Chapter 13
Moonlighting Functions of Heat Shock Protein 90

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Abstract Hsp90 is a highly expressed and ubiquitous chaperone in eukaryotes and bacteria. It works with hundreds of client proteins and is regulated by dozens of co-chaperones. Its functions in folding, stabilizing, assembling and disassembling proteins and complexes that are involved in many key processes in the cell, including antigen cross-presentation, stabilization of the cytoskeleton, signaling pathways, stabilization of steroid receptors and other transcription factors, assembly and disassembly of transcription machinery, DNA repair, and the cell cycle. This ubiquitous and versatile intracellular protein is found to have even more functions outside the cell. In this review we discuss the idea that Hsp90 is a moonlighting protein with roles as a secreted cytokine and as a cell surface apoptotic signal and receptor for bacterial cells and lipopolysaccharide.

Keywords Chaperone · Cytokine · HSP90 · Moonlighting protein · Receptor · Secretion

Abbreviations

A2MR	Alpha 2 macroglobulin receptor
BMDC	Bone marrow derived dendritic cells
CD11	Integrin subunit
CD18	Integrin subunit

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CXCR4	Chemokine receptor 4
ER	Endoplasmic reticulum
GDF5	Growth differentiation factor 5
HEp-2	Human epithelial type 2
Hsc70	Heat shock cognate 71 kDa protein
hsp75	Heat shock protein 75 kDa mitochondrial
Hsp90	Heat shock protein Hsp 90
HtpG	High temperature protein G/C62.5
LBP	Lipopolysaccharide-binding protein
LOX-1	Lectin-like oxidized LDL receptor-1
LPS	Lipopolysaccharide
LRP-1	LDL receptor-related protein 1/ CD91
MoonProt	Moonlighting proteins database
PTMs	Post-translational modifications
TLR4	Toll-like receptor 4
TRAP1	Heat shock protein 75 kDa mitochondrial

13.1 Introduction

Heat shock protein 90 (Hsp90) is a highly-expressed protein that acts as an ATP-dependent molecular chaperone (Csermely and Kahn 1991; Nadeau et al. 1993; Panaretou et al. 1998; Obermann et al. 1998). It is important for maintaining cellular proteostasis in physiological and stress conditions (reviewed in Schopf et al. 2017; Pearl 2016) through its function in general protein folding and stabilization, including assembly of complexes and aiding client proteins to interact with ligands. By interacting with a diverse set of client proteins, including kinases, nuclear receptors and hundreds of other proteins, it plays key roles in antigen cross-presentation, stabilization of the cytoskeleton, signaling pathways, stabilization of steroid receptors and transcription factors, assembly and disassembly of transcription machinery, DNA repair, the cell cycle and many other cell processes. These numerous and diverse interactions are enabled and regulated by a large number of co-chaperones that modulate its ATP binding and hydrolysis, conformational changes, and interactions with client proteins (reviewed in Schopf et al. 2017).

Hsp90 is highly conserved in eukaryotes and bacteria, but it is not found in archaea (Chen et al. 2006). Eukaryotes have two Hsp90 proteins in the cytoplasm, Hsc82 and Hsp82 in the yeast *S. cerevisiae*, and Hsp90α and Hsp90β in *Homo sapiens* (Rebbe et al. 1987). There are also versions in the endoplasmic reticulum (endoplasmin/Grp94/Hsp90B1), mitochondria (hsp75/TRAP1) (Song et al. 1995) and chloroplasts (Hsp90c). Bacteria usually contain only one Hsp90 gene. HtpG (High temperature protein G/C62.5) is the homologue in *E. coli* (Bardwell and Craig 1987). Most of the discussion below about the moonlighting functions of Hsp90 is about mammalian Hsp90α unless indicated otherwise.

In addition to its many roles inside the cell, Hsp90 is a moonlighting protein with several more functions outside the cell. Moonlighting proteins comprise a group of multifunctional proteins that have multiple biochemical or biophysical functions performed by a single polypeptide chain (Jeffery 1999). They do not include proteins that are considered multifunctional due to gene fusions, different versions of the protein due to multiple RNA splice variants, or pleiotropic effects. Taxon specific crystallins in the lens of the eye of several species were among the first proteins to be identified as moonlighting proteins. Zeta crystallin is the same protein as a quinone oxidoreductase, which catalyzes the conversion between quinone and semiquinone (Rao et al. 1992). Delta-2 crystallin is an arginosuccinate lyase, which cleaves arginosuccinate to produce arginine and fumarate in the urea cycle (Wistow and Piatigorsky 1990). Lambda crystallin is also an enzyme, L-gulonate 3-dehydrogenase, and catalyzes NAD-linked dehydrogenation in the urinate cycle (Ishikura et al. 2005). As another type of example, aconitase, an enzyme in the citric acid cycle that uses an iron-sulfur cluster in its active site, has a second function in which it binds to mRNA and regulates translation of proteins involved in iron uptake (Philpott et al. 1994; Banerjee et al. 2007; Kennedy et al. 1992).

