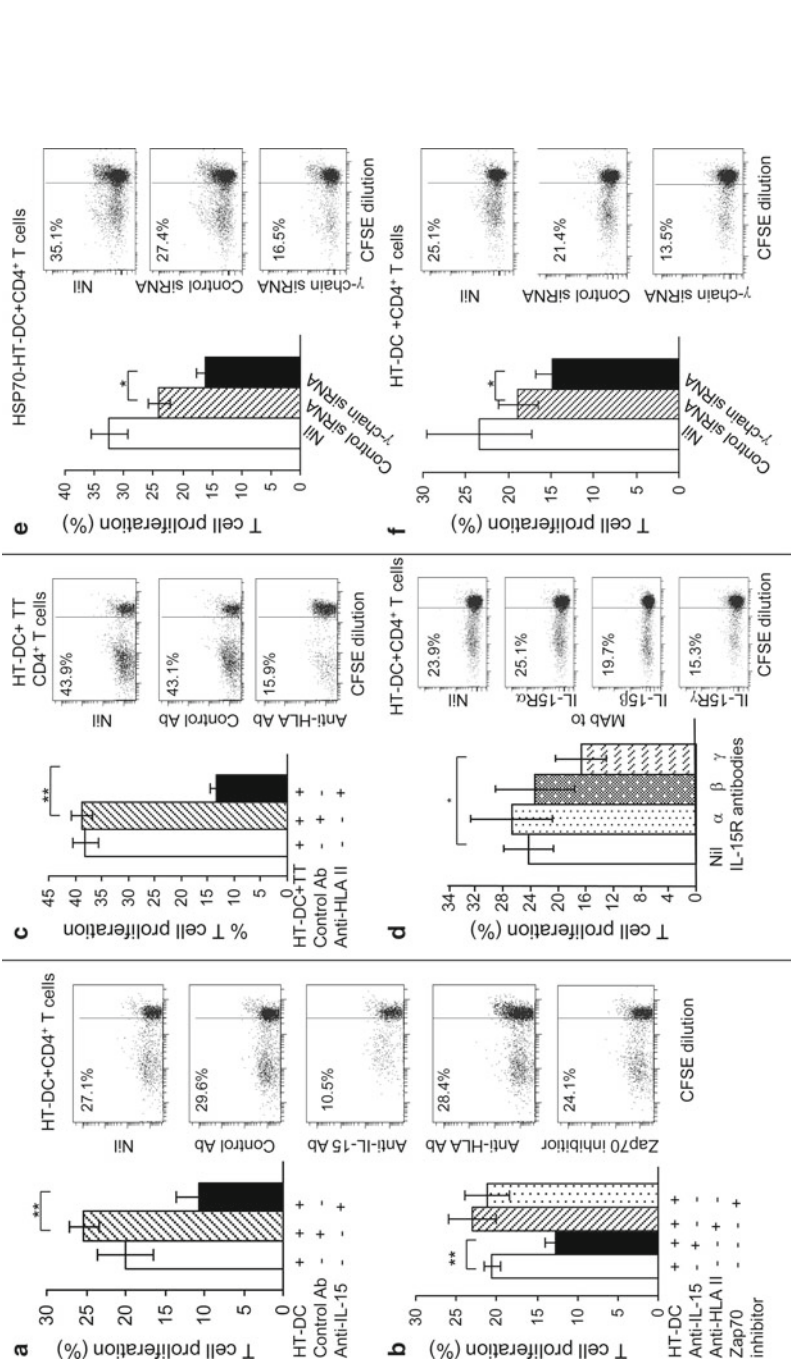

Fig. 13.3 (**a**) Inhibition of DC maIL-15 induced T cell proliferation by antibodies to IL-15, (**b**) but not by Zap70 inhibitor or antibody to HLA class II antigens, (**c**) inhibition of TT antigen stimulated CD4+ T cell proliferation by anti-HLA class II molecules; (**d**) The effect of antibodies to IL-15Rα, β and γ chains, (**e**) and (**f**) siRNA of IL-15R γ-chain on HSP70 or HT-DC, respectively induced CD4+ T cell proliferation. Three or more independent experiments were carried out in (**a–f**) and the results are expressed as mean ± sem. *p < 0.05, ** p ≤ 0.01 indicate significant differences between the bracketed columns by the paired Student's t test

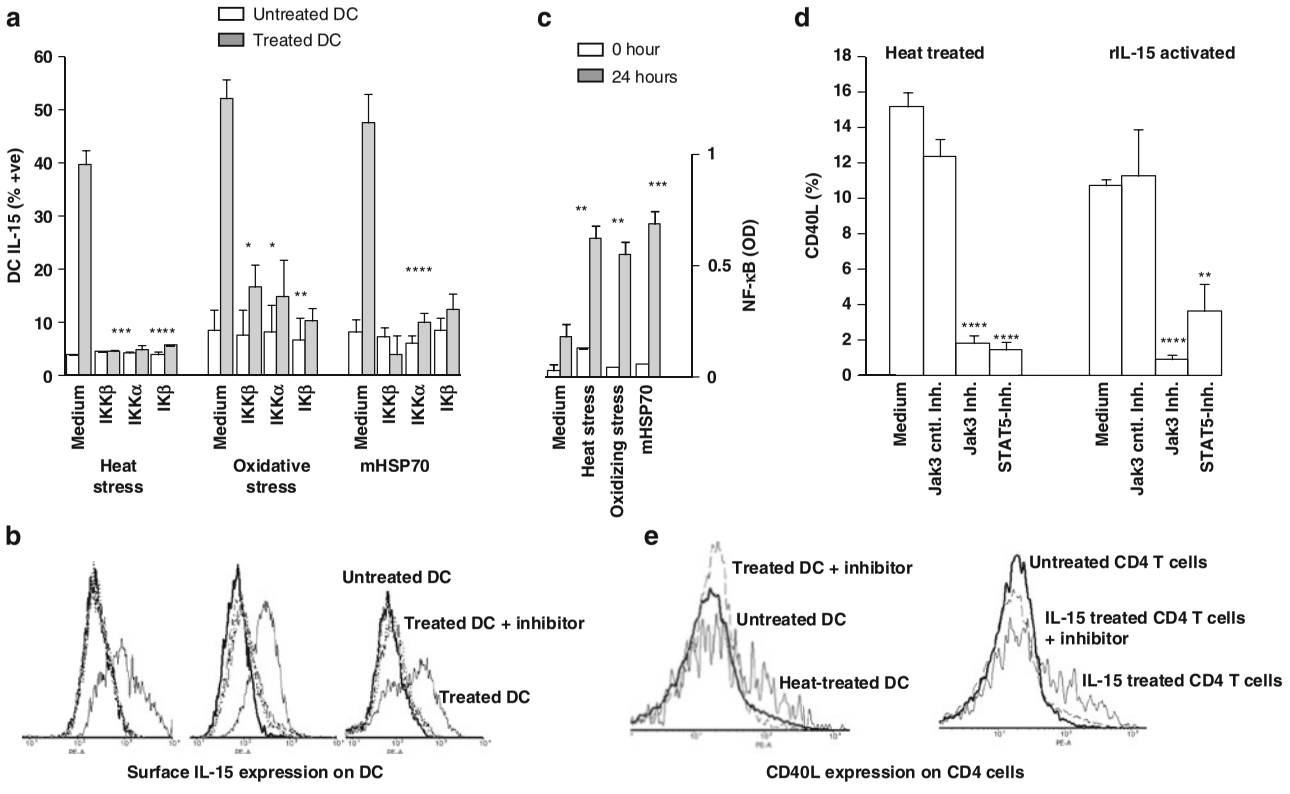

Fig. 13.4 (**a**) The effect of thermal or oxidative stress and microbial (m)HSP70 applied to DC on membrane associated IL-15 expression in the presence and absence of NF-κB signalling inhibitors, (*open columns* indicate the baselines), with (**b**) representative flow cytometry profiles. (**c**) Nuclear NF-κB levels after 0 and 24 h stimulation. (**d**) The effect of HT-DC or recombinant (r) IL-15 on the $CD4^+$ T cell expression of CD40L, in the presence and absence of Jak3 and STAT5 inhibitors and (**e**) corresponding flow cytometric profiles. Three or more experiments were carried out in **a**, **c**, and **d** *$p<0.05$, **$p=0.01$, ***$p=0.002$, ****$p=0.001$ calculated by the paired Student's t test

13.8 Re-activation of the DC-CD4+ T Cell Feed Back Circuit

To demonstrate that the feed-back circuit is re-activated by the CD40L molecules expressed by CD4+ T cells binding CD40 receptors on DC, CD40L+CD4+ T cells were co-cultured with autologous immature DC. CD4+ T cells activated by HT-DC which induce CD40L expression stimulated autologous immature DC to express maIL-15 and the maturation markers CD83, CD40, CD86 and HLA class II molecules (data not presented). An antigen-independent feed-back cycle is generated by activating the CD40 molecules on DC, which elicits maIL-15, this interacts with CD4+ T cells γc complex, inducing CD40L on CD4+ T cells, which in turn ligates CD40 and reactivates the DC. The data have been reported in reference (Wang et al. 2010).

13.9 The Role of Inducible HSP70 in Alum-Mediated Adjuvanticity

Alum is the only clinically proven adjuvant to elicit Th2 type immune responses and enhance antibody production in humans, although how alum mediates these effects on the immune system is still largely unknown. Recent studies suggest that alum activates the inflammasome NLRP3 leading to activation of caspase-1 and release of mature IL-1β in innate immune cells, including DC and monocytes (Li et al. 2008; Kool et al. 2008). The possibility that alum may act as a stress agent was then investigated *in vivo*, following co-administration of OVA with alum in four groups of 6–10 BALB/c mice per group, with an untreated and OVA-treated control group. Flow cytometry examination of splenic $CD11c^{high}$ cells, which are predominantly DC, excluding the $CD11c^{low}$ plasmablasts, showed significant increase in the cell-surface HSP70 ($p<0.01$) and to a lesser extent intra-cellular HSP70 (date not presented). The findings that alum upregulates HSP70 in splenic CD11c+ cells suggests a stress-mediated response.

13.10 MaIL-15 on DC and CD40L Expression in CD4+ and CD8+ T Cells

We have then evaluated the effect of alum on murine splenic CD11c+ DC following immunization with OVA and alum. OVA alone had no significant effect on maIL-15, from 8.2 ± 0.9 to 10.4 ± 1.4 % but with alum maIL-15 was upregulated to 17.8 ± 0.9 % ($p=0.001$; Fig. 13.5a, c). Since inducible HSP70 has not been reported to be involved in the mechanism of alum-induced functions, we used PES (phenylethynesulfonamide), which interacts with the inducible HSP70 peptide-binding domain and leads to disruption of HSP70 co-chaperones and substrate proteins (Leu et al. 2009).

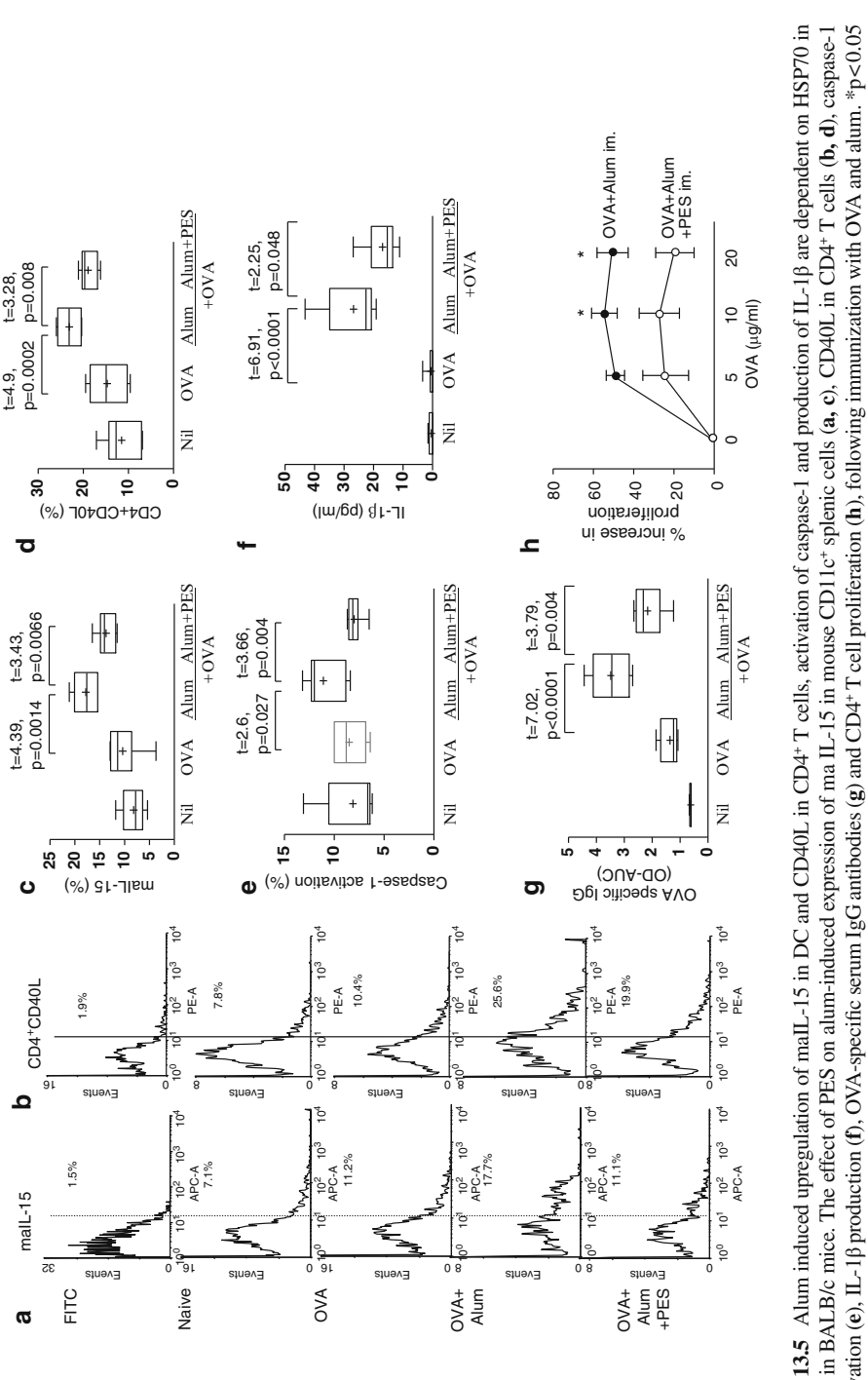

Fig. 13.5 Alum induced upregulation of maIL-15 in DC and CD40L in $CD4^+$ T cells, activation of caspase-1 and production of IL-1β are dependent on HSP70 in vivo in BALB/c mice. The effect of PES on alum-induced expression of ma IL-15 in mouse $CD11c^+$ splenic cells (**a, c**), CD40L in $CD4^+$ T cells (**b, d**), caspase-1 activation (**e**), IL-1β production (**f**), OVA-specific serum IgG antibodies (**g**) and $CD4^+$ T cell proliferation (**h**), following immunization with OVA and alum. *p<0.05

To this end we co-administered PES with alum in a further group of mice and compared the results with those treated with alum alone. PES significantly inhibited maIL-15 from 17.8 ± 0.9 % with alum + OVA to 13.7 ± 0.7 % with added PES ($p < 0.01$, Fig. 13.5c).

Expression of CD40L was evaluated in CD4+ T cells as maIL-15 of DC interacts with IL-15 receptor complex on CD4+ T cells and stimulates the CD40L activation marker. Indeed, CD40L was significantly upregulated in mice immunized with alum (23.1 ± 1.0), compared with the OVA immunized mice (14.7 ± 1.2; Fig. 13.5b, d). CD40L in CD4+ T cells was significantly inhibited with PES from 23.1 ± 1.0 to 18.9 ± 0.8 % ($p < 0.01$, Fig. 13.5b, d). Thus, alum significantly upregulated maIL-15 in CD11c+ DC and CD40L in CD4+ T cells, which may re-engage CD40 on DC and B cells and stimulate their functions. Both maIL-15 and CD40L expression were at least partly dependent on HSP70, as demonstrated by the significant inhibition with PES.

13.11 Activation of Inflammasomes Demonstrated by Caspase-1 and IL-1β

Caspase-1 is an integral part of the multiprotein NLRP3 inflammasome complex was activated by alum ($p < 0.05$; Fig. 13.5e). Production of IL-1β was also significantly upregulated ($p < 0.0001$; Fig. 13.5f). PES significantly decreased caspase-1 activity from 11.1 ± 0.8 to 8.0 ± 0.3 % ($p = 0.004$; Fig. 13.5e) and IL-1β expression (from 26.8 ± 3.7 to 17.0 ± 2.2 pg/ml ($p < 0.05$; Fig. 13.5f). Thus, alum activates caspase-1, which converts pro-IL-1β to the active form involved in stimulating adaptive immune responses. Both functions were partly inhibited with PES, consistent with HSP70 involvement in inflammasomes.

13.12 Alum Induces Adjuvanticity

The adjuvant function of alum has been amply demonstrated, but here we use it as a baseline to compare with the stress agents. OVA administered with alum elicited a very significant increase in serum IgG OVA specific antibody production from 1.37 ± 0.13 in the OVA immunized to 3.49 ± 0.27 ($p < 0.0001$) in the OVA + alum immunized mice, assayed 1 week after the 3rd and last immunization (Fig. 13.5g). IgG antibodies elicited with OVA and alum were significantly inhibited from 3.49 ± 0.27 with alum alone to 2.16 ± 0.2 ($p < 0.01$) with PES and alum immunized mice (Fig. 13.5g). Surprisingly, analysis of the effect of PES on the 3 IgG subtypes showed that only IgG2a was significantly inhibited, which is associated with Th1 response (data not presented). The antibodies are expressed as total absorbance (OD) of the serially diluted sera, calculated by the area under

the curve. Significant dose-dependent increase in proliferative responses of OVA specific $CD4^+$ T cells were recorded after restimulation *in vitro* with OVA (Fig. 13.5h). Treatment with PES again demonstrated significant decrease in OVA specific $CD4^+$ T cell proliferation ($p<0.05$, Fig. 13.5h). These inhibition studies argue in favour of the adjuvanticity of alum in murine OVA-specific B and T cell responses being at least partly dependent on inducible HSP70. It is important to note that these effects were observed *in vivo*, in the absence of any changes in the proportion of viable $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells and total splenic cell population after administration of PES, ruling out PES induced cytotoxicity. Altogether, the mechanism of $CD11c^+$ DC-$CD4^+$ T cell interaction, manifested in the serial expression of maIL-15, CD40L respectively, activation of caspase-1, IL-1β, inflammasome and adjuvant functions appears to be partly dependent on alum stimulated inducible HSP70.

13.13 Cell-Surface and Intracellular HSP70 Induced by the Stress Agents

These findings raised an intriguing question whether stress inducing agents can function as adjuvants? To this end we examined HSP70 expression *in vivo*, following co-administration of OVA with Na arsenite, Gramicidin or dithiocarbamate in three groups of 6–10 BALB/c mice per group, with an untreated and OVA-treated control groups as described above for alum. Flow cytometry examination of splenic $CD11c^{high}$ cells, showed significant difference in the cell-surface and intra-cellular HSP70 (Wang et al. 2012). These findings demonstrate that cell-surface and intracellular HSP70 in splenic $CD11c^+$ cells are upregulated by the three diverse stress agents consistent with that of alum.

13.14 Membrane Associated maIL-15 on DC and CD40L Expression in $CD4^+$ T Cells

All three stress agents significantly upregulated maIL-15 (ANOVA, $p<0.0001$); with Na arsenite, Gramicidin or dithiocarbamate ($p<0.001$; Fig. 13.6a, c), as was shown with alum. Significant differences of CD40L expression in $CD4^+$ T cells were also found in the entire cohort of mice (ANOVA, $p<0.01$). CD40L in $CD4^+$ T cells was upregulated in mice immunized with Gramicidin (18.4 ± 1.5 %, $p<0.01$), dithiocarbamate (18.8 ± 1.3, $p<0.05$) and Na arsenite (15.1 ± 1.5 %), but the latter failed to reach significant value as compared with the OVA immunized mice (14.7 ± 1.2; Fig. 13.6b, d). Thus, all stress agents significantly upregulated CD40L of $CD4^+$ T cells, as was found with alum.

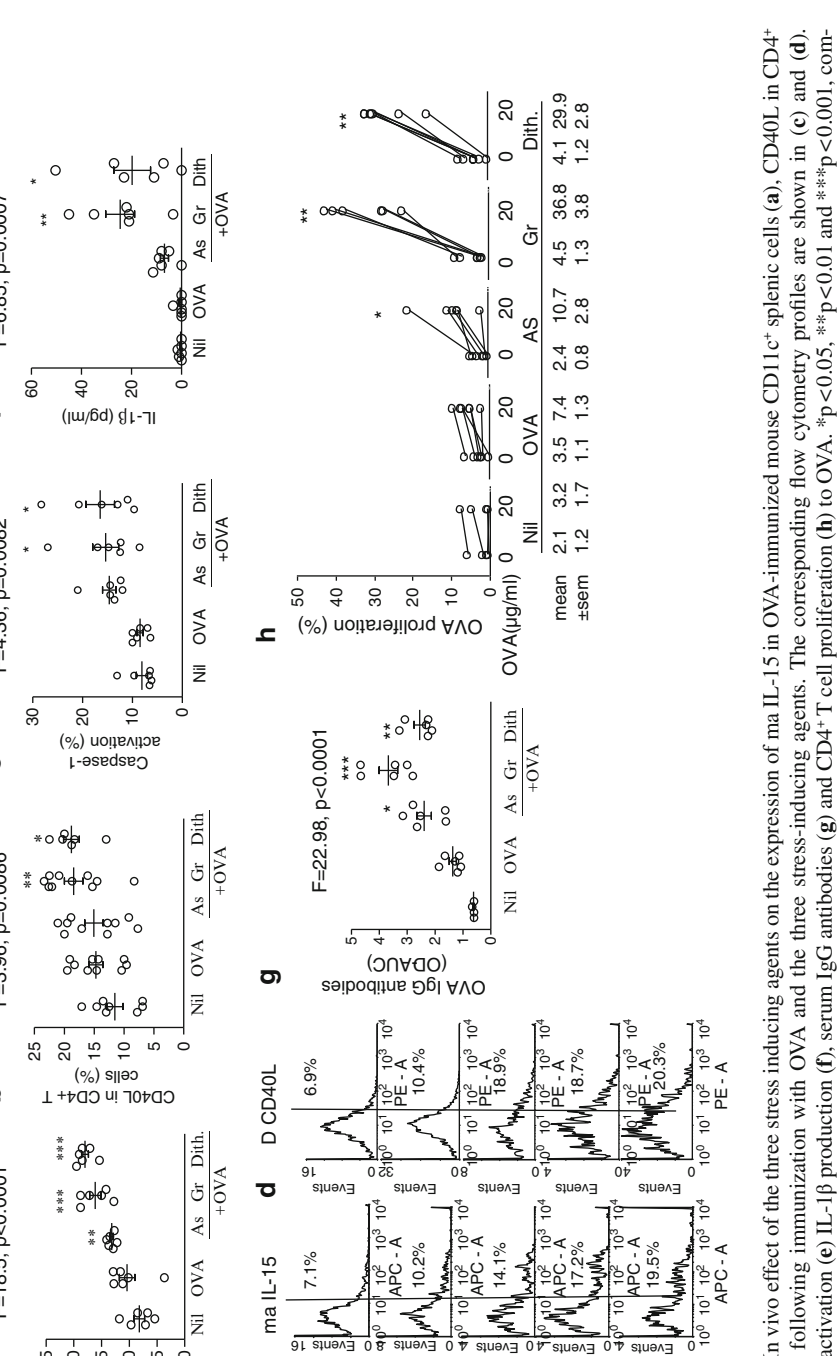

Fig. 13.6 In vivo effect of the three stress inducing agents on the expression of ma IL-15 in OVA-immunized mouse CD11c$^+$ splenic cells (**a**), CD40L in CD4$^+$ T cells (**b**) following immunization with OVA and the three stress-inducing agents. The corresponding flow cytometry profiles are shown in (**c**) and (**d**). Caspase-1 activation (**e**) IL-1β production (**f**), serum IgG antibodies (**g**) and CD4$^+$ T cell proliferation (**h**) to OVA. *p<0.05, **p<0.01 and ***p<0.001, compared with naïve mice

13.15 Activation of Inflammasomes Demonstrated by Caspase-1 and IL-1β

Activation of caspase 1 and production of IL-1β were demonstrated with each test agent ($p < 0.05$–0.01, Fig. 13.6e, f), except Na arsenite, though IL-1β was upregulated from 0.4 ± 0.3 to 6.9 ± 1.6 pg/ml. Thus, both alum and the stress agents activate caspase-1, which converts pro-IL-1β to the active form involved in stimulating adaptive immune responses.

13.16 Stress-Induced Adjuvanticity

The adjuvant function of stress-inducing agents has received limited attention in the past. Here we have examined the effect of administering the stress agents with OVA on antibody and $CD4^+$ T cell responses, as was done with alum. Serum IgG OVA specific antibody production assayed 1 week after the 3rd immunization showed significant difference in the 5 groups of mice (ANOVA, $p < 0.0001$) and with each stress agent (Fig. 13.6g). Mice immunized with OVA alone showed no significant increase in antibodies. Examination of the proliferative response of OVA specific $CD4^+$ T cells were then assayed after restimulation *in vitro* with 20 μg/ml OVA (Fig. 13.6h). Significant increases in proliferation were found in OVA immunized mice treated with each of the three stress agents (Fig. 13.6h).

13.17 Mechanism of Inducible HSP70 Involvement in Adjuvanticity

The mechanism of the inducible HSP70 mediating adjuvant function has yet to be established, although there is abundant evidence that HSP70 activates innate immune responses. HSP70 interacts with a number of proteins in cellular signal pathways, mRNA transcription and stress responses and plays a crucial role in cell survival and anti-apoptosis (Mayer and Bukau 2005; Brodsky and Chiosis 2006; Garrido et al. 2006; Schmitt et al. 2006). The inducible HSP70 is found in low level expression in normal cells, but is rapidly upregulated upon exposure to stress. Currently, the HSP70 mediated signalling pathways, important for alum stimulated innate responses, NLRP3 activation and adjuvanticity, are not clear and require further investigation. However, endogenous HSP70 plays an important role in the induction of immune responses *in vivo*. Oral application of Carvacrol, a major compound in the oil of many Origanum species, upregulates HSP70 expression in Peyer's patches of mice and specifically promotes T cell recognition of endogenous Hsp70 (Powers and Workman 2007).

Reducing expression of HSP70 expression by deletion of HSP70 genes in animals significantly inhibited immune responses and attenuated autoimmune diseases (Wieten et al. 2010).

13.18 Conclusions

Various agents, such as heat, inflammation, metabolites, K^+ efflux or oxidation can induce cell stress, a hallmark of which is rapid upregulation of inducible HSP70. The latter is also an important regulator of apoptotic signaling pathways, through multiple steps, including control of mitochondrial membrane integrity and caspase activation (Mayer and Bukau 2005; Brodsky and Chiosis 2006; Garrido et al. 2006; Schmitt et al. 2006; Powers and Workman 2007). Our studies have explored two important functions of stress and inducible HSP70 in stimulating memory cell homeostasis and adjuvant function.

Stress-activated DC express endogenous intracellular and cell surface HSP70, and maIL-15 molecules through activation NF-κB signaling pathway. Inhibition of iHSP70 diminishes maIL-15 induction. IL-15 mediates antigen-independent proliferation of T cells by interacting with the IL-15 receptor complex, promotes basal homeostatic proliferation and survival of $CD4^+$ and $CD8^+$ memory T cells and elicits $CD4^+$ effector memory T cells, generating cytokines and chemokines. IL-15 may function as an internal adjuvant, eliciting antigen specific $CD4^+$ and $CD8^+$ T cell and B cell responses. IL-15 expressed on stressed DC interacts with the IL-15 receptor complex on $CD4^+$ T cells, activating Jak3 and STAT5 phosphorylation signalling pathway and induces homeostatic T cell receptor independent $CD4^+$ memory T cell interacting circuit.

Furthermore, we have identified a novel function of Alum in stimulating HSP70 stress response in DC, which may be at least part of the mechanism of alum adjuvanticity. Upregulated iHSP70 elicitis maIL-15 expression in DC demonstrated both in vitro and in vivo. Inhibition of inducible HSP70 significantly impairs alum stimulated innate immune responses, adjuvant function, and antigen specific T and B cell responses. Conversely, stress inducing agents, such as arsenite, gramicidin or dithiocarbamate which upregulate iHSP70, maIL-15 and CD40L expression can function as adjuvants and elicit antigen specific immune responses, demonstrated in antibody induction and $CD4^+$ T cell responses.

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Chapter 14
Candida albicans Ssa: An Hsp70 Homologue and Virulence Factor

Sumant Puri and Mira Edgerton

Abstract *Candida albicans* is a member of the normal oral and gut microbiota and is also an opportunistic pathogen causing oral and genital infections in humans. Two of the Hsp70 proteins of this organism, Ssa 1 and Ssa 2, show unusual biological actions, presumably moonlighting actions, which contribute to the interaction of this yeast with its host. Both Ssa 1/2 are found on the outer surface of the fungus and this location provides novel functions for these proteins. It also appears to be an Achilles heel of this fungus. The Hsp70 proteins are highly immunogenic and so the surface location of Ssa 1/2 makes a good immunological target for innate and adaptive immune responses to this organism and also suggests these proteins could be vaccine candidates. Surprisingly, Ssa 1/2 binds to the antifungal peptide Histatin (Hst) 5 and enables this toxic molecule to be taken up by the yeast causing cell death. In spite of these findings, *C. albicans* lacking Ssa 1, but not Ssa 2, were significantly less virulent in infected mice and this was related to the loss of invasiveness of this fungus. Thus these Hsp70 proteins play unexpected roles in the lifestyle of *C. albicans.*

14.1 Introduction

Candida albicans expresses proteins belonging to both major families of conserved heat shock proteins, Hsp90 and Hsp70, as well as several small Hsps (sHsps). Hsp90 has been well studied, largely because of its classical response to elevated temperature that has a direct role in the yeast to hyphae transition when cells are shifted from 30 to 37 °C. Although also expressed as a cytosolic protein, *C. albicans* Hsp70 has perplexed researchers since its discovery within the fungal cell wall. Hsp70

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proteins are well known for their cytosolic role in protein quality control, where they perform the function of central chaperones. Here, we will discuss the role of Hsp70 with regards to its unconventional role as a cell wall Hsp, as well as its influence on *C. albicans* pathogenesis. We will contrast this role with respect to Hsp90, which has typical stress-induced functions as a feature true to classical Hsps. Readers should also compare the role of Hsp70 in the virulence of *C. albicans* with the role of Hsp60 in the virulence of another dimorphic yeast, *Histoplasma capsulatum*, which is described by Nosanchuk and Guimarães in Chap. 12.

14.2 Functions of *C. albicans* Hsp90 and Small Hsps

Although Hsp90 functions in a classical manner in response to temperature elevation, it is unique among eukaryotes in that many of its client proteins are signal transduction proteins (Young et al. 2001). Hsp90 engages a broad repertoire of client proteins, including telomerase, the actin organizer N-WASP, nitric oxide synthase, various nuclear hormone receptors, and a wide range of protein kinases (Pearl and Prodromou 2006). Consistent with established functions, most *C. albicans* Hsp90 client proteins, including MAPK Mkc1 and the protein phosphatase calcineurin (LaFayette et al. 2010), and very recently identified MAPK Hog1 and casein kinase CK2 (Diezmann et al. 2012), are all involved in cellular signaling processes. Moreover, a role for Hsp90 in candidial morphogenesis that accompanies temperature shift from 30 to 37 °C has been shown. Shapiro and coworkers demonstrated a temperature induced relief of Hsp90-mediated repression of Ras-PKA signaling, thereby allowing for yeast to hyphae transition (Shapiro et al. 2009). In addition to these binding partners, a major cell cycle regulator protein, Cdc 28, was identified as a novel client protein for Hsp90 (Senn et al. 2012), further bolstering its role in cellular morphogenesis.

Another important function of Hsp90, within the fungal world, is its ability to potentiate azole drug resistance in both *Saccharomyces cerevisiae* and *C. albicans*. It has been suggested that Hsp90 acts as a "buffer" to allow phenotypic variants of various proteins involved in signaling to be maintained and functional (Young et al. 2001). This pool of variants that would otherwise be degraded without Hsp90, eventually becomes a target for the process of natural selection and subsequent evolution. Thus, Hsp90 is proposed to have a role in promoting evolution by allowing accumulation of various stress-induced phenotypes even after relief of stress has occurred (Rutherford and Lindquist 1998). In the case of *C. albicans*, stabilization of calcineurin and the cell wall integrity (Pkc1) pathways' terminal MAPK (Mkc1) contribute to the promotion of drug resistance against azoles and echinocandins (LaFayette et al. 2010; Singh et al. 2009). Furthermore, post-translational modification of Hsp90 by acetylation has been shown to be necessary for evolution of drug resistance in both *S. cerevisiae* and *C. albicans* (Robbins et al. 2012).

All Hsp's are ATP-dependent chaperones, except the ATP-independent small heat shock proteins (sHsps) (Jaya et al. 2009). Four sHsps have been identified in

C. albicans, namely Hsp10, Hsp12, Hsp 30/31, and the most recently characterized Hsp21 (Mayer et al. 2012). Hsp10 inhibits the ATPase activity of another heat shock protein, Hsp60 (Dubaquie et al. 1997), and Hsp12 contributes to membrane stability in *S. cerevisiae* (Sales et al. 2000; Welker et al. 2010). However, few roles for sHsps have been characterized in *C. albicans*. Only recently a study by Myer and coworkers showed that Hsp21 is involved in adaptation to thermal and oxidative stresses in *C. albicans*, and like Hsp90, it mediates that adaptation through its effect on a MAPK signaling pathway (Mayer et al. 2012). Specifically, phosphorylation of Cek1 in response to temperature elevation was abrogated in an *hsp21*Δ/Δ mutant. Thus, much remains to be elucidated regarding the functions of sHsps in *C. albicans*.

14.3 *C. albicans* Hsp70 – Unconventional Roles and Moonlighting Activities

S. cerevisiae, the model yeast, has five subfamilies of the Hsp70 gene family, from SSA to SSE (Lopez-Ribot and Chaffin 1996). Six cytoplasmic Hsp70s exist in *S. cerevisiae*, including the Ssa family consisting of Ssa 1-4 and the Ssb family comprised of Ssb1 and Ssb2 (Peisker et al. 2010). Ssa-type Hsp70s are similar to the cytosolic Hsp70s found in other organisms including bacteria; whereas Ssb-type Hsp70s have been identified only in fungi (Shulga et al. 1999). Members of the Hsp70 family of proteins are involved in a variety of cellular processes, including the classical function of chaperoning polypeptides to different organelles such as the nucleus (Okuno et al. 1993; Shi and Thomas 1992; Shulga et al. 1996). The four members of the *S. cerevisiae* Ssa subfamily share 80–97 % of gene sequence similarity (Lopez-Ribot and Chaffin 1996). Ssa-type Hsp70s are involved in nuclear import (Quan et al. 2004) while Ssb-type Hsp70s are involved in nuclear export (Shulga et al. 1999). As a result, Ssa proteins are distributed both in the nucleus and the cytoplasm, while Ssb proteins are present only in the cytosol. Classical import of proteins into nuclei, a receptor-mediated process, is carried out by soluble nuclear localization signal (NLS) receptors (Mattaj and Englmeier 1998). *S. cerevisiae* Hsp70 has been shown to participate both in the targeting and the translocation phases of NLS-directed nuclear transport (Shulga et al. 1996; Mattaj and Englmeier 1998; Melchior and Gerace 1995).

Eroles and coworkers first discovered Hsp70 in the pathogenic yeast *C. albicans* in an immuno-screen of a mycelial expression library using polyclonal antibodies raised against mycelial cell wall (Eroles et al. 1995). The nucleotide sequence of the respective cDNA had 99.3 % identity to *C. albicans HSP70* and the mRNA expression levels were shown to increase in response to heat. The protein was present not only in cytosolic fractions, but also in the membrane particles. In the same year, La Valle and coworkers discovered a similar protein in a screen of yeast-form expression library, using serum directed against the whole fungal cell (LaValle et al. 1995). The gene sequence encoding this protein was 84 % similar to *S. cerevisiae* Hsp70

protein, Ssa 1. This study also found that expression of *C. albicans HSP70* as assessed by Northern blot and RT-PCR was increased in parallel with an increase in temperature from 22 to 37 °C, thus presenting a classical role in heat shock response.

Subsequently, Lopez-Ribot and coworkers identified a constitutively expressed 70kD protein that was also surface localized in *C. albicans*, similar to the previous reports (Lopez-Ribot et al. 1996). However, sequence analysis of the corresponding gene showed only 77 % homology to the previously described *C. albicans HSP70* gene, and homologies ranging from 65 to 76 % with SSA, SSB, SSC, and SSD subfamilies of Hsp70 in *S. cerevisiae*, as well as 56–61 % homology to the SSE subfamily. As a result, this constitutively expressed Hsp70 was named Ssa 2 and the previously discovered, temperature inducible, Hsp70 was named Ssa 1.

The cell surface localization of *C. albicans* Ssa 1 and Saa 2 was perplexing in that no classical secretion signal sequence exists in either Ssa 1 or Ssa 2 (Li et al. 2006), thus creating doubts about the true function of these proteins at the surface. However, surface localization of Hsp70 (Ssa 2) had previously been demonstrated by immunochemical methods (Lopez-Ribot et al. 1996). *C. albicans* Ssa proteins were shown to be recognized by affinity-purified antibody in beta-mercaptoethanol extracts containing cell wall components from both yeast and germtube forms of *C. albicans*. Indirect immunofluorescence also confirmed its cell surface localization. Furthermore, a 70 kD moiety was shown to be present outside the cell membrane, in association with the cell wall, when cells were treated with impermeable biotin to label proteins external to the cell membrane. Also, immunoelectron microscopy showed the presence of this protein at the cell surface and the outer surface of the plasma membrane, extending through the cell surface and at times forming channel-like connections between the inner and outer surfaces of the cell wall. Recently, we showed that Ssa 1 lies outside the cell membrane in live *C. albicans* cells by direct vital staining (Fig. 14.1) (Sun et al. 2010), thus confirming that cell wall localization of Ssa proteins is not secondary to cell lysis or other artifacts of preparation. The Ssa 2 protein has potential O-linked glycosylation sites, which upon being mutated affect the surface expression of this protein (Sun et al. 2008). This provided additional evidence of how these proteins may have evolved to allow for their surface expression, most likely through the secretion process involving the endoplasmic-reticulum-Golgi pathway.

14.4 Immunogenicity of Surface Hsp70 Proteins

The Hsp70 family of proteins is extremely immunogenic in nature and has been shown to play a role in eliciting immune response in various pathogenic organisms (LaValle et al. 1995). *Histoplasma capsulatum*, a pathogenic fungus, also expresses a surface localized Hsp70 that participates in cell mediated immunity (Gomez et al. 1992). See Chap. 12 for a detailed discussion of the role of Hsp60 in *H. capsulatum* virulence. *C. albicans* Ssa 1 whole protein and two of its N-terminal polypeptide fragments reacted with sera from normal healthy subjects

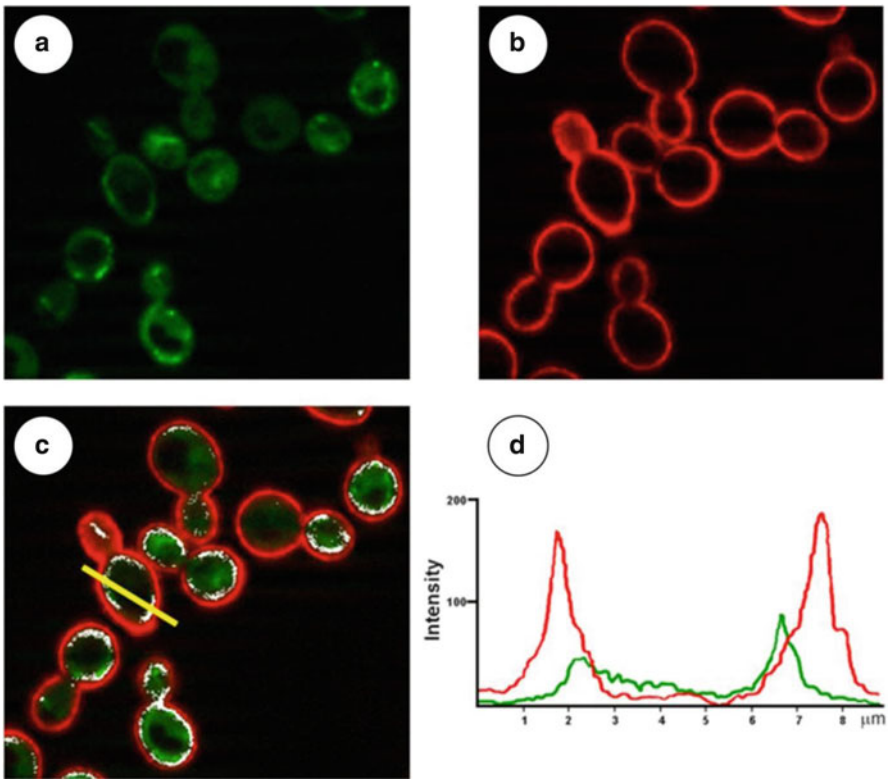

Fig. 14.1 Localization of Ssa 1 on *C. albicans* yeast cells expressing an Ssa 1-GFP fusion protein. Images of Ssa 1-GFP (**a**) and the fluorescent-labeled anti-*C. albicans* antibody (**b**). The merged image is shown in (**c**). (**d**) Graphs of fluorescent intensity at different cross sections of the yeast in panel (**c**)

(La Valle et al. 1995) and recombinant Ssa 1 was shown to induce T cell proliferation (Chaffin et al. 1998). Ssa 2 was recognized by sera from healthy human subjects as well as candidiasis patients (Lopez-Ribot et al. 1996). Together, these findings pointed towards an immune-protective role of Ssa proteins. Mice inoculated with recombinant Hsp70 showed both enhanced antibody and cell mediated immune responses (Bromuro et al. 1998). Surprisingly, there was no protection to infection in mice immunized with Hsp70; in fact, an enhancement in infection was observed. In this regard, however, it is interesting to note the role of a 47 kD fragment, a breakdown product of *C. albicans* Hsp90, in immunity. Antibodies against this fragment were shown to offer protection via the humoral branch of immunity in systemic candidiasis and therefore, Mycograb, a commercial antibody against Hsp90, is being tested for therapeutic use (Burnie et al. 2006). Thus, Hsps in *C. albicans* represent a class of proteins that could potentially be exploited as probable vaccine candidates against candidiasis.

14.5 Human Salivary Histatin 5 Binding to *C. albicans* Ssa Proteins

The most basic attribute that comes with the chaperoning function of a heat shock protein is the ability to bind to other proteins. Cell surface localization of *C. albicans* Ssa 1/2 proteins raised an important question as to the role of these proteins in that regard. Our group identified the binding partner of human salivary antifungal protein Histatin (Hst) 5 and Histatin 3 to be Ssa 1/2 using MALDI-MS analysis of protein complexes isolated by column chromatography (Li et al. 2003). Yeast two-hybrid analysis further demonstrated a strong interaction between Ssa 1 and Hst 5 (Li et al. 2006). Measuring Hst 5 binding to *ssa 1*Δ/Δ*ssa 2*Δ/Δ double mutant of *S. cerevisiae* tested the functional relevance of this interaction (Li et al. 2006). The choice of the organism was based on the high homology among Ssa proteins between *S. cerevisiae* and *C. albicans* and the viability of the *S. cerevisiae* double mutant. There was a two-fold decrease in the binding of iodine-labeled Hst 5 to mutant cells, corroborated by an almost 70 % loss in the fungicidal activity of Hst 5 against this mutant. These results were replicated in *C. albicans ssa 1*Δ/Δ and *ssa 2*Δ/Δ mutants (Li et al. 2006), thus illustrating the importance of yeast Ssa proteins for Hst 5 binding and also the close relationship between cell binding and fungicidal activity.

Another class of salivary antimicrobial proteins (AMPs) besides Histatins found in the oral environment, is the β-defensins. Ssa 1/2 proteins were shown to have a role in the candidacidal activity of β-defensins as well, as seen in the reduced killing of *C. albicans ssa 1*Δ/Δ and *ssa 2*Δ/Δ mutants when compared to WT cells (Vylkova et al. 2006). A recent finding showed an interaction between Hst 3 and a 70 kD heat shock-like protein, called heat shock cognate protein 70 in human gingival fibroblasts (Imamura et al. 2009). This suggests that heat shock proteins present in oral pathogens or cells of the oral cavity may have evolved to interact with salivary proteins, thereby performing a unique niche specific role. For example, Hst 3-mediated stabilization of Hsc 70 in complex with $p27^{Kip1}$(a cyclin dependent kinase inhibitor) suggested a role for Hst 3 in cell proliferation (Imamura et al. 2009). Hst 5-Ssa 1/2 interaction, on the other hand, is detrimental for *C. albicans* (Li et al. 2003, 2006); yet this interaction may have initially evolved to facilitate other undiscovered physiological and molecular interactions that were beneficial to *C. albicans* as an oral pathogen. Our subsequent discovery of the role of Ssa 1 as a cell surface invasin that is necessary for not only oral but systemic infection (Sun et al. 2010) (discussed later) is a good example of such beneficial roles for the pathogen.

14.6 Transcription of *C. albicans Ssa* Proteins

Over the past decade, an elaborate mechanism has been elucidated, explaining how Ssa and Hst 5 interact to drive AMP-mediated killing of *C. albicans*. As mentioned earlier, despite high homology (87 % identity), Ssa 1 and Ssa 2 proteins have very

different transcriptional profiles, with Ssa 1 largely being a heat inducible protein, whereas Ssa 2 is a constitutively expressed protein. When both proteins were compared in parallel for their ability to bind Hst 5 in an yeast-two hybrid assay, Ssa 2 showed a significantly higher binding interaction than Ssa 1 (Li et al. 2006). This pattern was consistent with the results of an *in vitro* pull down experiment using recombinant Ssa proteins (Li et al. 2006). While acting as molecular chaperones, heat shock proteins bind their client proteins only transiently. Ssa 1/2 binding pattern was similar to a classic heat shock protein, in terms of the transient nature of their binding with Hst 5 as an increase in binding was observed in the presence of a chemical cross-linker (Li et al. 2006). Thus Ssa proteins retain some properties unique to their class, even while participating in unconventional cellular functions in non-cytosolic locales such as the cell wall.

