Natalya Kurochkina *Editor*

SH Domains

Structure, Mechanisms and Applications

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Preface

Src-family protein tyrosine kinases (PTKs) are involved in cellular pathways of growth and differentiation in response to action of growth factors and other ligands that activate cell surface receptors. Regulation of these pathways is extremely important for cell life. Domain architecture of PTKs comprises catalytic and regulatory domains (SH domains) and plays an important role in its function. Structure and activation/deactivation mechanisms of PTKs were extensively studied. SH3 and SH2 domains are mainly involved in protein–protein interactions. Recognition of specific sequence motifs by these modules, polyproline sequences by SH3 and phosphotyrosines by SH2 in particular, contributes significantly to regulation of the kinase action.

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SH3 Domains as Suitable Models to Study Amyloid Aggregation

Bertrand Morel, David Ruzafa and Francisco Conejero-Lara

Abstract Protein aggregation and amyloid fibril formation are related to a variety of neurodegenerative diseases for which no effective therapies or prevention methods exists yet. The study of well-characterized model proteins, even unrelated to disease, is a potent approach to investigate amyloid fibrils and in fact many of the advances in this intricate problem have been achieved from the study of model systems. In this chapter, we review the use of SH3 domains in the study of amyloid aggregation. We especially focus on the interest of combining biophysical techniques to quantitatively describe the kinetics of the different steps leading to fibrillation. Indeed, SH3 domains have been studied not only to elucidate the structural determinants of amyloid fibrils but also to unveil the thermodynamic and kinetic determinants of their formation precursors. Such contributions have given insight into the forces driving amyloid aggregation and may be of useful interest in future development of novel therapeutic or preventive strategies for neurodegenerative diseases.

Keywords Protein aggregation · Oligomers · Thermodynamics · Kinetics · Mechanism

Abbreviations

Spc-SH3	Src-homology region 3 domain of chicken α-spectrin
PI3-SH3	SH3 domain of α-subunit of bovine phosphatidylinositol -3-kinase
NMR	Nuclear magnetic resonance
TEM	Transmission electron microscopy
AFM	Atomic force microscopy

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1 Introduction

In order to function, a protein must fold to a specific conformation following its synthesis on the ribosome. This folding process is therefore the second stage of the translation of genetic information into biological activity. For many years this phenomenon was considered a scientific curiosity, but is now recognised as central to an understanding of many aspects of molecular and cellular biology, including molecular trafficking and the control of the cell cycle. In addition, the failure of proteins to fold correctly or the misfolding of correctly folded proteins can have a dramatic impact. A group of diseases, most of them with tremendous social consequences, is related to the inability of a certain protein or peptide to adopt or maintain its native, functionally active conformation (Dobson 1999, 2004). Despite the existence of exigent mechanisms of control of protein aggregation in the cell, under certain metabolic circumstances (stress, cell aging or presence of exogenous or infectious agents) some misfolded proteins or peptides form highly organized aggregates, which become deposited in the form of fibrils or plaques known as amyloids (Chiti and Dobson 2006). Since 1907, when Alois Alzheimer described for the first time senile plaques and neurofibrillar tangles in a middle-aged woman affected by memory deficits (Watson et al. 1987), the number of diseases found to be related with amyloid aggregates has increased continuously. To date there are more than 30 diseases, some sporadic and/or hereditary and some transmissible, associated with amyloid deposits (Stefani and Dobson 2003). This type of diseases is known with the generic name of protein deposition diseases. Among these, there are some of the most devastating neurodegenerative diseases, such as Alzheimer, Parkinson and Huntington diseases. They infringe an enormous social and personal damage and it is crucial therefore to understand in detail the molecular mechanisms and the physicochemical factors governing their genesis in order to learn how to treat and prevent them. Deriving mechanistic information about the intricate protein aggregation processes is a daunting task that relies upon the establishment of theoretical models allowing an accurate and quantitative interpretation of the experimental aggregation data. However, most proteins related to disease are very difficult to handle because of their intrinsic properties and high aggregation propensity, making it extremely difficult to obtain reproducible experimental data. For this reason, the use of well-characterized model proteins, even though unrelated to disease, has constituted a potent approach to investigate the mechanisms of formation of amyloid fibrils thanks to the extensive information available about their folding mechanism and conformational stability.

SH3 domains have been extensively studied in the field of protein folding and protein interactions providing a wealth of thermodynamic, kinetic and structural information from both experimental and computational approaches (Martinez et al. 1998; Martinez and Serrano 1999; Sadqi et al. 1999; Vega et al. 2000; Ventura et al. 2002a, b; Sadqi et al. 2002a; Casares et al. 2003, 2004; Lindorff-Larsen et al. 2004;

Casares et al. 2007; Periole et al. 2007). This extended knowledge makes the SH3 domain a very suitable model system to investigate the mechanisms of protein misfolding and formation of amyloid fibrils.