Additional examples of protein moonlighting are found throughout the evolutionary tree – in plants, bacteria, archaea, insects, mammals, including several proteins in humans. Moonlighting proteins have been found that perform different functions when in different locations within a cell, expressed in different cell types, as part of different multimers, or upon binding of substrates, products, cofactors or other small molecule ligands (Jeffery 1999, 2004, Jeffery 2009). The Moonlighting Proteins Database (MoonProt, www.moonlightingproteins.org), launched online in 2014 (Mani et al. 2014), contains more than 300 moonlighting proteins. They cover diverse types of proteins including, but not limited to, enzymes, transcription factors, chaperones, receptors, and ribosomal proteins. Some perform their distinct functions at different times, but others perform multiple functions simultaneously. The ability of a single protein to participate in multiple cellular activities can be valuable to the cell, for example, in coordinating multiple biochemical processes or metabolic pathways. In addition to Hsp90, the known moonlighting proteins include many other heat shock proteins and chaperones, including DnaK, Hsp60/GroEL, Hsp70, DegP, FtsH, calreticulin, DegQ, peroxiredoxin, and protein deglycase 1 (reviewed in Jeffery 2018; Pockley and Henderson 2017; Chen et al. 2017).

13.2 Moonlighting Functions of Hsp90

13.2.1 Hsp90 on the Cell Surface

Some of the moonlighting intracellular chaperones, as well as dozens of other intracellular proteins, have been found to perform a second function on the cell surface. Most often, they act as adhesins that interact with other cell types, soluble host proteins, or extracellular matrix (Kainulainen and Korhonen 2014; Jeffery 2018),

including DnaK from *Bifidobacterium* (Ruiz et al., 2011), *Lactococcus lactis* (Yuan and Wong, 1995), *Neisseria meningititis* (Tzeng et al., 2008), and *Mycobacterium tuberculosis* (Kennaway et al., 2005), Hsp60 from *Legionella pneumophila* (Garduño et al., 1998) and *Listeria* (Kim et al., 2006), and human calreticulin (Saito et al. 1999). How or why these particular proteins are secreted and attached to the cell surface is not known; their physiochemical features are similar to other cytosolic proteins (Amblee and Jeffery 2015). Proteomics studies of proteins on the cell surface of dozens of species have identified many additional intracellular proteins on the cell surface (reviewed in Wang and Jeffery 2016), but it is not yet clear if those proteins also have a second function on the cell surface or the same function as inside the cell. In some cases, the proteins might have been identified in the surface proteomics studies due to experimental challenges such as being closely associated with cytoplasmic domains of transmembrane proteins. One such study identified Hsp90 on the surface of *E. coli* strain BL21 (Thein et al. 2010), although its function on the bacterial surface is unknown.

Hsp90 has been found to have additional functions when displayed on the surface of mammalian cells. The amount of Hsp90 secreted was shown to increase due to a number of factors, including activation of endothelial cells and interaction of cells with extracellular matrix components like fibronectin, with much more of the Hsp90α isoform than the Hsp90β secreted (Song and Luo 2010). One extracellular function of Hsp90 is as a signal on the surface of human apoptotic cells to trigger engulfment by dendritic cells. Apoptosis is important for development, differentiation and as a response to stresses that damage cells, but it's important to remove these dying cells to maintain tissue homeostasis. Zhu and coworkers found that the display of Hsp90, along with Hsp60 and Hsp70, is an early response in apoptotic process of EL4, E.G7, and HL60 cells to a variety of cell stresses including uv light and cisplatin (Zhu et al. 2016). The Hsp proteins appeared on the cell surface even before phosphatidylserine, an early apoptotic signal, became exposed on the surface. Once on the cell surface, the Hsp serve as an "eat me" signal to prompt bone marrow derived dendritic cells (BMDC) to phagocytose the dying cells (Fig. 13.1). The phagocytic cells also engulf latex beads covered with Hsp60, 70 or 90, but the internalization was prevented by the addition of soluble Hsp60, 70, or 90, presumably because they block the receptor for the Hsp on the dendritic cells. The receptor for Hsp90 on the dendritic cells was found to be the lectin-like oxidized LDL receptor-1 (LOX-1). Expression of LOX-1 on the surface of CHO cells enabled Hsp90 also to bind to those cells.