Our lab took an in-depth transcriptional analysis of *C. albicans SSA 1* and *SSA 2* genes in WT and mutant strains carrying only a single allele of either *ssa 1* or *ssa 2* in an *ssa 2*Δ/Δ or *ssa 1*Δ/Δ background, termed *ssa 2*Δ/Δ*sas 1*Δ and *ssa 1*Δ/Δ*sas 2*Δ, respectively. Unlike previous reports, we found that both transcripts are constitutively expressed (Li et al. 2006), although we refer to them as basal or uninduced levels. Heat shock for 1 h at 37 °C resulted in 2.7 and 1.8 fold increases in transcript levels of *ssa 1* and *ssa 2*, respectively. Expression levels of the single allele of *ssa 2* in *ssa 1*Δ/Δ*sas 2*Δ greatly increased upon heat shock, as compared to no increase in the expression of the only *ssa 1* allele in *ssa 2*Δ/Δ*sas 1*Δ (Li et al. 2006). This highlighted the importance of Ssa 1 as compared to Ssa 2, since a compensatory increase of *ssa 2* in the absence of the more important and highly expressed *ssa 1* could explain such an observation. Protein quantification by Western blotting paralleled the transcript levels as cell wall levels of Ssa 1 were observed to be 4–5 folds higher than Ssa 2 (Li et al. 2006). This clearly showed Ssa 1 to be the predominant Hsp70 member in *C. albicans* cell wall. Ssa 1/2 are also present in the cytoplasm, and the cytoplasmic levels remained unchanged upon heat shock (Li et al. 2006). In contrast, analysis of total cell-associated iodine labeled Hst 5 among WT and mutant strains showed that Ssa 2 was more critical for this Hst 5 binding process. However, heat shock increased the total cellular association of Hst 5 in all the strains, pointing towards the importance of the classical heat shock response of these proteins.

Cytosolic Ssa proteins play important roles in the binding and transport of their client proteins across membranes. Also, the importance of intracellular transport of Hst 5 for its killing ability has been previously reported (Li et al. 2006). Therefore intuitively, the role of cell wall Ssa 1/2 in translocating the bound Hst 5 was evaluated in our lab. Translocation rates of Hst 5, measured by the detection of cytoplasmic levels of labeled Hst 5, were extremely poor in *ssa 2*Δ/Δ*sas 1*Δ mutant, as compared to the WT, with *ssa 1*Δ/Δ*sas 2*Δ showing intermediary rates (Li et al. 2006). Thus, Ssa 2, as expected from its important role in Hst 5 binding, was also shown to be more critical for Hst 5 translocation. Candidacidal results for the same set of strains were in perfect agreement with all other observations. Greater contribution of Ssa 2 was observed in killing under non-heat shock conditions (Li et al. 2006). Heat shock increased killing for all the strains, consistent with the idea of increased presence of these and maybe some other functionally similar yet unknown proteins in the cell wall.

14.7 Mechanism of Non-conventional Histatin 5 Binding with *C. albicans* Ssa

Ssa 1/2, like classical chaperones, have two discrete functional domains, a more variable C-terminal peptide binding domain and a highly conserved N-terminal ATPase domain. The two domains are coupled, allowing for an ATP-dependent cyclical binding and release of peptide substrates. Such energy-dependent bindings are important for holding the substrate in the correct conformation and facilitating their passage through intracellular membranes. More details of the mechanism of action of Hsp70 proteins as molecular chaperones are to be found in Chaps. 1 and 2. Hst 5 killing has a requirement for cellular ATP (Koshlukova et al. 1999, 2000) and therefore it was initially hypothesized that ATP hydrolysis may play an important role in Hst 5-SSa 1/2 binding and translocation. *In vitro* Hst 5 binding studies with WT and truncated Ssa 2 indeed presented evidence for the requirement of the ATPase domain of Ssa 2 in Hst 5 binding (Sun et al. 2008). Binding analysis by comparison of K_d's for normal and non-hydrolysable ATP showed only a threefold difference, suggesting that ATP binding and not hydrolysis per se is necessary for Hst 5 binding. However, a tenfold increase in binding K_d in the presence of ATP (Sun et al. 2008) suggested an ATP binding-induced change in the ATPase domain, improving the efficiency of that domain to bind Hst 5. Thus, Hst 5 requires the Ssa ATPase domain for binding, but does not compete for conventional ATP binding sites within this region. Accordingly, cells carrying mutations in the ATPase domain, especially the TAEGF epitope, showed a pronounced reduction in Hst 5 translocation and toxicity, as compared to cells carrying WT Ssa 2 (Sun et al. 2008). The significance of this epitope in binding explained the previous anomalous observation that even though Ssa 1 is the most prominent cell surface Hsp70, Ssa 2 plays a major mechanistic role in Hst 5 antimicrobial activity since Ssa 1 lacks this epitope that is present only in Ssa 2. This epitope, marked in green in Fig. 14.2 (Sun et al. 2008), represents the regions from aa 128–135, and is completely surface accessible and hence perfectly placed for Hst 5 binding. Importantly, this surface binding site is distinct from the conventional C-terminal peptide binding domain whose binding of typical client proteins is driven by cycled ATP binding and hydrolysis. Interestingly, binding of the cationic AMP pyrrhocoricin by bacterial DnaK (a bacterial Hsp70 homologue) was mapped to the C-terminal variable region of the lid domain outside the conventional peptide binding region (Otvos et al. 2000), and was shown to be related to its intracellular uptake and killing functions (Kragol et al. 2001). Thus, binding of AMPs to non-conventional sites of Hsps may be a generalized mechanism for their transport into target cells.

14.8 *C. albicans* Ssa 1 is Involved in Virulence

The ability of microbial Hsps to bind cationic proteins or peptides such as AMPs, as well as the immunogenic capability of Ssa 1/2 led us to investigate their ability to mediate invasion and virulence in *C. albicans*. *C. albicans ssa*

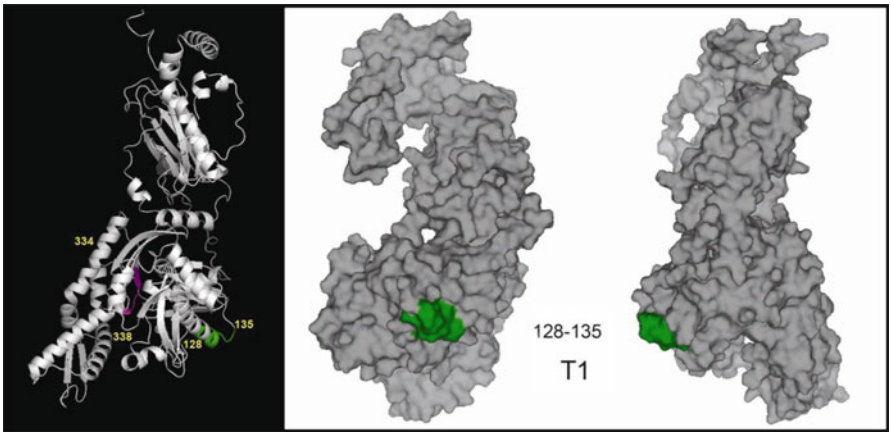

Fig. 14.2 Hst 5 binding sites within Ssa 2p lie near the nucleotide-binding cleft of the ATPase domain

*1*Δ/Δ and *ssa 2*Δ/Δ mutants were tested in a murine model of haematogenously disseminated candidiasis as well as in a murine model of oral candidiasis. The *C. albicans ssa 1*Δ/Δ mutant was found to have remarkably attenuated virulence in both systemic and oropharyngeal infections, that was reversed upon gene restoration (Sun et al. 2010). Strikingly, the *C. albicans ssa 2*Δ/Δ mutant had only mildly attenuated or no reduction in virulence in these models, highlighting the functional differences between these proteins.

Avirulence of the *ssa 1*Δ/Δ mutant was clearly evident from the lack of extensive infection with *ssa 1*Δ/Δ mutant cells as compared with WT cells in the kidney (in disseminated candidiasis; Fig. 14.3, top) and tongue (in oropharyngeal candidiasis; Fig. 14.3, bottom) sections (Sun et al. 2010). This could largely be attributed to the inability of *ssa 1*Δ/Δ to effectively bind to the endothelial N-cadherin and epithelial E-cadherin receptors that induce endocytosis of *C. albicans* by endothelial as well as oral epithelial cells, respectively. As a result of these observations, we have termed Ssa 1 as being an "invasin", adding yet another functional moonlighting attribute to this Hsp70 protein.

Besides heat shock, heat shock proteins are expressed in response to various environmental stress conditions including nutritional stress and oxidative stress (see Chap. 2 for further discussion on eukaryotic stress responses). It is interesting to note that various stressors, including oxidative (peroxide and menadione), osmotic (salt), cell wall (Calcofluor white), and plasma membrane (SDS), did not cause increased sensitivity in *ssa*Δ/Δ mutants, as compared to WT, as shown by normal growth of mutant cells under these stress conditions (Fig. 14.4) (Sun et al. 2010). Therefore, the significant attenuation of virulence of *C. albicans ssa 2*Δ/Δ mutant in hematogenously disseminated candidiasis and oral candidiasis is not likely to be a result of impaired viability in the presence of host stressors.

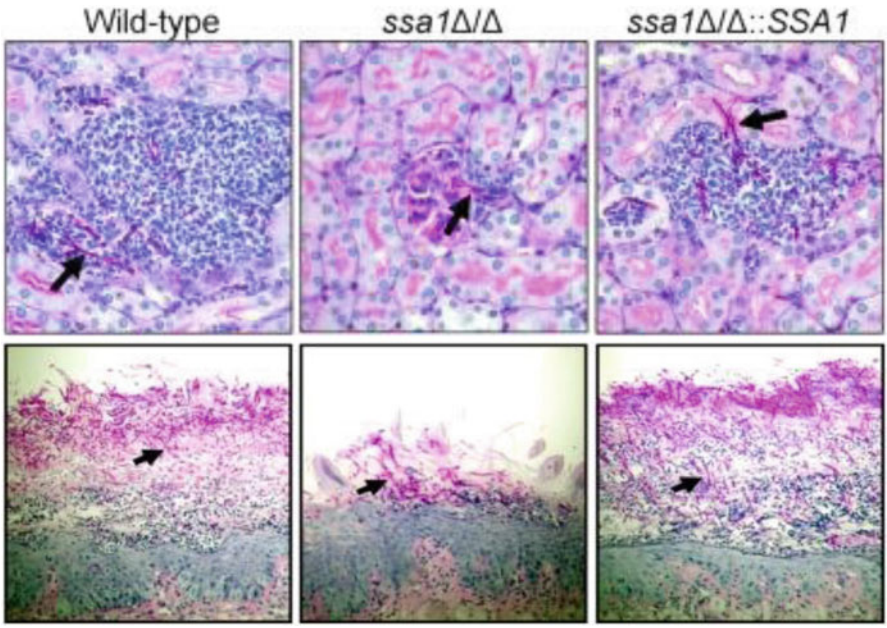

Fig. 14.3 Histopathology for disseminated candidiasis in kidney sections (*top*) and oropharyngeal candidiasis in tongue sections (*bottom*)

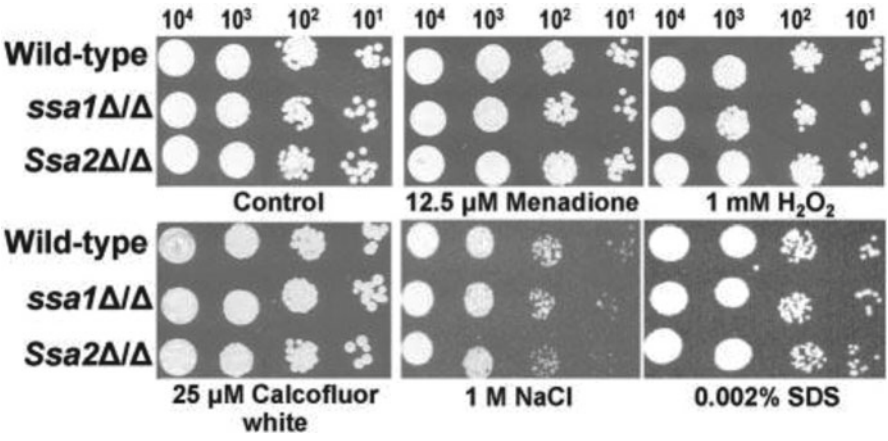

Fig. 14.4 Growth of *ssa* mutants under various non-heat stressors, as compared to WT

14.9 Conclusion

C. albicans Ssa 1/2(Hsp70) and Hsp90 behave like classical heat shock proteins with respect to temperature induction, ability to bind other proteins/peptides transiently in an ATP-dependent manner, and retain a certain level of immunogenicity; yet both

perform non-conventional functions. Hsp70s bind small cationic peptides at the microbial cell surface and help to transport them intracellularly, act as invasins, and are not inducible in response to non-heat stresses. Hsp90 has an entirely different repertoire of non-conventional functions in controlling drug resistance and cellular morphogenesis by interacting with the cellular signaling machinery. These differences between Hsp70 and Hsp90 in *C. albicans* are exquisitely reflected by the divergence in the nature of their client proteins. Young and coworkers reviewed these distinctions with clarity; Hsp70s are more promiscuous in their choice of client proteins that largely include short peptide segments such as those exposed in nascent polypeptide chains; while Hsp90 prefers proteins in near native states of conformation which are in the late stages of folding, and is best suited for ligand binding and interaction with other factors (Young et al. 2001), a feature of numerous signaling proteins. Hst 5 is a relatively small (24 amino acid residues) peptide and thus is likely to be an interacting client protein for candidal Hsp70s (Ssa 1/2), although it lacks the hydrophobicity of conventional Hsp70 client polypeptides. Similarly, interactions of Hsp90 with Mkc1, calcineurin, and Cdc 28 comply with Hsp90s generic choice of client proteins.

C. albicans is a dimorphic organism and its ability to undergo filamentation in response to temperature shifts experienced in the human host as well as its ability to withstand host innate immunity including AMPs such as histatins are key to its survival during an infection. Importantly, both of these phenomena are mediated by *C. albicans* Hsp90 and Hsp70 (Ssa 1/2), respectively. Fungal Hsps have evolved from merely protecting nascent polypeptides from degradation under stress, to performing key roles in pathogenesis. A recent report identified 226 "environmentally contingent" interactors of Hsp90 (Diezmann et al. 2012), and our own (unpublished) microarray data comparing *ssa 1*Δ/Δ cells with WT have identified numerous genes that are transcriptionally regulated in the mutant. Thus, the network of interacting partners among *C. albicans* Hsps is more extensive than previously thought, and it is likely that additional non-conventional roles for Ssa 1 and Ssa 2 will emerge in the future.

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Chapter 15
Hsp90 Plays a Role in Host-Bacterial Interactions: Insight Gained from *Acanthamoeba castellanii*

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Abstract The selective pressures they are faced with, both inside and outside their hosts, shape pathogens. Facultative intracellular organisms, including *Legionella, Mycobacterium* and many other pathogens, encounter amoebal predators in nature and this interaction leads to acquisition of novel genetic material that facilitates their ability to infect both amoebae and higher order species. Understanding these interactions that are continuously occurring in the environment provides an opportunity to obtain greater insight into the mechanisms of pathogenesis, particularly because these relationships are most often between single-celled organisms, making them more amenable to genetic analyses as compared to more complex multicellular creatures. We utilized the single celled virulence model for *Legionella* and *Mycobacterium*, the environmental amoeba *Acanthamoeba castellanii*, to identify bacterial and host genes required for pathogenesis and susceptibility/resistance to infection, respectively. In this chapter, we will focus on our intriguing observation that decreased expression of Hsp90, a molecular chaperone involved in host cell signaling and protein targeting, increases resistance to host cell infection by pathogenic bacteria.

15.1 Introduction

Many pathogenic organisms infect humans from environmental ecosystems where they have evolved the ability to efficiently infect numerous single-celled protozoal host cells that are present in biofilms (Cirillo 1999). Ecological pressure from protozoa that normally utilize bacteria as their primary food source results in

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acquisition of eukaryotic cell virulence mechanisms by some bacteria that both improves their fitness in this environmental niche and, as a side effect, increases their ability to cause disease in mammals. Several specific bacterial pathogens including *Vibrio cholerae, Listeria monocytogenes, Burkholderia cepacia, Chlamydia pneumoniae, Edwardsiella tarda, Francisella tularensis, Legionella pneumophila, Mycobacterium avium* and *Mycobacterium marinum* have been shown to persist in aquatic environmental reservoirs prior to infecting a human or animal host (Abd et al. 2003; Barker and Brown 1994; Cirillo 1999; Cirillo et al. 1997; Greub and Raoult 2004; Henke and Seidel 1986; Rowbotham 1980; Thom et al. 1992). The impact of environmental evolution in protozoa on virulence for mammals is most likely due to the similarity of environmental organisms, particularly amoebae, to the mammalian innate immune cells. In the case of *Acanthamoeba castellanii*, they produce oxygen radicals, have an oxidative burst, carry cell surface receptors and utilize phagocytic mechanisms that are very similar to mammalian macrophages (Allen and Dawidowicz 1990a, b; Brown et al. 1975; Davies et al. 1991; Davies and Edwards 1991; Lock et al. 1987). A key survival strategy exhibited by pathogens outside of mammalian hosts is their ability to thrive successfully during interactions with amoebae, most likely because amoebae constantly graze on bacteria and have the ability to eliminate bacteria from a water environment unless the bacteria develop amoebal killing or intracellular survival mechanisms. In fact, the genomes of *Legionella, Coxiella, Francisella* and *Mycobacterium* appear to have been at least partially shaped by exchange of genetic material during interactions with amoebae (Lamrabet et al. 2012). In order for pathogens to successfully infect mammalian hosts from aquatic environmental reservoirs, they must survive and replicate to large numbers in complex biofilms. Similar interactions with complex environmental ecosystems outside of mammalian hosts have also been observed in pathogenic fungi (Steenbergen et al. 2001). Among the 1.5 million known fungal species, only 0.01 % are pathogenic to humans (Casadevall 2012). Since some of these pathogenic fungi can persist in environmental reservoirs, it is possible that aspects of their virulence are due to selective pressures imposed by amoebal predators, also leading to acquisition of new traits. This interaction has been exploited to identify novel virulence factors from *Cryptococcus* through analysis of interactions with free-living amoebae (Mylonakis et al. 2007). Taken together, these observations suggest that genetically tractable protozoa represent potentially important virulence models that can be used to gain insight into both bacterial and fungal pathogens. Findings obtained in protozoa may be utilized to prevent mammalian infections through development of novel strategies to treat these environmental reservoirs so that pathogenic organisms can be better controlled within them.

In our own studies, we utilized one of these environmental pathogens, *Legionella pneumophila*, as a genetic selection for environmental amoebae that can resist killing by this pathogen (Yan et al. 2004), a result also observed in human macrophages, albeit less efficiently. Since *L. pneumophila* has the ability to kill nearly 100 % of *A. castellanii* in a typical tissue culture flask in the laboratory (Cirillo et al. 1994), the amoebicidal mechanisms of *L. pneumophila* represent a strong selective pressure for resistant amoebae in the laboratory. However, the polyploid nature of

A. castellanii (Byers 1986; Byers et al. 1990) and its ability to divide amitotically, dramatically decrease the stability of any genetic characteristic, despite the strength of pathogen selection. As a result of these issues, more than ten rounds of selection were required to produce stable *A. castellanii* mutant. Once stable mutants were isolated, we found that four of these mutants expressed reduced levels of Hsp90 and this decrease in expression correlates with decreased infection and intracellular survival in these amoebae. These observations indicate an important role for Hsp90 during host-pathogen interactions that warrants further study to better understand the mechanisms involved.

15.2 Studies in *A. castellanii* Identify Hsp90 as a Host Immune Protein

Amoebae are present in diverse environments and, since their discovery by August Johan Rosel Von Rosenhof in 1757, they have been extensively studied as important model organisms for understanding cell and molecular biology (Martinez 1985; Allen and Dawidowicz 1990b; Korn and Weisman 1967; Weisman and Korn 1967; Weisman and Moore 1969; Bowers 1977; Baines et al. 1995; Baines and Korn 1990; Neff 1957). Due to the similar phagocytic characteristics of *Acanthamoeba castellanii* (Ac) to macrophages and owing to its unicellular nature, amoebae offer an excellent genetic tool for analysis of pathogen-host cell interactions to researchers. These model systems have been exploited not only for the study of pathogenesis but also, much more frequently, for analysis of the biology of cytoskeletal proteins, mitochondria and cell biological analyses in general (Korn and Weisman 1967; Archer et al. 1994; Kelleher et al. 1995; Kong and Pollard 2002; Brzeska et al. 2001; Volkmann et al. 2001; Avery et al. 1995; Yin and Henney 1997; Wong et al. 1992; Schulze and Jantzen 1982; Nellen and Gallwitz 1982; Jones et al. 1993; Byers et al. 1991). In addition to being present in natural environments as saprophytes, *Acanthamoeba* are found associated with infections in humans that can manifest clinically as mild to severe keratitis (Illingworth and Cook 1998; Nagington et al. 1974; Schaumberg et al. 1998; Marciano-Cabral et al. 2000) or frequently fatal forms of meningitis (Culbertson 1958; Martinez 1980; Kenney 1971; Jager and Stamm 1972) particularly in immune compromised individuals (Martinez et al. 2000; Martinez 1983; Nagington 1975; Nagington et al. 1974; Sison et al. 1995). The importance of amoebae both as a model system and as a pathogen make it likely that studies geared toward understanding the mechanisms by which amoebae interact with pathogens will provide information relevant to both to fundamental cell biology and human infectious diseases.

Phagocytosis and the formation of phagosomes is the process by which cells internalize and digest components from the external milieu and the components involved are well conserved throughout evolution. Mechanisms used by predatory amoebae to internalize bacteria as food are similar to those of professional phagocytes in multicellular organisms (Rowbotham 1980). A recent study compared phagosomes from amoebae, *Drosophila* and murine macrophages, identifying an

ancient core of phagosomal proteins from which many immune mediators may have developed (Boulais et al. 2010). These observations suggest that ancestral bacterial populations may have exploited interactions with amoebae during their evolution and, as a side-effect, become more efficient pathogens of humans. Bacterial mutants that enter and replicate less efficiently inside macrophages also demonstrate a growth defect in amoebae (Cirillo et al. 2000, 2001, 2002; Danelishvili et al. 2007; Samrakandi et al. 2002).

Amoebae usually live as free moving trophozoites, becoming cysts when conditions are not favorable (Salah et al. 2009). This presents two very different environments where the bacteria must persist, should they remain within the amoebae. Bacteria must withstand the microbicidal actions of amoebae and resist severe nutrient limiting conditions within amoebal cysts. Amoebae can undergo a respiratory burst and produce reactive oxygen species similar to antimicrobial properties exhibited by professional phagocytes of multicellular eukaryotes (Davies et al. 1991; Davies and Edwards 1991; Allen and Dawidowicz 1990b). *Legionella* are known to inhibit phagosome lysosome fusion, a characteristic feature exhibited inside macrophages (Horwitz 1983). *Legionella* is also known to use type IV secretion system components to infect both amoebae and macrophages (Stone and Abu Kwaik 1998). Interactions with amoebae may have resulted in acquisition of ankyrin repeats from the host by *Legionella* (Price et al. 2009). Experimental evidence suggests that bacteria belonging to diverse genera, *Legionella* and *Mycobacterium* can persist together inside amoebae (Lamrabet et al. 2012). This suggests that amoebae provide natural environments for exchange of genetic material and directed evolution of certain pathogens. *Campylobacter jejuni,* a pathogen of food animals and humans, have been found to be more resistant to low pH when residing within amoebae, relative to bacteria that are not in contact with amoebae (Axelsson-Olsson et al. 2010). Similar to *C. jejuni, M. avium* subsp. *paratuberculosis,* a pathogen of ruminants, must overcome the acidic pH inside the rumen that has been shown to be rich in amoebae, before it can colonize gastro-intestinal tract (Sweeney 2011). *M. avium* passaged in amoebae is more virulent than that cultured in standard laboratory medium (Cirillo et al. 1997). It has been proposed that amoebae filled with *Legionella* are more infectious than free *Legionella* (Rowbotham 1980). Taken together, these data suggest that studying amoebal-bacterial interactions represents a key component of understanding how human disease occurs.

Amoebae are widely used as a model for virulence studies (Cirillo 1999). Virulence studies using this model can be accomplished by infecting the cells with mutant bacterial populations and characterizing the genetic components that confer a desired phenotype. Large-scale mutant screens to identify host components involved in pathogenesis are usually relatively difficult to perform. A favorable and economic approach would be to take advantage of amoebae to identify host components involved in immunity. We developed a strategy and isolated amoebal mutant clones that demonstrate enhanced microbicidal activity and a defect in bacterial uptake (Yan et al. 2004). Monolayers of amoebae are normally completely destroyed when infected with *Legionella* (Cirillo et al. 1994). Amoebal mutants that became resistant to killing by *Legionella* were isolated and cultured again. This selection procedure was performed for several rounds to ensure that stable mutants were

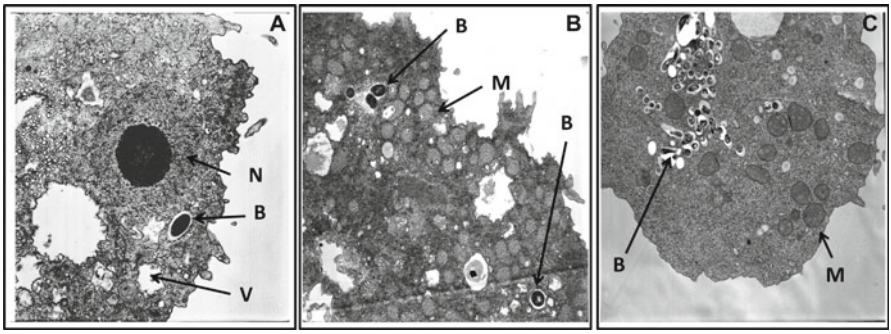

Fig. 15.1 Ultrastructural analysis of an *A. castellanii* Hsp90 mutant by transmission electron microscopy. Ultra structural analysis of *A. castellanii* wild type (**a** and **c**) infected with *M. avium* at 1 h and 14 days post infection respectively and the *A. castellanii* Hsp90 mutant infected with *M. avium* at 8 h post infection (**b**). Hsp90 mutant displays increased numbers of mitochondria. *M* mitochondria, *V* vacuole, *N* nucleus, *B* bacteria (*M. avium*)

obtained and clones were isolated by performing limiting dilution (Yan et al. 2004). Amoebal mutants demonstrated several intriguing phenotypic changes, including increased mitochondrial numbers (Fig. 15.1). Interestingly, activated macrophages have also been shown to display increased numbers of mitochondria, suggesting that this phenotype may play a role in resistance to infection with bacteria (Cohn and Benson 1965a, b). Our amoebal mutants also display enhanced microbicidal activity, most likely, at least partially, due to an increased frequency of phagosome-lysosome fusion in response to *Legionella* infection (Yan et al. 2004). Proteomic, molecular and phenotypic analyses found that resistance to bacterial infection in our amoebal mutants is due to reduced expression of heat shock protein 90 (Hsp90). Amoebal mutants display reduced uptake of *M. marinum* and *M. smegmatis* but not inert particles, latex beads, suggesting a specific reduction in bacterial uptake. The importance of Hsp90 in the amoebal phenotype was confirmed in macrophages treated with specific Hsp90 inhibitors, since macrophages treated with these inhibitors display enhanced microbicidal activity.

During bacterial-host cell interactions, several different signaling pathways synchronize in response to bacterial stimuli and in the bacteria themselves, in response to binding the host cell. Host cells undergo actin polymerization, activation of specific receptors, and activation of a signaling cascade, which results in internalization and maintenance of the bacteria initially within an intracellular vacuole. The process of phagosome formation leading to degradation of bacteria observed in higher order eukaryotes is similar to the nutrient uptake mechanisms found in unicellular organisms (Boulais et al. 2010). In order to efficiently regulate this enormous turnover of proteins by the host cell, in response to bacterial infection, several chaperones are induced. Furthermore, chaperone proteins are key components involved in triggering bactericidal activity and degradation of bacterial proteins as well as aiding in antigen presentation. There is a growing body of evidence indicating that Hsp90, via interactions with other host cell and potentially also bacterial proteins, is

critical for many aspects of bacteria-host cell interactions (Robert 2003; Tsan and Gao 2009; Ripley et al. 1999; Srivastava et al. 1994; Udono 2012). The role of Hsp90 family members in bacterial infection of mammals is described in later chapters in this volume.

15.3 Pathogen Interactions Involving Hsp90

Hsp90 is highly conserved throughout evolution, with homologues present in both eukaryotes and bacteria (Johnson 2012 – see also Chap. 1). In eukaryotes, several forms of Hsp90 can be found, including Hsp90 present in the cytosol, its homologue known as Trap-1 in mitochondria, Grp94/gp96(HSP90B1) in the endoplasmic reticulum and Hsp90c in chloroplasts (Richter and Buchner 2001; Young et al. 2001; Zuehlke and Johnson 2010). There is only one homologue of Hsp90 called HtpG (high temperature protein G) in bacteria (Heitzer et al. 1992; Zuehlke and Johnson 2010). Hsp90 functions in an ATP dependent manner and has a conserved ATP binding domain situated at the N-terminus (Pearl and Prodromou 2006). Details of how Hsp90 functions as a molecular chaperone is found in Chap. 2. As a chaperone, Hsp90 is induced in response to many different stresses, similar to conditions that bacteria would face during infection of host tissues (Robert 2003). Interactome analyses found that Hsp90 interacts with hundreds of proteins, underscoring the importance of Hsp90 in responding to stress (Echeverria et al. 2011).

Receptor-ligand interactions occur when pathogens first come in contact with host cells. Hsp90 has been shown to bind bacterial LPS and interact with specific receptors on the bacterial surface (Jin et al. 2003; Triantafilou et al. 2001; Montanari et al. 2012). The role of Hsp90 as a component of the LPS receptor complex is detailed in Chap. 18. In addition, Hsp90 is known to interact with a number of proteins in host cells that are involved in innate and adaptive immune responses (Srivastava 2002). Together, these observations suggest that Hsp90 is involved in both the interaction with and response to pathogens during infection of host cells. Hsp90 interacts with vitamin D receptor (VDR), a transcriptional factor involved in production of the antimicrobial peptide cathelicidin (Marcinkowska and Gocek 2010; Angelo et al. 2008), suggesting that at least in some cases this response is involved in protection from infection. Interestingly, the stability of erythropoietin-producing hepatoma (Eph) receptor EphA2 is dependent upon interaction with Hsp90 (Annamalai et al. 2009). In separate studies, we found that *ephA2*$^{-/-}$ mice infected with *M. tuberculosis* have more disorganized granulomas relative to *ephA2* sufficient mice and improved clearance during the chronic phase of infection (Khounlotham et al. 2009). These studies found that *M. tuberculosis* regulates expression of EphA2, which appears to help it survive during the chronic phase of infection in mice. These observations suggest that the role of Hsp90 observed *in vitro* translates into effects upon susceptibility of the host during disease. A yeast two hybrid screen found that Hsp90 also interacts with the nucleotide-binding oligomerization domain of the (Nod)-like receptor (NLR) family of proteins (Mayor

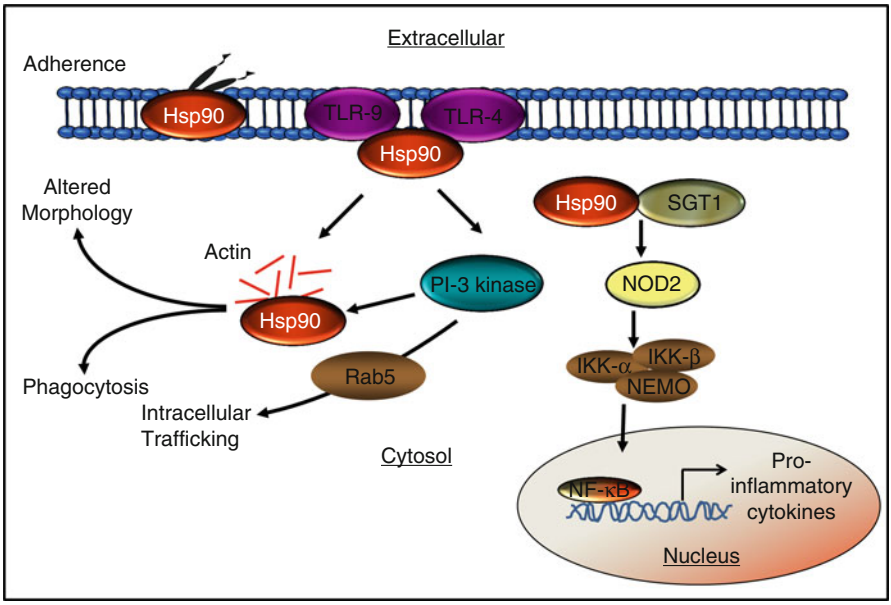

Fig. 15.2 Functions of Hsp90 in mammalian cells. Hsp90 can interact directly with bacteria at the host cell surface through binding to LPS or bacterial proteins. It can also interact indirectly with bacteria through protein-protein interactions with specific host cell surface receptors that are known to bind bacteria and trigger an Hsp90-dependent signal transduction pathway. Signaling through Hsp90 can affect phagocytosis, actin polymerization and intracellular trafficking. In addition to the role of Hsp90 in the signal transduction pathways that lead to these events, Hsp90 is also involved in signaling to and stabilization of proteins directly involved in phagocytosis, actin polymerization and trafficking. In addition, Hsp90 can disrupt the NOD2-SGT-1 complex and lead to pro-inflammatory cytokine production via NF-κB. Through impacts on multiple aspects of several critical host cell pathways, Hsp90 represents a key component of many aspects of host cell responses to pathogens. These roles offer an opportunity to use Hsp90 for modulation of host responses to better prevent infections

et al. 2007). NLRs are important for defense against pathogens, since they are involved in inflammasome formation and signaling within the proinflammatory cytokine cascade (Ogura et al. 2006; Martinon and Tschopp 2007). Reversible binding of Hsp90 with NALP3 in the absence of an appropriate stimulus is thought to prevent degradation and auto-activation of NALP3 (Mayor et al. 2007). Bacterial components such as esat-6 of *M. tuberculosis* can signal NALP3-mediated necrotic cell death (Wong and Jacobs 2011). Taken together, these observations suggest that through direct interactions with bacteria and indirectly through interactions with other host cell proteins, Hsp90 is critical for host responses to infection (Fig. 15.2).

Despite the similarities in structure and ATP-dependent activity between HtpG in bacteria and eukaryotic Hsp90, very little is known about the specific role of HtpG (Buchner 2010). Instead, a diverse set of seemingly disparate observations have been made regarding the functions of HtpG in bacteria. An *E. coli htpG* mutant

exhibits a modest defect for growth under elevated temperatures and a similar mutant in *Edwardsiella tarda* is hyper-susceptible to reactive oxygen species (Dang et al. 2011). In *Cyanobacterium,* HtpG is critical for the assembly of phycobilisomes, which function as light harvesting complexes in these bacteria (Sato et al. 2010). Interestingly, it has also been shown that HtpG is required for the activity of clustered regularly interspaces short palindromic repeats (CRISPR) which is a major defense system against foreign DNA in bacteria (Yosef et al. 2011). In *M. tuberculosis* secreted bacterial stress proteins that are not related to the family of eukaryotic Hsp90, facilitate adherence of the pathogen to macrophages (Hickey et al. 2010). Detailed description of the role of M. tuberculosis Hsp60 in bacterial virulence is provided in Chap. 8 and of M. tuberculosis Hsp70 in Chap. 13. The unifying themes found within these observations is that, similar to Hsp90, HtpG appears to play a role in stress responses and host-pathogen interactions, apparently mediated by key protein-protein interactions.

15.4 Conclusions

Single cell host virulence models are extremely valuable for providing insight into the dynamic interactions that occur at the host-pathogen interface. Environmental amoebae represent a natural environmental host for pathogens that has resulted in the acquisition of traits that increase virulence for mammals. Amoebae, at least partly due to their unicellular nature, are amenable to genetic analysis. The similarity of environmental amoebae to mammalian professional phagocytes makes them well-suited to analysis of innate immunity, in particular. Through the use of one such environmental amoeba, *A. castellanii*, we found that Hsp90 plays an important role during host cell interactions with pathogens. The observations made using environmental protozoa as a model are consistent with the observed function of Hsp90 in mammalian cells and its homologues in bacteria. Additional studies are needed to better understand the mechanisms of action of Hsp90 during bacterial infections.

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Part III
Host Cell Stress Proteins that Promote Bacterial Virulence

Chapter 16
Role of Peptidyl-Prolyl *cis/trans* Isomerases in Cellular Uptake of Bacterial Protein Toxins

Holger Barth

Abstract Binary actin ADP-ribosylating toxins consist of two proteins which are produced and secreted by pathogenic clostridia. The enzyme components ADP-ribosylate actin in the cytosol of mammalian cells which leads to destruction of the actin cytoskeleton. The separate transport components are heptameric ring-shaped molecules which bind to receptors on the surface of target cells, assemble with the enzyme components and trigger the subsequent receptor-mediated endocytosis of the toxin complexes. The enzyme components then translocate from acidified endosomal vesicles into the host cell cytosol. This step is also mediated by the transport components which change their conformation and form pores in the endosomal membranes due to the acidic conditions in the endosomes. The enzyme components translocate as unfolded proteins through these pores across endosomal membranes and their translocation and/or refolding is facilitated by cytosolic host cell factors including the chaperone Hsp90. We discovered that PPIases such as cyclophilin A and FK506-binding proteins 51/52 are also involved in membrane translocation of these toxins. The PPIases interacted with the enzyme components *in vitro* and in living cells and their pharmacological inhibition by cyclosporin A or FK506 inhibited the translocation of the enzyme components to the cytosol and thus protected living cells from intoxication with binary actin-ADP-ribosylating toxins. In conclusion, we have identified a novel Hsp90/PPIase-dependent translocation mechanism which might be selective for ADP-ribosylating toxins. The pharmacological inhibition of toxin translocation could lead to novel therapeutic strategies against diseases associated with such bacterial toxins.

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16.1 Introduction

Bacterial AB-toxins are extremely potent virulence factors which cause severe human and animal diseases such as diphtheria, cholera, anthrax, tetanus or botulism. AB-toxins are produced and secreted by pathogenic bacteria and are taken up into the cytosol of mammalian cells. There, they act as enzymes and modify their specific substrate molecules which results in characteristic cell damage and represents the reason for the typical clinical symptoms of the respective infections. Thus, each AB-toxin is closely linked to a certain disease and to a particular bacterium which produces this toxin. To enter cells, these toxins contain special binding- (B) and membrane translocation- (T) domains, which mediate the binding of the toxin to a receptor on the surface of its target cells. The receptor-mediated internalization of the toxin into endosomal vesicles and the translocation of the enzymatic active (A) domain across the membranes of such vesicles ensures entry into the cytosol. This intracellular membrane translocation of certain toxins is facilitated by host cell chaperones. The current knowledge on the role of peptidyl-prolyl *cis/trans* isomerases (PPIases) in the uptake of bacterial AB-toxins into mammalian cells is summarized in this chapter.

16.2 Uptake of Bacterial AB-Type Toxins into the Cytosol of Mammalian Cells

The cellular uptake of various AB-toxins has only been investigated in detail during recent years. It was discovered that following receptor-mediated endocytosis, the toxins exploit vesicular protein trafficking pathways of their host cells to deliver their A-domains into the cytosol (for review see, for example: Sandvig and Olsnes 1984; Montecucco et al. 1994; Olsnes et al. 2000; van der Goot and Gruenberg 2006). Depending on the organelles from where the A-moieties translocate to the cytosol, the toxins can be divided into two major groups. The *short-trip* toxins including diphtheria and anthrax toxins deliver their enzyme subunits from the lumen of acidified endosomal vesicles into the cytosol (Sandvig and Olsnes 1981; Olsnes et al. 1988). The *long-trip* toxins, such as cholera toxin, undergo a retrograde vesicular transport and deliver their enzyme subunit from the endoplasmic reticulum into the host cell cytosol (Majoul et al. 1996; for more detailed information see also Chap. 21). In any case, the A-subunits of the toxins must translocate across at least one intracellular membrane to reach the cytosol. Several groups have substantially contributed to the understanding of the molecular mechanisms underlying the translocation of bacterial toxins, in particular diphtheria and anthrax toxins, across cell membranes. It became evident that the toxins form trans-membrane pores in endosomal membranes which are involved in the translocation of the A-subunits from the lumen of acidified endosomes to the cytosol (for review see: Trujillo et al. 2006; Young and Collier 2007; Collier 2009; Murphy 2011). However, the

molecular mechanism underlying the intracellular membrane translocation is still poorly understood for most bacterial toxins and therefore the investigation of such processes is of particular scientific and also medical interest.

In our laboratory, we investigate the cellular uptake and the intracellular membrane translocation of binary actin ADP-ribosylating toxins, a special group of AB-toxins produced by *Bacillus* and *Clostridium* species (for review see: Barth et al. 2004; Aktories and Barth 2011; Stiles et al. 2011). Here, the A- and B-subunit are located on two different non-linked proteins which are called components (Ohishi et al. 1980). The single components are not toxic when applied to cells or animals but, in combination, the A- and B-components form very potent toxin complexes (Simpson 1982; Ohishi 1983a, b; Ohishi et al. 1984; Ohishi and Miyake 1985; Ohishi and Yanagimoto 1992; Sakurai and Kobayashi 1995). The assembled toxin complexes bind to cells via the B-component and are internalized by receptor-mediated endocytosis. Subsequently, the B-components form pores in the membranes of acidified endosomes and the A-components translocate through these pores across endosomal membranes into the cytosol (for review see, for example, Barth et al. 2004). In contrast to single chain toxins, where the enzymatic domain and the translocation domain are located on the same protein, pore formation and translocation can be analyzed separately and therefore binary toxins represent ideal models to study the molecular mechanisms underlying these processes (for review see Barth et al. 2004). We discovered that host cell chaperones such as Hsp90 (Haug et al. 2003a, 2004; for review on Hsp90 see Wandinger et al. 2008) and particular PPIases including cyclophilins (Cyps) (Kaiser et al. 2009, 2011) and FK506 binding proteins (FKBPs) (Kaiser et al. 2012) facilitate membrane translocation during the uptake of binary actin ADP-ribosylating toxins into mammalian target cells. In contrast, the binary anthrax toxins, which share structure homology and the overall cellular uptake mechanism with the actin ADP-ribosylating toxins, do not require these host cell factors to deliver their enzyme components to the cytosol (Dmochewitz et al. 2011). In conclusion, the current knowledge on the role of PPIases in uptake of bacterial protein toxins implies that the requirement for Hsp90, Cyps and FKBPs during membrane translocation might be specific and selective for ADP-ribosylating toxins.

16.2.1 Cellular Uptake of Clostridial Binary Toxins

Clostridial binary actin-ADP-ribosylating toxins are the *Clostridium botulinum* C2 toxin on the one hand and the iota-like toxins including *C. perfringens* iota toxin, *C. difficile* toxin (CDT) and *C. spiroforme* toxin (CST) on the other hand (Barth et al. 2004). The enzyme components of these toxins catalyze the transfer of one ADP-ribose moiety from NAD^+ onto arginine-177 of G-actin (Aktories et al. 1986; Ohishi and Tsuyama 1986; Popoff et al. 1988; Schering et al. 1988; Simpson et al. 1989) which induces the depolymerization of actin filaments as depicted in Fig. 16.1. This results in the rounding of adherent cells (Wiegers et al. 1991) and delayed caspase-dependent cell death (Heine et al. 2008).

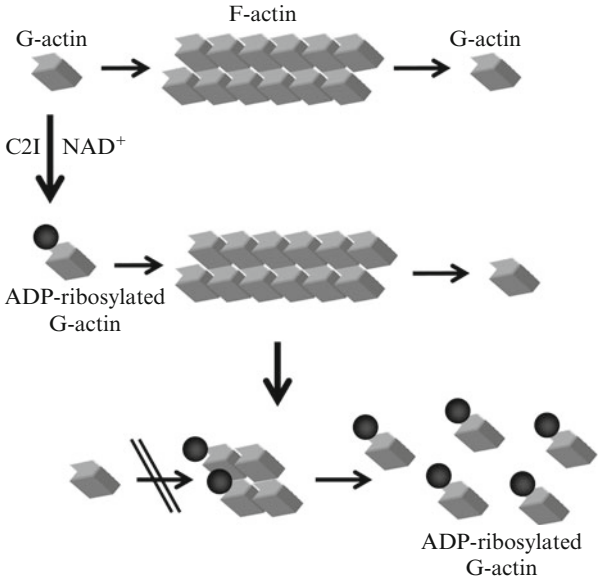

Fig. 16.1 Mode of action of *Clostridium botulinum* C2 toxin. The enzyme component C2I catalyzes the covalent transfer of ADP-ribose from NAD^+ onto G-actin in the cytosol of eukaryotic cells. Mono-ADP-ribosylated G-actin can assemble into actin filaments (F-actin) but then acts as a capping protein and prevents the assembly of further G-actin molecules to this end of the filaments. This mode of action results in depolymerization of F-actin and breakdown of the actin cytoskeleton

C2 toxin, the prototype of this toxin family, is produced by *C. botulinum* type C and D strains (Nakamura et al. 1978) and consists of the enzyme component C2I (49.3 kDa) (Fujii et al. 1996; Barth et al. 1998a) and the binding/translocation component C2II (80 or 100 kDa, depending on the strain) (Barth et al. 2000; Sterthoff et al. 2010). The finding that C2I ADP-ribosylates actin (Aktories et al. 1986; Aktories and Wegner 1989) introduced the new family of binary actin-ADP-ribosylating toxins. Later it was shown that the catalytic site in the C-terminal domain of C2I contains the highly conserved amino acid residues that are found among all bacterial mono-ADP-ribosyltransferases (Barth et al. 1998a; Masignani et al. 2000). Although purified C2 toxin is a very potent enterotoxin which rapidly kills animals after application of 1–2 pmoles and causes necrotic, haemorrhagic lesions in the intestinal wall (Simpson 1982; Ohishi 1983a, b), its role in disease is still unclear. One reason might be that all C2 toxin-producing *C. botulinum* strains also produce the extremely potent neurotoxins which cause botulism and therefore dominate the disease.