2 The Folding Landscape of SH3 Domains

Much of our current understanding of the molecular mechanisms, kinetics and thermodynamics of protein folding has been achieved by studying SH3 domains. This was motivated by their small size, ease of purification both in wild type and mutant forms, high solubility under a wide variety of conditions, and simplicity of their folding processes. In fact, the SH3 domains have been described as archetypical two-state folding proteins under many experimental conditions. Immediately after the determination in the early nineties of the high-resolution structures of a variety of SH3 domains, the SH3 domain of α-spectrin (Spc-SH3) was first described to fold and unfold in a two-state process (Viguera et al. 1994). Subsequent studies with other SH3 domains from drk (Farrow et al. 1995), and from Src (Grantcharova and Baker 1997), Fyn (Plaxco et al. 1998), PI3 (Guijarro et al. 1998a) or Abl (Filimonov et al. 1999) kinases confirmed the typical two-state folding behavior of SH3 domains. Subsequently, several of these domains were submitted to protein engineering analysis of the folding-unfolding kinetics (Matouschek et al. 1990; Fersht et al. 1992) to characterize the structural features of the folding transition state (Martinez et al. 1998; Martinez and Serrano 1999; Riddle et al. 1999; Northey et al. 2002a, b). The transition state is mainly organized around the β-hairpin made by the β3 and β4 strands, with additional small contributions depending of the specific SH3 domain (Fig. 1a). On the other hand, dissociation of strands β1 and β5 and other structural elements, such as the RT loop and the n-Src loop are early events of the unfolding process. These and other experimental studies prompted a number of theoretical and computational analyses that have provided deeper details about the two-state transition (Klimov and Thirumalai 2002; Shea et al. 2002; Lindorff-Larsen et al. 2003; Ollerenshaw et al. 2004). The folding nucleus of the SH3 domains appears to be structurally constrained by the native domain topology and is strongly polarized around the formation of the distal β3-β4 hairpin.

Recently, a larger complexity of the SH3 folding-unfolding landscape has been highlighted, being more rugged than anticipated. On one side, the unfolded state of some SH3 domains is significantly more compact that a random-coil chain. Early hydrogen-deuterium exchange measurements suggested that the Src-SH3 unfolded state retains certain residual structure (Grantcharova et al. 2000). A detailed NMR characterization of the unfolded state ensemble of Spc-SH3 in equilibrium with the native state also indicated residual structure resembling that of the folding transition state (Kortemme et al. 2000). Likewise, the relatively unstable drkN-SH3 domain has also shown to populate a variety of compact unfolded conformations in equilibrium with the native state under non-denaturing conditions (Mok et al. 2003).