Hsp90 on mammalian cell surfaces is also involved in sensing bacterial proteins and initiating an immune response. The cell surface protein JlpA on *Campylobacter jejuni*, a common cause of food poisoning, was found to interact directly and specifically with cell surface-exposed Hsp90α on human epithelial type 2 (HEp-2) cells (Jin et al. 2003). Binding resulted in the initiation of signaling pathways involving NF-κB and p38 MAP kinase that lead to activation of proinflammatory immune responses. The authors noted that because Hsp90α does not contain a transmembrane domain, there must be at least one additional cell surface protein involved that can transduce the signal into the cell. The cell surface protein NadA from *Neisseria*

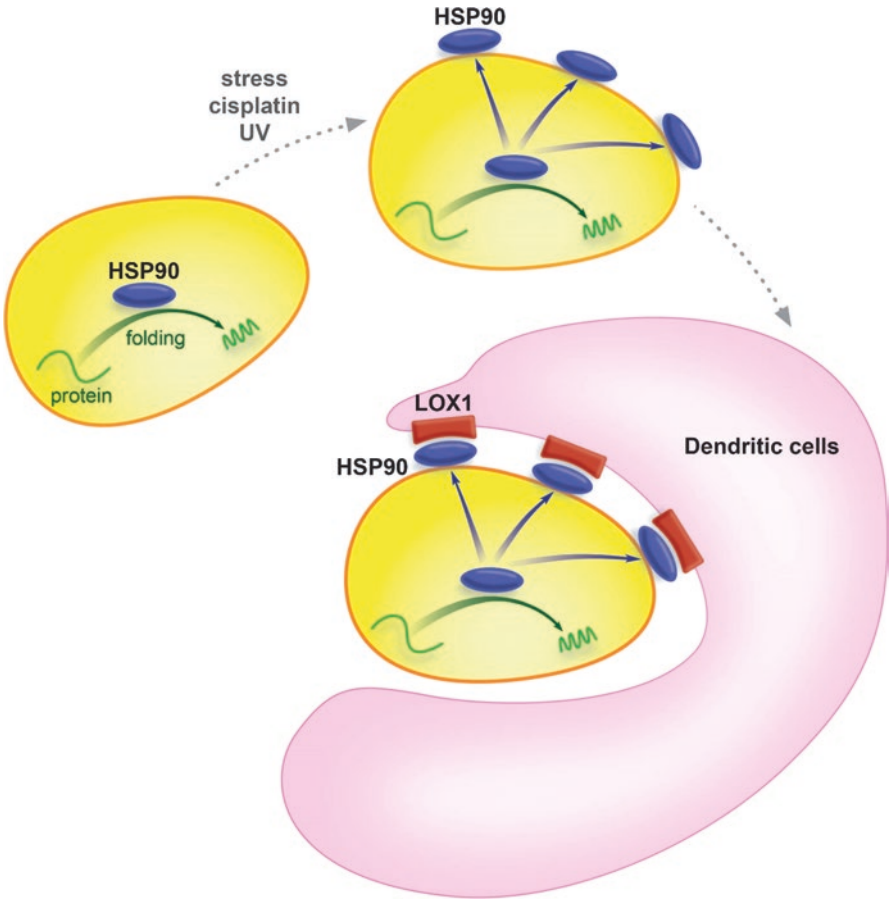

Fig. 13.1 Surface expression of Hsp90 triggers engulfment by dendritic cells. Hsp90 functions as a chaperone inside the cell under nonstress conditions. It becomes expressed on the cell surface as an "eat me" signal in response to stresses that trigger apoptosis, including UV light and cisplatin. The secreted Hsp90 binds to the LOX-1 receptor on dendritic cells (pink), and the dendritic cells engulf and destroy the cells displaying Hsp90

meningitidis also binds to Hsp90α on the surface of monocytes (Cecchini et al. 2011). Binding results in cell activation and the induction of cytokine and chemokine secretion.