The uptake of C2I into the cytosol of target cells is mediated by the activated binding/translocation component C2IIa (see Fig. 16.2). Proteolytic cleavage between Lys181 and Ala182 converts C2II into the biologically active C2IIa, ring-shaped heptamers, which form a complex with C2I (Ohishi 1987; Barth et al. 2000; Stiles

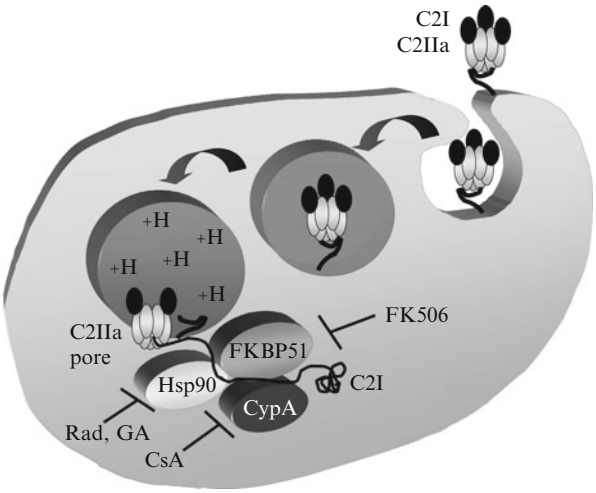

Fig. 16.2 Role of Hsp90, CypA and FKBP51 in cellular uptake of the binary C2 toxin from *C. botulinum*. The heptameric binding/translocation component C2IIa binds to a carbohydrate receptor on the surface of mammalian cells and forms a complex with the enzyme component C2I. After receptor-mediated endocytosis the toxin reaches acidified endosomal vesicles. Due to the acidic conditions in the endosomal lumen C2IIa inserts into the endosomal membrane and forms a trans-membrane pore. C2I translocates in an unfolded conformation through the C2IIa pore from the endosomal lumen into the host cell cytosol. Translocation and/or refolding of C2I is facilitated by the chaperone Hsp90 and the PPIases CypA and FKBP51. The cell-permeable pharmacological inhibitors of Hsp90 (Rad, GA) and the PPIases (CsA, FK506) prevent translocation of C2I to the cytosol and protect cells from intoxication with C2 toxin

et al. 2002). Most likely, three molecules of C2I can bind to one C2IIa heptamer (Kaiser et al. 2006). C2IIa/C2I complexes are either formed in solution prior to their binding to cells or C2IIa binds to the cellular receptor first and then C2I assembles to C2IIa (Kaiser et al. 2006). The receptor for C2IIa consists of an Asn-linked complex and hybrid carbohydrates which are present on all mammalian cell types (Eckhardt et al. 2000) and therefore all cell types tested so far are sensitive to C2 toxin. Following receptor-mediated endocytosis (Nagahama et al. 2009; Pust et al. 2010), C2IIa/C2I complexes reach early endosomal vesicles from where C2I is delivered into the cytosol (Barth et al. 2000). This step depends on the acidic conditions in the endosomal lumen which triggers a conformational change of C2IIa heptamers resulting in exposition of hydrophobic residues on the surface of C2IIa (Blöcker et al. 2003). This leads to insertion of C2IIa into endosomal membranes and the formation of trans-membrane pores, which serve as translocation channels for C2I (Blöcker et al. 2003). The translocation of C2 toxin is depicted in Fig. 16.2.

Although the overall cellular uptake mechanism of C2 and iota-like toxins is widely comparable, there are some differences regarding the cellular receptor as well as the pore structures and the translocation mechanism. In contrast to C2 toxin, iota-like toxins bind to a protein receptor on the cell surface (Papatheodorou et al.

2011, 2012). This lipolysis-stimulated lipoprotein receptor (LSR) is only expressed by certain cell types/lines and therefore the sensitivity to iota-like toxins is restricted to such cells (Papatheodorou et al. 2011).

16.3 Hsp90 and PPIases Facilitate Membrane Translocation of Binary Actin ADP-Ribosylating Toxins

The C2IIa translocation pore has an inner diameter between 2.7 and 3.2 nm (Schleberger et al. 2006) which is too narrow for translocation of unfolded C2I. Our earlier finding that C2I indeed translocates as an, at least, partially unfolded protein from the endosomal lumen to the cytosol (Haug et al. 2003b) addressed the question whether host cell chaperones might be involved in the translocation process of this C2I ADP-ribosyltransferase. The question whether host cell factors are crucial for membrane translocation was addressed before by other groups for diphtheria toxin, another ADP-ribosylating toxin which also delivers its catalytic domain from endosomes to the cytosol. Interestingly, the T-domain of diphtheria toxin mediates the transport of the catalytic domain across planar phospholipid bilayers in the absence of cellular proteins (Oh et al. 1999) indicating that *in vitro* the translocation is an autonomous process independent of host cell factors (Oh et al. 1999). This was different when the translocation process was investigated in isolated acidified endosomes. First, Lemichez and co-workers showed that the addition of cytosol increased the ATP-dependent translocation of the catalytic domain from purified acidified endosomes and identified β-COP as an essential cytosolic factor (Lemichez et al. 1997). Later, the group of John Murphy identified Hsp90 and thioredoxin reductase as components of a cytosolic complex that facilitates membrane translocation of the catalytic domain of diphtheria toxin (Ratts et al. 2003). In the same year, we showed that Hsp90 is involved in the translocation of the C2 toxin from early endosomes into the cytosol of living cells (Haug et al. 2003a). This was the first report that Hsp90 is crucial for cellular uptake of binary toxins.

The first hint that Hsp90 is involved in the mode of action of C2 toxin was that pharmacological inhibitors of Hsp90, radicicol (Rad) or geldanamycin (GA), protected cultured mammalian cells from intoxication with this toxin (Haug et al. 2003a). Pharmacological inhibition of Hsp90 activity prior to C2 toxin application had no effect on the enzymatic activity of C2I *in vitro* or in the cytosol of living cells but prevented the uptake of C2I into the cytosol and therefore the ADP-ribosylation of actin in such cells (Haug et al. 2003a). Inhibition of Hsp90 did neither affect formation of the C2IIa/C2I complex and binding of this complex to the receptor, nor the receptor-mediated endocytosis or the pore formation by C2IIa in endosomal membranes. Importantly, Rad and GA inhibited the translocation of C2I from early endosomes into the cytosol in intact cells (Haug et al. 2003a) as well as *in vitro* (Kaiser et al. 2012). The latter was demonstrated by addition of cytosol to C2 toxin-containing endosomal vesicles, according to the method established earlier by others (Lemichez et al. 1997; Ratts et al. 2003) to investigate *in vitro* translocation of

diphtheria toxin. The addition of cytosol dramatically increased the C2IIa-mediated release of C2I from the endosomes, indicating that cytosolic factors facilitate translocation of C2I *in vitro* (Kaiser et al. 2009, 2012). Moreover, pre-treatment of the cytosol with pharmacological Hsp90 inhibitors blocked the cytosol-triggered translocation of C2I (Kaiser et al. 2012). Collectively, the findings indicated that Hsp90 is crucial for translocation of C2I from acidified endosomes into the cytosol. Although we found that purified Hsp90 directly interacts with C2I *in vitro* and that Hsp90 was co-precipitated with C2I from lysates of cells that had been treated with C2 toxin prior to their lysis (Kaiser et al. 2012), the precise mechanism underlying the interaction between Hsp90 and C2I is, thus far, not completely known.

The next important question was whether Hsp90 alone is sufficient to facilitate translocation of C2 toxin or whether further factors are involved in this step. We focused on the role of PPIases, protein-folding helper enzymes which accelerate the *cis/trans* isomerization of peptide bonds after proline residues (for review see Göthel and Marahiel 1999 and Chap. 2 of this book), for two reasons. Firstly, PPIases have been identified as components of cellular Hsp90-containing multi-chaperone complexes such as steroid receptor complexes (see Chap. 2). Secondly, there are specific and cell-permeable pharmacological inhibitors against PPIases available which allow the performance of cell-based experiments. The family of PPIases was discovered in 1984 by Gunter Fischer and comprises Cyps, FKBPs and parvulins (Fischer et al. 1984, 1989; Lang et al. 1987; Schmid 1993; Schmid et al. 1993; Wang and Heitman 2005). The PPIase activity of Cyps is blocked by cyclosporin A (CsA), a cyclic undecapeptide from the fungus *Tolypocladium inflatum*, that of FKBPs by FK506 also known as Tacrolimus (Handschumacher et al. 1984; for review see Galat 2003). CsA as well as FK506 are immunosuppressive drugs which are well established therapeutics for patients after organ transplantation (Borel 1989). However, their immunosuppressive mode of action is independent of the inhibition of PPIase activity and is mediated by inhibiting the synthesis of certain cytokines including interleukin-2 in activated T-cells (Elliott et al. 1984; Krönke et al. 1984; Liu et al. 1991; Swanson et al. 1992; Weiwad et al. 2006; for review see Wang and Heitman 2005). In brief, in the cytosol of T-cells CsA/Cyp as well as FK506/FK506BP complexes inhibit the activity of calcineurin, a calcium-calmodulin-activated phosphatase. This prevents the calcineurin-catalyzed dephosphorylation of NFAT (nuclear factor of activated T-cells) and thereby the translocation of this transcription factor from the cytosol into the nucleus where it normally induces the transcription of cytokine genes resulting in production of certain pro-inflammatory cytokines.

Pre-treatment of cells with either CsA or FK506 prevented their subsequent intoxication with C2 toxin, i.e. the toxin-induced cell rounding was significantly delayed and the amount of ADP-ribosylated actin in inhibitor-treated cells was strongly reduced (Kaiser et al. 2009, 2012). CsA and FK506 did not interfere with the ADP-ribosyltransferase activity of C2I but inhibited the uptake of C2I into the cytosol (Kaiser et al. 2009, 2012), just as discovered before for the Hsp90 inhibitors. Again, when the influence of the PPIase inhibitors on the individual steps of toxin uptake into cells was analyzed, no effects on binding of the toxin to the cell surface

or endocytosis were detected. As expected, CsA as well as FK506 inhibited the membrane translocation of C2I from early acidified endosomes to the cytosol in living cells as well as from isolated endosomal vesicles in the *in vitro* translocation assay (Kaiser et al. 2009, 2012). Taken together, Cyps and FKBPs facilitate translocation of C2I across endosomal membranes (see Fig. 16.2) and thus play an essential role during uptake of C2 toxin into the cytosol of mammalian cells. Moreover, the combined application of PPIase- and Hsp90-inhibitors suggested that the PPIases might act in a synergistic manner with Hsp90 during toxin translocation (Kaiser et al. 2012).

Because CsA and FK506 inhibit various Cyps and FKBPs, respectively, specific antibodies were used to decipher which particular Cyps and FKBPs are involved in toxin translocation. *In vitro* translocation of C2I from endosomes was blocked when the applied cytosol was pre-incubated with a specific antibody against CypA, implying that CypA might be relevant for membrane transport of C2I (Kaiser et al. 2009). This was confirmed by pull-down experiments with lysates from C2 toxin-treated cells where CypA co-precipitated with C2I (Kaiser et al. 2009). Finally, C2I directly interacted with purified recombinant CypA *in vitro* as demonstrated by dot blot analysis (Kaiser et al. 2009). Collectively, there are various pieces of evidence that CypA specifically and essentially contributes to the membrane translocation of C2I *in vitro* and in living cells. This finding was plausible because CypA is the main cytosolic Cyp and a well established target for CsA. However, we can not exclude at present that further Cyps, for instance Cyp-40, might also be involved in the translocation of C2 toxin. By performing a set of comparable experiments, we identified FKBP51 and the closely related FKBP52 as novel and specific interaction partners of C2I *in vitro* (Kaiser et al. 2012). In intact cells, FKBP51 was associated with C2I as demonstrated by pull-down (Kaiser et al. 2009). However, it can not be excluded that FKBP52 is relevant for membrane translocation of C2I. Interestingly, FKBP12, which is most efficiently inhibited by FK506 among all FKBPs, did not interact with C2I *in vitro* (Kaiser et al. 2009). One possible explanation that C2I does not bind to FKBP12 but to FKBP51/52 might be that the interaction with Hsp90 occurs in the C-terminal domains of FKBP51 and FKBP52 where both proteins share high amino acid sequence homology among each others but not with FKBP12 (Chambraud et al. 1993; Callebaut et al. 1992). C2I might interact with the FKBPs via these domains as discussed recently (Kaiser et al. 2009).

16.3.1 ADP-Ribosyltransferase Domains Might Be Crucial for Interaction with Hsp90 and PPIases

Having established that C2I interacts with Hsp90, CypA and FKBP51, a more careful analysis of this interaction was performed. The enzymatic inactive N-terminal domain of C2I (C2IN, amino acid residues 1–225) interacts with C2IIa and mediates translocation of C2I into the cytosol (Barth et al. 1998b, 2002). Although recombinant C2IN showed binding to Hsp90 and the PPIases in dot

blot and co-precipitation experiments, the pharmacological inhibition of Hsp90, Cyps and FKBPs did not prevent the C2IIa-mediated uptake of C2IN into the cytosol of living cells (Kaiser et al. 2012). This implies that the interaction between C2IN and these host cell factors has no functional relevance, and suggests that the interaction which is relevant for membrane transport might occur via the ADP-ribosyltransferase domain of C2I. This interaction however can not be investigated directly because the C-terminal domain of C2I can not be expressed as an active ADP-ribosyltransferase. Instead, recombinant C2IN fusion proteins were used to address this question. C2IN serves as adaptor for the C2IIa-mediated transport of foreign cargo proteins into cells (Barth et al. 1998b, 2002). Interestingly, Hsp90 and PPIases were only involved in cellular uptake of C2IN fusions when the cargo proteins of such fusions were ADP-ribosyltransferases, such as the C3 transferase from *C. limosum* (Kaiser et al. 2009, 2012) or C/SpvB from *Salmonella enterica* (Pust et al. 2007). This was a strong hint that Hsp90, Cyps and FKPBs might specifically and selectively facilitate the intracellular membrane translocation of bacterial ADP-ribosylating toxins. This hypothesis is confirmed by results obtained for other toxins.

16.4 Role of Hsp90, Cyps and FKBPs in Membrane Translocation of Further Bacterial Toxins

All bacterial toxins for which a function for Hsp90 and PPIases in membrane translocation was discovered so far are ADP-ribosyltransferases. As described before, Ratts and co-workers identified Hsp90 as an essential cytosolic factor for membrane translocation of diphtheria toxin (Ratts et al. 2003) and the group of Ken Teter reported that Hsp90 is required for transport of the enzyme subunit from cholera toxin from the endoplasmic reticulum to the cytosol (Taylor et al. 2010; for more detailed information see Chap. 21 by Teter). We found recently that Hsp90, CypA and FKBPs are crucial for the uptake of iota toxin and CDT, further members of the family of binary actin ADP-ribosylating toxins and also facilitate translocation of their enzyme components across membranes of acidified endosomes into the cytosol (Kaiser et al. 2011). This implies a common Hsp90/PPIase-dependent translocation mechanism for the family of binary actin ADP-ribosylating toxins.

Interestingly, Hsp90 and CypA are not required for membrane translocation of the binary lethal toxin from *Bacillus anthracis in vitro* and in intact cells (Dmochewitz et al. 2011; Zornetta et al. 2010). However, other host cell factors were identified which facilitate the PA63-mediated translocation of LF across vesicular membranes *in vitro* (Tamayo et al. 2008). This is remarkable because the overall cellular uptake mechanisms of binary anthrax and C2 toxins are widely comparable as well as the structures of their translocation pores PA_{63} and C2IIa, respectively (for review see Barth et al. 2004). Lethal factor (LF), the enzyme component of anthrax lethal toxin which is transported by PA_{63}, is a metalloprotease that specifically cleaves MAPK kinase (Duesbery et al. 1998; Vitale et al. 1998; Tonello and Montecucco

2009). This confirms the hypothesis that exclusively ADP-ribosyltransferases require the activities of Hsp90 and PPIases for their membrane translocation and/or refolding. For proof of principle, a fusion toxin was used where the catalytic domain of LF was replaced by DTA (diphtheria toxin A chain), the ADP-ribosyltransferase domain of diphtheria toxin which mono-ADP-ribosylates and thereby inactivates elongation factor-2 (EF-2) in the cytosol of mammalian cells (Blanke et al. 1996; Wesche et al. 1998). DTA was fused to LF_N, the N-terminal adaptor domain of LF, which interacts with PA_{63} and thereby mediates membrane translocation of LF through PA_{63} pores (Blanke et al. 1996; Wesche et al. 1998). Importantly, the PA_{63}-mediated membrane translocation of LF_N-DTA from endosomal vesicles was facilitated by Hsp90 and CypA *in vitro* and Rad as well as CsA inhibited the PA_{63}-mediated uptake of LF_N-DTA into the cytosol of cultured cells (Dmochewitz et al. 2011). Moreover, purified Hsp90 and CypA specifically bound to LF_NDTA and DTA – but not to LF – *in vitro*, indicating that this interaction occurs via the ADP-ribosyltransferase domain DTA (Dmochewitz et al. 2011). In conclusion, there are several pieces of evidence that Hsp90 and PPIases specifically and selectively facilitate membrane translocation of ADP-ribosylating toxins, but not of others, such as the Rho-glucosylating toxins A (TcdA) and B (TcdB) from *C. difficile* which also deliver their enzyme domain through pores across the membranes of acidified endosomal vesicles into the host cell cytosol (Kaiser et al. 2009, 2012). It may be that the host cell chaperones recognize the catalytic domains of various bacterial mono-ADP-ribosyltransferases because of their similar tertiary structure which includes a highly conserved ß-strand and an α-helix in the catalytic-site (Collier 1995; Masignani et al. 2000, 2006).

16.5 Conclusions

Collectively, it became evident that besides Hsp90, PPIases are crucial for the uptake of certain bacterial protein toxins into mammalian cells. Particular Cyps and FKBPs facilitate the intracellular membrane translocation of the enzyme moieties of ADP-ribosylating toxins and this might occur in a synergistic mode of action. Although this new knowledge contributes to a better understanding of the molecular mechanisms underlying the membrane translocation step during uptake of bacterial toxins into the cytosol of mammalian cells, future work is necessary to investigate the interplay between toxins and host cell factors in more detail. In particular, it will be important to investigate whether further chaperones and PPIases besides Hsp90, CypA and FKBP51 are involved in membrane translocation of ADP-ribosylating toxins and whether the host cell factors interact with the toxins and among each other to facilitate translocation and/or refolding. Although Hsp90, CypA and FKBP51 co-precipitated with C2I from lysates of C2 toxin-treated cells it is not clear whether they bind individually to C2I in living cells or as part of multi-chaperone complexes which facilitate membrane translocation of C2I. It was reported that FKBP51, FKBP52 and Cyp-40 are components of Hsp90-containing

multi-chaperone complexes which interact with glucocorticoid receptors in mammalian cells and modulate their affinity for steroid hormones (Pirkl and Buchner 2001; Pratt and Toft 1997; Ni et al. 2010; Stechschulte and Sanchez 2011).

Finally, the targeted pharmacological inhibition of the PPIases which are involved in the uptake of bacterial toxins into mammalian cells could be considered as a novel therapeutic strategy to protect cells from intoxication and thus prevent the outbreak of the clinical symptoms caused by particular toxins in animals and humans. PPIase inhibitors act on the molecular level of the toxin uptake and thus should work when the intoxication was caused by isolated toxins (e.g. food poisoning) but also during infections with toxin-producing bacteria. In the latter case, they should also work even if the bacteria are resistant against antibiotics. The established substances CsA and FK506 are immunosuppressive drugs and thus should not be applied to a patient infected by toxin-producing bacteria but novel non-immunosuppressive PPIase inhibitors (Daum et al. 2009; Fischer et al. 2010) might be attractive substances for such cases (for review see Barth 2011).

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Chapter 17
Listeria monocytogenes and Host Hsp60 – An Invasive Pairing

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Abstract Microbial infection has a dramatic impact on host cell function and can induce host stress-response programs, including the heat shock response. *Listeria monocytogenes* is a human foodborne bacterial pathogen which interacts with the host gastrointestinal epithelium during the initial phase of the systemic disease, listeriosis. The early interaction of *L. monocytogenes* with the intestinal epithelium is a critical determinant of the outcome of infection, and is mediated by multiple bacterial factors, including *Listeria* adhesion protein (LAP). The epithelial receptor for LAP is human heat shock protein 60 (Hsp60), and the LAP-Hsp60 interaction facilitates bacterial adhesion to and translocation through intestinal epithelial monolayers. Interestingly, *L. monocytogenes* infection induces the expression of Hsp60 in epithelial cells, a phenomenon which renders host cells more susceptible to subsequent LAP-mediated *L. monocytogenes* infection. This chapter describes the importance of the host heat shock response during microbial infection, and highlights the role for LAP and host Hsp60 in mediating infection by *L. monocytogenes*.

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17.1 Introduction – Mammalian Heat Shock Proteins in Health and Disease

17.1.1 Role of HSPs in Cellular Homeostasis

Mammalian heat shock proteins (HSPs) are molecular chaperones that maintain cellular homeostasis by facilitating protein assembly, stabilization, transport, protein folding, trafficking and proteolytic degradation of damaged proteins (Feder and Hofmann 1999; Pockley 2003). Although often constitutively expressed at low levels within the cell, chaperone abundance and activity is highly influenced by homeostatic perturbation, caused by physiological stressors such as heat shock, oxidative stress, ultraviolet irradiation, chemicals, nutritional deficiencies, psychological stressors and infection (Belles et al. 1999; Feder and Hofmann 1999; Macario and Conway de Macario 2007; Malago et al. 2003; Padwad et al. 2009). The HSPs are proteins with diverse molecular masses and are designated based on such mass: Hsp100 (gp96), Hsp90, Hsp70, Hsp60, Hsp47, Hsp40, Hsp32, Hsp27, Hsp10, etc. (Pockley 2003). More details of the roles of molecular chaperones in the stress responses of bacteria and eukaryotic organisms is provided in Chaps. 1 and 2, respectively.

Historically, the chaperone activity of HSPs has been attributed to their intracellular location. For example, Hsp60 mediates protein folding primarily in the mitochondrial matrix (Itoh et al. 2002), Hsp70 (HSPA1A) performs chaperone functions in the cytoplasm (Park et al. 2007), and Hsp100 (gP96) is an ER-resident chaperone (Linderoth et al. 2001). However, a number of reports have shown that chaperones such as Hsp60, Hsp70, Hsp90 and Hsp100 (gp96), can also be localized on the plasma membrane or secreted from a variety of cell types (Bocharov et al. 2000; Burkholder and Bhunia 2010; Cabanes et al. 2005; Ferm et al. 1992; Fisch et al. 1990; Jones et al. 1994; Kaur et al. 1993; Soltys and Gupta 1997, 1999). This extracellular localization has been observed during both stress and unstressed conditions, suggesting that surface-expressed and secreted chaperones may fulfil roles beyond maintenance of homeostasis. Further discussion of plasma membrane-associated molecular chaperones is to be found in Chap. 18.

17.1.2 HSPs as "Danger" Signals and Immunomodulators

In contrast to intracellular HSPs, which have critical functions as molecular chaperones, secreted and surface-expressed HSPs can serve as immunomodulators that provide an important link between innate and adaptive immunity (Srivastava 2002). For example, extracellular Hsp60 interacts with cell surface receptors: CD14, CD40 and TLRs to induce either pro-or anti-inflammatory effects (Pockley et al. 2008). Surface-expressed HSPs can potentiate the immune response by binding to pathogen-associated

molecular patterns (PAMPs) to facilitate PAMP-induced TLR signaling (Byrd et al. 1999; Habich et al. 2005; Osterloh and Breloer 2008; Osterloh et al. 2007; Triantafilou et al. 2001; Vance et al. 2009). The role of cell surface molecular chaperones in binding the key PAMP, lipopolysaccharide, is dealt with in detail in Chap. 18. Cell surface HSPs can also trigger lymphocyte activation by directly participating in antigen presentation and cross-presentation (Binder et al. 2001; Callahan et al. 2008; Chen and Androlewicz 2001; Li et al. 2002; Ménoret et al. 2001; Tsuji et al. 2012; Wells et al. 1998). In fact, HSP-peptide complexes promote such strong activation of T-lymphocytes that HSPs are now viewed as potential candidates for use in vaccine development against tumors or microbial infection (Binder et al. 2007; Pockley et al. 2008; Udono and Srivastava 1994). In addition, secreted HSPs are found in the circulation where they may serve as endogenous warning signals that stimulate an immune response in the host (Merendino et al. 2010; Njemini and Mets 2010). Their potency as "danger signals" may be due to their structural similarities to microbial HSPs which are often released during infection (Pockley et al. 2008), or may occur because circulating HSPs are indicative of host cell damage or lysis (Davies et al. 2006). However, despite the growing number of reports linking HSPs to immune function, there is some controversy over whether immunomodulation is due to direct involvement of the HSPs themselves, or is a result of the antigenic peptides bound by their HSP chaperones (Tsan and Gao 2009). This potentially important criticism has been fully refuted by a group of workers in the field of secreted HSPs (Henderson et al. 2010).

17.1.3 Microbial Infection Modulates the Mammalian Heat Shock Response

Numerous reports show that bacterial and viral infection can impact host chaperone expression, which suggests an important connection between the host heat shock system and the response to microbial infection (Table 17.1). Belles et al. (1999) demonstrated that *in vivo* infection with *L. monocytogenes* increased plasma membrane expression of Hsp60 in lymphocytes isolated from spleen and liver of intravenously infected mice. *Salmonella enterica serovar* Enteritidis infection enhanced Hsp70 and Hsp90 expression in enterocyte-like intestinal epithelial Caco-2 cell line (Malago et al. 2003). In addition, Rotavirus infection of Caco-2 cells triggered host cell release of vesicles containing Hsp70 and inhibited T cell function for persistent viral infection (Barreto et al. 2010; Rodriguez et al. 2009). Similarly, Hepatitis B virus induced expression of Hsp60 from hepatocytes and activated regulatory T cells thus promoting persistent infection (Kondo et al. 2010). The role of cell surface stress proteins in viral infection is described in Chap. 19.

The impact of the HSP response during infection is complex, and varies between microbes. Several reports suggest that the host alters HSP expression during infection as a means of self-protection. For example, gastric epithelial cells down-regulate Hsp70 expression in response to *Helicobacter pylori* infection

Table 17.1 Role of HSPs during infection

General function	Heat shock proteins	Specific function	References
Immunomodulation	Hsp60; Hsp70; Hsp90	Binds to LPS to promote TLR signaling	Byrd et al. (1999), Habich et al. (2005), Osterloh et al. (2007), and Triantafilou et al. (2001)
Antigen presentation and cross-presentation	Hsp70; Hsp90; gp96 (Hsp100)	Enhances antigen presentation on MHC Class I molecules	Binder et al. (2001), Callahan et al. (2008), Chen and Androlewicz (2001), Ménoret et al. (2001), and Wells and Malkovsky (2000)
	Hsp90	Enhances antigen presentation on MHC Class II molecules	Tsuji et al. (2012)
	Hsp90	Enhances antigen cross-presentation by antigen presenting cells	Callahan et al. (2008)
Promotes infection of host cells by pathogens	Hsp60	Invasion receptor for *Staphylococcus aureus* on epithelial cells	Dziewanowska et al. (2000)
	Hsp60	Adhesion and translocation receptor for *Listeria monocytogenes* in intestinal epithelial cell lines	Burkholder and Bhunia (2010) and Wampler et al. (2004)
	Hsp70	Promotes Rabies viral transcription, translation and production	Lahaye et al. (2012)
	Hsp70	Invasion receptor for *Brucella abortus* on placental trophoblasts	Watanabe et al. (2008)
	Hsp70/Hsp90	Promotes invasion and intracellular replication of Dengue virus in neuroblastoma, macrophage and monocytic cell lines	Chavez-Salinas et al. (2008), Padwad et al. (2010), and Reyes-del Valle et al. (2005)
	Hsp90	Positively regulates Rotavirus infection	Dutta et al. (2009)

(Axsen et al. 2009), a mechanism which triggers apoptosis of infected cells and promotes bacterial clearance (Liu et al. 2011; Pierzchalski et al. 2006). Recently, the protective effect of the heat shock transcription factors, heat shock factor 1 (HSF1) was demonstrated during *L. monocytogenes* infection in mice. More details on HSFs are to be found in Chap. 2. HSF1 prevented overproduction of proinflammatory cytokines (TNF-α and IFN-γ) and septic shock induced by *L. monocytogenes* (Murapa et al. 2011). In other cases, pathogens benefit from the host heat shock response. *S. aureus* binds to surface-expressed Hsp60 on epithelial cells to promote its invasion (Dziewanowska et al. 2000). *Brucella abortus* uses Hsp70 to mediate invasion of placental trophoblasts, and administration of an anti-Hsp70 antibody to pregnant mice prevented *Brucella*-induced infectious abortion (Watanabe et al. 2008). Membrane expression of the host Hsp70-Hsp90 complex was shown to be critical for Dengue virus infection and replication within neuroblastoma, U937 and THP1 cells (Chavez-Salinas et al. 2008; Padwad et al. 2010; Reyes-del Valle et al. 2005). Similarly, during Rotavirus infection, Hsp90 positively regulated infection by modulating cellular signalling proteins (Dutta et al. 2009). Hsp70 also positively regulated rabies virus life cycle by aiding viral RNA transcription and protein translation (Lahaye et al. 2012).

17.2 *Listeria monocytogenes* and Mammalian Hsp60

17.2.1 L. monocytogenes – *An Opportunistic Foodborne Pathogen*

Listeria monocytogenes is a facultative, intracellular opportunistic human pathogen that causes the potentially life-threatening foodborne illness listeriosis (Freitag et al. 2009). Following consumption via contaminated food, *L. monocytogenes* first adheres to and invades host intestinal epithelial cells. Upon entry into epithelial cells, *L. monocytogenes* escapes from the host phagosome and avoids autophagic recognition to replicate intracellularly and spread from cell-to-cell (Lam et al. 2012; Ogawa et al. 2011). *L. monocytogenes* can exit the epithelium, and then it is phagocytosed by macrophages, which are also permissive to *L. monocytogenes* intracellular replication and serve as a key mechanism for bacterial dissemination and development of systemic listeriosis (Camejo et al. 2011). *L. monocytogenes* poses the greatest health threat to immunocompromised individuals. In these patients, the bacterium can cross the blood–brain barrier and infect the central nervous system (Disson and Lecuit 2012), where infection manifests as meningitis or encephalitis. In pregnant women, the bacterium penetrates the foeto-placental barrier, and foetal infection often results in spontaneous abortion, stillbirth, and neonatal listeriosis (Bakardjiev et al. 2006; Baud and Greub 2011; Lamont et al. 2011).

17.2.2 *Multiple* L. monocytogenes *Virulence Factors Drive the Gastrointestinal Phase of Infection*

The early interaction of *L. monocytogenes* with the intestinal epithelium is a crucial step for initiation of systemic infection. The bacterium uses numerous virulence factors to drive its initial association with host epithelial cells, including fibronectin binding protein (FbpA), ActA, autolysin amidase (Ami), LapB and CtaP (Camejo et al. 2011). FbpA binds host fibronectin, while ActA, a protein which enables actin-based motility during intracellular infection, promotes adhesion to surface proteoglycans (Alvarez-Dominguez et al. 1997). Ami, an autolysin amidase (Milohanic et al. 2004), and CtaP, a bacterial cysteine transport-associated protein (Xayarath et al. 2009), both contribute to adhesion via interaction with unknown host receptors. LapB also is involved in adhesion and invasion of epithelial cells (Reis et al. 2010).

The internalin (Inl) family of proteins promote adhesion to and invasion of a variety of host cells. InlB, InlC and InlJ interact with host intestinal mucin Muc2 (Lindén et al. 2008), and InlJ also binds to intestinal epithelial cells (Sabet et al. 2008). InlA, the best-studied *L. monocytogenes* virulence factor, interacts with its receptor E-cadherin, located in epithelial tight junctions (adherence junction), to drive invasion of host cells (Pizarro-Cerdá et al. 2012). Systemic spread of *L. monocytogenes* is often attributed to invasion driven by InlA, because InlA-deficient mutants exhibit less extraintestinal translocation in animal models of oral listeriosis (Lecuit et al. 2001). However, InlA is not the only virulence factor that drives systemic infection, as numerous *in vivo* studies have demonstrated animal morbidity and mortality following oral infection with InlA-deficient mutants or in animals with modified E-cadherin that fails to interact with InlA (Barbour et al. 2001; Czuprynski et al. 2003; Jaradat and Bhunia 2003). Indeed, it is now well-documented that additional virulence factors promote epithelial cell invasion and translocation independent of InlA. For example, InlB is another surface associated invasion protein that interacts with Met receptor present in human and mouse but not the receptor from rabbit or guinea pigs (Khelef et al. 2006). *Listeria* virulence protein (Vip) binds to surface-expressed chaperone gP96 (Hsp100) to promote bacterial invasion of epithelial cells, and Vip-mediated invasion is required for extraintestinal dissemination in animal models of oral infection (Cabanes et al. 2005). Therefore, *L. monocytogenes* translocation across the host intestinal epithelial barrier is a complex process that involves multiple bacterial virulence factors (Camejo et al. 2011; Pizarro-Cerdá et al. 2012).

17.2.3 Listeria *Adhesion Protein (LAP) Facilitates Bacterial Adhesion To and Translocation Through the Host Intestinal Epithelial Barrier*

We identified *Listeria* adhesion protein (LAP) as a bifunctional bacterial alcohol acetaldehyde dehydrogenase (*lmo1634*; 95 kDa) that also serves as a virulence

factor during the gastrointestinal phase of infection (Burkholder et al. 2009; Jaradat et al. 2003; Kim et al. 2006; Pandiripally et al. 1999; Santiago et al. 1999; Wampler et al. 2004). LAP is present in both pathogenic and non-pathogenic *Listeria*, except *L. grayi* (Pandiripally et al. 1999). LAP was initially characterized as an adhesion factor that promoted binding of *L. monocytogenes* to cultured epithelial cell lines, with greatest LAP-mediated adhesion occurring in cells derived of intestinal origin (Jaradat et al. 2003). During *in vivo* mouse infection, LAP was required for full virulence in orally-infected animals, but had no apparent role when animals were infected intraperitoneally (Jaradat et al. 2003). Together, these early studies suggested that LAP was important for promoting *L. monocytogenes* pathogenesis specifically during the gastrointestinal phase of infection. Further characterization of LAP's role during infection of Caco-2 intestinal epithelial cell lines revealed that, in addition to promoting adhesion, LAP also facilitated bacterial translocation across the epithelial monolayers, which could greatly contribute to development of systemic disease *in vivo*. Interestingly, cell invasion assays indicated no apparent role for LAP in bacterial invasion into host cells, which suggested that LAP might promote bacterial translocation via a paracellular route (between epithelial cells) rather than via a transcellular route (through epithelial cells) providing an alternative strategy for *Listeria* to cross epithelial barrier during infection (Burkholder and Bhunia 2010).

Although LAP is not controlled by the canonical *Listeria* virulence regulatory factor PrfA (Burkholder et al. 2009), its expression and subcellular localization are finely tuned by the external environment such as elevated temperature (>37 °C) (Santiago et al. 1999) and nutrient-limiting starving conditions (Jaradat and Bhunia 2002). Furthermore, LAP expression is greatly influenced by conditions which mimic those in the host gastrointestinal tract. For example, LAP expression and secretion are significantly enhanced by anaerobiosis, a hallmark characteristic of the distal small intestine and colon, the primary sites for *L. monocytogenes* infection (Burkholder et al. 2009). Not unexpectedly, anaerobiosis also promotes LAP-mediated infection both *in vitro* and *in vivo* (Burkholder et al. 2009). These findings emphasize the importance of LAP as a virulence factor during the gastrointestinal phase of *L. monocytogenes* infection.

17.2.4 Listeria *Adhesion Protein (LAP) Uses Human Hsp60 as a Receptor*

The epithelial cell receptor for LAP was identified as human Hsp60 (Wampler et al. 2004). Like many other molecular chaperones, Hsp60 primarily functions inside of the host cell, where it directs protein folding and transport in the mitochondria (Singh et al. 1990). However, we and others have observed that a small fraction of total cellular Hsp60 is associated with the plasma membrane in a variety of cell types (Burkholder and Bhunia 2010; Cappello et al. 2008). Hsp60 level also increased in colon mucosa of patients suffering from Crohn's

disease and ulcerative colitis (Rodolico et al. 2010) and is overexpressed during colorectal and other cancers (Cappello et al. 2003, 2008). We showed that in Caco-2 cells, plasma membrane-associated Hsp60 could be blocked with a specific antibody to decrease LAP-mediated binding, which indicated that *L. monocytogenes* interacted with Hsp60 on the cell surface (Wampler et al. 2004). The LAP-Hsp60 interaction is strong and specific; recombinant purified LAP bound with high affinity to purified Hsp60, but not Hsp70, in a plasmon resonance sensor (Kim et al. 2006). Purified Hsp60 immobilized on a microfluidic biochip (Koo et al. 2009) or paramagnetic beads (Koo et al. 2011) also aided specific detection of *L. monocytogenes*. We showed that the association of LAP with Hsp60 is critical for LAP-mediated adhesion and translocation through intestinal epithelial monolayers. Caco-2 cells in which Hsp60 mRNA was silenced via shRNA were significantly less susceptible to LAP-mediated bacterial binding and transepithelial translocation (Burkholder and Bhunia 2010). Caco-2 cells over-expressing Hsp60 also facilitated enhanced listerial epithelial translocation through paracellular route.

17.2.5 Molecular Interactions Between LAP and Hsp60

As a housekeeping alcohol acetaldehyde dehydrogenase, LAP performs essential metabolic functions within the bacterial cytoplasm. However, the cytoplasmic pool of LAP is not available to interact with host cells unless it is displayed on the surface. This was evidenced by the finding that non-pathogenic *Listeria* species, which possess abundant intracellular LAP but do not secrete it, exhibit no LAP-mediated interaction with host cells (Jagadeesan et al. 2010). Even though a genetically modified non-pathogenic *Listeria* was able to secrete higher levels of LAP, the strain was still unable to interact with mammalian Hsp60 indicating the existence of an alternative mechanism. It was later demonstrated that surface reassociation of LAP on the bacterial cell surface is critical for promoting bacterial interaction with mammalian cells and lack of its surface association prevented non-pathogenic *Listeria* from interacting with host cells (Burkholder et al. 2009; Jagadeesan et al. 2010). In pathogenic *Listeria* (i.e. *L. monocytogenes*), we found that a fraction of cellular LAP is secreted, although the protein contains no signal sequence to direct its extracellular localization. Like several other virulence factors lacking secretion signals, LAP is secreted by the accessory secretory system, SecA2 (Burkholder et al. 2009; Mishra et al. 2011). Although not necessary for bacterial survival, the SecA2 system transports a number of virulence proteins to the cell surface or into the extracellular space (Rigel and Braunstein 2008). As mentioned above, secreted LAP reassociates with an unknown molecule on the bacterial cell surface to allow interaction with host cells (Burkholder et al. 2009; Jagadeesan et al. 2010), a phenomenon also exhibited by other *L. monocytogenes* virulence factors such as InlB (Braun and Cossart 2000; Jonquieres et al. 1999).

The LAP protein (866 amino acids) consists of an N-terminal acetaldehyde dehydrogenase (ALDH) and C-terminal alcohol dehydrogenase (ADH) (Jagadeesan et al. 2010). Analysis of the LAP molecular structure revealed that a region on the N-terminal domain, between Gly_{224} and Gly_{411}, designated N2 subdomain interacts with Hsp60 (Jagadeesan et al. 2011). This N2- subdomain alone is sufficient to mediate binding to Hsp60, as inert microspheres coated with the purified N2 protein fragment exhibited greater binding to surface Hsp60 on cultured HCT-8 ileocecal epithelial cell line than did microspheres coated with other LAP domains (Jagadeesan et al. 2011). Furthermore, anti-Hsp60 antibody also significantly reduced N2 coated microspheres binding to HCT- 8 cells confirming involvement of N2 subdomain interaction with Hsp60 on intestinal cells.

17.3 Mechanism of LAP-Hsp60-Mediated Virulence

A previous report indicated that *L. monocytogenes* infection enhanced Hsp60 expression in lymphocytes from spleen and liver in mice (Belles et al. 1999). Similarly, a low infectious dose of *L. monocytogenes* (10^4 cfu/ml) also increased Hsp60 expression in enterocyte-like epithelial Caco-2 cells, although the impact of infection on Hsp60 induction was independent of LAP (Burkholder and Bhunia 2010). Infection-induced Hsp60 expression required bacterial contact with host cells, as no change in Hsp60 levels or localization was observed when cells were separated from bacteria by a semipermeable membrane. Interestingly, infection increased total cellular Hsp60 expression, and also enhanced its abundance in membrane protein fractions (Fig. 17.1). Further analysis by confocal microscopy revealed enhanced localization of Hsp60 in paracellular membrane borders in *L. monocytogenes-infected* monolayers (Fig. 17.1), which further suggests that Hsp60 might promote bacterial translocation through a paracellular route (Burkholder and Bhunia 2010). Interestingly, our recent work suggests that during *L. monocytogenes* infection, LAP alters expression of host tight junction proteins and increases expression of TNFα, both of which may compromise epithelial tight junction integrity through activation of NF-kB (Kim et al. 2012). These LAP-induced changes in tight junctions further promote paracellular bacterial translocation during intestinal infection (Fig. 17.2). The significance of LAP-Hsp60 interaction was further verified on a probiotic bacterial system. The probiotic bacterium, *Lactobacillus paracasei* expressing LAP interfered with *L. monocytogenes* interaction with epithelial cells thereby reducing infection by 46 % in an *in vitro* cell culture model (Koo et al. 2012). The interaction of the recombinant probiotic with Hsp60 on epithelial cells prevented *L. monocytogenes*-induced epithelial tight junction compromise and subsequent translocation through the epithelial barrier, and cell damage.

The finding that *L. monocytogenes* induces epithelial Hsp60 expression is intriguing, and suggests that *Listeria* may enhance the availability of its own receptor to promote infection. Indeed, our preliminary *in vitro* work suggests that the early upregulation of Hsp60 which occurs during initial exposure to *L. monocytogenes*

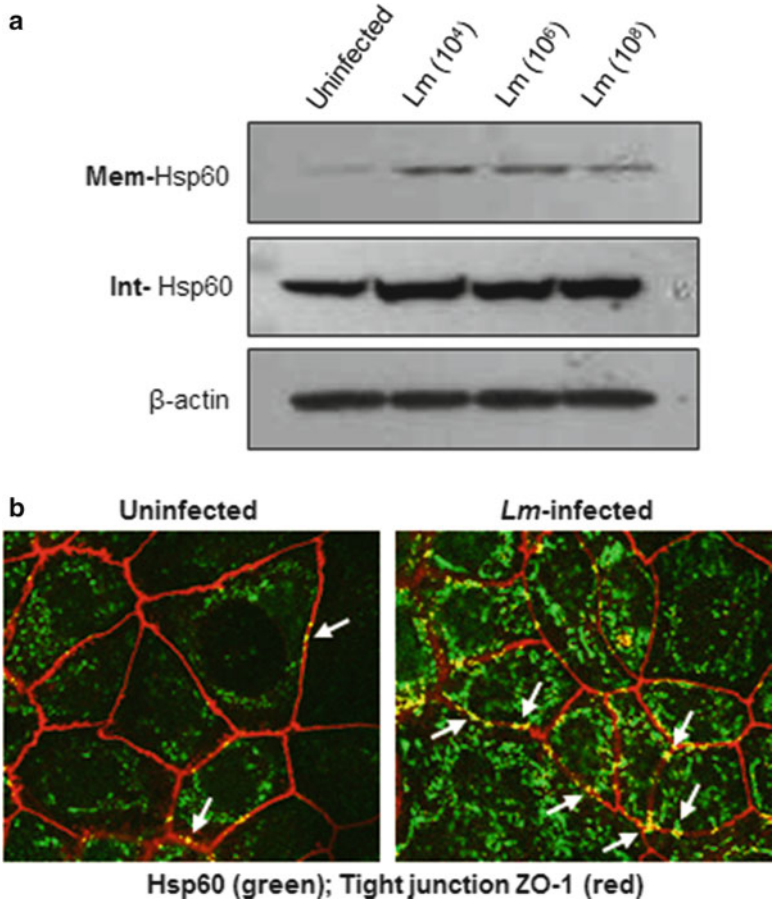

Fig. 17.1 *Listeria monocytogenes* infection increases Hsp60 expression and plasma membrane association in enterocyte-like Caco-2 epithelial monolayers. (**a**) Immunoblot showing membrane-associated (Mem-Hsp60) and intracellular (Int-Hsp60) Hsp60 in uninfected and *L. monocytogenes* infected Caco-2 cells. Cells were infected for 6 h with 10^4, 10^6 or 10^8 cfu/ml *L. monocytogenes*. **β**-actin was used as an internal control. (**b**) Confocal immunofluorescence micrograph showing Hsp60 (FITC; *green*) cellular localization in uninfected (*top panel*) or *L. monocytogenes*-infected Caco-2 monolayers. The tight junction protein ZO-1 was labelled (Cy5; *red*) for the purpose of visualizing paracellular borders. Cells were infected for 6 h with 10^6 cfu/ml *L. monocytogenes*. *Arrows* point to areas where Hsp60 colocalized with paracellular tight junctions (colocalized Hsp60 appears *yellow*). (Adapted from Burkholder and Bhunia 2010 with permission from ASM)

does render the host cells more susceptible to subsequent LAP-mediated infection (Burkholder and Bhunia 2010). This finding suggests an interesting interplay between *Listeria* and its host during infection. The host cell may mount a heat shock response during infection to protect against *Listeria*-induced damage, but because *L. monocytogenes* uses Hsp60 as a receptor, the host stress response actually benefits the pathogen and potentiates infection.