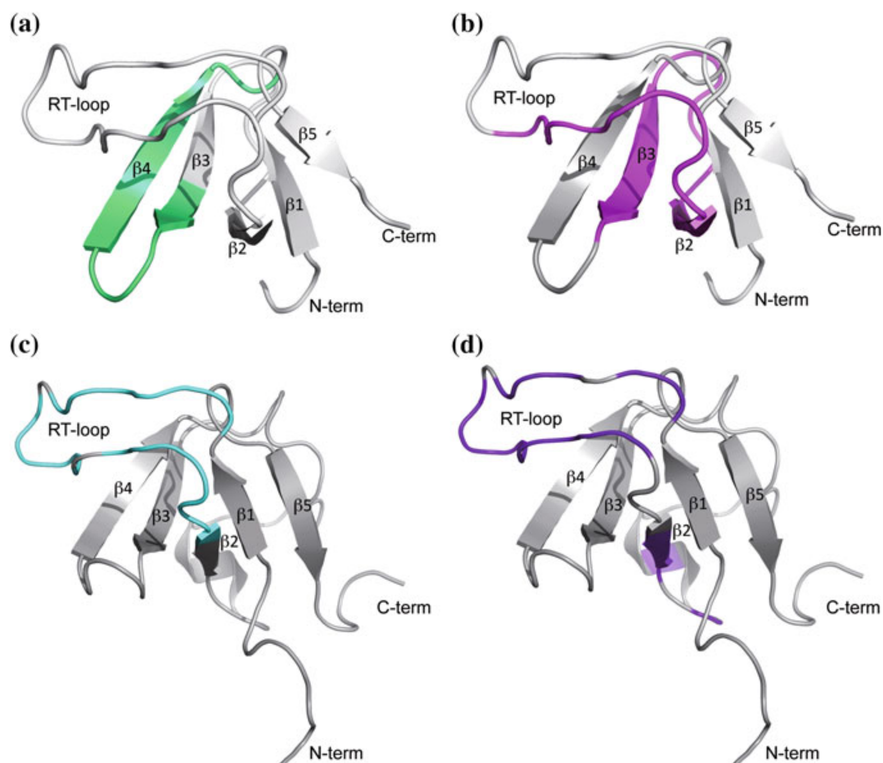

Fig. 1 Regions of Spc-SH3 involved in folding (**a**) and aggregation transition states (**b**). Regions of PI3-SH3 involved in oligomeric (**c**) and fibril core (**d**) structures. The important structural regions for each case are represented in *color*. They were determined using the results obtained by Martinez and Serrano (1999) (**a**), Ruzafa et al. (2014) (**b**) and NMR and MS H/D exchange results obtained by Carulla et al. (2009) (**c**, **d**). Only regions with relevant high ϕ-values are colored in *green* in *panel a* and residues with proton occupancy less than 0.25 are colored in *cyan* and *purple* in *panels c* and *d* respectively. The structure representations of N47A Spc-SH3 (pdb: 1QKX) and PI3-SH3 (pdb: 1PNJ) were done using Pymol (De Lano 2002)

Similarly, the native state is not a unique, rigid state but a dynamic and heterogeneous ensemble of conformations, in which a range of partial unfolding motions can occur. Hydrogen-deuterium exchange experiments followed by NMR demonstrated that the native-state ensemble of the Spc-SH3 domain undergoes broad variety conformational fluctuations extending even above the transition state (Sadqi et al. 2002a, b; Casares et al. 2007). According to computational studies, several selected mutants of Spc-SH3, some of them prone to aggregation, populate a native-like intermediate with unstructured and exposed strands β1 and β5 (Krobath et al. 2012). In the case of PI3-SH3, the native-state dynamics involves the formation of a native-like, partially-unfolded intermediate (Wani and Udgaonkar 2009b), which also manifests in unfolding kinetics together with a more unfolded intermediate (Wani and Udgaonkar 2009a). Molecular dynamics

simulations of the Src-SH3 unfolding indicate a hierarchical order in the decrease of native contacts, being the contacts between the N and C-terminal strands the first to disappear (Tsai et al. 1999), whereas contacts involved in the transition state persist well after the majority of the native contacts are lost. Moreover, a low populated, partially unfolded intermediate was suggested from computational studies in Fyn-SH3 (Ollerenshaw et al. 2004). This intermediate was stabilized by specific mutations, allowing its structural characterization by relaxation-dispersion NMR (Korzhnev et al. 2004; Neudecker et al. 2007). This on-pathway folding intermediate contains largely native-like structure, but strand β5 is disordered and the hydrophobic strand β1 becomes exposed and involved in non-native contacts. It has been suggested that these partially unfolded states may play a key role in the initiation of fibrillation (Neudecker et al. 2012).

In fact, partially unfolded intermediates of the Spc-SH3 domain, accessible from the native state, can establish intermolecular interactions, especially at high concentrations (Casares et al. 2004). One type of intermolecular interaction is domain swapping, in which protein monomers interact by exchanging some of their structural motifs recruiting nearly the same interactions as those stabilizing its monomeric form. A monomer-dimer equilibrium involving domain swapping occurs in Eps8-SH3 (Kishan et al. 2001), in which dimerization occurs by reciprocal exchange of about half of the domain structure, with the n-Src loop acting a flexible hinge. Likewise, c-Src-SH3 can form intertwined dimers in a similar swapped structure (Camara-Artigas et al. 2009). A similar mode of dimerization was predicted in silico for Spc-SH3 (Ding et al. 2002). Domain swapping was computationally analysed in Eps8-SH3 by Onuchic and coworkers (Yang et al. 2004), who concluded that under conditions favoring intermolecular interactions, swapping is way to reduce energy frustration and the mode of swapping is determined by the native-state topology rather than local signals at the hinge region.

The detection and characterization of less ordered oligomeric species is much more challenging due to their higher size and structural heterogeneity. However, single-molecule fluorescence techniques have detected directly the formation ensembles of oligomers with quite heterogeneous sizes as precursors of amyloid aggregation of the PI3-SH3 (Orte et al. 2008) and the Spc-SH3 (Paredes et al. 2012) domains at acidic pH.

In summary, under the appropriate conditions low-populated partially unfolded intermediates of SH3 domains can establish intermolecular interactions giving rise to oligomeric species that may drive the system to progress toward the amyloid aggregation cascade.