In addition to detecting bacterial proteins, Hsp90 also acts as part of a complex that binds to bacterial lipopolysaccharide (LPS) and triggers an immune response (Triantafilou and Triantafilou 2002). After lipopolysaccharide-binding protein (LBP) binds and transfers the LPS to membrane-bound CD14, the LPS is passed into the membrane where it binds to a complex of receptors (Triantafilou et al. 2001a, b). The complex includes chemokine receptor 4 (CXCR4), Hsc70, Hsp90α, and growth differentiation factor 5 (GDF5). This complex recruits Toll-like receptor

4 (TLR4) and/or integrins (CD11 or CD18) to act as transmembrane signaling proteins to activate cytoplasmic signaling pathways (Triantafilou et al. 2001a, b).

13.2.2 Hsp90 as a Cytokine

Another way for intracellular proteins to have additional functions outside the cell is as a soluble signaling protein. Many intracellular proteins moonlight as secreted cytokines and growth factors. Phosphoglucose isomerase, the second enzyme in glycolysis, is the same protein as neuroleukin, autocrine motility factor, and differentiation and maturation mediator (Gurney et al. 1986; Chaput et al. 1988; Faik et al. 1988; Xu et al. 1996; Watanabe et al. 1996). Secreted chaperones in particular have been found to have many roles in immunomodulation, angiogenesis, and cell migration (reviewed in Henderson and Pockley 2005; Henderson and Pockley 2010).

Hsp90 also acts as a cytokine. Cheng and coworkers showed that Hsp90 secretion can be triggered by TGF-α (Cheng et al. 2008). Secretion can also be triggered by hypoxia in human dermal fibroblasts (Woodley et al. 2009). How Hsp90 is secreted is not completely understood, but it has been found together with several other heat shock proteins (but not all chaperones) in the lumen of exosomes, and the amount found in exosomes increases under heat stress (Clayton et al. 2005). Once outside the cell, Hsp90 binds to the ubiquitously expressed surface receptor LRP-1 (LDL receptor-related protein 1), which is also known as CD91 or the alpha 2 macroglobulin receptor (A2MR) (Fig. 13.2). Receptor binding causes an increase in cell migration by epidermal cells, dermal cells and keratinocytes and promotes wound healing and angiogenesis (Cheng et al. 2008; Li et al. 2007; Song and Luo 2010; Jayaprakash et al. 2015), but without cell proliferation. The interaction with the alpha2 macroglobulin receptor CD91 is also important in another pathway. Hsp90, along with heat shock proteins gp96 and Hsp70, can form a complex with antigens and bind to CD91 for aiding uptake of the antigen by antigen-presenting cells (Basu et al. 2001).

An understanding of how Hsp90 performs its immunomodulatory, angiogenesis, and wound healing activities may aid in the development of novel treatments for promoting wound healing, which is especially needed for the treatment of the difficult to treat skin wounds and ulcers common in diabetic patients. It was shown that topical Hsp90α speeds up wound healing in mice (Li et al. 2007). The Cheng group also demonstrated that application of a purified 115 amino acid fragment of Hsp90α accelerated both acute and diabetic wound healing in mice (Chen et al. 2011). Interestingly, only the Hsp90α isoform promotes wound healing, and the other major isoform, Hsp90β, does not. As mentioned above, the Hsp90β isoform is not secreted at the same level as Hsp90α, and it does not have significant effects on cell migration (Jayaprakash et al. 2015). Hsp90's role as a cytokine is also often co-opted by cancer cells, as reviewed by Hance and co-workers (Hance et al. 2014). A