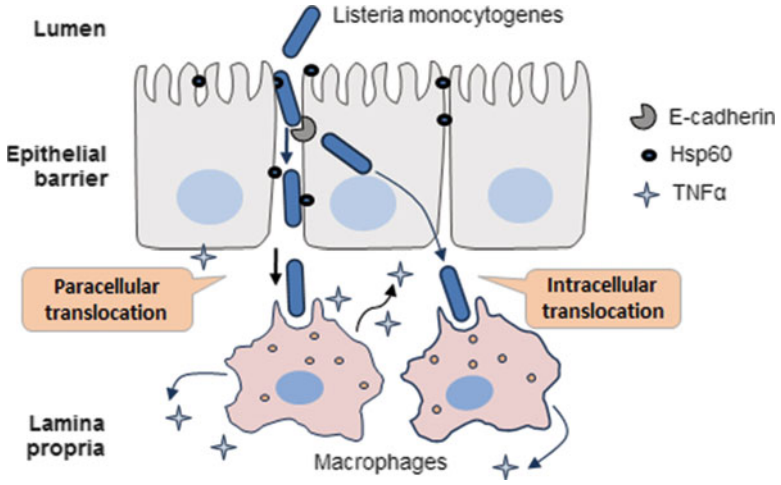

Fig. 17.2 Proposed model for *Listeria monocytogenes* translocation through epithelial barrier after interaction of LAP with Hsp60 during intestinal phase of infection. The interaction of LAP with Hsp60 directly promotes paracellular translocation, and may indirectly promote the association of *L. monocytogenes* InlA with its receptor E-cadherin in epithelial cell junctions for intracellular translocation. LAP-mediated paracellular translocation and TNFα production may enhance macrophage recruitment and bacterial phagocytosis, which could aid in dissemination of *L. monocytogenes* during systemic infection. (Adapted from Burkholder and Bhunia 2010 with permission from ASM)

17.4 Conclusions

L. monocytogenes is a well-adapted pathogen that uses many virulence factors to mediate the gastrointestinal phase of infection (Sleator et al. 2009). As such, LAP likely functions in concert with other bacterial virulence proteins, such as InlA, to penetrate the gastrointestinal barrier (Burkholder and Bhunia 2010). At this time, the precise role of LAP and Hsp60 in the broader context of *in vivo* gastrointestinal infection is unclear. However, because LAP is a critical virulence factor during oral *L. monocytogenes* infection, we speculate that the LAP-Hsp60 interaction promotes paracellular translocation across the gut barrier *in vivo*. By promoting bacterial translocation, and potentially via LAP-mediated TNFα production, the LAP-Hsp60 interaction could enhance macrophage recruitment and bacterial phagocytosis at the site of infection, to promote dissemination and development of systemic listeriosis (Fig. 17.2). Our studies provide an example of a pathogen which promotes and benefits from the host heat shock response; however, interplay between microbes and their hosts is complex, rarely uniform, and often differs between microorganisms. Therefore, many interesting questions remain regarding the role of the host heat shock response in the outcome of microbial infection.

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Chapter 18
Cell Surface Stress Proteins and the Receptor for Lipopolysaccharide

Kathy Triantafilou and Martha Triantafilou

Abstract One of the most powerful signals in Biology is the outer surface component of Gram-negative bacteria – the amphiphilic molecule known as lipopolysaccharide (LPS). Certain mammals, *Homo sapiens* being a good example, are exquisitely sensitive to this complex bacterial-host signalling molecule and LPS is a major cause of the pathology of Gram-negative bacterial infection, including being the major injurious agent in septic shock. How cells recognise LPS has long been a mystery which has only begun to be solved in the last decade or so. A curious facet of this story is that among the proteins which are involved in recognising LPS are the molecular chaperones, Hsp70 and Hsp90. The elucidation of the role of these, and other, cell stress proteins in LPS recognition is the focus of this chapter.

18.1 Introduction

As scientists, words are central to our lives, and have to be treated seriously. However, one of the most complex biological molecules, the molecule known as lipopolysaccharide (LPS), has the capacity to confuse the average biological scientist. This is because LPS is known as an endotoxin and suddenly, the term endotoxin and LPS become synonymous. However, it is important to be clear that when referring to LPS, the terminology introduced by Hitchcock and

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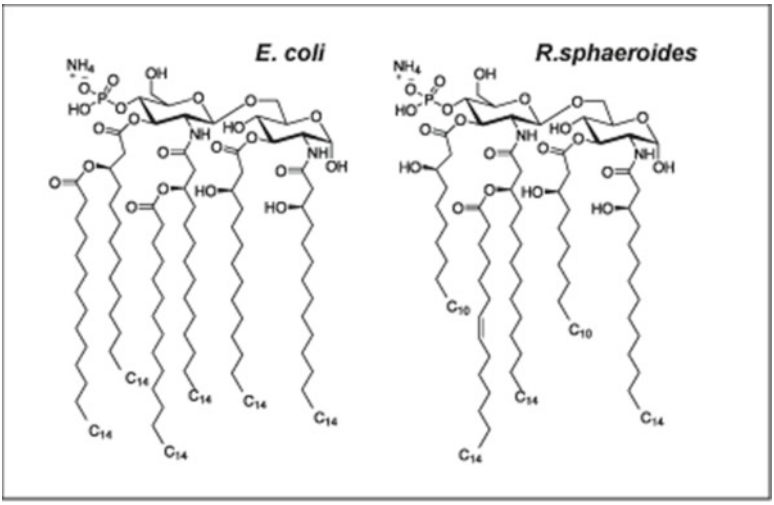

Fig. 18.1 The structure of the active component of LPS, lipid A showing a potent endotoxic species from E. coli, with six acyl chains and an antagonistic form of LPS from *Rhodobacter sphaeroides* which will antagonise the action of the *E. coli* LPS

co-workers is being used and this states that "the term LPS should be reserved for purified bacterial extracts which are reasonably free of detectable contaminants, particularly protein" (Hitchcock et al. 1986). This is the definition that will be followed in this chapter.

Lipopolysaccharide has a long and distinguished history. In the nineteenth century, bacteria were discovered and their role in human disease, particularly their ability to cause fever, identified. Richard Pfeiffer was the first to observe that the bacterium causing cholera contained a toxin tightly anchored to the bacterial cell wall and he named it endotoxin to discriminate it from the soluble toxin produced by *Vibrio cholera*e (Pfeiffer 1892). Substantial work on LPS was carried out in the intervening years, but it was the introduction of a phenol/water extraction method by Otto Westphal in the 1950s (Westphal et al. 1952) that opened the way to the structural elucidation of LPS. Work going on since the 1960s has revealed that the average *E. coli* cell contains 3.5×10^6 LPS molecules with ¾ of the cell surface being covered by this moiety (Raetz 1993). The LPS molecule consists of three chemically distinct regions: (i) lipid A; (ii) the LPS core region and (iii) the LPS O-specific side chain. The biological activity of LPS is a function of the complete molecule, but the cell stimulating, so-called endotoxic action, of this molecule is largely due to the lipid A portion (Fig. 18.1). Lipid A is a disaccharide composed of a range of sugars linked to a variable number of acyl chains by amide or ester links (Kabanov and Prokhorenko 2010). The diverse structure of lipid A, particularly the numbers of acyl chains, controls enormously, the endotoxic activity of LPS which can range in potency from pg/ml to μg/ml concentrations and above (Fujimoto et al. 2005). It is still not clear how this

enormous range of potency of the LPS molecule is achieved at the level of the 'LPS receptor'.

Purified lipopolysaccharide from *E. coli* or from *Salmonella* spp. can be active at nano- to picro-gram/ml quantities on the cells from sensitive animal species (e.g. *Homo sapiens*). It was therefore obvious that there must be a high affinity receptor for this protein on responsive cells. However, elucidating the nature of this receptor is still taxing the biological community. Proteins of molecular masses ranging from 18 to >90 kDa and including those of 70 kDa have been identified as receptors for LPS (reviewed by Henderson et al. 1998). It was not until the late 1980s/early 1990s, that some identifiable LPS binding proteins were found. These included LPS-binding protein (LBP) (Tobias et al. 1989) and CD14 (Wright et al. 1990). However, of these proteins, only CD14 could be on the cell surface while LBP seemed to function to solubilise LPS monomers from the LPS micelle and transfer it to CD14. Note that LBP is now seen as one member of a growing family of proteins which have been termed the TULIP (tubular lipid-binding) domain superfamily (Kopec et al. 2011). One problem that the identification of CD14 generated as a cell surface receptor for LPS was that it was a glycosylphosphatidylinositol (GPI)-linked protein and therefore unable to signal to the cell. This suggested that the CD14 had to interact with some other membrane component to allow for intracellular signalling.

The next step in the story depended upon the chance finding of mouse strains which were insensitive to LPS. These include the C3H/HeJ, C57BL/10ScNCr and C57BL/10ScCr strains which all have mutations at the so-called Lps locus (Mergenhagen and Pluznik 1984). Genetic analysis of the Lpsd allele of the C3H/HeJ mouse identified a single missense mutation resulting in the replacement of a proline with a histidine in the protein, Toll-like receptor (TLR)4, thus identifying this protein as the LPS receptor (Poltorak et al. 1998). Bruce Beutler, in whose lab this work was done, received the 2011 Nobel Prize for this work.

Did the identification of TLR4 as the LPS receptor answer all the burning questions about LPS biology? Surprisingly, it did not. Although TLR4 has been shown to be essential for LPS innate immune recognition, it has also been shown that accessory molecules are required. In addition to TLR4, CD14 (Wright et al. 1990) and MD2 (Shimazu et al. 1999) are also involved in signalling. CD14 has been shown to bind LPS and transfer it to TLR4, which transduces the signal. MD-2, a secreted glycoprotein that interacts with TLR4, enhances its sensitivity to the ligand. Without this molecule, TLR4 is still capable of binding LPS, but the sensitivity to this ligand is extremely low (Re and Strominger 2003; Visintin et al. 2003).

However, it also turns out that other proteins are involved in the LPS 'sensing apparatus' which may account for the enormous range of affinities reported for LPS-host cell interactions (Henderson et al. 1998). Included in this 'sensing apparatus' are a number of molecular chaperones. This ability of eukaryotic molecular chaperones to bind LPS has been a major problem in the literature that claims that specific molecular chaperones, if they are secreted, can function as intercellular signalling proteins (Henderson et al. 2010).

18.2 Molecular Chaperones and the Binding of LPS

The concept of molecular chaperones being involved in sensing LPS originated in a rather strange pairing of biological activities. This is the finding that the anti-cancer agent, from the yew tree, taxol, has effects on cells indistinguishable from that of LPS. To determine how this molecule promoted the LPS-like response the 'receptor' for taxol was isolated using affinity proteomics with macrophages. Two proteins bound to taxol and these were identified as Hsp70 and Hsp90 (Byrd et al. 1999). Of interest Byrd and coworkers also found that LPS inhibits Dengue virus binding to monocytes (Byrd et al. 1999) – see more details in Chap. 19. Now these molecular chaperones were isolated from cell extracts and it was not known from which cell compartment they came. Did LPS also bind to these proteins? Using a similar approach with LPS, rather than taxol, it was found that the cellular proteins which bound to LPS were the molecular chaperones, Hsp70, Hsp90, the chemokine receptor CXCR4 and the growth factor, growth differentiation factor (GDF)5 (Fig. 18.2).

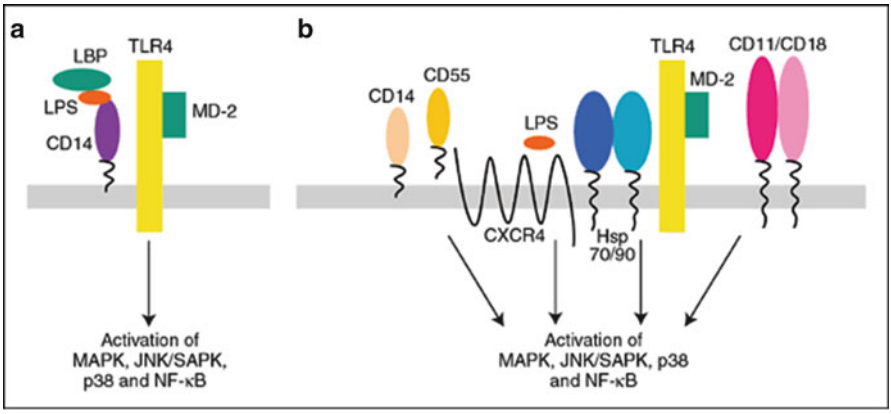

Fig. 18.2 Models of lipopolysaccharide (*LPS*) recognition. (**a**) Accepted model of LPS recognition, where LPS binds LPS-binding protein (*LBP*) in the serum, and then the LPS–LBP complex binds to CD14 on the cell surface of monocytes. CD14 lacks a transmembrane region and cannot transmit a signal; however, following its association with Toll-like receptor 4 (*TLR4*) and the accessory protein MD-2, signal transduction is triggered, leading to activation of multiple signalling molecules, such as MAPK, JNK/SAPK, p38 and NF-kB. (**b**) Proposed model of LPS recognition, where LPS binds pattern recognition receptors on the cell surface of monocytes, forming an activation cluster. Multiple signalling molecules are triggered from the multiple receptors that associate within the activation cluster. In addition to CD14, TLR4 and MD-2, other possible interacting molecules are CD55, chemokine receptor 4 (*CXCR4*), heat shock proteins (*Hsps*) and the integrin CD11/CD18. However, it might be expected that different combinational associations of receptors occur, enabling the innate immune system to respond to a wide range of microbial pathogens (*JNK/SAPK* c-Jun-N-terminal kinase/stress-activated protein kinase, *LBP* LPS-binding protein, *LPS* lipopolysaccharide, *MAPK* mitogen-activated protein kinase, *NF-kB* nuclear factor kappa beta (transcription factor), *p38* p38 MAPK. Reproduced from Triantafilou and Triantafilou (2004b), with permission)

However, these experiments failed to pull-out TLR4, which is supposed to be the LPS receptor (Triantafilou et al. 2001). Another cell surface protein found to bind to LPS was the complement regulatory protein, decay-accelerating factor or CD55 (Heine et al. 2001). This seems an odd collection of proteins to be binding to LPS and it was not clear what connection lay between them. Furthermore, the assumption was that they formed a complex on the surface of target cells. One obvious question was, whether they were involved in LPS signalling. This was tested by using blocking antibodies. Surprisingly, antibodies to each of these LPS-binding proteins inhibited the activation of a myeloid cell line (Mono-Mac-6) or human peripheral blood monocytes exposed to LPS (Triantafilou et al. 2001). It is believed that the blocking effect is due to steric hindrance of the complex or of the interactions of LPS with the complex (Triantafilou et al. 2001, 2002a, 2008; Triantafilou and Triantafilou 2002).

Having identified what appears to be a complex of proteins on the surface of LPS-responsive cells the question that arose is – how do these proteins interact with LPS? This question was addressed using the biophysical techniques of fluorescence recovery after photobleaching (FRAP) and fluorescent resonance energy transfer (FRET). These techniques also made use of a new technique to fluorescently label LPS (Triantafilou et al. 2000) and of fluorescently labelled antibodies to cell surface components such as CD14. FRAP allows measurement of the velocity of lateral movement of fluorescent molecules on the cell surface and FRET allows measurement of the interaction of two defined proteins at the cell surface. Using FRAP, with fluorescently labelled LPS and a labelled Fab fragment of an anti-CD14 binding antibody, revealed that the mobility of CD14 in the cell membrane is hardly influenced by binding LPS and that the LPS is rapidly transferred to a state in which it is immobilised. This immobilisation was found to be due to the LPS binding to Hsp70 and Hsp90, and antibodies to these two molecular chaperones, added prior to the LPS, block the progressive immobilisation of LPS and also inhibit LPS-induced cell activation, thus placing these two cell stress proteins centre stage in the LPS activation phenomenon (Triantafilou et al. 2002b). The use of FRET independently revealed the interaction, at the target cell surface of LPS with Hsp70 and Hsp90 (Triantafilou et al. 2001).

These findings implicating two heat shock proteins in the interactions of LPS with target cells raises all sorts of questions. The first is – exactly where is the Hsp70 and Hsp90 in the cell membrane? Now CD14 had been recognised to be present in the Triton-X100 component of plasma membrane fractions which suggested that it was part of a microdomain or lipid raft (Pugin et al. 1998). Such rafts can be disrupted by use of agents such as nystatin or methyl-beta cyclodextrin (MCD) and indeed, such reagents were able to inhibit LPS-induced production of TNFα by monocytes (Triantafilou et al. 2002c) suggesting that these proteins were resident in such lipid rafts.

Further analysis of the LPS interaction with target cells has revealed that Hsp70 and Hsp90 form a cluster with TLR4 within lipid microdomains following LPS stimulation. Moreover, Hsp70 and Hsp90 are involved in TLR4/LPS trafficking and targeting to the Golgi apparatus, revealing a complex orchestration of these proteins

to different cellular compartments (Triantafilou and Triantafilou 2004a). Thus, on the cell surface of LPS-responsive cells there is a complex ballet of interactions in which LPS is passed from one 'receptor' to another to finally induce its biological effect. It is assumed that it is in the combinatorial dynamics of these interactions that the affinity and efficacy of the LPS is defined (Triantafilou and Triantafilou 2005; Triantafilou et al. 2008). Evidence in support of this hypothesis was gained by comparing the complexes formed when cells were stimulated with different acylated versions of LPS or synthetic versions with lower potencies. Thus endotoxically active LPS was associated with the generation of receptor complexes containing TLR4, Hsp70, Hsp90 CXCR4, GDF5, CD11b/CD18, CD81 and CD55. With LPS of low activatory agonist activity the recruitment complex contained Hsp70, Hsp90, CD55 and a lower level of TLR4 but failed to contain CXCR4, GDF5, CD55, CD11b/CD18 or CD18. This suggests that the latter components might be involved in enhancing the signalling to target cells of a potent LPS species (Triantafilou and Triantafilou 2004a).

18.3 Is LPS Binding a Universal Phenomenon with Molecular Chaperones?

One obvious question that these findings raise is whether LPS binding is a natural function of molecular chaperones and protein-folding catalysts. An obvious place to look for an answer to this question would be the cell stress proteins of Gram-negative bacteria as these proteins would be constantly exposed to LPS. However, the only bacterial molecular chaperone reported to interact with LPS, and possibly act as an LPS chaperone, is Skp (Qu et al. 2009). So if molecular chaperones bind to LPS there may be some evolutionary explanation. In addition to Hsp70 and Hsp90 interacting with LPS, it has been reported that the human chaperonin (Hsp)60 protein binds LPS and that there is a small binding motif in the apical (protein binding) domain of this protein that recognises LPS (Habich et al. 2005). This actually is homologous to a binding region in the LPS-binding protein, factor C, from the horseshoe crab. Hsp60 can be found on the surfaces of cells under different sets of conditions and so it is possible that this protein could function as an independent 'LPS sensor'. As has been explored in Chap. 17, cell surface Hsp60 recognises the Gram-positive organism, *Listeria monocytogenes*. However, here the binding is not to bacterial teichoic acids, but to a bacterial moonlighting protein, acetaldehyde alcohol dehydrogenase. However in rabbits exposed to LPS it has been shown that this amphiphilic molecule induces the upregulation of cell surface Hsp60 expression (Wick et al. 2008). So the story is extremely complex and still requires detailed elucidation.

One molecular chaperone which needs mention in this context is a member of the Hsp90 family known as gp96. This protein was found to enhance the response of macrophages to LPS (Warger et al. 2006). Inactivation of the gene encoding gp96 was found to generate a mouse which was LPS-insensitive but

still responsive to *L. monocytogenes*. It turns out that gp96 is responsible for the cell surface location of the TLRs (Yang et al. 2007). Upregulation of gp96 induces elevated responses to LPS, mainly as a result of TLR4 expression (Liu et al. 2006). However, it also turns out that LPS can alter the regulation of cell surface gp96, revealing an additional pathway of LPS control of its own signalling (Kim et al. 2010).

18.4 Modulation of Hsp/LPS Interactions

If LPS is interacting with cell surface-associated Hsp70 and Hsp90 it would be expected that modulation of these proteins would influence LPS signalling. Now, Hsp90 is a major cell stress protein and has many intracellular functions. For this reason it has become a subject of substantial interest and there has been much use, over the past two decades, of the antibiotic, geldanamycin, which binds to and blocks the activity of Hsp90. This is now only one of a growing group of Hsp90 inhibitors (Neckers and Workman 2012). Geldanamycin was found to inhibit LPS activation of the murine macrophage cell line J774 and this was associated with a reduction in cell surface expression of CD14, but with no decrease in the transcription of the gene for this protein. Indeed, intracellular levels of CD14 were increased in geldanamycin-treated cells. This suggests some interference with the LPS 'sensing apparatus' when cells are exposed to geldanamycin, resulting in loss of cell surface CD14 (Vega and De Maio 2003). Indeed, Hsp90 inhibitors have anti-inflammatory effects both *in vitro* (Chatterjee et al. 2008; Yun et al. 2011; Luo et al. 2011) and *in vivo* (Ambade et al. 2011; Poulaki et al. 2007; Yun et al. 2011). Another possible way of looking at the importance of Hsp90 in LPS signalling would be by seeing if Hsp90, Hsp90 mutants or derived peptides could block LPS signalling. This has not yet been achieved but such experiments have been done with Hsp70.

Preincubation of human monocytes with soluble Hsp70, prior to addition of LPS, was able to significantly, but not completely, inhibit LPS signalling (Triantafilou et al. 2008). The Hsp70 protein has a N-terminal ATP-binding domain and a C-terminal domain which has a peptide binding function. Generation of C- and N-terminal mutants has revealed that LPS binds to the ATP-binding domain and, in particular, the base of the ATP-binding cleft (Triantafilou et al. 2008). Another group have shown that extracellular Hsp70 induces LPS tolerance (Aneja et al. 2006) which may fit with the inhibitory effect of Hsp70 preincubation.

It is of interest that the human Hsp70 protein and the Hsp70 (DnaK) protein of *Mycobacterium tuberculosis* both activate human monocytes but do so through different parts of the protein. Thus, the active site in the human protein is in the N-terminus (Becker et al. 2002), where the LPS binding site is found. In contrast, the agonist site in the bacterial DnaK protein is in the C-terminus, adjacent to the peptide binding site (Wang et al. 2005). In these studies the binding receptor for the Hsp70 protein is CD40.

18.5 Conclusions

It is becoming appreciated that signalling at cell surface receptors can be more complex than the simple unitary binding of an agonist to a single receptor subunit or the binding of the agonist leading to receptor dimerisation. A good example of an immunological receptor complex is the T cell receptor which is now recognised to involve many protein subunits including the T cell receptor (TCR) itself plus CD3, CD4/8, CD28 and LFA-1 as a minimum. This forms part of a larger multicellular cluster known as the immunological synapse, connecting the T lymphocyte with the antigen-presenting cell (Dustin and Depoil 2011). The function of the immunological synapse, and the potential reason for its complexity is to ensure that T cell clonal proliferation is very tightly controlled. It appears that the recognition of the Gram-negative outer coating, LPS, is under similar tight control, if the nature and complexity of the recognition complex can be used as an evolutionary measure of control. As innate immunity is regarded as the evolutionary simpler aspect of immunity it is not clear why similar complexity in the LPS and T cell antigen receptors should have evolved. Is the 'LPS receptor' actually something more complex, not designed to simply 'measure' LPS but a means of sampling many of the pathogen-associated molecular patterns (PAMPs) on the bacterial cell surface and providing the immune cell with a recognisable snapshot of what the innate cell is dealing with. Speculative as this is, it leaves open the question of why evolution should have evolved to utilise two key cell stress proteins in this process.

The answer to this question may be found in other chapters in this volume which reveal the role played by eukaryotic cell stress proteins in the recognition of specific bacteria and in the recognition of a range of viruses.

Gazing into the crystal ball and speculating on the fact that microbes and eukaryotes utilise many of the same cell stress proteins it is possible that these microbial proteins are utilised to inhibit the cell signalling actions of their eukaryotic homologues.

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Chapter 19
Host Molecular Chaperones: Cell Surface Receptors for Viruses

Tomoyuki Honda and Keizo Tomonaga

Abstract Molecular chaperones play important roles in maintaining cellular homeostasis under normal conditions. They also participate in a post-translational quality control system, maintaining the correct conformation of proteins under changing environmental conditions. While most molecular chaperones localize in the cytosol, some can exist outside the cell and are involved in moonlighting activities. It has been reported that some molecular chaperones at the cell surface act as receptors for viruses. Viruses using molecular chaperones as their receptors take advantage of these molecules to enable efficient introduction of their genomes into the cell and/or for selection of favorable target cells and of replication-competent virions.

19.1 Introduction

Molecular chaperones are essential for the correct functioning of cells and are found in every living organism. They are a large and diverse group of unrelated proteins that assist in the correct, non-covalent assembly and/or disassembly of other polypeptide-containing structures, but are not permanent components of these structures when they are performing their normal biological functions (Ellis 1997). For more details of the functions and mechanisms of molecular chaperones refer to Chaps. 1 and 2. Molecular chaperones are involved in the folding of a range of client proteins within the various cellular compartments. However, it is becoming clear that many molecular chaperones are present outside the cellular environment and

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Table 19.1 Molecular chaperones used for virus entry

Molecular chaperones	Virus	Reference
BiP	BDV	Honda et al. (2009)
	CAV-A9	Triantafilou et al. (2002)
	DENV	Cabrera-Hernandez et al. (2007)
Hsp70	DENV	Reyes-Del Valle et al. (2005)
	JEV	Das et al. (2009)
Hsp90	DENV	Reyes-Del Valle et al. (2005)
Hsc70	Rotavirus	Guerrero et al. (2002)
gp96	VSV	Bloor et al. (2010)
CypB	MV	Watanabe et al. (2010)

VSV, vesicular stomatitis virus

are involved in moonlighting activities. It has been reported that some molecular chaperones at the cell surface can act as receptors for viruses. Here, after a brief overview of the mechanisms of virus entry, we focus on the specific mechanisms by which molecular chaperones at the cell surface are used as receptors for viruses (Table 19.1). Then, we discuss the advantages to viruses of using molecular chaperones as their receptors.

19.2 Virus Entry

19.2.1 Virus Infection Cycles

Viruses infect their target cells by crossing cellular membranes. This is the key to initiating their infection cycles and involves a number of discrete steps, such as receptor binding and entry, capsid destabilization and genome uncoating, and the release of viral nucleic acids at the site of replication. Usually, progeny viral genomes, be they RNA or DNA, must transit the cell-free stage to access new host cells. While viral nucleic acids are protected by cellular membranes in the infected cells, they are potentially susceptible to damage in the environment outside the cell, during the cell-free stage. Viruses mitigate these risks by packaging their genomes into particles protected by a membrane and/or protein shell. Thus, viral particles must be sufficiently resilient to protect their genomes from environmental and/or immunological insults. On the other hand, viruses must be able to cross cellular membranes to initiate their infectious cycles. This means that viral particles must be appropriately labile to ensure the contents are released when they have reached a suitable environment for replication. Thus, viral particles are metastable structures that can be unlocked during entry by specific molecular and/or cellular environmental keys (Marsh and Helenius 2006). Receptors are keys to the unlocking process, triggering subsequent infection.

19.2.2 Virus Receptors

Initial encounters between a virus and a host cell are mediated through surface components of the virion, either membrane glycoproteins for enveloped viruses or sites on viral capsids, projections or indentations, for non-enveloped viruses (Marsh and Helenius 2006). These bind to glycolipid and/or glycoprotein attachment factors at the cell surface, such as heparan sulfate proteoglycans (de Haan et al. 2005; Vlasak et al. 2005). These attachment factors, whose interactions with viruses may lack specificity, concentrate virus particles on the host cell and provide an initial catch-hold from which viruses can then recruit specific receptors that drive the reactions leading to entry. Following attachment, receptor binding is often the second and more critical step in viral infection. Cellular receptors vary from one virus to the next and are in many cases cell specific. Receptors drive fusion/penetration events directly at the surface of target cells, or within endosomal compartments, by inducing conformational changes in key virus surface structures (Fig. 19.1). The use of specific cell surface components with restricted expression patterns is frequently responsible for virus tropism. A number of the cell surface components exploited by viruses have been identified and are reviewed elsewhere (Grove and Marsh 2011; Kalia and Jameel 2011; Gerlier 2011).

19.2.3 Membrane Fusion/Penetration

Enveloped viruses achieve entry into animal cells in two principal ways: by direct fusion with the plasma membrane or by an internalization process via the endosomal pathway (Fig. 19.1). For viruses using the first strategy, fusion between the viral and plasma membranes occurs after receptor binding and before the virus genome penetrates the cell. In the case of endosomal entry, the viruses fuse with the endosomal membrane in order to be released into the cytoplasm. While a detailed understanding of the entry process and fusion mechanisms is available for many enveloped viruses, much less is known about the entry of non-enveloped viruses. Because these viruses do not have a membrane, the entry mechanisms do not involve membrane fusion, and capsid-dependent mechanisms for penetrating the cellular membrane are likely to be involved. Most non-enveloped virus capsid proteins have short, membrane altering, amphipathic or hydrophobic sequences that mediate membrane penetration (Banerjee and Johnson 2008). On binding to the receptor, these capsid proteins may undergo conformational rearrangement. The sequences exposed by the conformational changes are then thought to form a transmembrane pore through which viral genomic nucleic acids may be extruded into the cytoplasm (Hogle 2002). Non-enveloped viruses may also achieve entry in two principal ways: by direct penetration at the plasma membrane or by an internalization process into endosomes. Most viruses, regardless of whether they are enveloped or not, prefer to enter cells via endocytosis because the endocytic machinery confers the additional advantages of specific localization within the cell, leading to successful infection (see below) (Pelkmans and Helenius 2003).

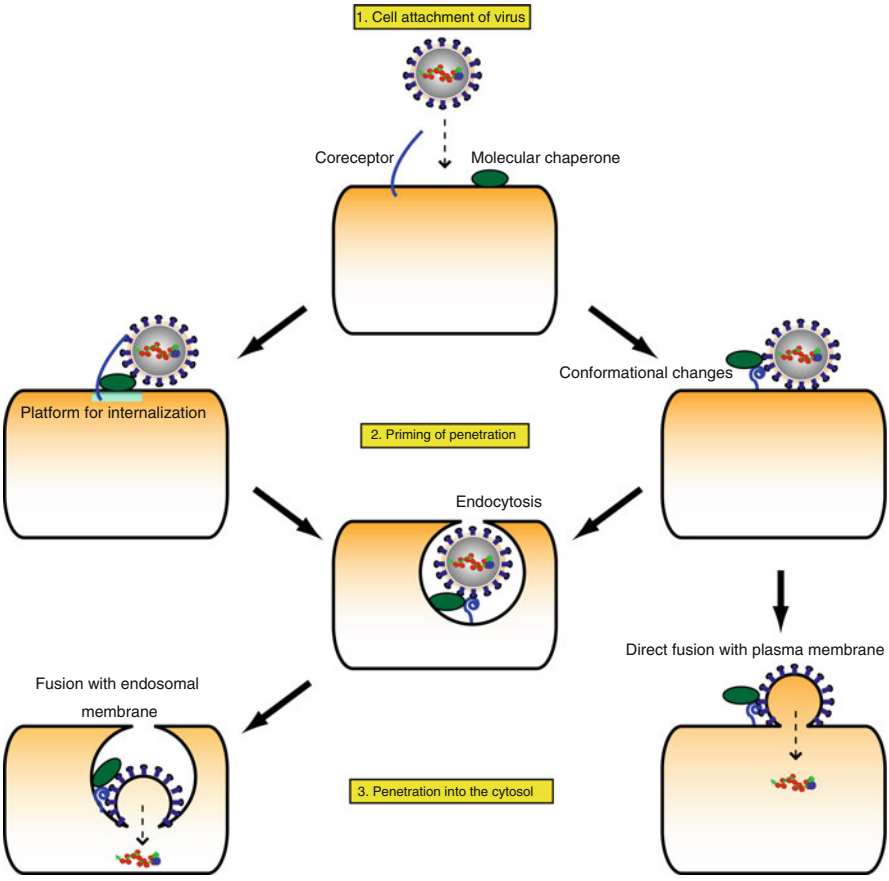

Fig. 19.1 A model of enveloped virus entry. The virus binds to cell surface receptors, such as molecular chaperones on the cell surface. After attachment of the virus, priming of virus penetration into the cytosol occurs, including recruitment of the virus-receptor complex to platforms, such as the LR, conformational changes of receptors, and endocytosis. Then, direct fusion with the plasma membrane or fusion with the endosomal membrane allows virus penetration into the cytosol

19.3 Regulation of Virus Entry by Molecular Chaperones at the Cell Surface

19.3.1 BiP (Grp78/HSPA5)

BiP (immunoglobulin heavy chain-binding protein), also called glucose-regulated protein 78 (GRP78), or in the new nomenclature HSPA5, is an endoplasmic reticulum chaperone whose function is generally thought to be restricted to controlling the structural maturation of nascent glycoproteins and the unfolded protein response (UPR) signaling cascade (Lee 2001; Ma and Hendershot 2004). However, BiP is

also expressed on the cell surface, where it functions as a receptor for a wide variety of ligands. These include activated α2-macroglobulin, the major histocompatibility complex I (MHC-1), plasminogen kringle 5 and microplasminogen, and cell-surface voltage-dependent anion channel (VDAC) (Misra et al. 1994, 2002; Gonzalez-Gronow et al. 2007). BiP is a receptor not only for host cellular molecules but also various pathogens (Henderson and Martin 2011).

Borna disease virus (BDV) belongs to the *Bornaviridae* family of nonsegmented, negative-stranded RNA viruses and is characterized by highly neurotropic and noncytopathic infection (Ludwig and Bode 2000; Tomonaga et al. 2002). BDV cell entry is mediated by endocytosis, following the attachment of the viral envelope glycoprotein (G) to the cellular receptor (Bajramovic et al. 2003; Gonzalez-Dunia et al. 1998). BDV G is translated as a precursor protein (GP) and is cleaved post-translationally by the cellular protease, furin, to generate two functional subunits of the N (GP1) and C (GP2) termini (Richt et al. 1998). Recent studies revealed that GP1 is involved in virus attachment to cells and that GP2 mediates a pH-dependent fusion event between the viral and cellular membranes (Bajramovic et al. 2003; Gonzalez-Dunia et al. 1998; Perez et al. 2001). We determined that BiP interacts with GP1 at the cell surface (Honda et al. 2009). We showed that treatment with anti-BiP antibody affects BDV infection, as well as GP1 binding to the cell surface (Honda et al. 2009). Furthermore, cell-surface BiP is distributed at synaptic sites in the hippocampal primary culture, through which BDV is believed to spread to new neurons (Honda et al. 2009). Previously, it was proposed that the kainite 1 (KA-1) receptor might act as the receptor for BDV in the CNS (Gosztonyi and Ludwig 2001). Because some glutamate receptors are known to bind to BiP (Rubio and Wenthold 1999), KA-1 receptors might interact with BiP and serve as a receptor complex for BDV.

Dengue virus (DENV) is an enveloped, positive-stranded RNA virus that belongs to the *Flavivirus* genus of the *Flaviviridae* family. DENV is an arthropod-borne virus transmitted by *Aedes* mosquitoes. DENV causes fever and hemorrhagic disorders in humans and non-human primates. The DENV genome encodes three structural proteins (envelope glycoprotein (E), membrane, and capsid). The E protein is found in the viral envelope and binds to receptors on the host cell membrane. The E protein contains three functional domains, termed domains I, II, and III. Domain I, the hinge region, links the other two domains. The high mobility of this region is responsible for changes in the E protein structure in response to variation in the pH. Domain II has a hydrophobic-rich peptide sequence with membrane fusion activity and also contributes to E protein dimerization (Modis et al. 2003, 2005; Zhang et al. 2003). Domain III is immunoglobulin-like and thought to be involved in binding to receptor molecules present on the host cell membrane. During viral infection, the adsorption of viral particles is initiated by binding of E protein to the receptor. Subsequently, the adsorbed viruses are taken into the cell by endocytosis. When the pH decreases inside endosomes formed by fusion with lysosomes, the viral membrane fuses with the endosomal membrane, through the action of the E protein fusion peptide. Eventually, the capsid enters the cell and the viral genome is released into the cytoplasm. BiP is the first non-Fc receptor for DENV identified on

liver cells (Jindadamrongwech et al. 2004). Treatment of liver cells with anti-BiP antibody affects DENV infection, as well as virus binding to the cell surface (Jindadamrongwech et al. 2004). Because antibody treatment inhibits virus infection partially, BiP probably functions as part of a receptor complex and additional protein elements may play a role in facilitating entry of the virus into the liver cells (Jindadamrongwech et al. 2004). Furthermore, it is likely that a variant form of BiP predominantly binds DENV (Jindadamrongwech et al. 2004), although whether this is a translational or posttranslational modification remains to be established.

Coxsackievirus A9 (CAV-A9) is a non-enveloped, positive-stranded RNA virus that belongs to the *Picornaviridae* family. CAV-A9 causes flaccid paralysis and chronic dilated cardiomyopathy. It is known that integrin αvβ3 is a receptor for CAV-A9 (Roivainen et al. 1994; Triantafilou et al. 1999). However, its presence alone is insufficient for CAV-A9 infection and a 70 kDa MHC-1-associated protein, BiP, is also required as a coreceptor for CAV-A9 (Triantafilou et al. 2002). Triantafilou (2002) proposed the following model for CAV-A9 entry: CAV-A9 utilizes integrin αvβ3 and BiP as receptor molecules for cell surface attachment. Once the virus has attached to the cell surface, it utilizes MHC-1, which is associated with BiP, as means of internalization. It has been reported that cross-linking of MHC-1 molecules using antibodies induces internalization (Huet et al. 1980; Machy et al. 1987). Similarly, by attaching to BiP, CAV-A9 cross-links BiP and MHC-1, inducing efficient internalization of the virus into the cytoplasm.

19.3.2 Heat Shock Protein 70 (hsp70) and Heat Shock Cognate Protein 70 (hsc70)

Hsp70 is critical for mounting cytoprotection against severe cellular stress, such as elevated temperature, and plays a role in disaggregation in protein quality control under such conditions of stress (Liberek et al. 2008). It also has been proposed that one of the functions of Hsp70 is the prevention of protein aggregation (Pelham 1986). Hsp70 possesses two functional domains, an ATPase domain (nucleotide-binding domain, NBD) and a substrate binding domain (SBD), capable of interaction with an extended polypeptide chain and equipped with a helical ‘lid’. The movement of the lid is controlled by a nucleotide bound to the NBD, resulting in either locking of the substrate chain within the SBD or opening of the lid, releasing the substrate and allowing the subsequent binding event (Mayer and Bukau 2005; Vogel et al. 2006). See Chaps. 1 and 2 for more details of this mechanism of protein folding. Hsc70 is a constitutively expressed molecular chaperone that belongs to the hsp70 family. Hsc70 can form a stable complex with newly synthesized hsp70 following heat shock (Brown et al. 1993). Hsc70 shares some structural and functional features with hsp70 (Ali et al. 2003), but also there are some differences between Hsp70 and Hsc70. For example, Hsc70 can decrease the functional surface expression of epithelial sodium channels, whereas Hsp70 has the opposite effect (Goldfarb et al. 2006). Both Hsp70 and Hsc70 can interact with lipid bilayers directly, but Hsc70

has a more dramatic effect in promoting membrane protein folding and polypeptide translocation (Ahn et al. 2005). Hsp70 and Hsc70 are required for cancer cell growth, but Hsc70 is also required for normal cell growth (Rohde et al. 2005). Both Hsp70 and Hsc70 are expressed at the cell surface and have been reported to act as receptors for certain viruses (see below).

In addition to BiP, as described above, DENV utilizes other host molecules as receptors. The 37-kDa/67-kDa high-affinity laminin receptor has been identified as a receptor for DENV on liver cells (Thepparit and Smith 2004). In monocyte-derived dendritic cells, DENV utilizes dendritic cell-specific ICAM 3-grabbing nonintegrin (or CD209) as a receptor (Navarro-Sanchez et al. 2003; Tassaneetrithep et al. 2003). Monocytes and macrophages are thought to be the major target cells for DENV infection and are responsible for dissemination of the virus after its initial entry. Hsp70 and Hsp90 have been identified as receptors at the surface of monocytes/macrophages (Reyes-Del Valle et al. 2005). Infection inhibition assays using monocytes/macrophages showed that Hsp70 and Hsp90 are part of a receptor complex required for DENV entry (Reyes-Del Valle et al. 2005). Hsp70 and hsp90 also have been reported to be cell surface receptors for lipopolysaccharide (LPS) on human monocytes/macrophages (Triantafilou et al. 2001). See Chap. 18 for a detailed description of this phenomenon. DENV infection in human monocytes is inhibited by bacterial LPS (Byrd et al. 1999). When monocytes are incubated with LPS prior to DENV infection, LPS may occupy Hsp70 and Hsp90 at the cell surface, preventing these proteins from interacting with DENV. Modis et al. (2004) proposed a model of conformational changes of the E protein required to promote membrane fusion. Hsp70 and Hsp90 might participate as molecular chaperones in this proposed conformational change of the E protein. Both Hsp70 and Hsp90 associate with the lipid raft (LR) in response to DENV infection (Reyes-Del Valle et al. 2005), suggesting that the LR is important for DENV entry.

Japanese encephalitis virus (JEV) is an enveloped, positive-stranded RNA virus that belongs to the *Flavivirus* genus of the *Flaviviridae* family. JEV is transmitted to humans by *Culex* mosquitoes. JEV genomic RNA is translated into a single large polyprotein at the endoplasmic reticulum (ER) membrane, then cleaved by host- and virus-encoded proteases into three structural proteins (capsid, precursor membrane, and envelope (E) proteins) and seven nonstructural proteins. JEV entry is inhibited by treatment with anti-Hsp70 and anti-Hsp90 antibodies (Thongtan et al. 2012). Pre-treatment with LPS also inhibits JEV entry, further supporting the idea that Hsp70 and Hsp90 constitute a cell surface receptor for JEV on microglial cells and play a role in the internalization of JEV (Thongtan et al. 2012). Hsp70 has been shown to interact with JEV through the E protein (Das et al. 2009). The RGD motif in the E protein and Leucine^{539} in Hsp70 form a stable interaction (Das et al. 2009). Furthermore, Hsp70 migrates into the LR after JEV infection and is co-fractionated with the E protein. The LR facilitates JEV entry, possibly by providing a convenient platform to concentrate JEV and its receptors, such as Hsp70, on the host cell membrane (Zhu et al. 2012).

Rotavirus is a non-enveloped, double-stranded RNA virus that belongs to the *Reoviridae* family. Rotavirus has a triple-layered protein capsid, and two proteins

on the surface; VP4 and VP7, are responsible for the initial interactions of the virion with the host cell. VP4, the viral attachment polypeptide, is cleaved by trypsin into subunits VP5 and VP8, and this cleavage is associated with the penetration of the virion into the cell. Rotavirus interacts with integrin αvβ3 in a post-attachment step (Guerrero et al. 2000), and also utilizes Hsc70 as a receptor at a post-attachment step (Guerrero et al. 2002). The interaction between rotavirus and Hsc70 was blocked by anti-VP4 and anti-VP7 antibodies (Guerrero et al. 2002). The rotavirus outer layer proteins, VP4 and VP7, also undergo conformational rearrangement during one or more of the multiple contacts that have been proposed to take place between the virus particle and molecules on the cell surface, or during the cell entry and/or uncoating of the virus. It is tempting to speculate that a protein with chaperone activity, such as Hsc70, might play a pivotal role in these processes (Guerrero et al. 2000; Hewish et al. 2000). Indeed, the Hsc70-treated virus reacted slightly differently with monoclonal antibodies and was more susceptible to heat and a basic pH than the untreated virus, suggesting that Hsc70 induces a subtle conformational change in the virus structure (Pérez-Vargas et al. 2006). Thus, Hsc70 is involved in the entry of rotavirus, acting in combination with other proteins, such as integrins.

19.3.3 Cyclophilins (Cyps)

In humans, there are three distinct families of peptidyl-prolyl isomerases (PPIases), also referred to as immunophilins: Cyps, FK506-binding proteins, and the parvulin family (Galat 2004) – see also Chap. 2. The three PPIase families are unrelated in sequence and three-dimensional structure. However, all catalyze the *cis*/*trans* isomerization of the peptide bond on the N-terminal side of proline residues in proteins (Fischer and Aumüller 2003). Typically, this PPIase activity serves as a chaperone to drive correct protein folding, as well as the conformational changes necessary for protein function (Schmid 1993). CypA is an abundant, cytosolic protein that binds to cyclosporine A (CsA) (Handschumacher et al. 1984). Homologues of CypA and other Cyp family members are highly conserved in all domains of life (Galat 2004; Wang and Heitman 2005). Cyps have been found to play a role in a wide range of diseases and cellular dysfunctions, such as cancer, angiogenesis, atherosclerosis, ER stress, and neurodegeneration (Lee and Kim 2010). Other chapters in this book highlight the role of PPIases in microbial infections (see Chaps. 5, 6 and 16).

Measles virus (MV) is a negative-stranded RNA virus that belongs to the *Morbillivirus* genus of the *Paramyxoviridae* family. MV infects more than ten million people worldwide each year, resulting in several hundred thousand deaths (Moss and Griffin 2006). MV is composed of six structural proteins: nucleoprotein (N), phosphoprotein, matrix protein, fusion protein (F), haemagglutinin protein (H), and large protein. Among these structural proteins, the H and F proteins are essential for viral entry. The H protein is responsible for cell attachment and its C-terminal globular head is folded into a six β-sheet blade propeller surmounting a stalk, transmembrane region and cytosolic tail (Hashiguchi et al. 2011). The F protein is synthesized as an

inactive precursor, F_0, which is cleaved to generate the disulfide-linked F_2 and F_1 subunits. Upon virus infection, following contact between viral particles and the MV receptors, the fusion peptide on the F protein is inserted into the target membrane, initiating virus-cell membrane fusion (Buchholz et al. 1996). The principal cellular receptor for MV is signaling lymphocyte activation molecule, SLAM (or CD150), which is expressed exclusively on immune cells (Tatsuo et al. 2000). However, MV infects various tissues in the body, including lung, kidney, gastrointestinal tract, vascular endothelium, and brain. This means that other receptors might exist, in addition to SLAM, CD46 and nectin-4 have been reported to be such receptor molecules (Sato et al. 2012). In addition to these H-binding receptors, CD147/EMMPRIN (extracellular matrix metalloproteinase inducer) has been reported to be a receptor for MV (Watanabe et al. 2010). CD147 mediates the H-independent entry of MV via interaction with incorporated CypB in the virus particles. Because CD147 is expressed at the surface of a variety of cells, including epithelial and neuronal cells, this molecule could serve as a receptor for MV in SLAM-negative cells.

19.4 What Are the Advantages to Viruses of Using Molecular Chaperones as Receptors?

As described above, various types of viruses use molecular chaperones at the cell surface as receptors. Virus entry is a complex process, involving a number of discrete steps such as receptor binding, internalization into cells, capsid destabilization and genome uncoating, and release of viral nucleic acids at the site of replication. Correct functioning and/or conformational changes of viral and host proteins are required to complete these steps. It is tempting to speculate that a receptor with chaperone activity could play a pivotal role. In addition to this, it is becoming clear that many molecular chaperones at the cell surface are moonlighting proteins and participate in non-folding activities (Henderson et al. 2006). In this section, we discuss the advantages to viruses, other than the chaperone activity of the receptor itself, of using molecular chaperones during virus entry.