3 Aggregation of SH3 Domains

At the other end of the aggregation cascade stand the amyloid fibrils. More than a decade ago, the PI3-SH3 domain was reported as the first protein unrelated to disease that forms amyloid fibrils (Guijarro et al. 1998b; Zurdo et al. 2001). Since

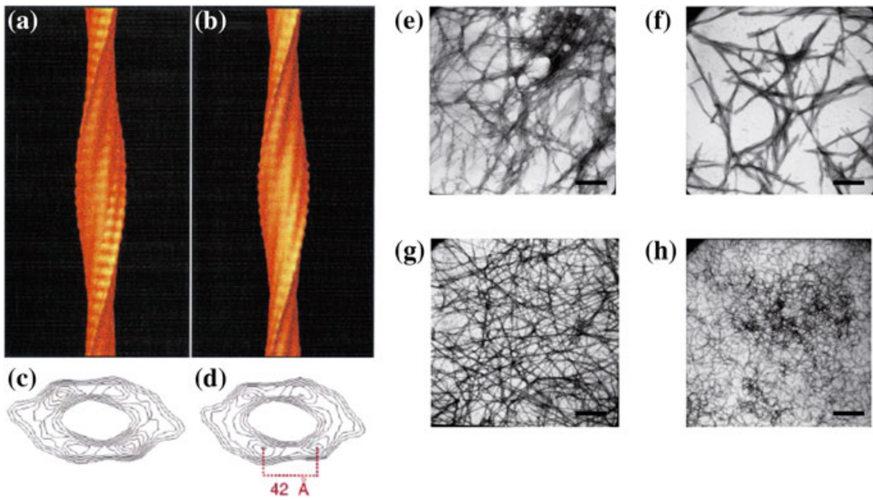

Fig. 2 Three-dimensional reconstructions and contoured density sections of the 610 Å (**a**, **c**) and the 580 Å form (**b**, **d**). The PI3-SH3 fibrils are shown as rendered surfaces in **a** and **b** and as contoured density cross-sections in **c** and **d**. Figures **a**–**d** were taken from Jimenez et al (1999) with permission. Transmission electron microscopy images of amyloid fibrils formed from different SH3 domains assembled under various experimental conditions (**e**–**h**). Fibrils were formed from Yes-SH3 at pH 3.0 **e**, N47A Spc-SH3 at pH 3.2 in the absence of salt incubated for one month and resuspended in 0.1 M glycine buffer containing 0.2 M NaCl pH 3.2 **f**, N47A Spc-SH3 domain incubated for one month at 37 °C in 0.1 M glycine pH 3.2 **g** and N47A Spc-SH3 domain incubated for 1 month at 37 °C in 0.1 M glycine, 0.2 M NaCl pH 3.2 **h**. The *black* segments in *panels e*–*h* correspond to 200 nm

then, a vast amount of data about the structure and biophysics of amyloid fibrils has been generated first with this model system and subsequently with many other SH3 domains such as Fyn-SH3 (Neudecker et al. 2012), c-Src-SH3 (Camara-Artigas et al. 2009), c-Yes-SH3 (Martin-Garcia et al. 2007), Abl-SH3 (Lapidus et al. 2012) and Spc-SH3 (Ventura et al. 2004; Morel et al. 2006).

The first three-dimensional model structure of an amyloid fibril was determined for the PI3-SH3 at 25 Å resolution using cryo-electron microscopy (Jiménez et al. 1999). The structure of the fibril is a double helix of two protofilament pairs wound around a hollow core (Fig. 2a, b). It shows a crossover repeat of $\sim$600 Å and an axial subunit repeat of $\sim$27 Å. These structural features indicate that the SH3 domain must partially unfold to adopt a longer, thinner shape to fit the amyloid form. The dimensions of the protofilaments (20 × 40 Å) cannot accommodate a folded SH3 domain but only a pair of flat β-sheets stacked against each other, with very little inter-strand twist (Fig. 2c, d). This structure has constituted a good model to better understand the assembly of other amyloid fibrils. More recent structural studies by magic-angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) spectroscopy described the molecular conformation of PI3-SH3 in amyloid fibrils (Bayro et al. 2010, 2011). The secondary structure in the PI3-SH3 fibrillar

state was found to be markedly different from the native fold. The three first native β-strands are preserved as part of longer β-strands in the fibrils, whereas residues that form parts of β-strands 4 and 5 in the native state adopt a less ordered conformation in the fibril with a short β-strand segment near the C-terminus (Bayro et al. 2010). Flexible regions in the native state, including the N-terminus and the RT and n-Src loops, adopt rigid β-strand conformations. These elements form a parallel, in-register β-sheet, extending along each protofilament of the fibril.

Amide hydrogen-deuterium studies by NMR and mass spectrometry (Carulla et al. 2005, 2009) have delineated similar regions of the polypeptide chain that form the core of the fibrils. Interestingly, these techniques have also established that amyloid fibrils are dynamic structures that can undergo a recycling of molecules at equilibrium between the bulk solution and the fibril ends (Carulla et al. 2005).

Despite the overall similarity in amyloid fibril core structures, there is a significant morphological variability between amyloid fibrils. This is related to a heterogeneity of the internal structure of the fibrils, which is influenced by a variety of factors related to the environmental conditions of their formation, such as temperature, pH, ionic strength, or mechanical factors such as the presence or absence of agitation (Zurdo et al. 2001; Morel et al. 2010) (Fig. 2e–h). These results suggest that, in contrast to the unique native conformation of natural proteins, amyloid fibrils can acquire a variety of structures corresponding to several energy minima in their conformational landscape, being the final conformation simply selected by the thermodynamic or in many cases kinetic factors that govern under each circumstance.