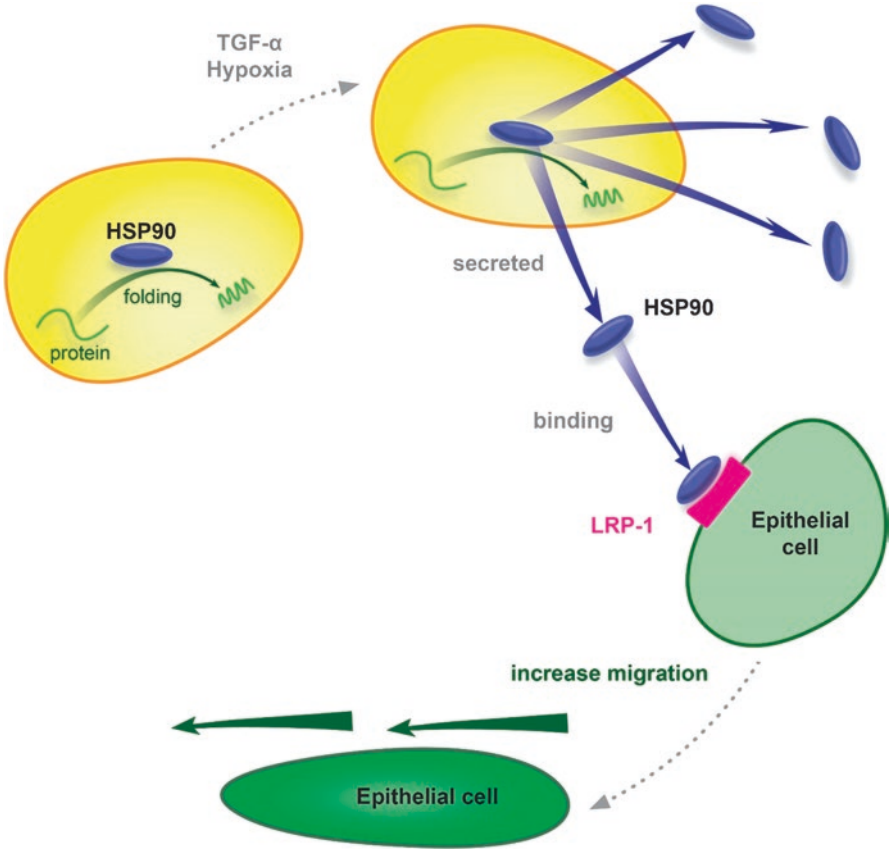

Fig. 13.2 Secreted Hsp90 interacts with the LRP-1 receptor on epithelial cells. Hsp90 secretion is triggered by TGF-α or HIF-1-α. Secreted Hsp90 binds to the LRP-1 receptor on epithelial cells (light green). Receptor binding causes an increase in epithelial cell migration (dark green cells and arrows)

better understanding of its roles in cancer cell migration could lead to novel therapeutics for metastasis.

13.2.3 Potential Kinase and Autophosphorylating Activity

It's interesting to note that in addition to using ATP during the chaperone function, several studies have shown that Hsp90 homologues can undergo autophosphorylation or even phosphorylate other proteins. This includes Hsp90 homologues from *Brassica napus* (rapeseed plant) (Park et al. 1998) and rat (Csermely and Kahn 1991; Langer et al. 2002). The chaperone function and the extracellular functions do not involve autophosphorylation of Hsp90 or phosphorylation of client proteins or

co-chaperones by Hsp90, so whether or not these activities have an as yet unknown role in any of the known functions or are part of an additional moonlighting function, is not clear.

13.3 Conclusions

Hsp90's many activities and interactions with client proteins, co-chaperones, receptors, and other macromolecules both inside and outside the cell are a very active area of research. Because of its key roles in many central cellular processes, it is important in both health and disease, and inhibitors of Hsp90 activity have been found to be effective anticancer therapeutics (Hance et al. 2014). Hsp90 is also a current target for developing treatments for other diseases as well, including diseases involving immune pathways (Neckers and Workman 2012; Verma et al. 2016). Although a great deal has been learned about its many roles as a chaperone inside the cell, there is less information about how Hsp90 performs its extracellular functions. Are ATP binding and hydrolysis needed? Are any additional proteins needed? Structural studies have identified multiple domains and conformations of Hsp90. Which domains are involved in the moonlighting function? Which amino acids are involved in binding to receptor? In what conformation, "closed", "open", extended, does it bind to the receptor? Many additional questions are common to the dozens of intracellular proteins that moonlight outside the cell and on the cell surface. How is Hsp90 secreted? How does it become attached to the cell membrane? Why is the Hsp90α isoform secreted from cells when the other major cytoplasmic isoform, Hsp90β, is not? Are the mitochondrial, chloroplast, and ER Hsp90 homologues also moonlighting proteins? Do other bacterial homologues have moonlighting functions outside the cell? What is the mechanism to increase secretion of Hsp90 under some cellular conditions? It undergoes phosphorylation by several kinases as well as other post-translational modifications (PTMs) (Mollapour and Neckers 2012), and PTMs have been found to play a role in switching functions in many other moonlighting proteins (Jeffery 2016). Do any of the PTMs affect secretion of Hsp90? Although much has been learned about the Hsp90 protein family in recent decades, there is still much to be learned. Understanding the molecular mechanisms of Hsp90's extracellular functions is needed to help clarify its roles in health and disease and could provide information about novel cellular pathways including how moonlighting proteins like Hsp90 are secreted. In addition, elucidating the connections between sequence, structure, and function in moonlighting proteins like members of the Hsp90 family is also needed to increase our general understanding of proteins, for example, improving our ability to predict all of the functions of a protein, understanding how protein functions evolve, and designing novel proteins.

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