19.4.1 Coupling to the Internalization Machinery

Although some viruses release their genomes into the cell by direct fusion with the plasma membrane, most, regardless whether enveloped or not, enter cells via endocytosis. One of the reasons for this is that the endocytic machinery confers the additional advantage of specific localization within the cell, enabling successful infection (Pelkmans and Helenius 2003). Multiple endocytic pathways operate at the cell surface and differ in their mechanisms of formation, molecular machinery, and cargo destination. Endosomes constitute a rapid transit system across the plasma membrane and through the cytoplasm. After internalization, virus particles are

sequestered in endocytic organelles until the appropriate conditions are met for release of the viral genomes. Endosomes also protect the virus against detection by host innate immunity. For viruses that replicate in the nucleus, the endosome can deliver its viral cargo to the vicinity of the nuclear pore, ready for translocation into the nucleoplasm (Whittaker and Helenius 1998). Thus, molecular chaperones at the cell surface may efficiently link cell attachment to virus internalization. The LR, where Hsp70 associates, plays important roles in the internalization of all viruses that utilize Hsp70 as a receptor (Mercer et al. 2010; Reyes-Del Valle et al. 2005; Zhu et al. 2012). Following initial attachment to the cell surface, BDV is also recruited to the LR prior to the internalization of the particles (Clemente et al. 2009). A characteristic of the caveolar/LR-dependent pathway, one of the virus internalization pathways, is that formation of primary endocytic vesicles depends on cholesterol and LR (Mercer et al. 2010). This suggests that viruses localized at the LR during their entry may use the LR as platforms to interact with additional host factors required for efficient internalization. The proteins that interact with BiP, such as MHC-1 and VDAC, associate with the LR in the plasma membrane (Kim et al. 2006; Misra and Pizzo 2008; Triantafilou and Triantafilou 2003), suggesting that BDV might use BiP to cross-link BiP-binding proteins at the LR for efficient internalization, as in the case of CAV-A9 described above.

19.4.2 Selection of Favourable Target Cells

Virus infection induces various kinds of cellular stresses that affect host cell viability, including oxidative stress, ER stress, and stress imposed by high temperature. For example, during JEV infection, the lumen of the ER rapidly accumulates substantial amounts of viral proteins for production of virus progeny (Su et al. 2002). Oxidative injury is a component of the demyelinating disease caused by MV (Valyi-Nagy and Dermody 2005). Conversely, cells with stress-resistance are favorable for virus infection and are able to produce viral progeny efficiently. To cope with stresses, the quality control systems of chaperone proteins are required to maintain the correct conformation of various proteins under stressed conditions. Indeed, *in vivo* models using mice expressing Hsp72 or hyper-thermal preconditioned mice reveal that Hsp72 levels can serve as a host determinant of viral neurovirulence in mice, reflecting the direct influence of Hsp72 on viral gene expression (Carsillo et al. 2004, 2006). The surface expression of this molecular chaperone ensures substantial amounts of chaperone protein expression in the target cells. Viruses that utilize molecular chaperones as receptors may select favorable cells to infect by targeting the surface expression of chaperones. DENV infectivity in monocytic cells is increased after heat shock treatment; this effect correlates mainly with an increase in viral entry attributable to the presence of Hsp70 and Hsp90 at the cell surface (Chavez-Salinas et al. 2008). Heat shock also modulates positively DENV replication in this condition (Chavez-Salinas et al. 2008), supporting the idea that the cells with surface expression of chaperones are favorable target cells for virus infection.

19.4.3 Selection of Competent Virions

The importance of molecular chaperones in the viral life cycle implies a global requirement for complete cellular functionality for optimal viral fitness. Thus, molecular chaperones incorporated into virions ensure that the virions are derived from the infected cells with substantial amount of chaperone protein expression, which means that the components of the virions are structurally matured. Both CypA and CypB interact with the MV N protein (Watanabe et al. 2010). CypA has been reported to participate in the uncoating of viral nucleic acids (Strebel et al. 2009; Ylinen et al. 2009). CypB plays a crucial role in the replication of JEV through an interaction with one of its nonstructural proteins, NS4A (Kambara et al. 2011). Similarly, CypA and CypB may be involved in the optimal functionality of the MV N protein. Thus, using CD147 on host cells as a receptor may be a strategy to select Cyp-positive MV virions, whose components are assumed to be competent for viral replication in the target cells.

19.5 Conclusions

It is clear that molecular chaperones are not only intracellular proteins whose only function is to help protein folding, but also are expressed on the surfaces of cells and have functions other than protein folding. One such function is as viral receptors for cell entry. Various types of viruses have been found to use molecular chaperones at the cell surface as receptors. These molecular chaperones might help viruses to shift from the cell attachment step to the internalization step, enabling efficient virus entry. Alternatively, by using molecular chaperones as receptors, these viruses might effectively target appropriate cells to replicate and/or ensure the quality of virus particles to infect. It is likely that molecular chaperones at the cell surface form part of a receptor complex essential for virus entry. Further studies are required for complete understanding of the virus entry processes that use molecular chaperones as receptors.

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Chapter 20
BiP (Grp78): A Target for *Escherichia coli* Subtilase Cytotoxin

Adrienne W. Paton, Hui Wang, and James C. Paton

Abstract Subtilase cytotoxin (SubAB) is the prototype of a recently discovered AB5 toxin family produced by certain strains of pathogenic *Escherichia coli*. Its A subunit is a serine protease belonging to the Peptidase_S8 (subtilase) family, while the pentameric B subunit binds to cell surface receptor glycans terminating in the sialic acid α2-3-linked *N*-glycolylneuraminic acid. Receptor binding triggers internalization of the holotoxin and retrograde trafficking to the endoplasmic reticulum (ER), where the A subunit cleaves its only known substrate, the essential Hsp70 family chaperone BiP (also known as GRP78/HSPA5). BiP is a highly conserved protein with diverse functions including the folding of nascent proteins in the ER and maintenance of cellular homeostasis as the master regulator of the ER stress response. BiP is essential for survival of eukaryotes from simple yeasts to higher organisms such as mammals. Consequently, SubAB-mediated BiP cleavage has devastating consequences for the cell, triggering a severe and unresolved ER stress response, ultimately leading to apoptosis. Apart from a likely role in pathogenesis of disease caused by bacteria that produce it, SubAB is also proving to be a useful tool for probing the role of BiP and ER stress in a variety of cellular functions and may have potential as a cancer therapeutic.

20.1 Role and Function of BiP

The Hsp70 family chaperone BiP (also known as GRP78 or in the new nomenclature HSPA5 – see Introduction to this volume) is an ancient molecule that is highly conserved among eukaryotes from simple yeasts to higher organisms such as mammals.

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It is increasingly being recognized for its essential role in cellular homeostasis, and it has a range of functions making it an archetypal moonlighting protein (Gething 1999; Hendershot 2004; Quinones et al. 2008). It is principally located in the ER lumen and is essential for the correct folding of nascent secretory proteins. BiP is approximately 72 kDa in size and comprises two functional domains, an N-terminal ATPase and a C-terminal protein-binding domain, connected by a short loop. The C-terminal domain recognizes and binds to hydrophobic regions of nascent unfolded secretory proteins, with subsequent release coupled to N-terminal domain-catalysed ATP-hydrolysis. A series of ATPase-driven C-terminal-mediated binding and release events enables the newly synthesized protein to adopt its final conformation (Gething 1999). BiP is also responsible for targeting of terminally mis-folded proteins to the Sec61 apparatus for retro-translocation into the cytosol and degradation by the proteasome, as well as for maintaining the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation (Hamman et al. 1998).

BiP also plays a crucial role as the ER stress-signalling master regulator (Kim and Arvan 1998; Rao et al. 2004 – see also Chap. 2 for a discussion of ER stress). ER stress can be induced by diverse perturbations in ER function, including calcium depletion, glucose or energy depletion, and accumulation of unfolded proteins (Quinones et al. 2008). Its purpose is to implement a series of changes in cellular activity, most notably the unfolded protein response (UPR), such that the ER stress is alleviated, enabling the cell to restore ER homeostasis and recover (Boyce and Yuan 2006). The UPR is a three-pronged response involving firstly, transcriptional up-regulation of ER chaperones including BiP and other proteins, creating an ER milieu in which protein folding capacity is optimized. Proteasome-dependent ER-associated degradation (ERAD) is also activated to remove unfolded proteins from the ER lumen. Furthermore, translation is modulated to slow down the traffic of nascent proteins into the ER compartment that require folding. However, if these responses fail to restore ER homeostasis, apoptosis may result (Boyce and Yuan 2006). In mammals, ER stress responses can be triggered by activation of three distinct ER membrane-spanning signalling molecules, namely PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). The luminal domains of all three of these sentinel proteins are known to interact with BiP, and the accumulation of unfolded proteins in the ER lumen is thought to recruit BiP away, allowing these sentinels to initiate signalling. By phosphorylation of its target eIF2α, activated PERK (pPERK) inhibits global protein synthesis, yet allows translation of mRNAs such as ATF4, a transcriptional activator for genes that ultimately assist in re-establishing ER homeostasis (Szegezdi et al. 2006). Release from BiP enables ATF6 to traffic to the Golgi, where it undergoes limited proteolysis, releasing a 50-kDa activated form, which then translocates to the nucleus and binds to the ER stress response element, thus inducing genes encoding ER chaperones such as BiP, GRP94 and protein disulphide isomerase, as well as the transcription factors C/EBP homologous protein (CHOP) and X-box-binding protein 1 (XBP1). Activated IRE1 splices XBP1 mRNA into a form that can be translated into XBP1 protein, which then up-regulates genes encoding ER chaperones and the

HSP40 family member P58^{IPK}. This protein provides a feedback loop by binding and inhibiting PERK, thereby relieving the eIF2α-mediated translational block, as the cell returns to normal function.

Notwithstanding its essential function as a chaperone, BiP also has a number of moonlighting roles outside of the ER (Quinones et al. 2008). It is expressed on the surface of many cell types, particularly cancer cells, as well as on endothelial cells and activated macrophages. It is involved in transduction of signals from a diverse range of ligands, as well as in virus entry (see Chap. 19 for detailed discussion of the role of BiP in virus uptake by cells) and antigen presentation. Surface expression may be a consequence of prolonged high level BiP expression exceeding the retention capacity of the KDEL retrieval system, which under normal circumstances confines BiP in the ER lumen (Ni et al. 2011). Surface BiP does not span the plasma membrane, and its signalling functions may be mediated through interaction with a complex comprising the transmembrane co-chaperone MTJ-1 and a cytoplasmic non-Ptx sensitive G protein, Gαq11, triggering an increase in intracellular calcium. IRE1, PERK and ATF6 may also be present in the plasma membrane in addition to the ER membrane, and interact with BiP on the extracellular surface (Quinones et al. 2008). An alternatively spliced BiP isoform that lacks the signal peptide has also recently been detected in the cytoplasm of leukaemia cells. This isoform is fully functional and is able to modulate ER stress responses in this compartment, potentially promoting cancer cell survival (Ni et al. 2011). BiP has also been detected in the nucleus, mitochondria and extracellularly when ectopically overexpressed or induced by ER stress (Ni et al. 2011).

20.2 Identification of BiP as the Target of Subtilase Cytotoxin (SubAB)

BiP is the first eukaryotic molecular chaperone to be identified as a target for a bacterial cytotoxin. This toxin, known as subtilase cytotoxin (SubAB), is the prototype of a novel family of bacterial AB5 toxins and is produced by certain strains of pathogenic *Escherichia coli* (Paton et al. 2004). AB5 toxins are characterized by enzymatic A subunits capable of disrupting critical host cell functions, non-covalently linked to pentameric B subunits that bind to specific glycan receptors on the eukaryotic cell surface, triggering toxin uptake (Beddoe et al. 2010; Fan et al. 2000). The best characterized AB5 toxin families are exemplified by Shiga toxin (Stx), cholera toxin (Ctx) and pertussis toxin (Ptx). The A subunit of Stx is an RNA-*N*-glycosidase which cleaves 28S rRNA, thereby inhibiting eukaryotic protein synthesis. The A subunits of Ctx and Ptx are ADP-ribosyltransferases which modify distinct host G proteins, resulting in alteration of intracellular cAMP levels and dysregulation of ion transport mechanisms (Beddoe et al. 2010). SubAB was discovered in a strain of Shigatoxigenic *E. coli* (STEC) responsible for an outbreak of haemolytic uraemic syndrome (HUS) in South Australia in 1998 (Paton et al.

1999, 2004). HUS is a life-threatening systemic sequelae of gastrointestinal infection with STEC, and is characterized by a triad of microangiopathic haemolytic anaemia, thrombocytopoenia and renal failure. Pathogenesis of HUS is largely attributable to Stx-mediated damage to microvascular endothelium (Paton and Paton 1998). Interestingly, anecdotal reports from the clinicians caring for the patients during the 1998 South Australian outbreak suggested that the clinical picture was somewhat atypical, with more marked neurological involvement than in previous cases of HUS seen at the same hospital. This led us to hypothesize that the infecting strain might produce an additional toxin capable of either augmenting the effects of Stx or causing additional pathology in its own right.

Analysis of a cosmid gene bank derived from the infecting STEC strain led to the cloning and characterization of the operon encoding SubAB (Paton et al. 2004). SubAB was then purified from recombinant *E. coli* and shown to be highly toxic for Vero cells *in vitro*, with a specific activity at least as potent, if not more so, than that reported for Stx (Lindgren et al. 1994; Noda et al. 1987; Paton et al. 2004; Yutsudo et al. 1987). Analysis of the deduced amino acid sequence of SubA revealed a 35-kDa secreted protein with a modest degree of similarity to members of the Peptidase_S8 (subtilase) family of serine proteases (pfam00082.8). This family is characterized by three conserved sequence domains, designated the catalytic triad (Siezen and Leunissen 1997). The SubA domain sequences match the consensus sequences for the so-called Asp, His and Ser subtilase catalytic domains at 11/12, 10/11 and 10/11 positions, respectively, including the known critical active site residues (in SubA these correspond to Asp_{52}, His_{89}, and Ser_{272}) (Paton et al. 2004). Significantly, mutagenesis of any one of these residues abolishes the cytotoxic activity of the respective SubAB holotoxin, confirming that proteolytic acivity is the basis for cytotoxicity (Paton et al. 2004; Talbot et al. 2005). The presence of the SubB pentamer (comprising 5 identical 13-kDa monomers), which mediates binding to target cells and internalization of the holotoxin, is also essential for cytotoxicity. The genes encoding SubA and SubB are co-transcribed and the AB5 holotoxin is assembled in the bacterial periplasm prior to release (Paton et al. 2004).

BiP was identified as the target of the SubA protease by proteomic comparison of extracts from Vero cell monolayers treated with either purified SubAB or the non-proteolytic mutant $SubA_{A272}B$ (Paton et al. 2006). 2-D gel analysis identified a new 28-kDa protein spot unique to the active toxin-treated cells. MALDI-MS analysis identified it as the C-terminal domain of BiP. Moreover, Western blot analysis using a polyclonal antibody raised against a 20 a.a. peptide from the C-terminus of BiP labelled a 72-kDa species in lysates of untreated Vero cells and those treated with the inactive toxin derivative. In contrast, the lysate of active SubAB-treated cells had a markedly diminished 72-kDa band, as well as a new antibody-reactive species at approximately 28 kDa, consistent with the proteomic data. Finally, to confirm that SubAB is directly responsible for cleavage of BiP, purified bovine BiP was tested *in vitro* as a substrate for purified SubAB or $SubA_{A272}B$. Untreated bovine BiP migrated as a single 72-kDa species, and was not affected by exposure to $SubA_{A272}B$. However, in the presence of active SubAB, BiP was cleaved into 44-kDa and 28-kDa fragments. This provided confirmation that SubAB directly cleaves BiP at a single position. Cleavage also occurred when BiP was treated with the purified catalytic

subunit SubA. N-terminal sequence analysis of the 28-kDa fragment indicated that cleavage occurs between two leucine residues at positions 416 and 417 in the 654 a.a. BiP sequence; these are located in the exposed hinge that connects the ATPase and protein-binding domains (Paton et al. 2006; Rao et al. 2002). SubAB was not able to cleave BiP *in vitro* when Leu_{416} was mutated to Asp. Moreover, transfected Vero cells co-expressing this protease-resistant BiP derivative were refractory to SubAB-mediated cytotoxicity, directly confirming the central role of BiP cleavage in the cytotoxic mechanism (Paton et al. 2006).

Most subtilase family enzymes are non-specific proteases that exhibit a preference to cleave after hydrophobic residues. However, proteomic analysis did not detect any other substrates in SubAB-treated Vero cells. Moreover, SubAB did not cleave purified human Hsp70 and Hsc70 proteins *in vitro*. These two members of the Hsp70 family are the closest homologues of BiP, and are identical at 7 of 11 amino acid positions flanking the cleavage site, including the di-leucine motif. The molecular basis for this exquisite specificity was revealed by solving the crystal structure of SubA to 1.8 Å resolution. In comparison with subtilisin Carlsberg, a subtilase family member with broad specificity, the catalytic His-Asp-Ser triad of SubA lies at the bottom of an uncharacteristically deep cleft, which would impose major steric constraints on substrate docking (Paton et al. 2006).

20.3 Interactions of SubAB with Host Cells

20.3.1 Receptor Specificity

SubAB intoxication of host cells commences with recognition of specific glycans displayed on cell surface receptors by the SubB pentamer. Glycan array analysis identified a high degree of specificity for glycans terminating with α2-3-linked *N*-glycolylneuraminic acid (Neu5Gc) with little discrimination for the penultimate moiety (Byres et al. 2008). Roughly 20-fold weaker binding was seen with otherwise identical glycans that terminate in α2-3-linked *N*-acetylneuraminic acid (Neu5Ac), which differs by one hydroxyl group from Neu5Gc. Binding was reduced over 30-fold if the Neu5Gc linkage was changed from α2-3 to α2-6, and 100-fold if the terminal sialic acid was removed (Byres et al. 2008). This high specificity for Neu5Gc-terminating glycans is, to the best of our knowledge, unique amongst bacterial toxins.

Crystallographic analysis of SubB-Neu5Gc complexes showed that Neu5Gc binds to a shallow pocket halfway down the sides of the SubB pentamer, whereas identical experiments using Neu5Ac failed to show any binding (Byres et al. 2008). Neu5Gc makes key interactions with the side chains of Asp_8, Ser_{12}, Glu_{36} and Tyr_{78}. The hydroxyl group that distinguishes Neu5Gc from Neu5Ac makes additional crucial interactions with $Tyr_{78}{}^{OH}$ and also hydrogen bonds with the main chain of Met_{10}. These key interactions could not occur with Neu5Ac, thus explaining the marked preference for Neu5Gc. Mutagenesis of either Ser_{12}, Glu_{36} or Tyr_{78} in SubB significantly reduced cell binding and specific cytotoxicity of the respective

holotoxin, confirming the biological relevance of the structural analysis. Of these, the Ser_{12} mutation had the greatest impact, reducing cytotoxicity by 99.98 %. Importantly, mutagenesis of Tyr_{78}, which interacts only with the OH group unique to Neu5Gc, reduced cytotoxicity by 96.9 % (Byres et al. 2008).

20.3.2 Uptake and Intracellular Trafficking

SubB-mediated binding to surface receptors triggers internalisation and intracellular trafficking, such that the SubA protease gains access to its substrate. Fluorescence co-localization with sub-cellular markers established that after endocytosis, SubAB-receptor complexes enter a retrograde transport pathway and traffic via the *trans-Golgi* network (TGN) and Golgi to the ER (Chong et al. 2008). SubAB internalisation and trafficking is exclusively clathrin-dependent, whereas Stx or Ctx can also engage the lipid raft transport pathway (Chong et al. 2008). The route through the Golgi is also distinct, with SubAB exploiting a novel p115/golgin-84-independent, COG/Rab6/COPI-dependent mechanism, and unlike Stx, retrograde transport is not dependent on the endosomal sorting nexins SNX1 and SNX2 (Smith et al. 2009). For SubAB, the ER lumen is the end of the journey and it now has unfettered access to its substrate BiP. However, the substrates of other AB5 toxins are located in the cytoplasm, so their catalytic subunits must also be retro-translocated across the ER membrane. This occurs via subversion of the Sec61 translocon (Lencer and Tsai 2003; Yu and Haslam 2005), and at least for StxA, retro-translocation is believed to occur following interaction with BiP and another chaperone HEDJ/ERdj3 (Yu and Haslam 2005). SubAB is also known to inhibit ERAD, presumably through reduced Sec61-mediated trafficking of substrates (Lass et al. 2008). This raises the intriguing possibility that SubAB-mediated BiP cleavage might interfere with entry of StxA into the cytosol, and modulate the *in vivo* consequences of Stx intoxication in patients infected with a bacterial strain producing both toxins.

Despite its complexity, the receptor binding, internalization and trafficking processes for SubAB occur rapidly *in vitro*, with BiP cleavage evident by Western blotting as early as 15 min after addition of purified toxin to Vero cell cultures (Paton et al. 2006). Thus, intoxication is rapidly irreversible and removal of exogenous SubAB within a few minutes after treatment does not prevent cell death (Paton et al. 2004). Nevertheless, it takes 1–2 days for signs of cytotoxicity to be evident by microscopy, and the cytopathic effect is maximal after 3–4 days (Paton et al. 2004).

20.3.3 Consequences of BiP Cleavage for the Cell

From the above, it is evident that cellular injury inflicted soon after exposure to SubAB triggers physiological changes from which the cell cannot recover. Such changes include activation of all three ER stress signalling pathways (Wolfson et al. 2008). In

SubAB-treated Vero cells, pPERK-dependent phosphorylation of eIF2α occurred within 30 min, and correlated with inhibition of translation. Activation of IRE1 was demonstrated by splicing of XBP1 mRNA, while ATF6 activation was demonstrated by depletion of the 90-kDa un-cleaved form, and appearance of the 50-kDa cleaved form. At least for PERK and IRE1, the response was very rapid, with little time for accumulation of unfolded proteins in the ER lumen; this suggests that cleavage by the toxin is sufficient to cause BiP to dissociate from the signalling molecules. However, SubAB-mediated ATF6 activation appeared to be somewhat slower. BiP has been reported to form a stable interaction with ATF6, with dissociation requiring direct triggering mediated by an ER stress-responsive sequence in the luminal domain of ATF6 (Shen et al. 2005). Thus, accumulation of unfolded proteins in SubAB-treated cells may be required before the ATF6 pathway is activated. Downstream consequences of BiP cleavage that were detected during the following 24 h period included up-regulation of GRP94, ATF4, EDEM, CHOP, and GADD34. BiP itself was also up-regulated at the mRNA level, but at the protein level, it continued to be degraded by SubAB in the ER lumen, presumably preventing restoration of ER homeostasis. Thus, SubAB treatment induced a severe and sustained ER stress response in Vero cells, and at 30 h, there was evidence of apoptosis, as judged by DNA fragmentation (Wolfson et al. 2008). Matsuura et al. (2009) also demonstrated SubAB-induced apoptosis in Vero cells by annexin V labeling, caspase activation and cytochrome c release from mitochondria.

Links between UPR signalling and cell death pathways via Bcl-2 family proteins are well-established (Li et al. 2006). Activated IRE1 directly facilitates Bax/Bak oligomerization at the ER membrane, and SubAB-induced apoptosis in mouse embryo fibroblasts and HeLa cells has been shown to be Bax/Bak-dependent (May et al. 2010; Yahiro et al. 2010). On the other hand, upregulation of CHOP by the ATF6 and PERK pathways did not appear to be essential, at least in HeLa cells (Yahiro et al. 2010). Nevertheless, the PERK pathway appears to play an important role in triggering apoptosis through phospho-eIF2α-mediated translational blockade of anti-apoptotic proteins (Yahiro et al. 2012).

In a separate study, Zhao et al. (2011) demonstrated that SubAB-mediated activation of the PERK and IRE1 pathways in renal epithelial cells caused phosphorylation of JNK, ERK, and p38 MAP kinases. This was associated with activation of AP-1 and induction of AP-1-dependent transcription. Such MAP kinase activation has previously been associated with Stx-induced HUS in humans. Morinaga et al. (2008) also reported the toxin induced cell cycle arrest in G1 phase, possibly through down-regulation of cyclin D1 due to a combination of translational inhibition and proteasomal degradation.

20.4 *In Vivo* Effects of SubAB

There are relatively few published studies on the *in vivo* effects of SubAB, and these have been confined to murine models. Gut colonisation with recombinant *E. coli* carrying the *subAB* operon on a low copy-number plasmid did not cause obvious

diarrhoea, but nevertheless resulted in dramatic weight loss (approximately 15 %) over a 6-day period. In contrast, mice colonised with a clone expressing the non-toxic mutant *subA*$_{A272}$*B* operon continued to thrive. Interestingly, toxin-affected mice appeared to recover and gained weight after about 6 days, and this correlated with sero-conversion against the toxin (Paton et al. 2004). In a separate study, immunisation with purified SubA$_{A272}$B also protected mice from weight loss induced by subsequent colonisation with *E. coli* expressing active SubAB (Talbot et al. 2005).

The amount of SubAB released into the gut by wild type STEC bacteria *in vivo* is unknown. However, intraperitoneal (IP) injection of as little as 200 ng of purified SubAB is fatal, with survival time inversely related to toxin dose (Paton et al. 2004). *In vivo* BiP cleavage and induction of ER stress, as indicated by CHOP induction, was evident in the livers of SubAB-treated mice (Paton et al. 2006). Interestingly, IP injection of SubAB resulted in microangiopathic haemolytic anaemia, thrombocytopoenia and renal impairment, fulfilling the triad of features that defines Stx-induced HUS in humans. There was also extensive microvascular thrombosis and other histological damage including interstitial haemorrhages in the brain, kidneys and liver. Peripheral blood leukocytes were raised at 24 h and there was also significant neutrophil infiltration in the liver, kidneys and spleen, as well as toxin-induced apoptosis at these sites (Wang et al. 2007). A later study reported similar findings, as well as haemorrhages and inflammatory changes in the small intestine after IP administration of SubAB (Furukawa et al. 2011). In our earlier study, we found that SubAB injection also induced dramatic splenic atrophy (Wang et al. 2007). More detailed examination revealed a marked redistribution of leukocytes, with increases in leukocyte sub-populations in the blood and peritoneal cavity. There was also a significant increase in the apoptosis rate of CD4+ T cells, B lymphocytes and macrophages (Wang et al. 2011). These findings indicate that apart from direct cytotoxic effects, which partially overlap those induced by Stx, SubAB interacts with cellular components of both the innate and adaptive arms of the immune system *in vivo*.

Interestingly, surface-localized BiP has been reported to bind to and inhibit the activation/release of tissue factor (TF), the major initiator of extrinsic coagulation (Watson et al. 2003). Significantly, *in vitro* treatment of human macrophages and endothelial cells with SubAB leads to induction of TF mRNA, as well as release and activation of TF (Wang et al. 2010). TF-dependent factor Xa generation activates thrombin, which in turn increases fibrin deposition and platelet aggregation, leading to thrombotic microangiopathy and thrombocytopoenia. Such perturbation of the coagulation cascade would not only account for the profound microvascular thrombosis and interstitial haemorrhages observed in SubAB-injected mice, but may also be critically important in triggering HUS in humans (Wang et al. 2010).

SubAB may also have more subtle effects on disease pathogenesis, perhaps through immune modulation. SubAB has been shown to preferentially inhibit secretion of immunoglobulins by activated murine B lymphocytes, leaving cytokine secretion relatively unscathed. The toxin preferentially cleaved newly synthesized BiP in these cells, and the C-terminal BiP fragment remained tightly bound to nascent immunoglobulin light chains, trapping them in the ER compartment (Hu

et al. 2009). SubAB may also have pro-inflammatory properties. SubAB treatment caused transient phosphorylation of Akt and activation of NF-κB in rat renal tubular epithelial cells, and this was mediated via the ATF6 branch of the UPR (Yamazaki et al. 2009). Activation of NF-κB is believed to play an important role in HUS and renal injury. However, at sub-cytotoxic concentrations, SubAB has actually been shown to inhibit LPS-mediated NF-κB activation in a murine macrophage cell line, and to protect mice from LPS-induced endotoxic lethality and experimental arthritis (Harama et al. 2009). Interestingly, this capacity of SubAB to inhibit LPS-mediated NF-κB activation has recently been shown to enhance survival of STEC bacteria in macrophages through reduced NO generation (Tsutsuki et al. 2012).

Notwithstanding the above *in vivo* consequences of SubAB exposure, the extent to which the toxin contributes to disease in humans is unknown, and is complicated by the high specificity of the toxin for receptor glycans terminating in Neu5Gc. Humans cannot synthesise this sugar, because they lack the CMP-*N*-acetylneuraminic acid hydroxylase (Cmah) that converts CMP-Neu5Ac to CMP-Neu5Gc, due to a mutation that occurred about two million years ago after evolutionary separation of the *Hominin* lineage from the great apes (Varki 2001). Thus, humans may be less susceptible to the toxin than other mammals, which produce an active Cmah enzyme, as uptake would be dependent on lower affinity interactions with Neu5Ac glycans. Nevertheless, humans can assimilate Neu5Gc from dietary sources and incorporate it into glycoconjugates expressed on epithelial and endothelial surfaces (Tangvoranuntakul et al. 2003), thereby conferring full susceptibility to SubAB. Indeed, *in vitro* binding of SubAB to human gut epithelium and microvascular endothelium has been demonstrated (Byres et al. 2008). Ironically, the richest dietary sources of Neu5Gc are red meat and dairy products, and these foods are also the commonest source of STEC contamination. This is a unique paradigm of bacterial pathogenesis, whereby humans may directly contribute to disease through dietary choices, simultaneously exposing themselves to the risk of STEC infection and sensitising their tissues to SubAB.

20.5 SubAB as a Cell Biological Tool and Potential Therapeutic Agent

Investigations of the roles of BiP in diverse cellular processes have been complicated by its essential nature. BiP$^{-/-}$ cell lines and knock-out mice are not available, and alternative approaches such as RNA knock-down or use of neutralizing antibodies are not 100 % efficient. In this context, the capacity of SubAB to rapidly cleave and inactivate BiP *in vitro* and *in vivo*, but without affecting any other cellular protein, provides a unique opportunity for functional investigations. Treatment with SubAB results in rapid degradation of virtually all cellular BiP in a wide variety of cell lines, effectively mimicking a knock-out phenotype. The toxin has already been used to examine the role of BiP and/or ER stress in ERAD in HeLa cells (Lass et al. 2008), modulation of T-cell activation and inflammatory responses of a variety of

cell types (Du et al. 2009; Hayakawa et al. 2008, 2009; Okamura et al. 2009; Takano et al. 2007) and expression and regulation of gap junction function in mesangial cells (Huang et al. 2009). A direct role for BiP in limiting leakage of Ca^{2+} from the ER through the Sec61 channel has also been demonstrated using SubAB digestion (Schäuble et al. 2012). SubAB has also been employed to identify the critical role of BiP in assembly and egress of cytomegalovirus virus from infected cells (Buchkovich et al. 2008, 2009) and in production and processing of dengue virus proteins (Wati et al. 2009). Both viruses upregulate BiP during infection, and SubAB treatment significantly reduces infectious virus release. A recent study also used SubAB to demonstrate that during SV40 virus infection, binding of BiP to the capsid is required for exit of partially disassembled virus particles from the ER on their way to the nucleus, which is the site of virus replication (Goodwin et al. 2011).

A wide range of tumours up-regulate BiP in response to ER stress induced by rapid growth, hypoxia and/or glucose deprivation and this is a crucial anti-apoptotic mechanism. BiP up-regulation is linked to metastatic potential and resistance to chemotherapy (Li and Lee 2006). Thus, SubAB has the potential to strike at an Achilles' heel in tumourigenesis. However, an appropriate targeting strategy would be required to prevent collateral damage to normal tissues if holotoxin were to be deployed. Alternatively, the A subunit alone could be delivered via a more tumour-specific targeting molecule. In one example of this strategy, a fusion of SubA with epidermal growth factor (EGF) was used to specifically target EGF receptor-(EGFR-) positive tumours. The EGF-SubA fusion protein killed EGFR-positive rat glioma and human breast and prostate cancer cells at picomolar concentrations (Backer et al. 2009). Cell lines expressing moderate to high levels of EGFR, which is associated with invasiveness and metastatic potential, were the most susceptible. Moreover, EGF-SubA acted synergistically with drugs such as thapsigargin that induce ER stress, enabling effective deployment at concentrations well below the cytotoxicity threshold for each component. EGF-SubA was also efficacious *in vivo*, significantly inhibiting tumour growth in mouse xenograft models of human breast and prostate cancer (Backer et al. 2009). In melanoma cells, which are notoriously resistant to chemotherapy, SubAB holotoxin induced cell death in its own right, but also acted synergistically to increase the death of melanoma cells in response to fenretinide or bortezomib (Martin et al. 2010). EGFR-positive melanomas should therefore be amenable to targeting with these drugs in combination with EGF-SubA.

As mentioned previously, many tumours not only upregulate BiP in the ER to manage ER stress, but also express it on the cell surface. Here it acts as a co-receptor and mediates signalling events that resist apoptosis and promote tumour cell proliferation and metastasis (Quinones et al. 2008; Ni et al. 2011). Interestingly, Ray et al. (2012) have recently shown that the isolated catalytic subunit SubA cleaves surface BiP in several cancer cell lines, releasing the 28-kDa C-terminal fragment into the culture medium. Since the SubB pentamer is absent, there is no internalization and trafficking of SubA, and hence no digestion of BiP in the ER compartment. This provides a unique opportunity to study the role of surface BiP-mediated signalling in cancer cell survival and metastasis, in isolation from its role in ER

homeostasis. This also raises the possibility of developing cancer therapeutics that target surface BiP, without the risk of collateral damage to normal cells that do not express BiP on their surface. This could also have protective effects against viruses requiring cell surface BiP for cell infection (see Chap. 19).

20.6 Conclusions

Studies of SubAB and its interactions with host cells have provided two new paradigms in bacterial pathogenesis. It is the first example of a bacterial cytotoxin whose mechanism of action involves covalent modification (in this case proteolytic cleavage) of a host chaperone protein (in this case BiP). It is also the first example of a bacterial toxin whose uptake by host cells (at least in the case of humans) is dependent upon dietary assimilation of a glycan (Neu5Gc) that the host cannot synthesise. The cytotoxic potency of SubAB underscores the critical role that BiP plays in host cell survival. BiP is an ancient molecule that is highly conserved among diverse eukaryotes from simple yeasts to higher organisms such as mammals. There is negligible sequence variation in the vicinity of the cleavage site, suggesting that SubAB has the potential for toxicity against a broad spectrum of life forms. It is increasingly apparent that BiP is more than just a protein-folding chaperone, and as summarized in this chapter, it has diverse roles in cellular homeostasis and stress signalling. Moreover, whilst principally located in the ER lumen of normal cells, it is now recognised that it may participate in important signalling functions at other sites, particularly on the cell surface. In view of its exquisite substrate specificity, SubAB is proving to be a valuable cell biological tool for investigating the diverse functions of BiP in the life and death of eukaryotic cells.

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Chapter 21
Cholera Toxin Interactions with Host Cell Stress Proteins

Ken Teter

Abstract Cholera toxin (CT) travels as an intact AB_5 protein toxin from the cell surface to the endoplasmic reticulum (ER) of an intoxicated cell. In the ER, the catalytic A1 subunit dissociates from the rest of the toxin. Holotoxin-associated CTA1 is held in a stable conformation, but the isolated CTA1 subunit is an unstable protein that assumes a disordered state at physiological temperature. Unfolding identifies the dissociated CTA1 subunit as a substrate for ER-to-cytosol export through the host quality control system of ER-associated degradation (ERAD). The translocated pool of CTA1 then interacts with host factors to regain an active conformation for the modification of its cytosolic G protein target. Thus, the intrinsic conformational instability of the CTA1 polypeptide drives host-toxin interactions related to the translocation event. As discussed herein, these interactions involve both standard and atypical functions for a variety of host chaperones.

21.1 Introduction

Many plant and bacterial toxins share a common structural organization that consists of an enzymatic A moiety and a cell-binding B moiety (Sandvig and van Deurs 2002). The A and B subunits can be different domains of the same protein or can be distinct proteins organized in various stoichiometries (e.g., AB, AB_2, AB_5). All AB toxins have intracellular targets, but they are initially present in the extracellular milieu. The toxins must therefore cross a membrane barrier in order to function. This only occurs after the holotoxin undergoes receptor-mediated endocytosis from the surface of a target cell. Some toxins such as diphtheria toxin and the Clostridial

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neurotoxins have an intrinsic pore-forming capacity that is activated by acidic pH. Delivery of these toxins to the acidified endosomes triggers a conformational change in the toxin B chain that produces a membrane-spanning conduit which allows the A chain to access the cytosol. Other AB toxins such as cholera toxin (CT) cannot form pores and must therefore utilize a pre-existing pore in the host endomembrane system for A chain entry into the cytosol. The endoplasmic reticulum (ER) is the only endomembrane organelle that contains such a protein-conducting channel. Thus, several AB toxins travel from the endosomes to the ER in a series of vesicle-mediated trafficking events collectively termed retrograde transport. These toxins enter the ER as intact holotoxins, but conditions in the ER promote the dissociation of the catalytic subunit from the rest of the toxin. The isolated A chain then moves through a translocon pore to enter the cytosol in a process involving the quality control mechanism of ER-associated degradation (ERAD).

AB toxins bind to distinct surface receptors, follow a variety of intracellular trafficking pathways, and use several catalytic mechanisms to attack a range of cytosolic targets. Multiple aspects of the host cell are thus exploited by the family of AB toxins. In fact, AB toxins are often used as tools to study eukaryotic cell biology (Schiavo and van der Goot 2001; Aktories 2005 – see also Chap. 20). For example, the multiple endocytic mechanisms and retrograde transport routes from the cell surface to the ER have been elucidated in large part with the use of bacterial toxins (Doherty and McMahon 2009; Sandvig et al. 2010). Studies on the cell biology of AB toxins have likewise generated important molecular insights into both bacterial pathogenesis and host chaperone function.

This chapter will focus on host-toxin interactions involving CT, an AB-type ER-translocating toxin produced by *Vibrio cholerae* that is primarily responsible for the profuse and potentially life-threatening diarrhoea of cholera (De Haan and Hirst 2004; Sack et al. 2004; Sanchez and Holmgren 2008). Recent reviews have covered the retrograde transport of CT to the ER (Wernick et al. 2010; Cho et al. 2012). Here, we will concentrate on translocation and post-translocation events which contribute to intoxication. Host chaperones are critical for both events, and they interact with CTA1 though both established and novel mechanisms.

21.2 Structure and Function of Cholera Toxin

CT is a prototypical AB_5-type protein toxin that contains an enzymatic A1 subunit, an A2 linker, and a homopentameric, cell-binding B subunit (Fig. 21.1). CTA1 and CTA2 are initially synthesized as two components of a single polypeptide chain. Nicking by *Vibrio cholerae* or host proteases generates an A1/A2 heterodimer that is linked by a single disulfide bond and extensive non-covalent contacts. CTA2 extends into the central pore of the ring-like CTB pentamer and thus maintains extensive non-covalent interactions with the cell-binding subunit as well.

CT binds to GM1 gangliosides on the surface of a target cell. This triggers toxin endocytosis through a mechanism involving cholesterol-rich lipid rafts. The majority

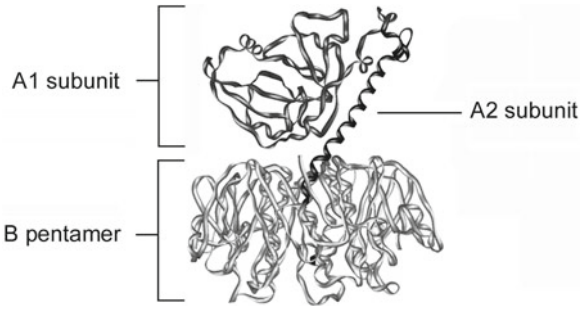

Fig. 21.1 CT structure. The catalytic CTA1 subunit is anchored to the CTA2 linker by a single disulfide bond and numerous non-covalent interactions. CTA2 extends into the central pore of the ring-like CTB homopentamer and thus maintains extensive non-covalent contacts with CTB. Separation of CTA1 from CTA2/CTB_5 is required for the ER-to-cytosol translocation of CTA1 and activation of its latent enzymatic activity. The image was derived from PDB 1S5F

of internalized toxin is delivered to the lysosomes for degradation, but a minor pool of toxin follows the productive route of retrograde transport to the endoplasmic reticulum (ER). Reduction of the CTA1/CTA2 disulfide bond occurs in the ER, which subsequently facilitates the chaperone-assisted displacement of CTA1 from CTA2/CTB_5. CTA1 then crosses the ER membrane, enters the cytosol, and initiates a cytopathic effect.

CTA1 is an ADP-ribosyltransferase that modifies the stimulatory α subunit of the heterotrimeric G protein (Gsα) at the cytoplasmic face of lipid rafts which float in the plasma membrane. The ADP-ribosylated form of Gsα cannot hydrolyze its bound GTP nucleotide and is therefore locked in an active state. This leads to the continual stimulation of adenylate cyclase and a corresponding elevation of intracellular cAMP. A chloride channel in the apical plasma membrane of intoxicated intestinal epithelial cells opens in response to the resulting cAMP-dependent signaling cascade. The osmotic movement of water which follows chloride efflux into the intestinal lumen generates a profuse, potentially life-threatening, watery diarrhoea.

21.3 ERAD-Mediated Toxin Translocation

Secretory proteins and proteins residing within the organelles of the endomembrane system are co-translationally inserted into the ER. These proteins enter the ER in an unfolded state as they are threaded through the Sec61 translocon pore. Proper folding of the ER-localized protein is then facilitated by a wide array of chaperones, oxidoreductases, and glysosyltransferases. The folding machinery of the ER is also a component of the ERAD quality control system which recognizes misfolded or misassembled proteins in the ER and exports them to the cytosol through protein-conducting "translocon" channels. The translocated, cytosolic substrate is subsequently appended with polyubiquitin chains which serve as a signal for degradation

by the 26S proteasome. ERAD thus prevents the accumulation of potentially toxic protein aggregates in the ER and ensures only properly functioning proteins will be delivered to the distal secretory compartments and/or the extracellular space (Smith et al. 2011; Guerriero and Brodsky 2012).

The ERAD provides CTA1 with a method of entry into the cytoplasm (Teter and Holmes 2002; Teter et al. 2003). As discussed below, CTA1 shifts to a disordered conformation upon its separation from the rest of the toxin. This activates the ERAD pathway and results in the ER-to-cytosol export of CTA1. However, CTA1 avoids the usual degradative fate of ERAD substrates. A strong arginine-over-lysine amino acid bias in the CTA1 polypeptide limits the number of potential sites for ubiquitin conjugation. This bias, which is not found in the CTB subunit, prevents the rapid turnover of CTA1 by the 26S proteasome (Hazes and Read 1997; Rodighiero et al. 2002). The toxin is eventually degraded in a ubiquitin-independent manner by the core 20S proteasome, but it persists in the cytosol long enough to elicit a cytopathic effect (Pande et al. 2007).

Interestingly, some endogenous host proteins also persist in the cytosol after the ER-to-cytosol translocation event. Calreticulin, for example, is an ER-localized chaperone that can be exported to the cytoplasm where it is active in cell adhesion and translation (Afshar et al. 2005; Petris et al. 2011). Nuclear export of the glucocorticoid receptor is regulated by an additional pool of calreticulin that apparently moves from the cytoplasm to the nucleoplasm (Holaska et al. 2002). Protein disulfide isomerase (PDI) is another ER resident protein that can be exported to the cytoplasm and nucleoplasm where it may regulate protein turnover and transcription factor activity (Turano et al. 2002). Thus, there may be a dual function for the ERAD translocation mechanism itself; it has a well-characterized role in the export and disposal of terminally misfolded proteins, but it also appears to play a poorly understood role in shuttling active proteins from the ER to the cytoplasm and/or nucleoplasm.

21.4 Conformational Instability of the CTA1 Polypeptide

CTA1 was originally thought to be a highly stable protein that activated the ERAD system by masquerading as a misfolded protein (Hazes and Read 1997; Tsai et al. 2001; Sandvig and van Deurs 2002). However, more recent structural studies have shown that the isolated CTA1 subunit actually *is* an unfolded protein. CTA1 is held in a stable, protease-resistant conformation by its assembly in the CT holotoxin (Goins and Freire 1988). The disulfide-linked CTA1/CTA2 heterodimer is also more stable than the isolated CTA1 subunit (Surewicz et al. 1990). However, the free CTA1 polypeptide exhibits an intrinsic instability that results in its partial unfolding at the physiological temperature of 37 °C (Pande et al. 2007). Conformational instability is only apparent after CTA1 separates from the rest of the toxin. Thus, CTA1 unfolding would only occur after it dissociates from CTA2/CTB_5 in the ER. The isolated, disordered CTA1 subunit would then be treated as an

ERAD substrate and exported to the cytosol. Stabilization of the isolated CTA1 polypeptide should therefore prevent its recognition by the ERAD system and, consequently, its export to the cytosol. In support of this model, we have demonstrated that CTA1 translocation to the cytosol and CT intoxication are blocked by conditions that prevent the thermal unfolding of CTA1 (Massey et al. 2009; Banerjee et al. 2010; Taylor et al. 2011a).

The intrinsic conformational instability of CTA1 drives host-toxin interactions related to the translocation event. CTA1 must first dissociate from its holotoxin in order to attain the disordered, translocation-competent conformation that is recognized by ERAD. The unfolded CTA1 subunit then interacts with ERAD chaperones and is delivered to a translocon pore for export to the cytosol. Extraction of unfolded CTA1 from the ER is accomplished by the cytosolic chaperone Hsp90. However, the translocated pool of CTA1 would remain in an unfolded state at 37 °C. CTA1 has little or no enzymatic activity at this temperature (Murayama et al. 1993). Continued instability in the cytosol may also explain why CTA1 is prone to ubiquitin-independent degradation by the 20S proteasome, which can only act upon unfolded substrates (Pande et al. 2007). The cytosolic pool of CTA1 must therefore interact with host factors to regain a folded, active conformation that can evade proteasomal degradation. Thus, four major events are linked to CTA1 instability and CTA1 translocation: (i) holotoxin disassembly; (ii) processing by ER-localized chaperones and the ER translocon pore; (iii) chaperone-assisted extraction from the ER; and (iv) refolding by factors in the host cytosol. Each event is considered in the following sections.