It is nowadays well established that the conversion of a peptide or protein into amyloid fibrils has a kinetic mechanism of nucleation and growth, typical of crystallization. In most cases, the time course of fibril formation shows a typical lag phase of variable length followed by an exponential growth (Ferrone 1999). Amyloid formation by PI3-SH3 at sub-millimolar concentrations is preceded by a very pronounced lag phase, typically several days in duration, in which fibrils are not detectable. The strong dependence of the fibrillation process on solution conditions (Zurdo et al. 2001) is consistent with the assumption that the destabilisation of the native state is a prerequisite for fibril formation, whereas the substantial lag phase in the early stages of the aggregation kinetics is a strong indication of a nucleation polymerisation mechanism. The lag phase or nucleation phase is the time required for the formation of the nuclei of aggregation, being this the rate limiting step of the aggregation process. Once a nucleus is formed, it progresses toward the fibrillation by a series of elongation steps with addition of protein molecules to the ends of the fibril (Wetzel 2006). Although it is assumed that the fibrils do not appear in a significant amount during the nucleation phase, this is a crucial stage in the overall aggregation process, in which a variety of oligomeric species are formed, many of them rich in β-sheet structure, providing nuclei for the assembly of larger species and finally the fibrils. A great effort is being made to identify, isolate and characterize the oligomeric and prefibrillar species present in solution before the appearance of the fibrils. The main reason of this active research is that it is increasingly evident that these

oligomeric species are involved in the neurotoxic mechanisms of amyloid-related neurodegenerative diseases (Haass and Selkoe 2007).

The aggregation of PI3-SH3 is preceded by formation of a series of non-fibrillar, metastable oligomeric species, known as protofibrils, visible by TEM or AFM as globular beads of 2–5 nm of diameter, chains of beads or annular structures (Bader et al. 2006). These structurally dynamic oligomers are capable of nucleating efficiently the formation of amyloid fibrils, reducing the lag phase of aggregation. Pulse-labeling H/D exchange experiments have shown that these protofibrillar oligomers are structurally heterogeneous and evolve progressively from relatively disordered species to more ordered aggregates, in which residues in and near the RT-loop form the first persistent structure (Fig. 1c) (Carulla et al. 2009).

It has also been evidenced the high toxicity of these oligomeric species in fibroblast and neuron cultures, whereas the amyloid fibrils show little if any toxicity (Bucciantini et al. 2002; Bolognesi et al. 2010).

4 Structural and Stability Determinants of Amyloidogenic Precursors

Despite the abundant details about the structural and morphological characteristics of the amyloid fibrils and protofibrils described above, the molecular mechanisms by which the first aggregation nuclei are generated and initiate the aggregation cascade are much less established. As reported earlier, Spc-SH3 domain has also been extensively explored to understand amyloid aggregation process. It has been found that the single mutation N47A, which destabilizes the folding nucleus of the Spc-SH3, favors the rapid formation of amyloid fibrils under mild acidic conditions, where the majority of the protein is in its native state (Morel et al. 2006; Varela et al. 2009). Fibrillation of Spc-SH3 is strongly accelerated by temperature and NaCl concentration, with thin and curly filaments favored by conditions that increase the rate of nucleation and thick mature amyloid fibrils formed under conditions of slow nucleation (Morel et al. 2010). Such results pointed toward an alteration of the protein solvation layer that dramatically modifies the protein conformational landscape and, as a consequence, alters the accessibility of amyloidogenic states. As a result, early oligomers of different sizes accumulate at diverse rates depending on the conditions, and effectively determine the final morphological and (possibly) structural properties of the protofibrils and amyloid fibrils.

Under conditions of rapid aggregation, the absence of lag phase indicates a rapid formation of amyloid oligomeric nuclei, which further condense into fibrillar species. This rapid aggregation follows a high order irreversible kinetics, suggesting that a rapid conformational change and oligomerization pre-equilibrium precedes the rate-limiting step of amyloid nucleation.