21.5 Holotoxin Disassembly in the ER

The CTB pentamer acts as a vehicle to deliver CTA1 to its site of translocation (Fujinaga et al. 2003). The CTA2/CTB_5 complex also serves as a scaffold to maintain CTA1 in a stable, protease-resistant conformation. However, CTA1 must dissociate from CTA2/CTB_5 in order to attain a translocation-competent conformation. Reduction of the CTA1/CTA2 disulfide bond is necessary for holotoxin disassembly, but reduction alone is insufficient for the separation of CTA1 from CTA2/CTB_5; the numerous non-covalent interactions between CTA1 and the rest of the toxin result in a stable macromolecular complex even in the absence of a disulfide bond (Mekalanos et al. 1979). The displacement of reduced CTA1 from its holotoxin requires the additional action of PDI, an ER-localized stress protein (Tsai et al. 2001).

PDI is a member of the thioredoxin superfamily which performs linked but independent roles as an oxidoreductase and a chaperone (Wilkinson and Gilbert 2004). These activities allow it to facilitate the proper folding of nascent secretory proteins as well as the disposal of terminally misfolded proteins through ERAD. The structure and function of PDI is regulated by its redox status; it is a dynamic, flexible molecule which assumes a compact conformation in the reduced state and a more

open conformation in the oxidized state (Tian et al. 2008; Kozlov et al. 2010; Nakasako et al. 2010; Serve et al. 2010). Only the reduced form of PDI will interact with CTA1. PDI thus acts as a redox-dependent chaperone in its interaction with CTA1 (Tsai et al. 2001). Although this interpretation has been controversial (Lumb and Bulleid 2002), PDI has since been shown to exhibit redox-dependent interactions with other substrates as well (Fu and Zhu 2009; Wang et al. 2012).

PDI was originally thought to actively unfold the holotoxin-associated CTA1 subunit and to thereby displace CTA1 from the rest of the toxin (Tsai et al. 2001). An alternative explanation for the "unfoldase" activity of PDI was suggested by our work which demonstrated the intrinsic instability of CTA1 will allow it to spontaneously unfold upon its separation from CTA2/CTB_5 at 37 °C. PDI could thus trigger toxin unfolding simply by removing CTA1 from the CT holotoxin. In support of this possibility, our biophysical analysis demonstrated that PDI does not directly unfold CTA1 (Taylor et al. 2011b). PDI must therefore separate CTA1 from its holotoxin by another mechanism. This mechanism does not appear to require the oxidoreductase activity of PDI, as: (i) reduction of the CTA1/CTA2 disulfide bond alone is insufficient for holotoxin disassembly (Mekalanos et al. 1979); (ii) no disulfide-linked intermediates between PDI and CTA1 have been identified (Tsai et al. 2001); and (iii) PDI-deficient cells are completely resistant to CT (Taylor et al. 2011b). The third observation indicates other ER-localized oxidoreductases cannot replicate the function of PDI in toxin disassembly, which emphasizes the unique and essential role of PDI in CT intoxication. Since this role does not involve oxidoreductase activity, it is likely linked to the chaperone function of PDI.

PDI binds to the folded conformation of CTA1 that is present in the CT holotoxin, and this interaction results in the displacement of CTA1 from CTA2/CTB_5. Unfolding of the dissociated CTA1 subunit then results in the release of its PDI binding partner (Taylor et al. 2011b). The association of PDI with folded, but not unfolded, conformations of CTA1 is unusual for a chaperone, which would be expected to recognize the surface-exposed hydrophobic amino acid residues of a misfolded protein. PDI was initially thought to recognize the hydrophobic C-terminal domain of CTA1 (Lencer and Tsai 2003), but more recent binding studies have demonstrated that PDI-CTA1 interactions do not require this region of the toxin. The binding site for PDI instead appears to be located in the 133 N-terminal amino acids of CTA1 (Taylor et al. 2011b). A more precise identification of the PDI binding site(s) in CTA1 should help further elucidate the holotoxin disassembly mechanism and the unusual chaperone activity of PDI which results in the disassembly, rather than assembly, of a macromolecular complex.

Recent structural studies using isotope-edited Fourier transform infrared spectroscopy have indicated that PDI itself unfolds upon contact with CTA1 (Taylor et al. 2013, manuscript in preparation). The toxin-induced unfolding of PDI provides a molecular explanation for holotoxin disassembly; the expanded hydrodynamic radius of unfolded PDI would act as a lever to dislodge reduced CTA1 from the rest of the toxin. In support of this model, we found the displacement of reduced CTA1

from CTA2/CTB_5 does not occur when PDI is locked in a folded conformation. Holotoxin disassembly was also blocked by chemical inactivation of the chaperone activity of PDI. In contrast, chemical inactivation of the oxidoreductase activity of PDI did not inhibit its ability to separate reduced CTA1 from the rest of the toxin. Two other ER-localized oxidoreductases (ERp57 and ERp72) did not unfold in the presence of CTA1 and did not displace reduced CTA1 from its holotoxin. Substrate-induced unfolding thus appears to be a novel property of PDI that is linked to its function as a chaperone.

21.6 CTA1 Interactions with ER-Localized Chaperones and the Translocon Pore

CTA1 appears to be processed as a typical ERAD substrate after PDI-mediated disassembly of the CT holotoxin. In ERAD, Hsp40 and ATP-dependent Hsp70 chaperones prevent protein aggregation by binding and occluding the solvent-exposed hydrophobic amino acid residues of their substrate (Brodsky 2007; Otero et al. 2010). This process also occurs for CTA1. ERdj3, an ER-localized Hsp40, binds to the unfolded conformation of CTA1 at 37 °C. Loss of ERdj3 function prevents CTA1 translocation to the cytosol and CT intoxication (Massey et al. 2011). BiP, an ER-localized Hsp70 which is also required for CTA1 translocation, prevents aggregation of the disordered CTA1 subunit (Winkeler et al. 2003). Hsp40 and Hsp70 family members often act on the same substrate in sequential fashion, with the initial substrate binding to Hsp40 resulting in the recruitment of Hsp70 and the stimulation of its ATPase activity to generate a high affinity Hsp70-substrate complex (see Chap. 2 for more details). Thus, ERdj3 and BiP likely work in concert to keep unfolded CTA1 in a soluble, translocation-competent state. A complex of CTA1, ERdj3, and BiP has yet to be identified, however.

The ER-localized oxidoreductase ERp72 also plays a role in CTA1 translocation. Depletion of ERp72 by RNAi results in a toxin-sensitive phenotype, which suggests ERp72 normally prevents CTA1 translocation into the cytosol (Forster et al. 2006). Thus, it was proposed that the chaperone function of ERp72 maintains CTA1 in a folded conformation that cannot engage the ERAD system for export to the cytosol. It is also possible that ERp72 competes with PDI for binding to the CT holotoxin; in this model the loss of ERp72 would produce a toxin-sensitive phenotype by enhancing the rate of PDI-mediated holotoxin disassembly. Further studies on the interactions between CTA1 and ER-localized chaperones should clarify the potential unfolding/folding cycle for dissociated CTA1 and the delivery of free CTA1 to the translocon pore.

CTA1 was originally thought to exit the ER through the Sec61 translocon. This model was based upon the physical association of CTA1 with Sec61 in ER-derived microsomes, but no functional correlation was provided (Schmitz et al. 2000). Loss of Derlin-1 function due to RNAi or expression of a dominant negative mutant was

later shown to partially block CTA1 translocation and/or CT intoxication (Bernardi et al. 2008; Dixit et al. 2008). Dixit et al. (2008) also established a physical interaction between Derlin-1 and CTA1. Derlin-1 is an ER resident protein with multiple transmembrane domains that is involved with ERAD and was thought to represent another translocon pore (Lilley and Ploegh 2004; Ye et al. 2004). An emerging model now suggests Derlin-1 plays an accessory role in translocation by accepting non-glycosylated ERAD substrates from BiP and transferring them to an oligomer of Hrd1 which forms a translocon pore (Okuda-Shimizu and Hendershot 2007; Horn et al. 2009; Carvalho et al. 2010). A supporting, rather than essential, role for Derlin-1 in CTA1 translocation would explain why this protein is not required for CT intoxication of zebrafish and some mammalian cell types (Saslowsky et al. 2010). The accessory role of Derlin-1 and primary role of Hrd1 in CTA1 translocation is further supported by a recent study that indicated CTA1 is transferred from Derlin-1 to Hrd1 before passage into the cytosol (Bernardi et al. 2010). This study further demonstrated that the loss of Hrd1 activity restricts CTA1 export to the cytosol. Hrd1 is a multi-pass ER transmembrane protein that was first identified as an E3 ubiquitin ligase (Bays et al. 2001; Deak and Wolf 2001). Its function as a ubiquitin ligase is required for substrate interaction, but the substrate is not necessarily the target of ubiquitination; *in trans* mono-ubiquitination of Hrd1 is thought to maintain the stability of the oligomeric Hrd1 translocon pore (Carvalho et al. 2010). Hrd1 may also modify the ERAD substrate with polyubiquitin chains as it emerges at the cytosolic face of the translocon pore, thus targeting the exported substrate for rapid proteasomal degradation (Bays et al. 2001; Deak and Wolf 2001). Hrd1 thus plays a dual role in ERAD, acting as both the translocon channel and an E3 ubiquitin ligase. CTA1 appears to exploit the former function while avoiding the latter due to its lack of lysine residues for ubiquitin conjugation.

21.7 CTA1 Translocation to the Cytosol

Unidirectional movement through the translocon pore will not occur spontaneously, so a host protein must provide the driving force for CTA1 dislocation from the ER. Most ERAD substrates are extracted from the ER in a process involving the homohexameric AAA ATPase p97 and its affiliated Ufd1-Npl4 complex (Wolf and Stolz 2012). However, p97 plays a minimal role in CTA1 translocation: inactivation of p97 with RNAi or the expression of a dominant negative mutant only produced a minor inhibitory effect on CT intoxication (Kothe et al. 2005; McConnell et al. 2007). In contrast, CTA1 translocation to the cytosol was strongly suppressed when RNAi or drug treatment was used to inactive the cytosolic chaperone Hsp90. This, in turn, blocked CT activity against both cultured cells and ileal loops (Taylor et al. 2010).

Hsp90 is an abundant, dimeric cytosolic chaperone that uses cycles of ATP binding and hydrolysis for substrate processing (Taipale et al. 2010; Zuehlke and Johnson 2010 – see Chap. 2). Client proteins follow a general pathway involving

sequential interactions with Hsp40, Hsc70, Hop, and, finally, Hsp90. Refolding or proteasome-mediated degradation of the client protein is ultimately determined by which co-factors are recruited to the substrate-chaperone complex. Hsp90 is also involved with the processing of a membrane-embedded ERAD substrate (Youker et al. 2004; Wang et al. 2006). However, our work provided the first demonstration of a role for Hsp90 in the extraction of a soluble ERAD substrate from the ER (Taylor et al. 2010). We also found that Hsp90 could bind directly to CTA1 in an ATP-dependent manner with a K_D of 7 nM. Thus, Hsp90-CTA1 interactions do not appear to follow the typical pathway of sequential chaperone interactions for substrate processing.

A preliminary biophysical analysis has indicated that Hsp90 can induce a gain-of-structure in the disordered, 37 °C conformation of CTA1 (H. Burress et al., unpublished observations). The Hsp90-assisted refolding of CTA1 provides a possible mechanistic basis for the Hsp90-mediated translocation of CTA1: by refolding CTA1 as it emerges at the cytosolic face of the ER membrane, Hsp90 could prevent the refolded CTA1 polypeptide from sliding back into the translocon pore. In this model, CTA1 would exit the ER by a ratchet mechanism which couples protein refolding with protein extraction. The ER-to-cytosol export of CTA1 could thus rely on both standard (refolding) and novel (extraction) functions of Hsp90.

Ufd1 and Npl4 also play a role in the ER-to-cytosol export of CTA1. The two proteins form a heterodimeric complex that binds polyubiquitin chains and assists the p97-dependent extraction of ERAD substrates from the ER (Meyer et al. 2002). As such, they would not be expected to be active in CTA1 translocation. Yet depletion of either protein by RNAi resulted in a toxin-sensitive phenotype (McConnell et al. 2007). It was therefore suggested that the Uf1d-Npl4 complex acts independently of p97 as a negative regulator of CTA1 translocation. Since CTA1 ubiquitination (Rodighiero et al. 2002) and functional polyubiquitin machinery (Pande et al. 2007) are not required for CTA1 passage into the cytosol, the novel regulatory function of Uf1d-Npl4 is likely distinct from its ubiquitin-binding activity.

21.8 CTA1 Refolding and Activation in the Cytosol

CTA1 will enter the cytosol in an unfolded state as it passes through a translocon pore. Since CTA1 is an unstable protein, it will not spontaneously refold in the cytosol. The translocated CTA1 subunit must therefore interact with one or more host factors to attain a folded, active conformation in the cytosol. Hsp90 is likely to play a role in this process, as we have found that it can induce a gain-of-structure in the disordered, 37 °C conformation of CTA1. In further support of this model, Giodini and Cresswell (2008) have demonstrated that Hsp90 can return luciferase to an active state after the chemically denatured, exogenously applied protein has moved from the ER to the cytosol. Yet Hsp90 inactivation did not block the cAMP response

to a CTA1 construct that was synthesized directly in the cytosol of cultured cells transfected with a CTA1 expression plasmid (Taylor et al. 2010). This result suggests additional host factors can provide a redundant refolding function for the disordered, cytosolic CTA1 subunit.

ADP-ribosylation factors (ARFs) were initially thought to return disordered CTA1 to an active conformation (Pande et al. 2007; Ampapathi et al. 2008; Cho et al. 2012). The six mammalian ARF proteins are normally involved with remodeling the composition of membrane lipids and regulating vesicular transport (Donaldson and Jackson 2011). However, as indicated by their name, ARF proteins were first isolated from cell extracts as allosteric activators of CTA1 (Welsh et al. 1994). An interaction with ARF6 induces a conformational change in CTA1 that provides NAD (the donor molecule for the ADP-ribosylation reaction) better access to the toxin active site (O'Neal et al. 2005). This, in turn, enhances the catalytic activity of CTA1. An interaction with ARF6 also stabilizes the CTA1 polypeptide, preventing both its temperature-driven shift to an unfolded conformation and its degradation by the 20S proteasome at 37 °C (Pande et al. 2007). However, ARF can only stabilize the folded conformation of CTA1; CTA1 retains its enzymatic activity at 37 °C when ARF is added to the folded toxin before heating to 37 °C, but no gain-of-function and no gain-of-structure occurs when ARF is added to the disordered CTA1 polypeptide after heating to 37 °C (Banerjee et al. 2013, manuscript in preparation). Other factors in the host cytosol are therefore required to place disordered CTA1 in a folded conformation that can be further modified by ARF proteins for maximal *in vivo* toxin activity.

We have recently found that the lipid raft environment where Gsα is located plays a key role in the refolding and activation of cytosolic CTA1 (Ray et al. 2012). Exposure to lipid rafts induces a gain-of-structure and a gain-of-function in the disordered, 37 °C conformation of CTA1. ARF can consequently enhance the activity of raft-associated CTA1 at physiological temperature (Banerjee et al. 2013, manuscript in preparation). *In vivo*, intact lipid rafts are required for optimal ADP-ribosylation of Gsα by the cytosolic pool of CTA1 (Ray et al. 2012). CT thus exploits a novel "lipochaperone" function of lipid rafts as well as novel functions of host cell stress proteins.

21.9 CTA1 Activity and the Unfolded Protein Response (UPR)

The UPR is usually engaged when stress conditions overwhelm the quality control capacity of ERAD (Ron and Walter 2007; Moore and Hollien 2012). There are three branches of the mammalian UPR, and they act in a coordinated fashion to (i) stimulate the expression of ER stress protein and ERAD activity; (ii) reduce overall protein synthesis; and (iii) expand the size/volume of the ER through phospholipid synthesis. These responses allow the cell to recover from

environmental insults that generate substantial levels of misfolded proteins in the ER. However, we recently found that UPR activation also results in cellular sensitization to CT. Moreover, CT itself activates the UPR (VanBennekom et al. 2013, manuscript in preparation).

CT induction of the UPR occurs through a G protein-dependent but cAMP-independent mechanism that apparently involves a p38 MAPK signaling pathway. UPR activation enhances the rate and extent of CTA1 delivery to the cytosol, possibly by stimulating retrograde toxin transport to the ER as well as ERAD-mediated toxin translocation to the cytosol. Although elevated levels of cAMP could be detected 90 min post-intoxication, UPR activation only occurred ~4 h after toxin exposure. The preliminary, relatively inefficient stage of CT intoxication is thus enhanced by the UPR activation which occurs later in the process. UPR activation usually involves a signaling pathway that is initiated in the ER, so G protein-dependent induction of the UPR is an unusual event.

Dixit et al. (2008) have also documented the toxin-induced activation of a host signaling pathway which leads to higher levels of expression for a subset of ER-localized cell stress proteins. Like UPR activation, this signaling mechanism generated a toxin-sensitive phenotype. However, unlike UPR activation, this pathway was activated by the CTB subunit and appeared to regulate protein expression at the level of translation. Thus, in addition to exploiting novel properties of host cell stress proteins, CT also up-regulates the expression of those proteins through atypical transcriptional and post-transcriptional mechanisms.

21.10 Conclusions

To reach its cytosolic Gsα target, CTA1 undergoes what has been described as an order–disorder-order transition (Ampapathi et al. 2008). The ordered CTA1 subunit moves from the cell surface to the ER as part of a stable CT holotoxin. In the ER, reduction of the CTA1/CTA2 disulfide bond permits the PDI-mediated displacement of CTA1 from the rest of the toxin. The free CTA1 subunit then shifts to a disordered conformation which engages the ERAD system for translocation to the cytosol. Hsp90 extracts CTA1 from the ER and may also, along with lipid rafts, facilitate the refolding of cytosolic CTA1. The ordered CTA1 polypeptide can subsequently interact with host ARF proteins to maximize its ADP-ribosyltransferase activity against the raft-localized Gsα target. The translocation and post-translocation processing of CTA1 involves both novel and established functions of host chaperones (Table 21.1). Other ER-translocating toxins employ a similar strategy of exploiting host cell stress proteins for productive intoxication, although the exact mechanisms vary from toxin to toxin (Spooner and Lord 2012). Further elucidation of A chain interactions with host chaperones will continue to enhance our understanding of both bacterial pathogenesis and eukaryotic cell biology.

Table 21.1 CTA1 translocation and post-translocation events

Event	Process
General pathways	
ERAD*	ERAD-mediated translocation is uncoupled from rapid, ubiquitin-dependent proteasomal degradation in the cytosol
UPR*	G protein activation of the UPR by a cAMP-independent mechanism enhances CTA1 delivery to the cytosol
Toxin disassembly	
PDI*	Binds to the stable, holotoxin-associated CTA1 subunit and then unfolds to displace CTA1 from the rest of the toxin
ERAD interactions	
ERdj3	Recognizes and masks the exposed hydrophobic amino acid residues of dissociated, disordered CTA1
BiP	Maintains CTA1 in a translocation-competent state by preventing aggregation of the isolated CTA1 subunit
ERp72	Limits the extent of CTA1 translocation, possibly by refolding the free CTA1 polypeptide
Translocon pore	
Derlin-1	A multi-pass ER transmembrane protein that binds to CTA1 and transfers it to Hrd1
Hrd1*	A multi-pass ER ubiquitin ligase that serves as an oligomeric channel for CTA1 export to the cytosol
Extraction from the ER	
Hsp90*	Pulls CTA1 out of the ER by refolding the toxin as it emerges at the cytosolic face of the translocon pore
Uf1d-Npl4*	Acts as a negative regulator of translocation independently of p97
Refolding in the cytosol	
Hsp90	Partially refolds the cytosolic pool of CTA1
Lipid rafts*	Exhibits a lipochaperone activity which partially refolds CTA1 in the environment of its G protein target
Activation in the cytosol	
ARF*	GTPase that regulates vesicle transport and serves as an allosteric activator for folded, but not disordered, CTA1

Asterisks indicate an event that involves an atypical or dual function for the host factor

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Chapter 22
Endoplasmic Reticulum Stress-Associated gp96 Chaperone is a Host Receptor for Adherent-Invasive *E. coli*

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Abstract Crohn's disease (CD) is a chronic intestinal disease in humans that develop due to an abnormal inflammatory response to the intestinal microbiota in genetically susceptible hosts. Ileal lesions of CD patients are abnormally colonized by Adherent-Invasive *Escherichia coli* (AIEC) producing outer membrane vesicles (OMVs) that contribute to the bacterial invasion process. Endoplasmic reticulum (ER) stress is observed at the intestinal mucosa in CD patients with increased expression of ER-localised stress response proteins. In particular in patients with ileal CD, the ER-stress response chaperone gp96 is strongly expressed on the apical surface of ileal epithelial cells and acts as a host cell receptor for OMVs. As a consequence, this chaperone contributes to increased bacterial invasion of host cells by AIEC.

22.1 Introduction

Gp96 (Srivastava et al. 1986), also known as Grp94 (Lee et al. 1981), endoplasmin and ERp99, is a paralogue of the heat shock protein 90 (HSP90) (Yang and Li 2005). It resides in the endoplasmic reticulum (ER), where its chaperone function is critical for the proper folding of many substrates. This protein is, for example, the

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master immune chaperone for Toll-like receptors (Yang et al. 2007). Interestingly, pathogenic bacteria have evolved mechanisms to utilise this moonlighting protein to aid in the infectious process and gp96 appears now to be a key mediator of the innate immune response due to its ability to bind pathogenic bacteria or their products. Indeed, gp96 is a plasma membrane receptor for Vip, a *Listeria monocytogenes* virulence factor that is required for cell invasion and downstream signalling events (Cabanes et al. 2005). Human colonocyte gp96 serves as a plasma membrane binding protein that enhances cellular entry of *Clostridium difficile* toxin A, participates in cellular signalling events in the inflammatory cascade and facilitates cytotoxicity (Na et al. 2008). A gp96 homologue, Ecgp96, the expression of which is increased during meningitis-associated *Escherichia coli* (*E. coli*) K1 infection of human brain microvascular endothelial cells, promotes invasion of pathogenic bacteria (Mittal and Prasadarao 2011 and discussed in detail in Chap. 23). In this chapter, we will describe a new function of gp96, as a plasma membrane receptor for Crohn's disease-associated adherent-invasive *E. coli* (AIEC) (Rolhion et al. 2010, 2011).

Crohn's disease (CD) is a multifactorial disease, occurring in individuals with genetic predisposition in whom an environmental or infectious trigger causes abnormal immune response (Man 2011; Strober et al. 2007; Xavier and Podolsky 2007). Genetic studies, in particular, genome-wide association studies, have identified mutations that are reproducibly associated with inflammatory bowel diseases (IBDs) (for reviews: Cho 2008; Kaser et al. 2010). Association of CD with polymorphism in *NOD2* (encoding an intracellular pattern recognition receptor), in *ATG16L1* and *IRGM* (encoding autophagy-related proteins) and in *XBP1* (encoding an ER stress response-related transcriptional factor), indicate the importance of innate immunity and autophagy in the pathogenesis of CD. Autophagy is an innate defense mechanism that acts as a cell-autonomous system to eliminate intracellular pathogens.

Although the precise pathophysiology of CD remains incompletely understood, recent studies link ER stress to these inflammatory conditions (for reviews: Hasnain et al. 2012; Kaser and Blumberg 2010; Morito and Nagata 2012). In addition, increased numbers of mucosa-associated *E. coli* with invasive properties or the presence of intra-mucosal *E. coli* have been reported in CD patients (Baumgart et al. 2007; Conte et al. 2006; Darfeuille-Michaud et al. 2004; Kotlowski et al. 2007; Martin et al. 2004; Sasaki et al. 2007). *E. coli* is the predominant aero-anaerobic Gram negative bacterial species of the normal intestinal flora, where it plays an important role in promoting the stability of the luminal microbial flora and maintaining normal intestinal homeostasis. As a commensal, *E. coli* co-exists with the mammalian host in harmony and rarely causes disease except when the normal gastro-intestinal barrier is breached. However, pathogenic *E. coli*, compared with commensal *E. coli*, have acquired specific virulence factors that increase their ability to adapt to new niches and allow them to cause disease (Clements et al. 2012; Kaper et al. 2004). *E. coli* strains associated with the intestinal mucosa from CD patients are highly adherent to intestinal epithelial cells (IECs) and are also invasive (Boudeau et al. 2001; Darfeuille-Michaud et al. 1998). On the basis of the pathogenic traits

of CD-associated *E. coli*, a new potentially pathogenic group of *E. coli*, was designed AIEC for Adherent-Invasive *E. coli*.

In this chapter, we will summarize recent findings on the link between ER stress and CD and provide a synopsis of current data on AIEC virulence and how the ER-associated gp96 acts as a host receptor for AIEC.

22.2 Endoplasmic Reticulum Stress and Crohn's Disease

22.2.1 Regular Functions of ER Stress Proteins and ER Stress

The ER is the starting point of the protein secretory pathway. Secretory or membrane proteins are first co-translationally transported into the ER from cytosolic ribosomes, then captured by ER molecular chaperones (e.g. gp96) and protein folding enzymes and are properly folded (Fig. 22.1a). The folded polypeptides are transported into the Golgi apparatus through the transporting machinery and are finally secreted to the cell surface through a secretory vesicle. However, the proteins that are improperly folded are specifically recognized and isolated by the ER-associated degradation (ERAD) recognition machinery (Araki and Nagata 2011; Smith et al. 2011). ERAD is a multi-step mechanism, which can be divided into the following three steps: (i) the substrate is recognized and isolated (ii) it is dislocated from the ER into the cytosol through a putative narrow pore, and (iii) it is then ubiquitinated by ERAD ubiquitin ligases and finally degraded by the proteasome in the cytosol. See Chap. 21 for further discussion of the ERAD pathway and bacterial toxins.

Under specific cellular conditions, such as heat shock, glucose depletion or inflammation, unfolded proteins can accumulate in the ER lumen leading to an ER stress and initiate the unfolded protein response (UPR) (for reviews, Mori 2000; Walter and Ron 2011). This response is orchestrated by three major stress sensors (ATF6p90, IRE1 and PERK) and their associated transcription factors (ATF6p50, XBP1 and ATF4) (Fig. 22.1b). The three stress sensors detect the protein-folding conditions in the ER lumen and transmit this information, resulting in production of transcription regulators that enter the nucleus to drive transcription of UPR target genes. Each pathway uses a different mechanism of signal transduction: ATF6 by regulated proteolysis, PERK by translational control, and IRE1 by nonconventional mRNA splicing. In addition to the transcriptional responses that largely serve to increase the protein-folding capacity in the ER, both PERK and IRE1 reduce the ER folding load by down-tuning translation and degrading ER bound mRNAs, respectively. So the UPR targets: (i) transcriptional activation of genes coding for ER chaperones and ERAD proteins to enhance ER folding and ERAD capacities; and (ii) translation attenuation to limit further entry of proteins in ER. If cells cannot re-establish homeostasis but continue to experience prolonged and unmitigated ER stress, they apoptose.

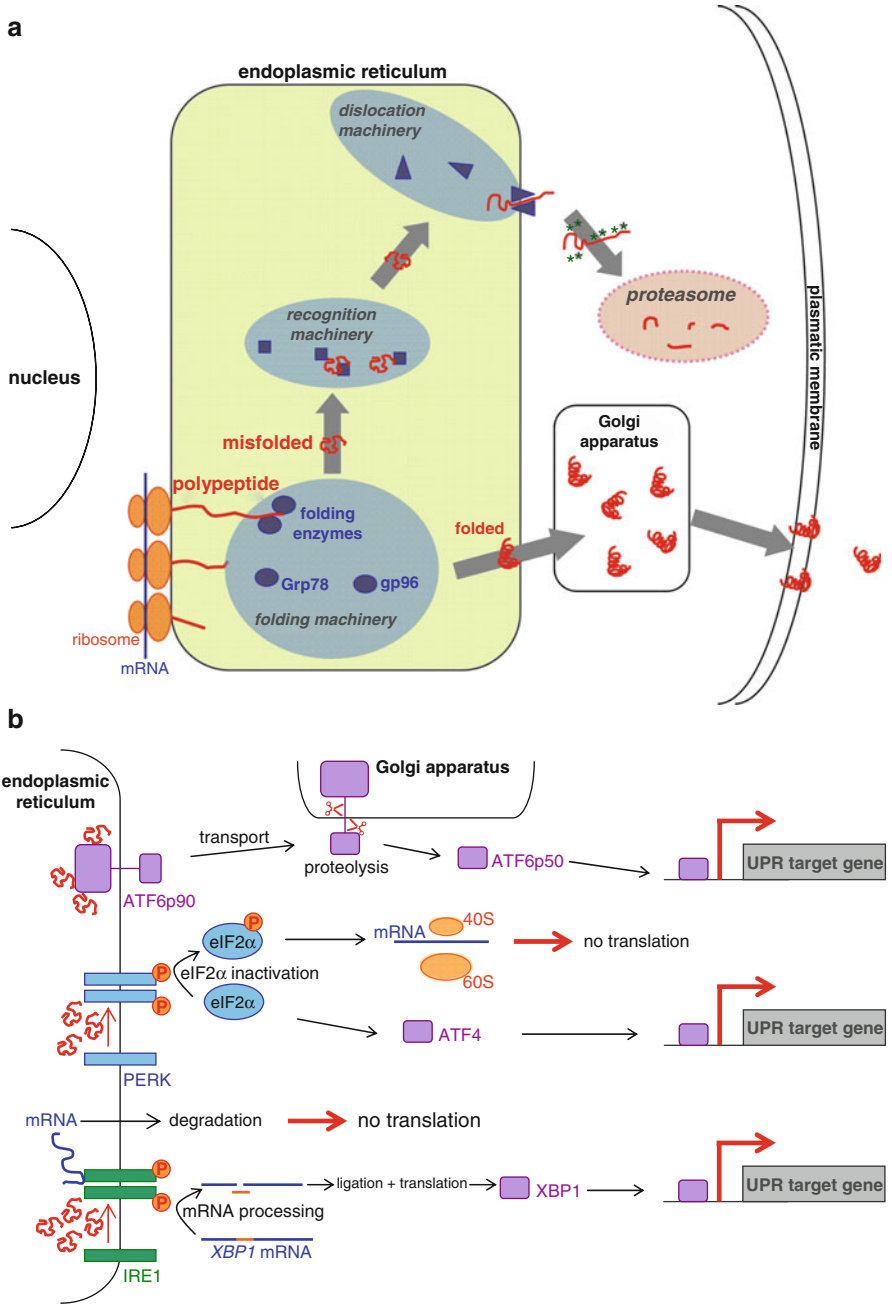

Fig. 22.1 Endoplasmic reticulum protein folding and ER stress. (**a**) Unfolded polypetides, newly synthesized by cytoplasmic ribosomes, are captured by molecular chaperones and protein folding enzymes. The folded polypeptides are transported into the Golgi and finally to the cell surface. Misfolded proteins are specially identified by the ERAD machinery, transferred to the dislocation

22.2.2 ER Stress and Inflammatory Bowel Diseases

The idea that ER stress is involved in IBD (CD and ulcerative colitis (UC)) is quite novel; however, it is now well recognised that unresolved ER stress, as the consequence of genetic abnormalities of the UPR or of a variety of secondary (inflammation and environmental) factors, leads to IEC, goblet cell and Paneth cell dysfunction (Heazlewood et al. 2008; Kaser et al. 2008).

22.2.2.1 Genetic Links

ER stress has been genetically associated with both forms of IBDs through an *XBP1* candidate gene study (Kaser et al. 2008) as well as more recently through the identification of the *ORMDL3* locus (Barrett et al. 2008; McGovern et al. 2010).

Genome wide linkage studies associate *22q12*, the region where *XBP1* resides, with genetic susceptibility to IBD (Barmada et al. 2004; Hampe et al. 1999; Vermeire et al. 2004). Multiple single nucleotide polymorphisms (SNPs) in *XBP1* were found to be associated with both UC and CD (Kaser et al. 2008). Indeed, a deep sequencing effort in >1,000 patients and controls discovered three fold more rare SNPs in patients with IBD compared to healthy controls. Functional studies on rare non-synonymous SNPs (i.e. SNPs that lead to an amino acid exchange; 'nsSNPs') revealed that IBD-only XBP1 nsSNP variants exhibited decreased transactivation of XBP1-regulated UPR target genes. *XBP1*$^{-/-}$ mice developed spontaneous small intestinal inflammation and hence closely mimicked the histological features of human IBD. *Xbp1* deletion in the epithelium resulted in substantial ER stress in IECs, and a marked pro-inflammatory hyper-reactivity of IECs towards microbial and cytokine stimuli.

In addition, XBP1 levels are increased in both inflamed and non-inflamed CD ileum and colon mucosa compared to those from healthy individuals, indicating the presence of ER stress in CD patients (Kaser et al. 2008).

22.2.2.2 HSPs Increased Expression in IBD Patients

So far, the over-expression of two ER-localised stress response chaperones, Grp78 (BiP) and gp96, has been described in the gastrointestinal tract of IBD patients. Grp78 expression is increased in inflamed CD and UC mucosa (Kaser et al. 2008;

Fig. 22.1 (continued) machinery, ubiquitinated and finally degraded by the proteasome (Adapted from Morito and Nagata 2012). (**b**) Three families of signal transducers (ATF6p90, PERK and IRE1) sense the protein-folding conditions in the ER lumen and transmit *via* a specific and independent mechanism that information to transcriptional factors (ATF6p50, ATF4 and XBP1) that enter the nucleus to drive transcription of UPR genes. In addition, PERK and IRE1 are down turning translation (Adapted from Walter and Ron 2011)

Rolhion et al. 2010; Shkoda et al. 2007) and gp96 protein expression is strongly increased in the ileal intestinal epithelium of patients with CD (Rolhion et al. 2010). Indeed, immunohistochemistry showed a very strong staining of gp96 in the ileal epithelium of 50 % of patients in the acute phase of CD and of 34 % patients in the quiescent phase of CD, in contrast to control ileal biopsies that showed very weak or no gp96 expression.

In patients with CD, gp96 and Grp78 were mainly observed at the apical plasma membrane of the epithelium. These observations are consistent with limited published precedents in the literature, demonstrating that the ER stress inducers trigger the redistribution of ER chaperones from the ER lumen to the cytoplasm, membrane and nuclear fractions, as well as to the cell surface (Hendershot et al. 1995; Morris et al. 1997; Shkoda et al. 2007; Triantafilou et al. 2001; Xiao et al. 1999).

22.3 Adherent-Invasive *E. coli* and Crohn's Disease

22.3.1 Links Between AIEC Bacteria and Ileal CD Pathogenesis

The criteria for inclusion in the AIEC group are: (i) the ability to adhere to and to invade IECs with a macropinocytosis-like process of entry dependent on actin microfilaments and microtubule recruitment; (ii) the ability to survive and replicate extensively in large vacuoles within macrophages without triggering host cell death; and (iii) the ability to induce the release of large amounts of TNF-α by infected macrophages (Boudeau et al. 1999; Glasser et al. 2001; Rolhion and Darfeuille-Michaud 2007). AIEC strains are isolated from ileal specimens of 36.4 % of CD patients *vs* 6 % of controls (Darfeuille-Michaud et al. 1998). The higher prevalence of AIEC bacteria in CD patients might arise from an inability of the intestinal mucosa to control this infection, such as from defects in Paneth cell function and subsequent decreased secretion of antimicrobial peptides. A loss of control of AIEC infection could also be related directly to autophagy deficiencies because of mutations in the genes *NOD2*, *ATG16L1*, or *IRGM*; functional autophagy restricts the replication of AIEC intracellular bacteria (Brest et al. 2011; Lapaquette et al. 2010). AIEC strains are preferentially found in early recurrent CD lesions after surgery, thus suggesting a possible role in the initiation of inflammation and not only as secondary invaders.

The interaction between AIEC and cultured IECs induces inflammatory responses such as upregulated expression of interleukin-8 and CCL20, leading to transmigration of polymorphonuclear leukocytes and dendritic cells as shown in co-culture models (Eaves-Pyles et al. 2008). In an *in vitro* model of human granuloma, AIEC-infected macrophages aggregate and fuse to form multinucleated giant cells, very similar to the early stages of epithelioid granulomas observed in CD patients (Meconi et al. 2007). AIEC can also disrupt the integrity of the polarized epithelial cell monolayer, allowing bacteria to breach the intestinal barrier and to

penetrate into the gastrointestinal epithelium (Denizot et al. 2012; Wine et al. 2009). In addition, recurrent ileal CD originates with small erosions in the follicle-associated epithelium that lies over Peyer's patches; AIEC strains interact with mouse and human Peyer's patches and then translocate across membranous/microfold cells (M cells) monolayers (Chassaing et al. 2011). Interestingly, it can be speculated that, in the presence of *NOD2* variants as observed in CD, higher numbers of AIEC bacteria could interact with Peyer's patches because an increased number of M cells have been observed in *NOD2* knock-out mice (Barreau et al. 2007). Finally, AIEC bacteria are able to potentiate an inflammatory mucosal immune response in dextran sulfate sodium-treated mice (Carvalho et al. 2008) and to induce colitis in transgenic mice expressing human CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6) (Carvalho et al. 2009). All together, these observations provide important links between AIEC bacteria, the intestinal cell barrier and ileal CD pathogenesis.

22.3.2 Adhesion and Invasion Factors of AIEC

Analysis of the AIEC reference strain, LF82, genome indicated the presence of a number of gene clusters and pathoadaptative mutations that may play a role in the virulence of AIEC strain LF82 (Miquel et al. 2010). So far, several virulence factors involved in the ability of AIEC bacteria to adhere to and to invade IECs have been identified (Fig. 22.2). Among them, type 1 pili are essential to promote bacterial adhesion (Boudeau et al. 2001) through the recognition of the glycoprotein CEACAM6, which is abnormally expressed on the apical surface of IECs in CD patients (Barnich et al. 2007). Flagella conferring bacteria mobility and regulating type 1 pili (Barnich et al. 2003), the outer membrane protein OmpC regulating the expression of many virulence factors (Rolhion et al. 2007), long polar fimbriae allowing interaction with M cells (Chassaing et al. 2011) and outer membrane vesicles (OMVs) (Rolhion et al. 2005), also play an important role in the invasive capacity of AIEC strain LF82.

OMVs, which are 50–200 nm proteoliposomes released by Gram-negative bacteria, can deliver bacterial factors to or into host cells (for reviews, Kuehn and Kesty 2005; Kulp and Kuehn 2010). The shedding of OMVs during the growth of bacteria is a common phenomenon and OMVs arise from the bacterial surfaces and consist of outer membrane and entrapped periplasmic components. Analyses of OM vesicle components have demonstrated that vesicles contain a wide variety of virulence factors (for review see Ellis and Kuehn 2010). These virulence factors include protein adhesins, toxins, and enzymes as well as nonprotein antigens such as lipopolysaccharide. Interestingly, the secretion of some virulence factors *via* OMVs is necessary for their full maturation, as reported for the activation of the pore-forming cytotoxin ClyA before its delivery to epithelial cells (Wai et al. 2003).

OMVs contribute to the invasion process of IECs by AIEC LF82 (Rolhion et al. 2005). Indeed, deletion of a gene involved in OMVs release in AIEC strain LF82

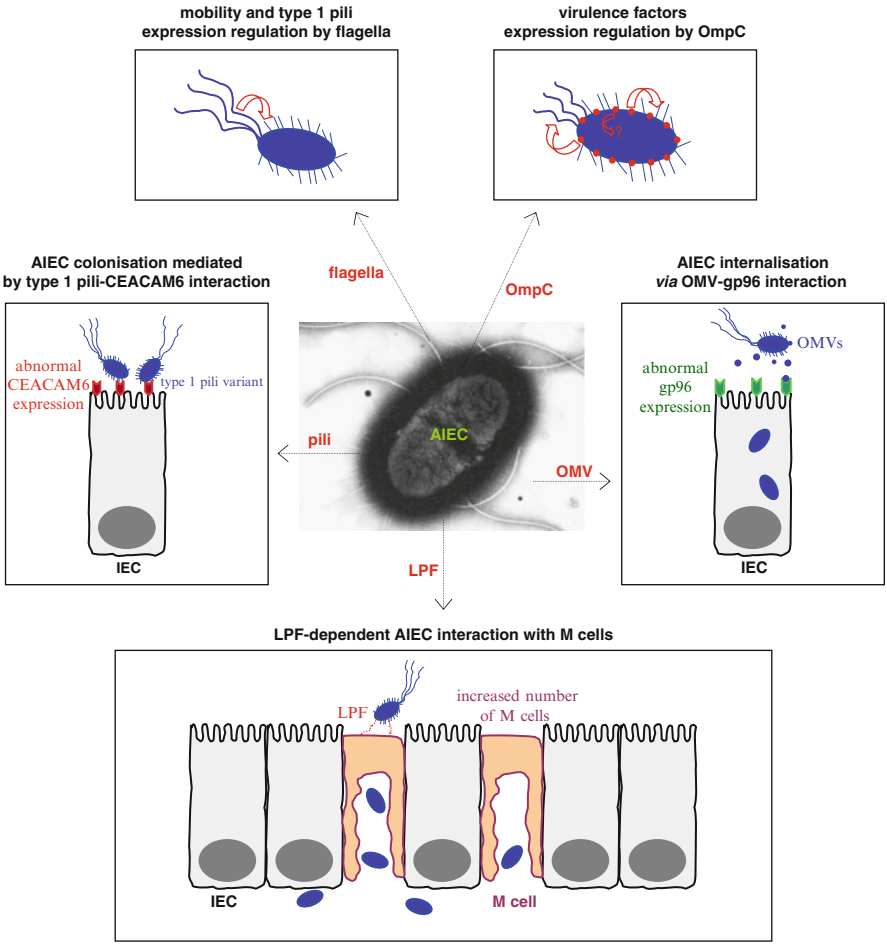

Fig. 22.2 Main virulence factors of AIEC bacteria. Several virulence factors involved in the ability of AIEC bacteria to colonise the gastro-intestinal tract have been identified. AIEC expressed type 1 pili variants that bind to CEACAM6, which is expressed at high levels on the apical surface of IECs in patients with ileal CD. Flagella conferring bacteria mobility and regulating type 1 pili and the outer membrane protein OmpC regulating the expression of many virulence factors also play an important role in the virulence of AIEC strain LF82. In addition, AIEC produce OMVs that interact with gp96, whose expression is increased on the apical surface of IECs in patients with CD. This interaction is essential for AIEC internalisation. Finally, AIEC express long polar fimbriae, which allow the bacteria to interact with Peyer's patches, whose numbers are increased in *NOD2* variants, and to translocate across monolayers of M cells (Adapted from Chassaing and Darfeuille-Michaud 2011)

resulted in a decreased ability of the bacteria to invade IECs. Pretreatment of IECs with AIEC LF82 OMVs partially restores the invasion level of this non-invasive mutant and the effect of LF82 OMVs is specific, since no increase in the invasion level is observed when cells were pretreated with OMVs from non pathogenic

E. coli K-12, suggesting that LF82 OMVs contain bacterial effectors involved in the invasion process that are not present in K-12 OMVs. Other than outer membrane proteins A, C and F, the composition of the AIEC OMVs and potential effector proteins delivered by this mechanism are unknown and further studies are needed to analyse LF82 OMVs composition and content and to identify the delivered bacterial effector(s) involved in the invasion process.

The ability of native bacterial OMVs to fuse with host cells has been illustrated, so far, for neisserial OMVs binding to the CEACAM1 receptor expressed on the surface of T-lymphocytes (Lee et al. 2007), and for OMVs from *Pseudomonas aeruginosa* fusing with respiratory epithelial cells (Bomberger et al. 2009). In addition, AIEC LF82 OMVs can fuse with IECs after interaction between the eukaryotic gp96 receptor and the bacterial protein OmpA at the surface of AIEC OMVs (Rolhion et al. 2010). OmpA expressed by LF82 bacteria binds gp96 protein expressed by IECs. OmpA is a major protein of OMVs and is an AIEC virulence factor involved in the invasion process. Indeed, deletion of the *ompA* gene in AIEC LF82 strain induced: (i) a significant decrease in the ability of the bacteria to invade IECs compared to a wild-type strain; (ii) a loss of fusion of OMVs with the host cell membrane; and (iii) a loss in the ability of OMVs to restore invasion of a non-invasive mutant.

22.4 Gp96 and CD-Associated AIEC

As gp96 is over-expressed in CD patients and as it interacts with AIEC virulence factor OmpA, experiments have been conducted to address the biological function of gp96 in the ability of AIEC to invade IECs (Rolhion et al. 2010). Invasion experiments, either in the presence of antibodies raised against gp96 (hiding gp96 epitopes and so preventing the interaction between gp96 and OmpA), or after transfection of IECs with *gp96* small interfering RNA (siRNA) (decreasing gp96 expression), showed a decreased invasion of AIEC bacteria, indicating that gp96 promotes AIEC invasion (Rolhion et al. 2010). In addition, in the presence of anti-gp96 antibodies, AIEC OMVs were unable to fuse with the host cell membrane suggesting that gp96 acts as the host membrane OMV receptor. This idea is reinforced by confocal microscopy by the observation that gp96 was recruited to the OMV fusion sites.

All these findings indicate that the AIEC bacteria, associated mainly with the ileal form of CD, are able to take advantage of the gp96 overexpression, as gp96 acts as a host cell receptor for AIEC invasion *via* OMVs rich in OmpA protein. It has previously been reported that CEACAM6 acts as a receptor for *E. coli* type 1 pili and therefore allows AIEC bacteria to colonize the ileal mucosa (Barnich et al. 2007). A colocalization of gp96 and CEACAM6 in CD patients has been observed (Rolhion et al. 2010). Increased expression of gp96 and CEACAM6 are observed at the same site leading to increased AIEC virulence, since the bacteria are able to colonize the mucosa by binding to CEACAM6 and can better invade the ileal

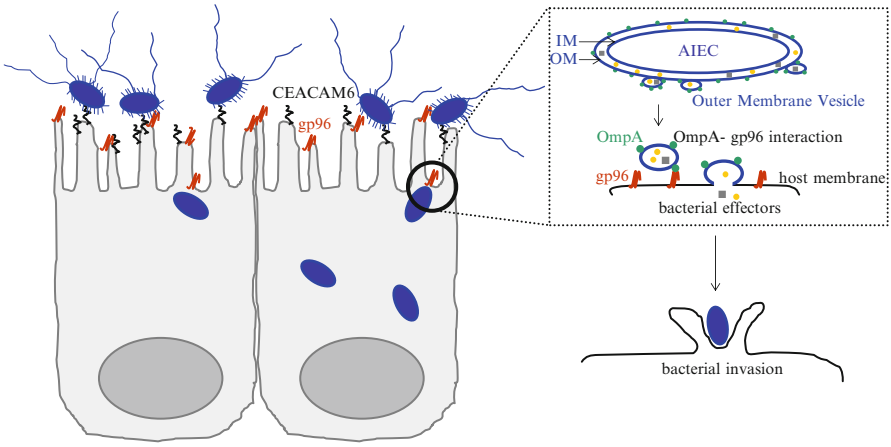

Fig. 22.3 Interplay between intestinal epithelial cells and Adherent-Invasive *E. coli*. Type 1 pili of AIEC mediate the ability of the bacteria to adhere to IECs through the recognition of abnormally expressed CEACAM6 in CD patients. OMVs, rich in OmpA protein (●), released by AIEC mediate the ability of the bacteria to invade IECs through the interaction with abnormally expressed gp96 in CD patients. OMVs are able to fuse with the host cell membrane promoting bacterial invasion through putative delivery inside host cells of bacterial effector(s) (● and ■) that remain to be identified (From Rolhion et al. 2011)

epithelium through AIEC OMV-gp96 interaction (Fig. 22.3). In addition, as gp96 participates in the folding and assembly of many secretory and membrane proteins, we can therefore wonder whether it participates in the cell membrane targeting of CEACAM6. One hypothesis is that patients at high risk for developing severe ileal CD are those who, in addition to expressing CEACAM6 (Barnich et al. 2007), overexpress gp96 in the ileal mucosa.

22.5 Conclusions

Gp96 was described as an ER-chaperone involved in the proper folding of many substrates, but since 2005, several studies reported that pathogenic bacteria, such as *Listeria monocytogenes*, *Clostridium difficile*, *E. coli* K1, use gp96 as a receptor to aid in the infectious process. Gp96, which is overexpressed on the apical surface of IECs in patients with Crohn's disease, acts as a host cell receptor for OMVs released by CD-associated AIEC, promoting invasion by AIEC. Therefore, AIEC bacteria could take advantage ot the ER stress occurring in patients with IBD. Because microbiota have an important role in the establishment of the ER stress response (Kaser et al. 2011), AIEC, as opportunistic pathogens, take advantage of such changes in the host innate immune responses.

Gp96 is overexpressed at the apical plasma membrane of IECs in CD patients. Unlike CEACAM6 expression (Barnich et al. 2007), no increased gp96 expression

was observed in response to AIEC pathogenic bacteria infection or following pro-inflammatory cytokine stimulation (TNF-α or IFN-γ) of IECs (Rolhion et al. 2010), suggesting that increased gp96 expression in CD might only be related to ER stress. This latter observation is in good agreement with the fact that gp96 was found to be strongly expressed in patients with both acute and quiescent phases of CD.

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Chapter 23
Escherichia coli K1 Meningitis and Heat Shock Protein, gp96

Nemani V. Prasadarao

Abstract Evidence is emerging rapidly that heat shock protein, gp96, plays a critical role in various infectious diseases. Herein, I emphasize the role of gp96 in the pathogenesis of *E. coli* K1 meningitis. *E. coli* K1 is the most common neonatal meningitis-causing Gram-negative bacterium, which interacts with gp96 via outer membrane protein A (OmpA) on both neutrophils and human brain microvascular endothelial cells (HBMEC). *E. coli* K1 infection induces the surface expression of gp96 in neutrophils, using it as a receptor for entering cells and suppressing the production of reactive oxygen species. Thus, the bacterium survives and multiplies inside neutrophils to achieve high-grade bacteremia. *E. coli* K1 subsequently interacts with HBMEC gp96 (Ecgp96), to induce a variety of signaling pathways for bacterial invasion of the blood–brain barrier and temporarily disrupts the tight junctions between the cells. Of note, *E. coli* K1 attachment to HBMEC promotes the interaction of Ecgp96 with TLR2 to form a complex, Ecgp96/TLR2, which then translocates to the cell surface. The binding of *E. coli* K1 OmpA to Ecgp96/TLR2 enhances the production of inducible nitric oxide, which is, in turn, responsible for more Ecgp96 expression at the cell surface and subsequent tight junction disruption.

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23.1 Introduction

Bacterial meningitis is a serious brain disease which causes significant morbidity and mortality rates particularly in the pediatric population and is characterized by marked inflammation of the meninges and subarachnoid space (Grandgirard et al. 2007). The mortality rates reach 100 % without treatment and even with effective antibiotics therapy many of the patients suffer neurological consequences such as loss of vision, loss of hearing and deficiency in learning and memory. Although the incidence of meningitis in the United States dropped from 1.9 to 1.5 cases per 100,000 live births in recent years, it is still widespread in developing countries (Hsu et al. 2009; Schuchat et al. 1997; Thigpen et al. 2011). Aseptic meningitis, also known as non-bacterial meningitis, is commonly due to enteroviruses and requires no direct treatment; supportive therapy is, however, useful in limiting the morbidity (Bamberger 2010). Bacterial meningitis, on the other hand, can be caused by a number of pathogens and no one particular clinical feature exists to definitely diagnose this disease. Strong suspicion should be harbored, however, when encountered with the combination of fever, seizures, meningeal signs and altered consciousness. The most common method of diagnosis is through cerebrospinal fluid (CSF) analysis, obtained via lumbar puncture, by counting WBC, protein and glucose levels. Gram stain of CSF is also useful in visualizing the pathogen in most cases (Tunkel and Scheld 1993). However, lumbar puncture is challenging because the procedure is invasive and painful. Nonetheless, the techniques for the diagnosis of meningitis are not readily available in third world countries.

Several bacterial pathogens which infect humans exhibit a propensity to enter the central nervous system (CNS) by crossing the blood–brain barrier (BBB) (El Bashir et al. 2003). Only a few pathogens, however, such as Group B Streptococcus, *E. coli* K1 and *Listeria monocytogenes* are responsible for 75 % of all neonatal meningitis (Ferrieri et al. 1980; Kim 2003). Although *Klebsiella* species and *Citrobacter* species rarely cause CNS infections, their presence can exert fatal outcomes. Neonates acquire bacterial infections from the mother during delivery or from nosocomial sources in the first week of life. Subsequently, the pathogen colonizes nasopharyngeal, upper respiratory or gastrointestinal mucosal surfaces followed by survival and propagation in the perivascular spaces, leading to high-grade bacteremia ($>10^3$ cfu/ml of blood) (Kim 2003). Achieving a high degree of bacteremia is a prerequisite for bacterial translocation across the BBB. During this traversal, the bacterial pathogens also disrupt tight junctions between the endothelial cells that form a single cell lining of the BBB, thereby leading to brain edema (Fig. 23.1). The invading pathogen consequently enters the subarachnoid space to multiply further, and elicits inflammatory responses in the host. Neuronal apoptosis in the dentate gyrus of the hippocampus and necrosis of the cortex commonly occur in bacterial meningitis (Tunkel and Scheld 1993; Scheld et al. 2002). Small vessel vasculitis and focal ischemia, or venous thrombosis, are other notable pathological features of the brain in this disease. Although antibiotic therapy is the primary

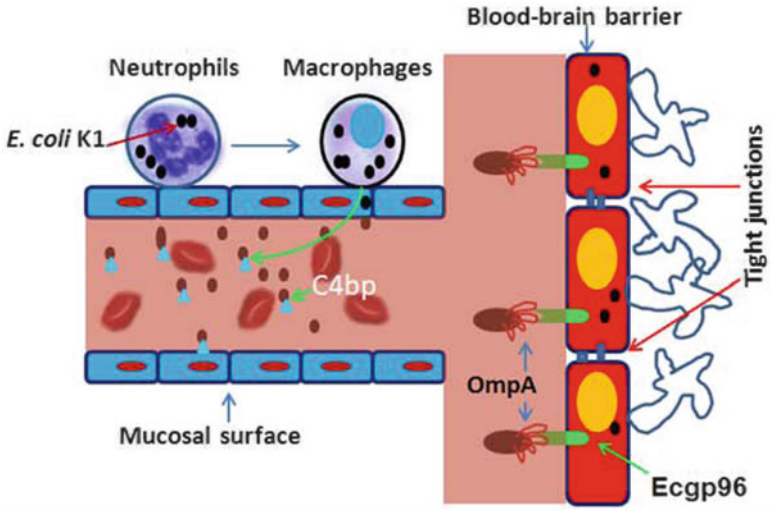

Fig. 23.1 Interactions of *E. coli* K1 with various host tissues to cause meningitis. Colonization of *E. coli* K1 with nasopharyngeal or gastrointestinal mucosa initiates the interaction of the bacterium with PMNs. *E. coli* K1 binding to gp96 in PMNs allows the bacteria to enter, survive and multiply in the cells. Since PMNs are short lived cells; the bacteria transfers to macrophages during phagocytosis of PMNs and further multiply in the cells. The bacteria release into the blood in enormous numbers, which situation is called bacteremia. Complement activity controls the number of *E. coli* K1 during early stages of infections, however, as the bacterial load increases, the serum bactericidal activity nullifies. *E. coli* K1 that binds to C4b-binding protein (C4bp) evades complement attack and consequently, the bound C4bp prevents the bacteria interacting with the receptors on the blood–brain barrier to invade. However, when the *E. coli* K1 numbers increase, there is insufficient C4bp in neonates to block all the bacterial binding sites and thus, *E. coli* K1 get an opportunity to interact with Ecgp96 for binding and invading brain microvascular endothelial cells to cause meningitis

option to treat the patients, corticosteroids and glycerol as adjuvant intervention markedly reduced the inflammation and showed beneficial effects in meningitis patients (Schut et al. 2008). A potential problem with antibiotic treatment, during high-grade bacteremia, is the release of large quantities of endotoxin, which often causes septic shock and, eventually, organ dysfunction. Therefore, removal of the bacteria from the circulation without releasing large amounts of proinflammatory products would provide an effective therapy for this disease.

Escherichia coli K1 is the leading pathogen that causes meningitis in premature infants (46 %), whereas it is the second most common bacterial pathogen in full-term neonates (15 %) (Kim 2003). Bacterial sepsis and meningitis caused by *E. coli* K1 remain a deadly disease, despite advanced empiric antimicrobial therapy and the provision of supportive care. Approximately 5 % mortality rates are noted in children in the developed world. In developing countries, however, the mortality rates increase to 30 %.

23.2 *E. coli* K1 Virulence Factors Responsible for the Pathogenesis of Meningitis

Bacterial pathogens express several surface structures, which are general components of the outer membrane of the bacterium, that potentially interact with host structures during the establishment of disease (Silhavy et al. 2010). One of the most prevalent molecules in Gram-negative bacteria is lipopolysaccharide (LPS) also, incorrectly, termed endotoxin (a mixture of LPS and outer membrane proteins), which is responsible for septic shock. Although a variety of LPS structures exists among different *E. coli* strains, O18 LPS is the predominant serotype in episodes of *E. coli* meningitis (Andreishcheva and Vann 2006; Silver et al. 1981). Capsular polysaccharide is another notable virulence factor of *E. coli* K1 and has been shown to be responsible for enabling the organism to avoid serum bactericidal activity (Kim 1992). Similar to LPS, diverse forms of capsular polysaccharides exist in *E. coli*; K1 capsular polysaccharide is commonly associated with meningitic isolates and is a polymer of sialic acid that covers the whole bacterial surface. Targeting K1 capsular polysaccharide, however, is challenging for the development of immunotherapies against *E. coli* K1 meningitis, due to the similarity of sialic acid linkage between K1 capsular polysaccharide and host glycoproteins, introducing the risk of damaging cross-reactivity (Finne et al. 1983).

Other pertinent surface structures of *E. coli* are the fimbriae. A variety of fimbriae present on diverse bacterial pathogens shows different binding specificities to mono- or oligo-saccharides moieties present on host glycoproteins. S-fimbriae, which bind terminal sialyl lactose (NeuAc2-3Gal1-4Glc) sugars, frequently associate with meningitic *E. coli* K1 strains (Parkkinen et al. 1988). S-fimbria protrudes like a needle out of the bacterial membrane and contains different protein subunits such as sfaA, sfaB, sfaC and sfaS (Prasadarao et al. 1993). The tip of S-fimbriae is capped with sfaS protein that exhibits affinity towards sialyl lactose moieties. In addition, type-1 fimbriae specific to mannose residues also exist in *E. coli* K1 (Stahlhut et al. 2009).

Outer membrane proteins (Omp) of *E. coli* K1 also provide opportunities to interact with a variety of host cell surface molecules. One of the major outer membrane proteins is OmpA, a 325 amino acid protein, which accounts for 1 % of the total protein mass of *E. coli* K1 (Sugawara et al. 1996; Pautsch and Schultz 1998, 2000; Arora et al. 2001; Bond et al. 2002). The first 177 amino acids of OmpA form a barrel in the membrane of *E. coli* K1 with eight transmembrane domains and four extracellular loops (Fig. 23.2). Although the OmpA structure is conserved throughout evolution, Smith and co-workers demonstrated the presence of two alleles of the *ompA* gene in Gram-negative bacteria (Smith et al. 2007). With respect to the function of OmpA, allele 2 (*ompA2*) tends to present in virulent strains of *E. coli*, and exhibits slight differences in the amino acid sequence of the extracellular loops. Although OmpA is a structural protein, it also serves as a receptor for several bacteriocins, such as colicin U, colicin L and bacteriocin 28b, and for several bacteriophages, such as K3, Ox2 and M1 (Morona et al. 1984; Cole et al. 1983; Manoil and Rosenbusch 1982). However, mounting evidence shows that OmpA acts as a virulence factor in many pathogenic bacteria.

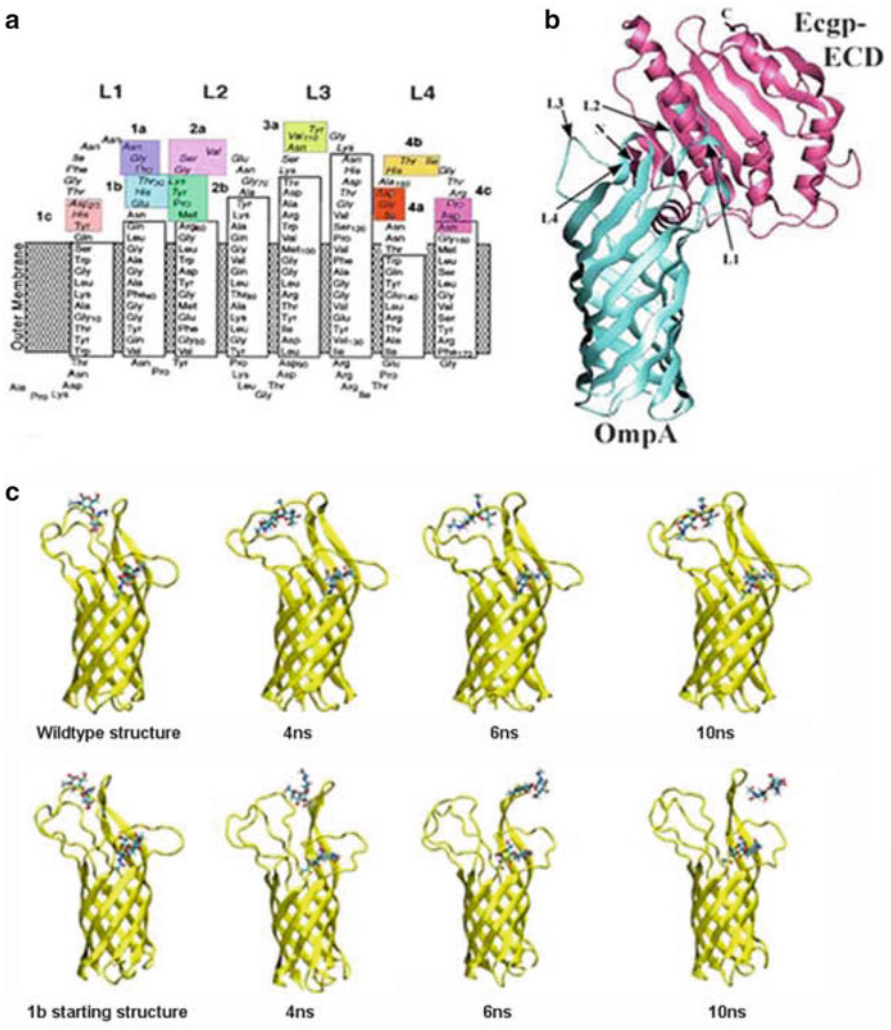

Fig. 23.2 Computer modeling of OmpA interaction with the carbohydrate epitopes and peptide regions of Ecgp96. OmpA is a 325 amino acid protein with the first 177 amino acids inserted into the outer membrane of the bacteria with four extracellular loops (L1–L4) and eight transmembrane domains. Three to four amino acids in each loop were mutated to alanines to evaluate the role of each region (color coded) in *E. coli* K1 invasion of HBMEC (**a**). The interaction of OmpA with the extracellular domain of Ecgp96 shows that OmpA loops prefer peptide regions around N-glycosylation sites (**b**). OmpA initially interacts with two GlcNAc1-4GlcNAc epitopes present on the Ecgp96, the first in a loop region formed by loops 1 and 2 and the second in the barrel region formed by loop 1, 2 and 4 close to the membrane region. Computer simulations of OmpA interaction with GlcNAc1-4GlcNAc epitopes using OmpA mutant 1b revealed that the carbohydrate structure is not stable in mutant OmpA and, therefore, kicked out of the protein (**c**). (Some of the figures were previously published in Pascal et al. 2010)

23.3 Role of gp96 in Immune Cells for Entry and Survival of *E. coli* K1

Strategic survival in the face of host immune defenses is essential to several intracellular bacteria, allowing them to establish infections. Therefore, bacterial pathogens have developed tactics to avoid or divert host defenses (Sal-Man et al. 2011; Finlay and Bonas 2011). A certain threshold of *E. coli* K1 is critical to cause meningitis and, thus the bacterium needs to survive in host tissues until it multiplies and achieves sufficient numbers. Serum complement is the first line of defense against invading microorganisms killing them by opsonizing with complement proteins that form the membrane attack complexes (MACs), which subsequently lyse the bacteria. *E. coli* K1 avoids serum bactericidal activity by binding to C4b-binding protein (a classical complement pathway inhibitor) via OmpA and triggering the cleavage of C3b, which is a critical component of complement for the opsonization of bacteria and the formation of MAC (Prasadarao et al. 2002; Wooster et al. 2006; Maruvada et al. 2008a).

Neutrophils (PMNs) are key components of innate immune defense that phagocytose invading bacteria in tissues and destroy them using an array of anti-microbial mechanisms (Arruda and Barja-Fidalgo 2009; Nauseef 2007). The respiratory burst, a sudden increase in oxygen consumption releasing oxygen-derived radicals, is one of the antimicrobial mechanisms utilized by PMNs to eradicate bacteria. The enzyme responsible for the respiratory burst is NADPH oxidase, made up of a multicomponent complex with individual components separated into distinct compartments in the resting stage. The major component of NADPH oxidase is, flavocytochrome b558, a heterodimeric heme protein consisting $gp91^{Phox}$ and $gp22^{Phox}$. Other essential proteins required for NADPH oxidase activity reside in the cytosol of PMNs, which include $p47^{Phox}$, $p67^{Phox}$ and rac2. The NADPH oxidase assembled on the phagosome membrane transfers electrons from the cytosol, sequentially through the two non-equivalent hemes in flavocytochrome b558, across the membrane to the electron acceptor, molecular oxygen, and thereby generates superoxide anion. Superoxide anion produced in the phagosome can interact with itself, thereby producing more toxic reactive oxygen species (ROS) including H_2O_2 and $\cdot OH$.

23.3.1 The Entry of E. coli *K1 Into Neutrophils Requires gp96 Expression*

An interesting observation is that depletion of PMNs makes newborn mice resistant to *E. coli* K1 infection (Mittal and Prasadarao 2011). This indicates that interaction between PMNs and *E. coli* K1 is a critical step in the pathogenesis of neonatal meningitis. As the bacterial numbers did not increase in PMN-depleted mice, PMNs may act as prime safe havens for the multiplication during initial stages of infection required to achieve high-grade bacteremia. In addition, PMNs containing *E. coli* K1

may disseminate bacteria to distal sites or to present to macrophages for subsequent rounds of multiplication. Concomitantly, depletion of PMNs 24 h post-infection in mice could not protect them from the onset of meningitis. Thus, one of the important questions during the pathogenesis of *E. coli* K1 meningitis is: what mechanism of *E. coli* K1 entry offers the bacteria a non-hostile environment in neutrophils to survive and thrive? The pathogen recognition receptors such as TLRs, although known to activate antimicrobial responses, are not involved in the entry mechanisms. Heat shock proteins such as gp96 can chaperone antigenic peptides and promote their delivery to antigen-presenting cells for presentation to T cells (Multhoff 2006; Harding 2007; Ni and Lee 2007). Two forms of heat shock proteins, HSP90α and HSP90β, exist in cells and gp96 is an endoplasmic reticulum paralogue of HSP90β form. Despite its general localization in the endoplasmic reticulum, gp96 gains extracellular access after cell activation, infection or necrotic cell death. Interestingly, *E. coli* K1 interaction with PMNs mediated by OmpA increases gp96 expression at the cell surface (Mittal and Prasadarao 2011). Suppression of gp96 expression by siRNA not only inhibited the surface translocation of gp96 upon infection with *E. coli* K1, but also prevented the entry of bacteria into the cells. Gp96 knockdown PMNs efficiently kill the invaded bacteria, indicating that interaction of OmpA of *E. coli* K1 with gp96 provides a survival strategy for the bacteria inside PMNs.

With this *in vitro* observations in mind, one wonders whether limiting access of the bacteria to PMNs would prevent the onset of meningitis in animal models. In agreement with this speculation, suppression of gp96 expression, by siRNA, in newborn mice followed by infection with *E. coli* K1, allows the animals to survive beyond the experimental period (Fig. 23.3). In contrast, wild type mice succumbed by 96 h post-infection. The gp96 knockdown mice showed no bacteremia, although the bacteria entered the circulation at earlier stages. Furthermore, these animals showed no signs of brain damage or blood–brain barrier leakage. Since PMNs are the first responders against invading pathogens, the data obtained from gp96 knockdown mice suggest that surface expression of gp96 in PMNs may be necessary for survival inside cells during initial round of multiplication. It is not yet fully known whether gp96 expression in other immune cells and/or at the blood–brain barrier in animal models play a role in this infectious process.

23.3.2 Survival of E. coli *K1 Inside Neutrophils*

Given the concept that ROS production combats invading pathogens, *E. coli* K1 infection of human PMNs, however, suppresses the production of ROS and allows the pathogen to survive efficiently inside the cells (Mittal and Prasadarao 2011). In contrast, lack of OmpA in *E. coli* causes the neutrophils to produce significantly greater levels of ROS and thereby, ensures that the bacterium cannot survive inside cells. Pretreatment of *E. coli* K1 with anti-OmpA antibody induced the production of ROS in PMNs. In addition, opsonization with serum of *E. coli* K1 had no effect on the suppression of ROS production, indicating that complement receptors in

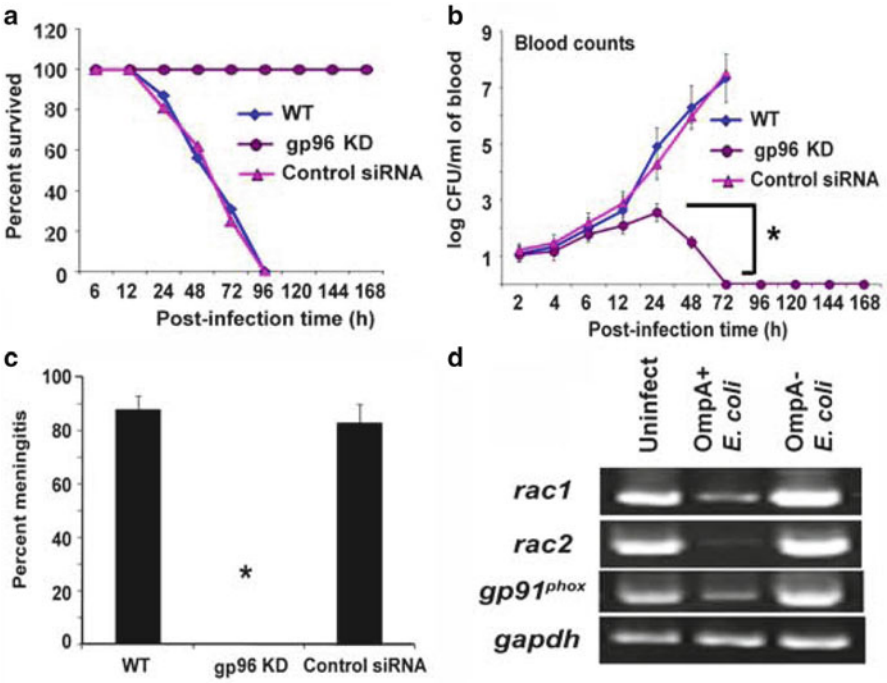

Fig. 23.3 Suppression of gp96 expression in newborn mice prevents *E. coli* K1-induced meningitis. (**a**) The expression of gp96 was reduced in 3 day old mice, by injecting gp96-siRNA along with Invivofectamine reagent, and then infected with 10^3 cfu of *E. coli* K1. Wild type and control siRNA injected mice served as controls. The survival of the animals was monitored for 7 days. (**b**) At various time periods, a small amount of blood was collected, dilutions made, and plated on agar containing antibiotics to enumerate bacterial numbers. (**c**) Cerebrospinal fluid (CSF) samples were collected when the animals were in a moribund state and directly inoculated into Luria broth containing antibiotics. The occurrence of meningitis is determined if the CSF cultures were positive. (**d**) PMNs were isolated from spleens of newborn mice infected with OmpA+ *E. coli* or OmpA– *E. coli,* total RNA prepared, and subjected to RT-PCR analysis using *rac1, rac2, gp91*^{phox} or *gapdh* primers. The data revealed that newborn mice with reduced expression of gp96 are resistant to *E. coli* K1-induced meningitis, which could be due to the inability of *E. coli* to survive in PMNs (The figures were previously published in Mittal and Prasadarao 2011)

PMNs are playing a minimal role in modifying the neutrophil function. Only viable *E. coli* K1 prevented the production of ROS while heat-killed bacteria generated robust quantities of ROS. Continuous suppression of oxidative burst, even in the presence of purified LPS and PMA, potent agonists of ROS generation, indicates that *E. coli* K1 hijacks the PMN cellular machinery. The underlying mechanism of oxidative burst suppression by *E. coli* K1 is due to downregulation of Rac1, Rac2 and gp91^{phox} expression at both the mRNA and protein levels in PMNs (Fig. 23.3). The ROS production in gp96 knockdown PMNs was unexpectedly similar to the levels produced by exposure to *E. coli* K1 and could be due to inefficient interaction of the bacteria with the neutrophils. In contrast, pretreating the PMNs with

anti-gp96 antibodies followed by infection with *E. coli* K1 generated large quantities of ROS. It is still to be determined whether gp96 expression levels control other surface structures such as TLRs in PMNs that are essential to interact with LPS and other bacterial products.

23.3.3 The Extracellular Loop 2 Interaction with gp96 is Vital for E. coli *K1 Survival in Neutrophils*

As OmpA contains four extracellular loops, they may engage in gp96 interaction on PMNs for *E. coli* K1 entry. Mutations in loop 2 of OmpA unexpectedly increased the *E. coli* K1 entry by fourfold into PMNs in which gp96 expression was suppressed by siRNA compared with other mutations in other loops (Mittal and Prasadarao 2011). However, loop 2 mutant *E. coli* K1 could not survive efficiently due increased production of ROS in gp96 knockdown PMNs. One reason that loop 2 mutants enter PMNs in high numbers is because of increased expression of complement receptor 3 (CR3) and the interaction of loop 2 mutant OmpA with CR3. These studies, therefore, suggest that the intact loop 2 interacts with gp96 for entering and surviving in PMNs. In contrast, mutation of three amino acids in loop 2 enables the bacteria to interact with CR3, which induces anti-microbial activity in PMNs.

23.4 Role of gp96 in Breaching the Blood–Brain Barrier

After survival of host defense mechanisms, *E. coli* K1 interacts with the BBB, which is composed of a lining of microvascular endothelial cells (BMEC), to enter the central nervous system. *E. coli* K1 uses an array of virulence factors to bind to and invade human BMEC (HBMEC). OmpA was the first virulence factor shown to promote the invasion of *E. coli* K1 into HBMEC (Prasadarao et al. 1996a, b). Loss of OmpA expression resulted in considerable attenuation (approximately 25-fold decrease) in the invasive ability in HBMEC in tissue culture, and complete loss of virulence in animal models. Complementation with the *ompA* gene restored the invasive capability of OmpA– *E. coli* to the level of the OmpA+ *E. coli*. In addition, purified OmpA reconstituted into liposomes as well as the anti-OmpA antibodies inhibited the invasion of OmpA+ *E. coli* into HBMEC. Two short synthetic peptides (a hexamer, Asn-27–Glu-32, and a pentamer, Gly-65–Asn-69) generated from the amino acid sequences of loops 1 and 2 of OmpA exhibited significant inhibition of OmpA+ *E. coli* invasion. In agreement with this, mutations (only three amino acids at a time) of these residues prevented the invasion of *E. coli* K1 in HBMEC (Pascal et al. 2010; Mittal et al. 2011). These studies prove that OmpA loops are critical for interaction and subsequent crossing of the BBB.

23.4.1 Endothelial Cell gp96 (Ecgp96)

Ecgp96, a homologue of endoplasmic reticulum specific gp96, was identified as the receptor for OmpA in HBMEC (Prasadarao 2002). The gene for Ecgp96 encodes for a protein with 803 amino acids and translates into 95.4 kDa with a signal peptide sequence and a putative single span transmembrane domain (Prasadarao et al. 2003). Although Ecgp96 contains a C-terminal KDEL motif similar to that of gp96, which is a signature marker for ER retention signal, it is still expressed on the cell surface of HBMEC. The amino acid sequence from 280 to 297 residues in Ecgp96, however, showed considerable difference compared with gp96 at both the DNA and the protein levels. Interestingly, Ecgp96 expresses only on brain endothelium, not in umbilical or aortic endothelium, providing reason for the neuroinvasive capability of *E. coli* K1 (Prasadarao 2002). The OmpA– mediated *E. coli* K1 invasion occurs via the interaction of N-terminal loops of OmpA with GlcNAcβ1, 4-GlcNAc epitopes of Ecgp96 (Prasadarao et al. 1996b). The chito-oligomers (GlcNAcβ1, 4-GlcNAc polymers) blocked *E. coli* K1 invasion of HBMEC both *in vitro* and in the newborn rat model of hematogenous meningitis. Furthermore, simulation of GlcNAcβ1, 4-GlcNAc sugar interaction with OmpA using molecular models revealed that these sugar moieties fit into the canyon formed by the loops 1 and 2 of OmpA, which showed favorable energy levels and conformations compared to any other area (Datta et al. 2003) (Fig. 23.3). *E. coli* K1 does not invade HUVEC despite the presence of glycoproteins containing GlcNAc1, 4-GlcNAc epitopes due to the existence of fucose linked to internal GlcNAc residue. The interaction of the protein backbone of the Ecgp96 with OmpA also plays a significant role in the invasion process. After initial identification of gp96 as a receptor for *E. coli* K1, several other pathogens such as, *Listeria monocytogenes*, *Clostridium*, adherent invasive *E. coli* (see Chap. 22), and *Candida albicans* have shown to interact with gp96 for binding to various cells (Cabenes et al. 2005; Rolhion et al. 2010; Liu et al. 2011; Na et al. 2008).

23.4.2 Ecgp96 Interacts with Toll-Like Receptor 2 During E. coli K1 Invasion of HBMEC

Toll-like receptors (TLRs) are pathogen recognition receptors, which play an essential role in innate immune responses against microbial pathogens (Abdelsadik and Trad 2011; Wells et al. 2011). Although a variety of TLRs has been described in literature, TLR2 and TLR4 associate with gp96 for proper folding and surface expression (Vabulas et al. 2002; Harding 2007; Saitoh and Miyake 2006; Tsan and Gao 2009; Yang et al. 2007). Lack of gp96 expression in macrophages rendered adult mice resistant to *Listeria* infection, and less responsive to TLR ligands, indicating that gp96 is crucial for TLR mediated signaling (Yang et al. 2007). The expression of gp96 on the surface of LPS stimulated B cells is critical for the

activation of Th2 cells (Banerjee et al. 2002). Furthermore, a physical interaction of gp96 and TLRs in B cells was demonstrated in a mouse model (Randow and Seed 2001). Despite the requirement of gp96 interaction with both TLR2 and TLR4, studies suggest that OmpA+ *E. coli* selectively upregulated and recruited Ecgp96/TLR2 complex to the membranes, whereas OmpA– *E. coli* induced TLR4 (Krishnan et al. 2012). Overexpression of a TIR domain-deficient, dominant-negative (DN) TLR2, inhibited the invasion, while DN-TLR4 had a marginal effect. *E. coli* K1 could not invade HBMEC expressing DN-TLR2 or Ecgp96Δ200 (C-terminal 200 amino acids deleted), indicating that the C-terminal portions of these molecules are essential for the invasion.

Furthermore, TLR2$^{-/-}$ newborn mice exhibited resistance to *E. coli* infection, while TLR4$^{-/-}$ animals became extremely sick by 48 h post-infection and succumbed by 72 h (Krishnan et al. 2012). Wild type mice infected with *E. coli* K1 normally survive for 96 h and brain sections of these animals revealed a significant number of neutrophil infiltration and signs of gliosis in the cortex. However, TLR2$^{-/-}$ mice displayed normal brain morphology similar to that of control uninfected mice, indicating that *E. coli* K1 did not cause any brain damage to these animals. In contrast, *E. coli* K1 caused greater damage in the brains TLR4$^{-/-}$ mice, similar or worse than that appeared in the control infected animals within 48 – 72 h post-infection. These findings along with gp96 knockdown experiments in newborn mice demonstrate that both TLR2 and gp96 are critical for the onset of meningitis in newborn mouse model.

23.4.3 Cellular Signaling Through Ecgp96

The binding of *E. coli* K1 to Ecgp96 via OmpA triggers a zipper-type mechanism to invade HBMEC (Prasadarao et al. 1999). This process, involves direct contact of bacteria with HBMEC membranes, which then sequentially enclose the organisms, resembles *Yersinia* and *Listeria* entry into epithelial cells (Yam and Theriot 2004; Seveau et al. 2007). The induction of a phagocytosis-like endocytic mechanism by *E. coli* K1 depends on local condensation of polymerized actin that is associated with the bacterium (Fig. 23.4). Focal adhesion kinase (FAK) plays a critical role in actin remodeling (Tomar and Schlaepfer 2009). Tyrosine phosphorylation and kinase activity of FAK increased during *E. coli* K1 invasion of HBMEC (Reddy et al. 2000a, b). Upon binding of *E. coli* K1 to HBMEC, FAK is autophosphorylated at Tyr397, which is subsequently responsible for the phosphorylation of additional tyrosine residues on FAK, including Tyr925. The autophosphorylation site of FAK (Tyr397) is necessary for association with the SH2 domain of Src family kinases and/or of the p85 subunit of PI3 kinase. Furthermore, Ecgp96 co-localizes at actin condensation sites during the invasion process, indicating that the recruiting actin filaments to the sites of bacterial entry, requires Ecgp96 signaling.

PI3-Kinase (PI3K) is a heterodimeric protein consisting of a regulatory subunit (p85) and a 110-kDa catalytic subunit (p110). The phosphotyrosine residues

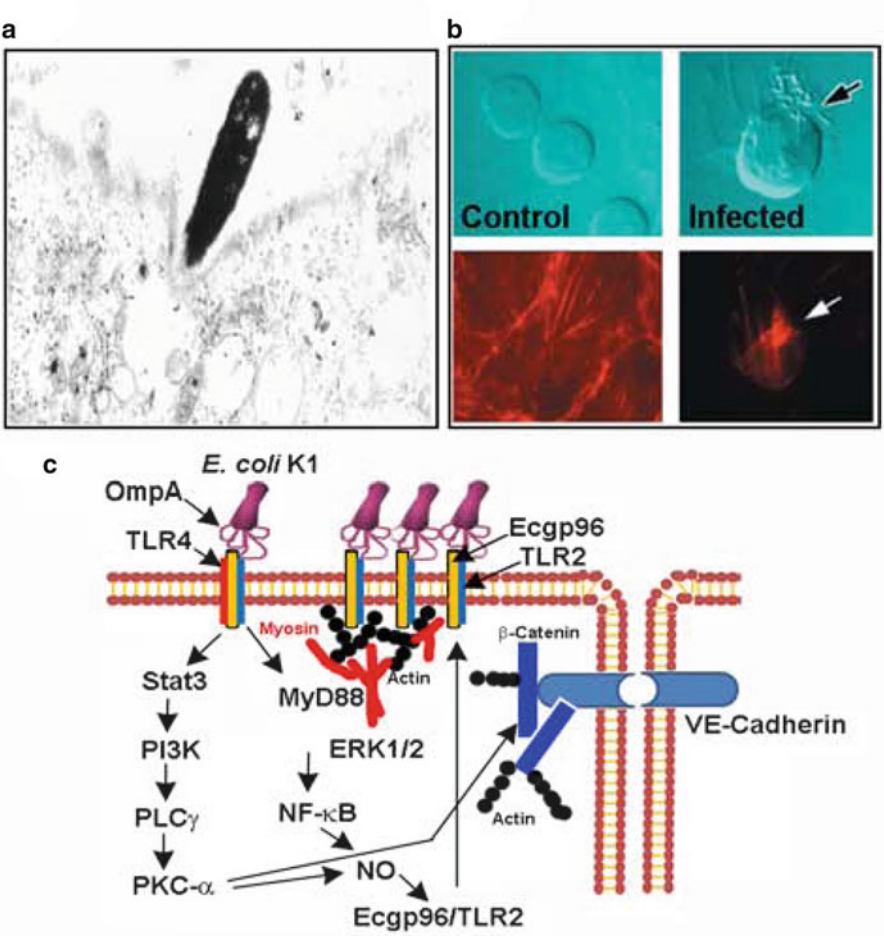

Fig. 23.4 Signaling events induced by *E. coli* K1 mediated by OmpA and Ecgp96 interaction in HBMEC. (**a**) Transmission electron micrograph showing the invasion of *E. coli* into HBMEC. (**b**) *E. coli* K1 adherence to HBMEC induced actin condensation beneath the bacterial attachment sites. Arrows indicate either bacteria or actin filament condensation. (**c**) In a resting stage, HBMEC cell surface contains Ecgp96 along with TLR2 and TLR4 as a complex, however, upon infection *E. coli* K1 induces a bipartite signals, one via Ecgp96 and second one via TLR2. Ecgp96 mediated signaling promotes PKC-α activation, which in turn regulates inducible NO production. The TLR2 mediated signaling occurs through MyD88, ERK1/2 and NF-κB, which also produces NO. These two signaling events converge at NO to enhance the complex formation of Ecgp96 and TLR2, which is then translocates to the cell surface. These additional Ecgp96/TLR2 complexes become adherence sites for more number of *E. coli* K1. Simultaneously, the activated PKC-α phosphorylates VE-cadherin at the adherence junctions to dislodge β-catenin and associated actin filaments. The released actin relocates to Ecgp96/TLR2 sites beneath the invading bacteria. Other signaling mechanisms (Not depicted in the cartoon) contribute to myosin condensation underneath the bacteria, which pulls down *E. coli* K1 into endosomes. The disruption of VE-cadherin at the adherence junctions causes leakage of the blood–brain barrier (Panels (**a**) and (**b**) were previously published in Prasadarao et al. 1999)

on tyrosine kinases such as Src and FAK interact with the SH2 domains of p85 subunit leading to activation of PI3K. Pre-treatment of HBMEC with an inhibitor of PI3K, LY294002 significantly prevented *E. coli* K1 invasion, indicating the critical role played by PI3K in the bacterial entry process (Reddy et al. 2000a, b). In agreement, *E. coli* K1 invasion activates a Ser/Thr kinase Akt, which is a downstream effector protein of PI3K. This activated PI3K associates with FAK in HBMEC infected with OmpA+ *E. coli*, not with OmpA– *E. coli*. Of note, PI3K involvement is independent of FAK in *L. monocytogenes* invasion of epithelial cells, which is strikingly different from *E. coli* K1 invasion process (Bierne et al. 2000).

PKC-α is a phospholipid-dependent serine/threonine kinase involved in major signaling events and ubiquitously present in all cells (Reyland 2009). PKC-α is autophosphorylated and translocates to the membrane upon *E. coli* K1 invasion of BMEC (Sukumaran et al. 2002). The activated PKC-α co-localizes with actin condensation points induced by invasive *E. coli* K1 at the bacterial entry site. Of note, phosphorylated PKC-α interacts with Ecgp96 to initiates the invasion process and overexpression of Ecgp96Δ200 inhibited the phosphorylation of PKC-α (Maruvada et al. 2008b). PI3K, which associates with FAK during *E. coli* K1 invasion, also converts PI (4, 5) P2 into PI (3, 4, 5) P3 at the plasma membrane, which has an affinity for PLC-γ and thus anchors it. *E. coli* K1 interaction with HBMEC induces the phosphorylation of PLC-γ at Tyr783, thereby activating and recruiting it to the membrane at bacterial attachment sites (Sukumaran et al. 2003a). These studies suggest that PLC-γ activation is critical for the influx of Ca^{2+} in HBMEC in response to OmpA+ *E. coli* infection. *E. coli* K1 invasion of HBMEC selectively increases ICAM-1 expression, which occurs only in cells invaded by the bacteria. OmpA interaction with Ecgp96 in HBMEC was also critical for the enhanced expression of ICAM-1 and was dependent on PKC-α and PI3K signaling (Selvaraj et al. 2007). Signal transducer and activator of transcription 3 (Stat3) plays a pivotal role in multiple cellular functions including actin cytoskeleton reorganization via RhoA, Rac1 and Cdc42 (Maruvada et al. 2008b). Stat3 phosphorylation and its association with Ecgp96 occur in HBMEC infected with *E. coli* K1, which phenomena are vital for activation of PI3K, PLC-γ and PKC-α.

E. coli K1 interaction with Ecgp96 induces the formation of caveolae in which the bacterium traverses the HBMEC (Sukumaran et al. 2003b). The activated PKC-α recruited to the plasma membrane upon infection also interacts with caveolin-1, a marker of caveolae. Of note, most of these signaling molecules condense beneath the *E. coli* K1 attachment sites along with actin. Therefore, a supramolecular complex formation occurs underneath the bacteria for efficient transcytosis of the pathogen. The force required to pull the bacteria present in caveolae into the cytoplasm needs the interaction of myosin and actin. Myosin filament formation and contractility is regulated by phosphorylation of myosin light chains (MLC) by Ca^{2+}-dependent MLC kinase (Rudrabhatla et al. 2006).

23.4.4 Nitric Oxide Induces Ecgp96 Expression and Tight Junction Disruption of HBMEC

Cerebral edema is a characteristic pathophysiological feature of neonatal meningitis caused by a variety of Gram-negative bacteria. Actin filaments at the periphery of endothelial cells are linked to cell-to-cell adherence junctions. Transmembrane calcium-dependent adhesive proteins (cadherins) integrated into the cell junctions connects to a complex network of cytoskeletal proteins inside the cytoplasm that, in turn, promote anchorage to actin filaments. Vascular-endothelial cadherin (VEC; cadherin-5) is an endothelium-specific cadherin that forms a complex with β-catenin via the cadherin domain (Harris and Nelson 2010). β-catenin binds to α-catenin, which in turn, interacts with actin filaments. Thus, β-catenin serves as a linker molecule to cadherin and actin. OmpA+ *E. coli* affect the HBMEC permeability by disrupting VEC and β-catenin interactions at the adherence junctions. This disassembly was totally abolished in HBMEC overexpressing a dominant negative form of PKC-α, suggesting that phosphorylation of VEC by PKC-α contributes to integrity of the adherence junctions. Of note, *E. coli* K1-induced adherence junction disruption was also prevented by treating the cells with anti-OmpA or anti-Ecgp96 antibodies, confirming that the interaction of these two molecules is critical for increased HBMEC permeability.

Nitric oxide (NO) is an essential modulator of cerebral vascular permeability (Linscheid et al. 1998; Hauser et al. 2005). Moreover, several observations suggest that NO levels increase in animal models of bacterial meningitis as well as in human patients with the disease (Azumagawa et al. 2003). NO is a pleiotropic mediator with antimicrobial capability against intracellular pathogens in the host (Bogdan 2001; Chakravortty and Hensel 2003). Experiments performed using nitric oxide synthase (NOS) knockout mice and NOS inhibitors revealed that NO is a major factor controlling the fate of pathogens by directly inhibiting the growth of various microbes *in vitro* (Chakravortty and Hensel 2003). Interestingly, OmpA+ *E. coli* induced higher levels of NO production due to efficient invasion of HBMEC compared with OmpA– *E. coli* by triggering the transcription of inducible NOS (iNOS) and to some extent of endothelial NOS (Mittal and Prasadarao 2010). The increased production of NO activates cGMP signaling in HBMEC, which in turn upregulates Ecgp96 expression at the plasma membrane. The additional Ecgp96 molecules provide OmpA+ *E. coli* extra binding sites for additional bacteria to invade. Of note, cGMP production also activates PKC-α and thereby increases HBMEC monolayer permeability by disassembling the VEC from adherence junctions.

23.4.5 Ecgp96 is Responsible for the Production of Biopterin During E. coli K1 Invasion

Vasodilation due to increased iNOS-dependent NO production during sepsis is responsible for persistent hypotension (Hauser et al. 2005). In addition, increased

levels of pterins observed in CSF of pediatric patients with bacterial meningitis indicate that NO and pterin production might have an interdependent relationship (Azumagawa et al. 2003). Tetrahydrobiopterin (BH4), a pterin analogue and an obligate co-factor for all the isoforms of NOS, primarily controls NO production (Alderton et al. 2001). GTP cyclohydrolase (GCH1, EC 3.5.4.16) is the first and rate-limiting enzyme that catalyzes the synthesis of guanosine triphosphate (GTP) for the formation of pterins (biopterin and neopterin) (Thony et al. 2000). Human GCH1 exists in its native form (~28 kDa) and assembles to a homo-decamer to catalyze GTP to pterins (Swick and Kapatos 2006). Furthermore, elevated levels of pterin are used as a diagnostic marker in infections caused by intracellular pathogens and in malignant tumors (Murr et al. 2002). *E. coli* K1 infection of HBMEC enhanced the expression of GCH1 to generate greater levels of biopterin (Shanmuganathan et al. 2013). GCH1 associates with Ecgp96 during the *E. coli* K1 invasion and, in agreement, suppression of GCH1 expression prevented bacterial entry into HBMEC. Similarly, silencing Ecgp96 expression inhibited *E. coli* K1-induced pterin synthesis by reducing GCH1 levels. DAHP (2, 4-diamino hydroxyl pyrimidine), a specific inhibitor of GCH1 blocked biopterin and NO production and, the invasion of *E. coli* K1 in HBMEC. Newborn mice pre-treated with DAHP are resistant to *E. coli* K1 meningitis substantiating the role of GCH1 and Ecgp96 interaction in the pathogenesis of this deadly disease.