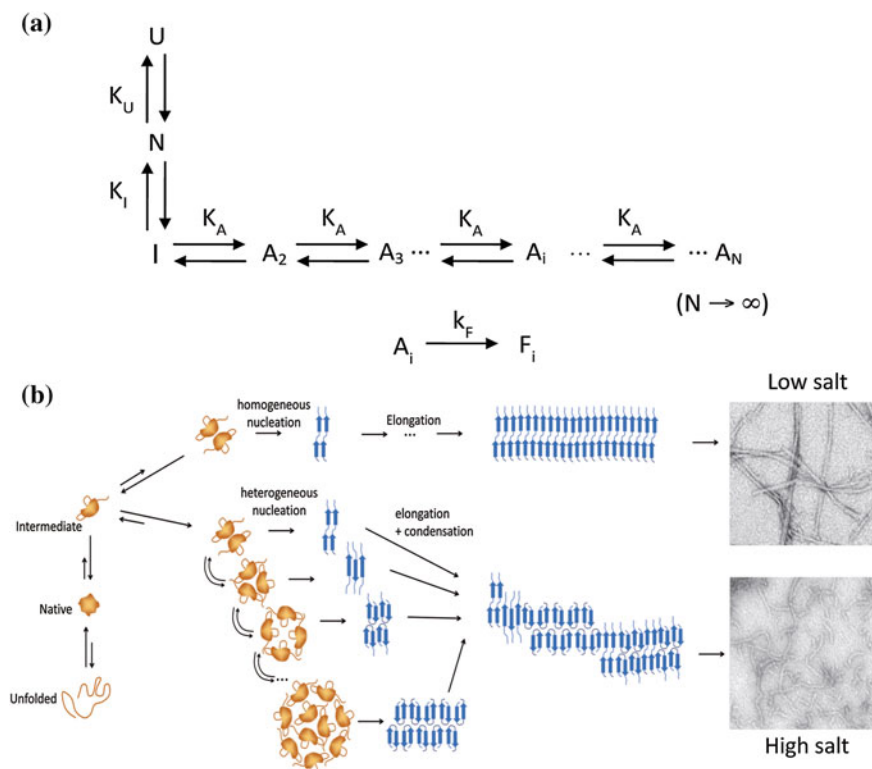

Fig. 3 **a** Kinetic nucleation model. N, I and U represent respectively the native, intermediate and unfolded monomeric states. A_i states represent oligomers of i size, where i can take any value from two to infinite. F_i represent oligomeric amyloid nuclei. *Double arrows* indicate rapidly-equilibrated reversible processes and the *single arrow* indicates a rate-limiting irreversible process. **b** Schematic illustration showing how the factors governing a conformational-oligomerization pre-equilibrium determine the type, abundance and heterogeneity of amyloid nuclei and the final morphological properties of amyloid fibrils. Figures were adapted from Ruzafa et al. (2012)

Equilibrium and kinetic folding-unfolding experiments with the WT and N47A Spc-SH3 under amyloidogenic conditions allowed the detection of ANS-binding partially unfolded species, which become more populated by factors favouring amyloid aggregation. The variable apparent order of the aggregation kinetics together with the detection of oligomers with a variety of sizes suggests that amyloid nucleation occurs by conformational conversion (Serio et al. 2000) within a heterogeneous distribution of oligomers (Paredes et al. 2012).

To interpret quantitatively these observations, a simple kinetic model has been developed (Ruzafa et al. 2012) (Fig. 3a). This model accounts very well for variation of the initial rates of aggregation with the protein concentration and the experimental kinetic orders and has allowed the derivation of kinetic and thermodynamic parameters characterizing the different nucleation steps (Fig. 3b).

Recently, this model was applied to analyse the effect of the salts on the nucleation kinetics of the Spc-SH3 (Ruzafa et al. 2013). Diverse salt ions influence aggregation rates to different extents without altering the overall mechanism and the high apparent order of the experimental kinetics (Ruzafa et al. 2013). In particular, anions have a drastic effect on the rate of nucleation of amyloid fibrils of the N47A mutant of the Spc-SH3 domain, whereas cations do not have an important role under the experimental conditions analysed. The increase of the activity of anions reduces the energy of a low-populated amyloidogenic intermediate, thereby increasing its population and stabilizing the oligomeric precursors of nucleation thus accelerating the subsequent fibrillation processes.

To further understand the molecular details of the nucleation process, a site-directed mutagenesis approach was combined with the kinetic model to analyse rapid amyloid aggregation of the N47A Spc-SH3 domain (Ruzafa et al. 2014). The changes in the stability of the native state produced by a series of mutations placed on each structural element of the domain did not correlate with the changes in the aggregation rates, although the overall aggregation mechanism was not altered. Despite this lack of correlation, the results demonstrated that the inhibitory or potentiating effect of amyloid formation exerted by the mutations is mainly related to the relative efficiency in the formation of early dynamic oligomers, which precedes the formation of amyloid nuclei. A robust kinetic and thermodynamic analysis allowed the identification of the regions of the polypeptide chain involved in the structure of the amyloidogenic intermediate state and in its intermolecular self-association process. The residues defined as major contributors to the stability of the amyloidogenic intermediate are located at several structural elements, most of them unrelated with the folding transition state (Fig. 1a) i.e., the RT loop, the diverging turn, strand β2, the n-Src loop and strand β3. In contrast, the amyloidogenic intermediate appears to have strand β1, strand β4, the 3_{10}-helix and strand β5 mainly unstructured (Fig. 1b). In addition, the highly hydrophobic strand β1 was found to be involved in the formation and stabilization of dynamic oligomers that act as precursors of amyloid nucleation. These results were striking because they indicated that, in order to form the kinetically critical amyloidogenic precursor, the protein needs to acquire a markedly different topology to that found along the native folding–unfolding pathway. Accordingly, native folding and amyloid nucleation of the Spc-SH3 domain take place on different regions of the conformational landscape (Fig. 1a, b).

Studies performed on the PI3-SH3 domain (Ventura et al. 2004; Carulla et al. 2009) agree well with these conclusions. Using protein engineering, Ventura and co-workers reported that the RT loop (residues L11-D23) and the adjacent diverging turn (residues L24-D28) of PI3-SH3 are specific regions influencing the amyloidogenic behavior. More recently, an interesting study based on pulse-labeling hydrogen–deuterium (HD) exchange monitored by mass spectrometry and NMR spectroscopy on amyloid fibrils of PI3-SH3 (Carulla et al. 2009) reinforced the crucial role of residues comprising the RT loop and the diverging turn in the ordered aggregates during fibrillation (Fig. 1c, d). This indicates that a large structural

reorganization must occur before oligomer formation. Similar implication of the RT loop in oligomerization preceding amyloid aggregation was also suggested for Spc-SH3 from computational analysis (Ding et al. 2002).

5 Conclusion

In this chapter, the use of SH3 domains as model protein to study the different stages in protein amyloid aggregation was summarized. It has been shown during the last two decades that the multidisciplinar studies of such modular domains provide useful information about structural, kinetics, thermodynamics and toxicity determinants along the aggregation cascade.

Significant insights into this field have been achieved using SH3 domains, such as the development of a novel kinetic methodology based on previous polymerization models. Such kinetic model has allowed the rationalization of the existence of diverse amyloid aggregation regimes modulated by the aggregation conditions, which govern the morphologies and properties of the final amyloid aggregates. In addition, the thermodynamic magnitudes characterizing the critical steps of amyloid nucleation have been obtained for the first time. Such understanding has enriched the existing framework for the development of rational strategies to design therapeutic compounds able to interfere with the complex protein self-association processes.

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SH Domain Proteins in Plants: Roles in Signaling Transduction and Membrane Trafficking

Xiaohong Zhuang and Liwen Jiang

Abstract The molecular mechanisms of signaling network molecules and dynamics are important topics in cell biology research. SH2 and SH3 are small scaffold molecules functioning in protein-protein interactions to mediate signal transduction pathways that are activated by protein kinases. In plants, several studies have uncovered the novel functions of SH2 or SH2-like domain containing proteins that are similar to the signal transducers and activators of the transcription (STAT) family. The *Arabidopsis thaliana* genome also contains SH3 domain-containing proteins (SH3Ps), but little is known about their functional roles in plant development and growth. In this chapter, we will summarize and discuss the evolutionary conservations of the plant SH2 and SH3 domain proteins with particular emphasis on their roles in regulating signaling transduction and membrane trafficking in plant cells.

Keywords SH2 domain · SH3 domain · Signaling transduction · Protein trafficking · BAR domain · Autophagy

1 Introduction

Plants and animals share the same habitat, but react to environmental cues differently. It is thus reasonable to expect that unique molecules and processes would be involved to mediate plant cell signal transduction. Key features of signaling

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processes in plant, like receptor dimerization, phosphorylation, kinase activation, also appear similar to those in animals, indicating that the mechanisms of receptor-mediated signaling are conserved within eukaryotic kingdoms (von Zastrow and Sorkin 2007). In plants, leucine-rich repeat receptor-like protein kinases (LRR RLKs) represent the largest plasma membrane-localized subfamily of receptors in Arabidopsis and function in diverse signaling pathways for plant development and pathogen defense (Shiu and Bleecker 2001; Antolin-Llovera et al. 2012; De Smet et al. 2009). LRR RLKs regulate the activity of downstream components through different downstream effectors to allow signaling transduction to take place in a scenario quite reminiscent to that in animals, such as transcriptional level of signaling molecules regulation or ubiquitin-mediated degradation and kinase activity to control the signal strength.