23.5 Conclusions

Identification of gp96 as a receptor for not only *E. coli* K1 but also for other bacterial pathogens to bind to and/or invade host tissues indicates that these invaders utilize "danger signals" to their own advantage. Recent data obtained by studying the pathogenesis of meningitis by *E. coli* K1 point out the possibility of developing therapeutic strategies targeting gp96 against neonatal meningitis. For now, a deeper understanding of how *E. coli* K1 manipulates the function of immune cells, by binding to gp96 and for finding a niche for initial survival and the role of major virulence factors for effective establishment of the infection is vital for developing successful alternative preventative strategies to combat this pathogen that affects the newborns. Since gp96 helps TLR2 and TLR4 for proper folding and function, further studies are needed to assess whether targeting gp96 would evolve into a broad spectrum therapy. It is also essential to understand whether gp96 chaperones other important surface structures to expand the range of targets via gp96. One can envision that potent gp96 inhibitors would emerge to treat prevalent conditions other than bacterial infections, such as Parkinson's disease, Alzheimer's disease and fungal infections. Although gp96 is not a magic bullet, it will provide additional arsenal for developing combination therapies and treating difficult diseases. The probability is that we have only seen the tip of the iceberg in this expanding knowledge about the biology of gp96 in various diseases.

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Part IV
Novel Interactions of the Cell Stress Response in Infection

Chapter 24
Bacterial Cell Stress Protein ClpP: A Novel Antibiotic Target

Heike Brötz-Oesterhelt and Peter Sass

Abstract Clp proteases play important roles in maintaining the protein homeostasis in bacterial cells, particularly under stress conditions. On the one hand they perform central functions in general protein quality control by degrading mis-translated, denatured or otherwise malfunctioning proteins. On the other hand, they direct cellular differentiation and development programs by temporally and spatially precise degradation of key regulatory proteins. On top of their already diverse functions in the physiological context, an additional role was discovered over the last years for this class of protein, namely to serve as a target for antibiotic action. Especially the conserved proteolytic core component of the protease machinery, designated ClpP, is important here. ClpP is capable of killing bacteria via uncontrolled proteolysis after deregulation by a potent class of novel antibiotics.

24.1 Composition and Operation of the Clp Protease Machinery

ClpP is conserved and widespread in eubacteria and was detected in all eubacterial genomes sequenced to date except for mollicutes (Yu and Houry 2007). It is also present in the apicoplast of *Plasmodium falciparum*, reminiscent of the cyanobacterial origin of this specialized organelle (Lin et al. 2009; Rathore et al. 2010), as well as in mitochondria and plastids of plants (Yu and Houry 2007). Although archaea and the eukaryotic cytoplasm lack a ClpP homologue, they possess, with the 20S

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core particle of the proteasome, a structure of analogous architecture and function (Maupin-Furlow 2012).

Crystal structures were solved for a variety of ClpP orthologues, including the bacterial species *Escherichia coli*, *Bacillus subtilis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* as well as the human mitochondrial ClpP, and revealed a conserved general organisation (for exemplary structures see Geiger et al. 2011; Gribun et al. 2005; Ingvarsson et al. 2007; Kang et al. 2004; Lee et al. 2011; Wang et al. 1997; Zeiler et al. 2011). ClpP is a cylindrical self-compartmentalized serine protease, composed of 14, in most cases identical, subunits. Two heptameric rings of seven subunits each stack face-to-face to form a barrel shaped structure roughly 90 Å in both height and diameter. The interior of the barrel holds a spacious chamber approximately 50 Å wide. Fourteen catalytic triads with the canonical residues typical for serine proteases (Ser, His, Asp) reside within this chamber, effectively shielded from potential protein substrates by the secluded compartment (Fig. 24.1a). Access to the proteolytic chamber is regulated by small pores in the apical and distal (top and bottom) surfaces of ClpP (Fig. 24.1b). All ClpP monomers investigated to date possess a rather compact body ("head region") and a prominent protruding α/β unit ("handle region") (Fig. 24.1c). The head regions establish mostly hydrophobic contacts among each other and build up the heptameric ring. The seven handles of one 7-mer intercalate with the seven handles of the opposite ring to form the tetradecamer. In the active 14-mer, the handle-regions of the two vis-à-vis members of the two rings are connected via hydrogen bonds and recent structural studies suggest that the joining of the two rings induces conformational changes within the catalytic triad that are a prerequisite for enzymatic activation (Geiger et al. 2011; Kimber et al. 2010; Lee et al. 2011). This activation process is a safety measure to ensure that the active catalytic triads have no access to the cytoplasm, because ClpP by itself is almost free of substrate specificity. Any protein substrate that enters the proteolytic chamber is processed to short peptides (reviewed in Baker and Sauer 2012). Thus, in the case of ClpP, proteolysis is regulated via restricted access of substrates to the proteolytic chamber and not via sequence preference of the active sites as such. As outlined above, the axial entrance pores to the proteolytic chamber are small. In some crystal structures a well resolved axial channel could be detected, in other structures this area was less well defined. However, as a general feature, the diameter of the entrance pores was too small to allow for the entry of proteins. Accordingly, in biochemical studies, purified *E. coli* ClpP did only manage to degrade small peptides, which can readily diffuse through the pores, but it was inactive with polypeptides or protein substrates (Thompson and Maurizi 1994). Consequently, ClpP by itself is no more than a peptidase and should be perceived as the dormant core of a larger proteolytic machinery.

For the degradation of proteins, Clp-ATPases of the Hsp100 family act in concert with ClpP (Fig. 24.1d). Many species possess more than one Clp-ATPase; for instance, *E. coli* expresses ClpA and ClpX, and *B. subtilis* harbors ClpX, ClpC, and ClpE (reviewed in Kirstein et al. 2009b). One function of the Clp-ATPases is to

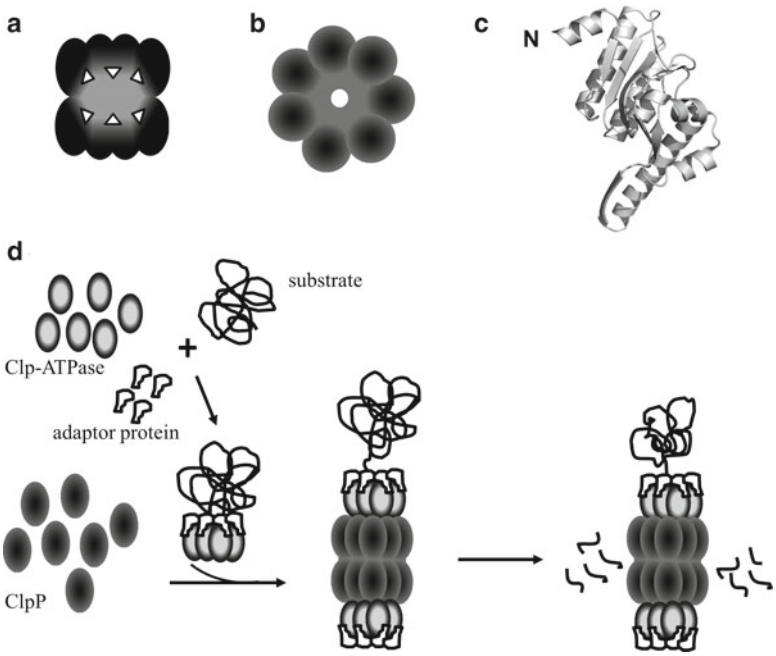

Fig. 24.1 Architecture and function of the Clp protease system. (**a**) Cross section of a barrel shaped ClpP 14-mer revealing the inner proteolytic chamber that accommodates the catalytic triads (*open triangles*) of the Clp proteolytic core unit. (**b**) Top view of the ClpP 14-mer showing the small entrance pore to the proteolytic chamber that restricts access to the catalytic triads. (**c**) Crystal structure of a *Bacillus subtilis* ClpP monomer (Adapted from Alexopoulos et al. 2012). (**d**) Concerted function of ClpP with its associated Clp-ATPases and substrate-specific adaptors for the controlled degradation of substrate proteins. Clp-ATPases (often supported by adaptor proteins) recognize and bind substrates and transport them to ClpP. In addition, they unfold the substrates and thread them through the entrance pores of the ClpP barrel to be cleaved inside the proteolytic chamber

recognise protein substrates that should be degraded by ClpP, to bring them to ClpP, to unfold them using energy generated by ATP-hydrolysis, and to thread them into the entrance pores of the ClpP barrel. As different Clp-ATPases prefer different substrates, this is a first means of achieving substrate specificity. A second regulating level is established by the so-called adaptor proteins, which raise the affinity of particular Clp-ATPases for specific substrates (reviewed in Kirstein et al. 2009b). Clp-ATPases do also form oligomers, but, interestingly, their rings are only hexameric (Baker and Sauer 2012; Kirstein et al. 2009b). By stacking to the apical and/or the distal surface of ClpP they generate a symmetry mismatch. As form follows function in nature, it can be assumed that this asymmetry has evolved for a particular reason, which is, however, not understood so far. Contacts between the Clp-ATPases and ClpP are established at two prominent sites. One important interaction is mediated by loops that protrude from each Clp-ATPase monomer (Kim and Kim

2003) and which possess a conserved tripeptide at the tip (i.e. IGF in *E. coli* ClpX or IGL in *E. coli* ClpA). Mutation studies and investigations using the antibiotics of the ADEP class (see below) indicate that these Clp-ATPase loops insert into large peripheral grooves in the apical and distal surfaces of ClpP (reviewed in Baker and Sauer 2012; Yu and Houry 2007). In addition, axial contacts occur close to the central entrance pores between a pore-2 loop of the Clp-ATPase and N-terminal stem-loop of ClpP. The fact that the ClpP/Clp-ATPase interactions are mediated through flexible loops and receiving loops or grooves, rather than through contacts between larger surface regions, is probably a prerequisite for building a functional machinery in the light of the described symmetry mismatch.

24.2 Functions of the Clp Proteases in the Physiological Context

The functions of Clp proteases in bacteria are manifold. Of note, the term Clp proteases is meant here as ClpP in concert with its diverse Clp-ATPases and adaptor proteins. The second Clp protease system in eubacteria consisting of the threonine protease ClpQ in concert with its ATPase ClpY (Ramachandran et al. 2002) is not a subject of this review.

When misfolded or aggregated proteins appear in the bacterial cell, e.g. under stress conditions, Clp-ATPases, which can also act independently as chaperones, work on their refolding together with other chaperone systems. If unsuccessful, the damaged protein is destined for degradation by different proteases, among them ClpP. Likewise, if ribosomes stall during translation in *E. coli*, the 11 amino acid SsrA-tag is attached to the C-terminus marking the defective protein for degradation by the ClpXP pair (Gottesman et al. 1998). In proteomic studies more than 100 intracellular substrates and five classes of degradation tags (synonym "degrons") were identified for *E. coli* ClpXP, two at the C- and three at the N-terminus (Baker and Sauer 2012; Flynn et al. 2003; Neher et al. 2006). Among these tags were also signal sequences for protein secretion, marking the substrate as an extracellular protein that went astray in the cytoplasm. Some substrates require a preceding cleavage event, before the degradation signal is exposed (Flynn et al. 2004; Neher et al. 2003) and adaptor proteins increase the affinity of Clp-ATPases for particular tags. For many bacterial species, deletion or inactivation of *clpP* results in severe phenotypes or even growth inhibition under stress conditions, demonstrating the important general function of the Clp proteases in ensuring correct functioning of proteins. In addition, Clp proteases have a variety of regulatory functions based on the proteolysis of key elements such as transcription factors. For instance, in *B. subtilis* they regulate motility, spore formation, exoenzyme synthesis and the development of genetic competence (Msadek et al. 1998; Pummi et al. 2002; Turgay et al. 1998). In *Caulobacter crescentus* they play a role in cell cycle regulation (Jenal and Fuchs 1998) and in *E. coli* they control e.g. the level of RecN and thereby

the duration of the SOS response (Neher et al. 2006). In *M. tuberculosis* and actinomycetes, ClpP is even essential for growth under moderate conditions *in vitro* (Gominet et al. 2011; Raju et al. 2012; Sassetti et al. 2003).

Clp proteases can also play important roles in eukaryotic cells. ClpP is indispensable for growth of *P. falciparum*, due to its essential function in the development of the apicoplast, a specialized organelle, which the parasites require for survival (Rathore et al. 2010). In addition, we are only beginning to understand the functions of the large number of different ClpP paralogues in plant cells. For instance, the model plant *Arabidopsis thaliana* contains no less than 10 ClpP-like proteins and the same number of Clp-ATPases (reviewed in Yu and Houry 2007). Some of these ClpP paralogues, designated ClpR, have inactive catalytic triads and are assumed to perform regulatory functions. The structure of the ClpP tetradecamer in the chloroplast stroma of *A. thaliana* was solved and contained the subunits ClpP1, ClpP3, ClpP4, ClpP5, ClpP6, ClpR1, ClpR2, ClpR3 and ClpR4 (Peltier et al. 2004). It is discussed that this diverse assortment of ClpP proteins helps plants to cope with the various types of environmental stress associated with their stationary lifestyle (Yu and Houry 2007).

24.3 ClpP and Clp-ATPase as Antibiotic Targets

The following paragraph shall present the unexpected discovery that ClpP can serve as an antibiotic target, meaning that binding of an antibacterial agent to this target prevents bacterial growth under moderate growth conditions *in vitro* (e.g. free bacteria cultivated on Petri dishes or in culture flasks in full media at 37 °C). This kind of discovery was not expected for ClpP, which is not essential for *in vitro* growth of most bacterial species under moderate conditions and cannot be explained by a simple inhibition of the physiological functions of ClpP. Rather, the antibacterial agents in question, designated “ADEPs” for acyldepsipeptides, use ClpP in a complex and elaborate fashion in order to bring about bacterial death.

The ADEP class of antibiotics goes back to the natural product complex 54556A that is produced by *Streptomyces hawaiiensis* NRRL 15010 and was already described in the early 1980s in a patent (Michel and Kastner 1985). However, this original report contained only the proposed structure of the natural products and a basic description of their antibacterial activity *in vitro*. Thereafter, the compounds were not further explored for 15 years, until researchers of Bayer HealthCare revisited them in an attempt to re-evaluate known but underexplored antibiotics. They revised the reported structures, established a modular and versatile route for total synthesis (Hinzen et al. 2006), and initiated a broad chemistry program, in the course of which many liabilities of the natural products were overcome. Even the most active natural product ADEP1 (Fig. 24.2) displayed only moderate activity against *S. aureus* and was not effective in rodent models of bacterial infection, due to chemical as well as metabolic instability. In contrast, improved synthetic

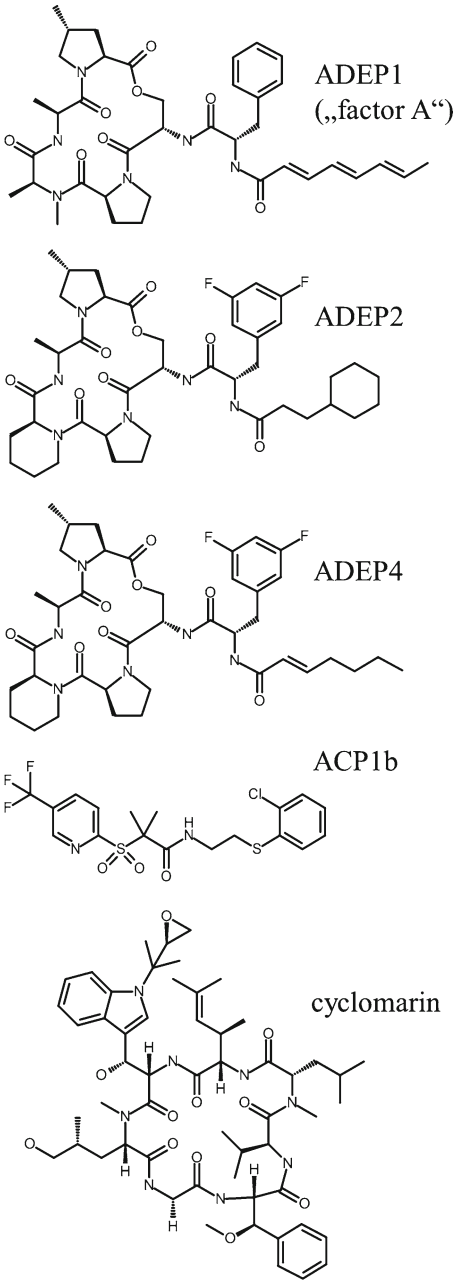

Fig. 24.2 Chemical structures of antibacterial agents that act by dysregulating the Clp protease

derivatives such as ADEP2 and 4 (Fig. 24.2) demonstrated impressive minimal inhibitory concentrations in the sub-µg/ml range against multi-resistant clinical isolates of staphylococci, streptococci and enterococci as well as an *in vivo* efficacy that surpassed that of the marketed antibiotic linezolid in mice (Brötz-Oesterhelt et al. 2005).

Another focus of the discovery team was the elucidation of the mechanism of antibacterial action of the ADEP class. The lack of cross-resistance to marketed antibiotics had already raised some hope of finding a new target, but its discovery was no easy task. Several attempts to search for the target in one of the classical metabolic pathways failed. ADEPs inhibited neither RNA nor DNA nor protein nor peptidoglycan nor fatty acid biosynthesis. Finally, the target was identified via two independent approaches. Selection of ADEP-resistant laboratory mutants revealed mutations in ClpP and affinity chromatography using immobilized ADEP trapped ClpP selectively (Brötz-Oesterhelt et al. 2005). In depth biochemical studies demonstrated that ADEPs deregulate ClpP in a multi-layered fashion (Kirstein et al. 2009a) and X-ray crystallography efforts by two independent groups yielded structures of ADEP in complex with ClpP from *B. subtilis* as well as from *E. coli* (Lee et al. 2010; Li et al. 2010), which provided explanations for the observed phenomena. It turned out that ADEPs bind to ClpP in a 1:1 stoichiometry (Fig. 24.3a), resulting in 14 ADEP molecules per ClpP barrel. The binding site is located at the outer rim of the apical and distal surfaces of ClpP and the hydrophobic cavity, where each ADEP is located, is formed by two adjacent ClpP monomers. Each ADEP molecule establishes contacts to both neighbouring subunits, thereby helping to stabilize the oligomeric ring structure. Indeed, *B. subtilis* ClpP is converted from a monomeric to a tetradecameric state in the presence of ADEP and some genetically engineered ClpP mutants with oligomerisation defects can still form 14-mers after ADEP addition (Lee et al. 2010). However, the ClpP barrels that assemble in the presence of ADEPs are no longer functional in the physiological sense (Fig. 24.3b). Interaction with all Clp-ATPases investigated to date is abolished and digestion of known natural ClpP substrates is prevented (Kirstein et al. 2009a). ADEPs were even shown to disassemble a preformed complex of ClpCP/MecA, with MecA being an adaptor for ClpC (Kirstein et al. 2009a). Instead, ADEP binding bestows independent proteolytic activity to ClpP (Fig. 24.3b). The isolated ClpP core that is normally incapable of protein degradation can now digest casein and other proteins *in vitro*, with the common feature that these non-natural substrates are either flexible, unstructured or have unstructured regions (Brötz-Oesterhelt et al. 2005; Kirstein et al. 2009a; Leung et al. 2011). The ADEP-activated ClpP core is also able to degrade nascent polypeptides in the course of translation (Kirstein et al. 2009a). The X-ray structures revealed that ADEP-binding leads to major conformational changes in the N-terminal segment of each ClpP subunit. As the N-termini line the pore, this movement enlarges the entrance pores (Fig. 24.3c), which are now wide enough to accommodate poorly structured regions of non-native substrates, but not stably folded proteins. Such unspecific destructive action of ADEP-activated ClpP towards a variety of unstructured protein regions is already a plausible explanation for the antibacterial activity of the ADEPs and most probably contributes to bacterial killing at higher ADEP-concentrations. However, follow-up studies showed that it is neither the sole nor the primary reason for bacterial death. At compound levels close to the minimal inhibitory concentration, ADEP-treated bacteria continue to produce biomass, but fail to divide, suggesting that ADEP-activated ClpP might have a preferential substrate related to cell division. Indeed, the essential cell

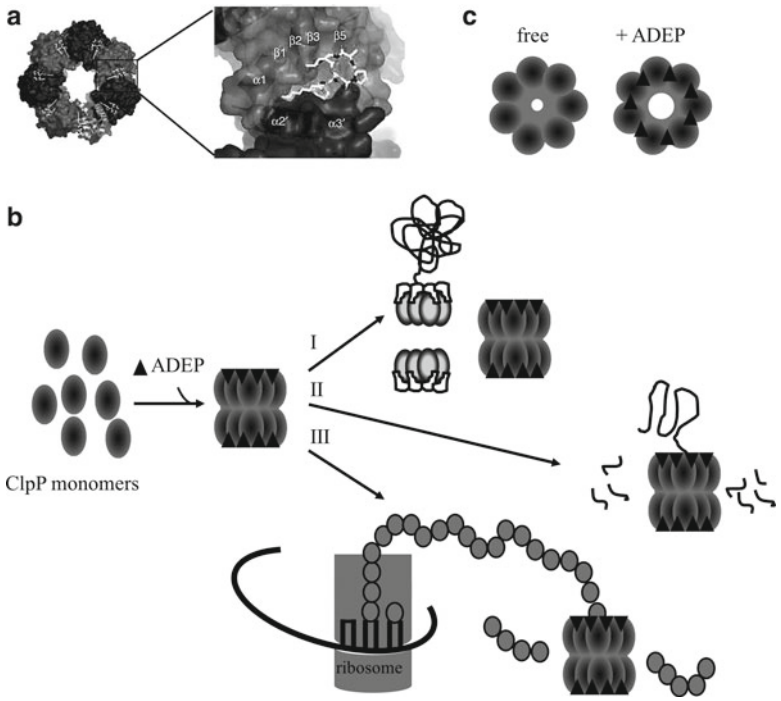

Fig. 24.3 Dysregulation of ClpP as a mechanism of antibiotic action. (**a**) Top view of the ClpP 14-mer where antibiotic acyldepsipeptides (ADEPs) bind to the interface of two adjacent ClpP subunits in a 1:1 stoichiometry. (**b**) Model of the ADEP mechanism of action. ADEPs alter the physiological functions of ClpP in a multifaceted manner. ADEPs not only abrogate the interaction of associated Clp-ATPases with ClpP and therefore inhibit all natural functions of the Clp protease system in the cell (*I*); they even bestow independent proteolytic activity to ClpP in the absence of Clp-ATPases to allow the degradation of flexible or unstructured proteins (*II*) as well as nascent polypeptides at the ribosome (*III*). (**c**) ADEP binding results in a conformational shift in the N-terminal region of ClpP that leads to opening of the entrance pore to the proteolytic chamber of the ClpP barrel (*top view*)

division protein FtsZ was shown to be rapidly and easily degraded by ADEP-activated ClpP *in vitro* as well as in ADEP-treated cells (Sass et al. 2011), explaining the phenotype of cell division inhibition and adding another level of complexity to the mechanism of action of this novel antibacterial class.

This new and fascinating mechanism of action motivated independent research groups to explore the potential of the ADEPs class further. Socha and coworkers described a structural modification that slightly improved the already impressive activity of previous ADEP-derivatives against enterococci (Socha et al. 2010) and Ollinger and colleagues reported some, albeit weak, antibacterial activity against *M. tuberculosis* (Ollinger et al. 2012). Another group performed a screening campaign to search for structurally unrelated compounds with the same mechanism of action. This effort yielded ACP1b (Fig. 24.2) as the most advanced structure that

deregulated ClpP in the same way as ADEPs. However, ACP1b was still substantially less potent than ADEP1 and lacked specificity due to unidentified off-target effects (Leung et al. 2011).

Although the ADEPs were the first class of antibiotics shown to kill bacteria by deregulating and over-activating rather than inhibiting its target, they are no longer the only one. The natural product cyclomarin (Fig. 24.2) is a potent antitubercular agent that is bactericidal against *M. tuberculosis* replicating in culture broth as well as in macrophages. Cyclomarin was recently reported to bind to the mycobacterial ATPase ClpC1 and to stimulate the proteolytic activity of the ClpC1/ClpP complex (Schmitt et al. 2011).

24.4 Conclusions

In the light of the worldwide increasing prevalence of bacterial resistance to diverse antibiotic agents with different modes of action, antibacterial drug discovery is more important than ever. Generally, two major strategies are currently followed to cope with the menace of antimicrobial resistance, one by adding new properties to well-known antibiotics that help to overcome already established resistance mechanisms to these compound classes, and another by searching for drugs that act by new mechanisms and targets that are unrelated to those currently used and therefore insensitive to already established bacterial resistance mechanisms. In the past, both strategies exclusively aimed at the inhibition of targets to reduce growth of or even kill bacteria. In recent years, the bacterial ClpP protease has emerged as interesting new drug target, and besides the promising antibacterial activity of the corresponding inhibitors/activators, these studies clearly indicate that there are more ways to counteract bacterial infections than just by simple inhibition of an essential bacterial protein or process. In fact, targeting the bacterial protease ClpP as described here allows an interference with the normal bacterial lifestyle in various manners. As ADEPs do not only inhibit the natural functions of ClpP but exert effective antimicrobial activity through over activation of their target, they revealed new options in antibacterial drug discovery. Hence, ADEPs are the forerunners to bring this innovative concept of target over activation for antimicrobial activity to our attention and the activity of cyclomarins suggests that nature has used this principle more often and in different ways.

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Chapter 25
Host Neuroendocrine Stress Hormones Driving Bacterial Behaviour and Virulence

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Abstract In recent years there have been striking advances in our understanding of not only intra-bacterial communication but also the existence of inter-kingdom crosstalk between bacterial pathogens and their eukaryotic hosts. The intimate co-evolution of host and bacterial chemical communication systems appears to have generated an array of specialised signalling molecules which enable this dialogue. Bacterial pathogens are able to eavesdrop on their host and constantly adjust their metabolic and virulence status in order to survive and cause disease. Host neuroendocrine stress hormones like adrenaline and noradrenaline play a key role in modulating the response of many pathogens during host infection. The huge benefits of unravelling such a complex interplay in inter-kingdom signalling merits additional efforts in reaching the ultimate goal of decoding and interfering with the dialect of hormones.

25.1 Introduction

Bacterial pathogens use an array of molecular sensors to perceive and facilitate adaptation to changes in their environment. Mechanisms which allow bacterial pathogens to eavesdrop on mammalian host signalling systems such as neuroendocrine (NE) stress hormones may aid towards their successful adaptation and survival within the host (Pacheco and Sperandio 2009). Upon entering the host, pathogens come in contact with a wide range of chemical signals including the NE stress hormone noradrenaline which is abundant in the gut as well as adrenaline which is

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found mostly in the bloodstream (Aneman et al. 1996; Eisenhofer et al. 1996; Furness 2000). Interestingly, bacterial lipopolysaccharide has the ability to signal the formation of adrenaline and noradrenaline by macrophages in the bloodstream (Flierl et al. 2007, 2009). It was therefore suggested that the phagocytic system represents a diffusely expressed adrenergic organ (Flierl et al. 2007, 2009).

Faced with such a wide repertoire of host signals and environments, bacteria employ collective decision making, synchronizing their responses efficiently, in order to circumvent host defences and survive. Successful pathogens have, therefore, evolved a communication protocol which employs producing and sensing the concentration of autoinducer molecules in a process called quorum sensing (QS) (Pacheco and Sperandio 2009; Williams 2007). Following detection of the autoinducer, bacteria coordinate their gene expression in such a way as to behave in a "multicellular" fashion. Thus, organised bacterial attack against host defences ensures maximum chances of survival. More recently, a bacterial autoinducer, AI-3, was shown to cross talk with the host NE stress hormones adrenaline and noradrenaline (Sperandio et al. 2003).

Host-pathogen interactions are extremely important in determining the overall outcome of an infection. There is increasing evidence to suggest that bacteria can sense host NE stress hormones such as adrenaline and noradrenaline to modulate their virulence (Karavolos et al. 2008b, 2011a, b; Pacheco and Sperandio 2009; Spencer et al. 2010). In this chapter we will present the latest evidence supporting this inter-Kingdom signalling hypothesis and discuss aspects of the newly evolving concepts of bacterial-host communication.

25.2 Autoinducers and Hormones

In Gram-negative bacteria QS is usually mediated by *N*-acylhomoserine lactones (AHLs), 2-alkyl-4-quinolones (AQs) and furanones such as autoinducer-2 (AI-2) (Bassler et al. 1994; Winson et al. 1995). QS presents an advantage to the bacteria in terms of allowing coordinated expression of mechanisms aiding bacterial survival whilst simultaneously modulating metabolic fitness (Fuqua and Greenberg 1998; Miller and Bassler 2001; Winzer et al. 2002, 2003; Winzer and Williams 2001).

AHLs represent a class of freely diffusible autoinducers produced solely by Gram-negative bacteria. AHLs mediate signalling via critical concentration-mediated activation of LuxR family transcriptional regulators via the LuxNUO signal transduction system (Taga and Bassler 2003; Xavier and Bassler 2003). In Gram-positive bacteria, the autoinducers are actively secreted, post-translationally modified, autoinducing peptides resulting from the cleavage of larger precursors. Signalling is achieved by interaction with membrane receptors using the classical two-component signal transduction system, or intracellularly following internalisation by oligopeptides permeases (Dunny and Leonard 1997; Lazazzera 2000; Lyon and Novick 2004; Schauder and Bassler 2001).

AI-2 is a signal molecule deriving from rearrangement of 4,5-dihydroxy-2,3-pentanedione (DPD) which is itself a by-product in a reaction catalysed by the enzyme LuxS (Surette et al. 1999). It has been suggested that different bacteria use a variety of rearranged forms of DPD as AI-2 (Xavier and Bassler 2005). For example, in *Vibrio harveyi* AI-2 is a furanosyl borate diester (Chen et al. 2002), while *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) produces a different form of AI-2, (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF), lacking boron (Miller et al. 2004). In *S*. Typhimurium, AI-2 is thought to freely diffuse and accumulate extracellularly where upon reaching a critical concentration it is internalised and activated by phosphorylation via the Lsr system (Xavier and Bassler 2005). LuxS-dependent AI-2 activity has been detected in spent culture supernatants of a wide variety of bacteria leading to the hypothesis that AI-2 represents a "universal" bacterial communication signal (Miller and Bassler 2001; Xavier and Bassler 2005). In a number of species studied so far LuxS and AI-2 have been shown to affect the regulation of genes encoding a wide variety of virulence factors, motility, cell division, antibiotic production, biofilm formation, and carbohydrate metabolism (Sircili et al. 2004; Vendeville et al. 2005; Xavier and Bassler 2003, 2005). In *S*. Typhimurium, AI-2 only affects the expression of the Lsr system involved in its own uptake (De Keersmaecker et al. 2005; Taga et al. 2003; Xavier and Bassler 2003). LuxS exhibits quorum sensing-independent activity in *S*. Typhimurium by modulating flagellar phase variation to favour expression of the more immunogenic phase-1 flagellin (Karavolos et al. 2008a).

The LuxS enzyme, due to its pleiotropic effects on bacterial metabolism, has also been indirectly implicated in the production of another autoinducer (AI-3) in *Escherichia coli* (Sperandio et al. 2003; Walters et al. 2006). In enterohemorrhagic *Escherichia coli* (EHEC), AI-3 acts synergistically with adrenaline and noradrenaline to regulate motility and virulence via the two-component signal transduction systems QseBC and QseEF (Pacheco and Sperandio 2009; Rasko et al. 2008). In spite of AI-3 being described almost 10 years ago, its structure is still unknown, and its role in quorum sensing is currently under investigation.

25.3 Role of Host Neuroendocrine Stress Hormones in Bacterial Growth and Virulence

The mammalian endocrine system represents a very fine and sensitive tool that synchronises the responses of host cells to a vast array of internal or external signals. Many forms of stress have profound effects on the functions of the gastrointestinal tract (Elenkov and Chrousos 2006). The intestinal mucosa, therefore, with its large commensal bacterial population, represents a highly interactive niche where bacteria-host communications can potentially thrive (Freestone et al. 2008; Lyte et al. 2011).

NE stress hormones have been shown to influence the growth of bacteria (Freestone et al. 2007, 2008). Indeed, recently it was demonstrated that NE stress hormones, can contribute to the ability of Gram-negative pathogens to replicate in iron-restrictive media that may reflect the conditions of the gastrointestinal tract (Freestone et al. 2007). This action of NE stress hormones is related to their natural ability to remove iron from mammalian iron-sequestering proteins like transferrin and lactoferrin and hence make it available for bacteria to use in growth-related processes (Freestone et al. 2000; Sandrini et al. 2010). NE stress hormones improve growth of coagulase-negative *Staphylococci* but have no significant effect on the growth of the important Gram-positive pathogen *Staphylococcus aureus* (Beasley et al. 2011; Neal et al. 2001).

While there has been extensive coverage in the literature of the ability of NE stress hormones to affect bacterial growth, this is by no means the only effect of NE stress hormone exposure on bacterial physiology and ability to cause disease. In enterotoxigenic *Escherichia coli*, NE stress hormones influence the expression of the virulence-associated K99 pilus adhesin (Lyte et al. 1997). Additionally, NE stress hormones affect expression of Shiga-like toxins produced by *Escherichia coli* O157:H7 (Lyte et al. 1996). Bacterial exposure to NE stress hormones increases the adherence of EHEC to bovine intestinal mucosa (Vlisidou et al. 2004) and upregulates Type 3 secretion in *Vibrio parahaemolyticus* (Nakano et al. 2007). Exposure of *Campylobacter jejuni* to NE stress hormones increases invasion of epithelial cells and breakdown of epithelial tight junctions (Cogan et al. 2007). Furthermore, there is evidence to suggest that *Borrelia burgdorferi* may intercept host NE stress hormone signalling to modulate its virulence (Scheckelhoff et al. 2007). Most recently, exposure of *Porphyromonas gingivalis* to NE stress hormones increased expression of the protease arg-gingipainB, a major virulence factor (Saito et al. 2011). These observations put forward a possible role of NE stress hormones in the establishment of infection via the induction of bacterial virulence factors.

In *S*. Typhimurium NE stress hormones have been reported to affect motility (Bearson and Bearson 2008; Moreira et al. 2010) and Type 3 secretion (Moreira et al. 2010). These observations have introduced a measure of controversy in the field since other groups have been unable to replicate such findings (Karavolos et al. 2008b, 2011a; Pullinger et al. 2010). It is possible that these observations may constitute an indirect effect of the natural ability of NE stress hormones to provide iron to the cells and hence affect motility and Type 3 secretion (Bearson et al. 2010; Ellermeier and Slauch 2008; Teixidó et al. 2011; Troxell et al. 2011).

The major feature of the *S.* Typhimurium adrenaline response is the upregulation of genes involved in metal homeostasis and oxidative stress (Karavolos et al. 2008b). When *S*. Typhimurium, is exposed to NE stress hormones there is an induction of key metal transport systems within 30 min of treatment (Karavolos et al. 2008b). The oxidative stress responses employing manganese internalisation were also elicited. Cells lacking the key oxidative stress regulator OxyR showed reduced survival in the presence of adrenaline and complete restoration of growth upon addition of manganese. Hence, through iron transport, adrenaline may affect the oxidative stress balance of the bacteria requiring OxyR for physiological growth. Adrenaline

sensing may therefore provide an environmental cue for the induction of the *Salmonella* stress response in anticipation of imminent host-derived oxidative stress.

Furthermore, a significant reduction in the expression of the *pmrHFIJKLM* antimicrobial peptide resistance operon reduced the ability of *Salmonella* to survive polymyxin B following addition of adrenaline (Karavolos et al. 2008b). Resistance to antimicrobial peptides has been shown to contribute to persistence of *S*. Typhimurium in a variety of niches ranging from the phagosomes within macrophages to the *C. elegans* intestine (Alegado and Tan 2000; Prost et al. 2007). In *S*. Typhimurium, the *pmr* locus is under the control of the BasSR two component system (Gunn et al. 1998; Hagiwara et al. 2004). According to the above, we have a direct reduction of bacterial antimicrobial peptide resistance by a mammalian hormone and hence a novel "antibacterial" role for adrenaline. However, we note that *Salmonella* may have adapted to this negative effect of adrenaline within mammalian hosts by increasing lipid A deacylation and palmitoylation, thus favouring survival via reduced TLR-4 receptor-based bacterial signalling (Kawasaki et al. 2004b, 2005). Systemic or macrophage produced adrenaline may therefore regulate the fine balance between the host and *Salmonella* defence mechanisms, and impact upon the development of disease.

To add to the above observations, treatment of *S*. Typhimurium with NE stress hormones reduces its resistance to the peptide cathelicidin LL-37, a human antimicrobial peptide. LL-37 is produced in the gastrointestinal tract, bone marrow and macrophages, and has antimicrobial activity against many Gram-positive and Gram-negative bacteria (Bals et al. 1998). A NE stress hormone-mediated increase in sensitivity to LL-37 may act as a host defence system to combat infection, suggesting that bacterial sensing of stress hormones may be a double-edged sword: although bacteria can sense and exploit these molecules, the host can use the same signals to manipulate the bacteria (Spencer et al. 2010).

Exposure of *S*. Typhimurium to NE stress hormones also affects expression of *virK* and *mig14*, two genes involved in survival and persistence within the host. Genetic deletion of either gene reduces the virulence of *S*. Typhimurium in a mouse infection model, and also reduces survival in macrophages, signifying a possible role in the late stages of infection (Brodsky et al. 2005; Detweiler et al. 2003). The down-regulation of *mig14* by NE stress hormones may hence reduce levels of persistent infection and promote clearance of bacteria (Spencer et al. 2010). All the above paradigms highlight the dual role of NE stress hormones in mediating host-bacterial interactions.

Salmonella enterica serovar Typhi (*S*. Typhi) is an exclusively human pathogen causing typhoid fever which is physiologically non-haemolytic (Huang and DuPont 2005). Exposure of *S*. Typhi to NE stress hormones marks a significantly increase in haemolytic activity (Karavolos et al. 2011a, b). The haemolytic response is specific to outer membrane vesicles containing the haemolysin HlyE. Mechanistically, NE stress hormones interact with the CpxAR putative adrenergic sensory system to downregulate outer membrane protein A (OmpA) levels via upregulation of the sRNA *micA*. Reduced OmpA levels increase outer membrane vesicle shedding and hence haemolysis via increased release of HlyE (Karavolos et al. 2011a, b).

The significantly increased ability of this important systemic pathogen to produce haemolysis upon interaction with NE stress hormones, may ultimately aid in enhancing its host invasiveness and survival.

25.4 Signaling Through Bacterial Adrenergic Receptors

In EHEC adrenaline and noradrenaline can substitute for the bacterial autoinducer AI-3 implying the existence of cross talk between the two signalling systems (Sperandio et al. 2003). This observation raised the possibility of the presence of adrenergic receptors in bacteria (Sperandio et al. 2003).

Indeed, the sensor kinase QseC is autophosphorylated on binding either adrenaline or noradrenaline, demonstrating the existence of adrenergic receptors in bacteria (Clarke et al. 2006). Furthermore, these adrenergic responses can be inhibited by mammalian α- and β-adrenergic antagonists like phentolamine and propranolol. Remarkably, there is strong specificity in the antagonistic effect with QseC only being blocked by phentolamine (Clarke and Sperandio 2005). In *Escherichia coli* O157:H7 and *Salmonella* the QseBC system has been proposed as the adrenergic receptor. However, new emerging evidence supports the existence of alternative adrenergic receptors. For example, in *S*. Typhimurium it has been demonstrated that QseBC is not required for norepinephrine-enhanced enteritis or intestinal colonisation in calves (Pullinger et al. 2010).

In another example of adrenergic receptor antagonist inhibition, increased expression of *virK* and *mig14* in *S*. Typhimurium was reversed by the addition of the β-adrenergic antagonist propranolol. Some adrenergic phenotypes in bacteria are associated with altered iron uptake via the siderophore enterobactin (Burton et al. 2002; Freestone et al. 2003). A *tonB* mutant, defective in siderophore uptake, showed the same differential gene regulation upon exposure to NE stress hormones as the parent strain, suggesting that the adrenergic regulation is mediated through a mechanism independent of TonB. Furthermore, in *S*. Typhimurium, QseBC does not mediate the adrenergic signalling cascade leading to increased sensitivity to the antimicrobial peptide LL-37 (Spencer et al. 2010). Hence, it is likely that a different putative adrenergic receptor is involved in this response.

Additionally, the adrenaline-induced reduction in the ability of *Salmonella* to resist polymyxin B was fully reversible by the β-adrenergic blocker propranolol. This effect was dependent on the BasSR two component signal transduction system which is the likely putative adrenaline sensor mediating the antimicrobial peptide response (Karavolos et al. 2008b). Adrenaline may, therefore, exert its effect on the *pmr* locus of S. Typhimurium via the reversible interaction of the β-adrenergic blocker with the BasS membrane sensor in a manner similar to the interaction of adrenaline with QseC in *E. coli*. The low (31 %) amino acid sequence identity between BasS and QseC may provide a clue as to why we observe β-blockage in *Salmonella* as opposed to α-blockage in *E. coli*.

The physiological phenotypes of NE stress hormones described above are not linked with QseBC or QseEF signalling (Karavolos et al. 2008b, 2011a, b; Spencer et al. 2010). This may reflect differences in pathogenesis between *S*. Typhimurium, an invasive pathogen infecting macrophages and epithelial cells and *E. coli*, a mainly non-invasive pathogen which remains in the host intestine. The significant divergence in niches occupied by these two pathogens requires different gene expression patterns for maximum infection efficiency; hence NE stress hormones may modulate different genetic pathways to the advantage or disadvantage of the pathogen.

The inhibition of NE stress hormone-mediated haemolysis by the adrenergic β-blocker propranolol in the exclusively human pathogen *S*. Typhi is another example of the existence of an additional putative novel bacterial adrenergic receptor. In *S*. Typhi, NE stress hormone-mediated haemolysis is clearly independent of the known *E. coli* O157:H7 adrenergic receptor QseBC and is mediated via the CpxAR two component system (Karavolos et al. 2011a, b)

Based on the above observations, it is evident that natural selection has ensured that there is no monopoly in bacterial adrenergic signalling. Millions of years of evolution have culminated in a fine tuned bacterial sensing system composed of different adrenergic receptors, which through their fastidious specificities, orchestrate the strategic responses of pathogens within their host milieu.

25.5 Conclusions

In response to acute stress, the sympathetic nervous system is activated due to the sudden release of NE stress hormones in a response known as the "fight-or-flight" reflex (Cannon 1915 – for more details see Chap. 2). This stress reaction evolved from our ancestral survival needs and causes immediate physical reactions in preparation of the muscular activity needed to fight or flee an imminent threat.

Recently, a number of groups, including ourselves, have made compelling observations that bacteria can also sense and respond to these host stress signals. Specifically, work from our laboratory has shown that *Salmonella* has evolved multiple specialised systems for directly sensing NE stress hormones. We have demonstrated that even brief exposure of *Salmonella* to physiological concentrations of stress hormones can result in marked changes in expression of virulence factors. These combined observations are summarised briefly in Fig. 25.1.

The effects of adrenaline and noradrenaline are likely to be complex, involving multiple effects on both bacteria and host gene expression signatures to actively influence the outcome of the infection. It is possible that the adrenergic modulation of these genes may confer an advantage to the bacteria under certain *in vivo* conditions but an unavoidable disadvantage in others; for example down-regulation of the lipopolysaccharide (LPS) modifying enzymes PmrF and PagL causes an increase in sensitivity to polymyxin B, but also concomitantly reduces activation of the TLR-4 Toll-like receptors, reducing the host inflammatory response to infection (Kawasaki et al. 2004a; Miller et al. 2005).

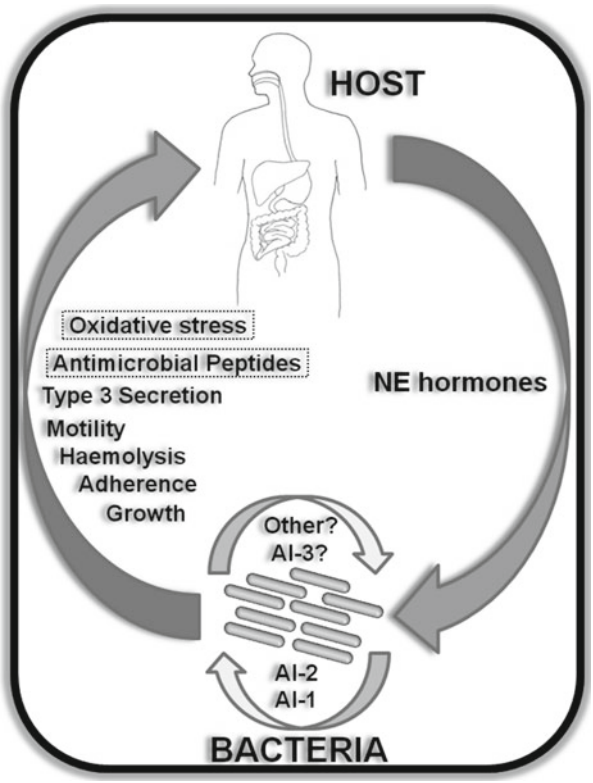

Fig. 25.1 Host-Pathogen communication via NE hormones. Host-produced NE stress hormones are sensed by bacterial pathogens which use them as environmental cues and, in combination with intrabacterial signalling through autoinducers (AIs), adjust their pathophysiology to their benefit. Bacterial sensing of NE stress hormones can also be a double-edged sword since it may exert a detrimental effect by weakening pathogen defences against the host arsenal (*dotted text boxes*)

Thus, host NE stress hormones can provide vital environmental cues for bacterial pathogens to navigate their way through their specific infectious cycle. On the other hand, NE stress hormones can provide the host with a unique tool to manipulate bacterial pathogens. The observations described in this Chapter provide important insights into the intriguing pathways leading to host-pathogen cross-talk and illustrate some of the unique ways bacterial pathogens intercept host communication signals to their advantage or detriment.

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Index

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