During the past decades, extensive studies on yeast and animal cells have revealed that protein-protein interactions via domain recognition provide a fundamental framework for signaling network assembly, which in turn controls the specificity of signaling transduction. Src homology-2 (SH2) and SH3 domain are the two well-studied modules for binding to receptor tyrosine kinases (RTKs) in animals (Pawson et al. 2001). These RTKs are activated by phosphorylation to recruit proteins containing SH2 domains for binding to the receptors, and subsequently regulate the activity of the downstream effectors. However, and different to animal cells, where a large population of the receptor kinases possess tyrosine kinase activity, most of the RLKs identified so far in plant are predicted to belong to the serine/threonine class of protein kinases. In recent years, the tyrosine phosphorylation site in plant RLKs has been detected and functions as an important regulator for the plant signaling transduction process, raising the question whether plants also use similar adaptors to animals for signaling regulation (de la Fuente van Bentem and Hirt 2009). Although SH2 and SH3 domain-containing proteins have been reported in plants, their exact role in which signaling pathway(s) has not yet been demonstrated experimentally. Here, we will discuss the evolutionary conservations of plant SH2 and SH3 domains and their potential functions in mediating plant signaling transduction. In particular, we will also discuss the roles of a plant SH3 domain-containing protein subfamily in regulating protein trafficking and organelle biogenesis.

2 SH2 Domain-Containing Proteins in Plants

As an evolutionary module, the SH2 domain usually contains 100 amino-acid residues and has multiple roles in regulating different cellular events (Pawson et al. 2001). The SH2 domain was first discovered in the Src tyrosine kinase protein and then found in many other adaptors or signaling related proteins. SH2 domain repeats contribute to the binding ability of SH2 domain to the phosphorylated substrates. In animal cells, signal transducers and activators of transcription (STATs) are other SH2 domain-containing members, which function as the second

messengers in the JAK (Janus kinase)-STAT pathway by selectively binding to a DNA sequence through their SH2 domain (Levy and Darnell 2002; Bromberg and Darnell 2000).

So far, based on sequencing blast and domain analysis, there are only several putative STAT type SH2 domain-containing proteins in Arabidopsis (Fig. 1). Based on the sequencing blast and comparisons using the SH2 domain of a *Dictyostelium* STAT protein, two SH2 domain-containing proteins are predicted (Williams and Zvelebil 2004) (Fig. 1a). However, their molecular functions are unreported.

In another study, two STAT-type SH2 domain proteins have been found in Arabidopsis and one in Rice (Gao et al. 2004) (Fig. 1b). Although they lack a DNA binding domain, in vitro pull down experiments have shown that one Arabidopsis STAT-type SH2 domain protein might be associated with a tyrosine-phosphorylated protein, implying that the SH2 module might have conserved roles in a tyrosine-dependent manner (Gao et al. 2004). Plant SPT6L (Suppressor of Ty insertion 6-like) proteins are predicted by secondary structural comparison to yeast SPT6 (Gao et al. 2004). They have been shown to control apical–basal polarity during embryogenesis in Arabidopsis (Gu et al. 2012). One notes that SPT6L also contains a putative WG/GW-repeat that is plant-species

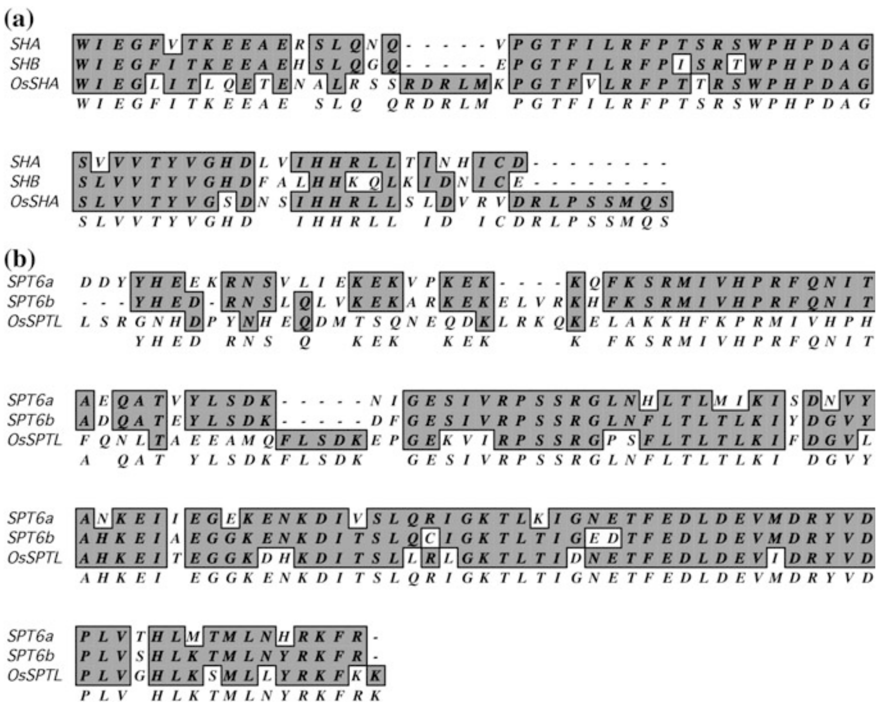

Fig. 1 Conserved SH2 domain-containing proteins in plants. **a** Sequence alignment of STAT type SH2 domain-containing proteins. **b** Sequence alignment of SPT6 type SH2 domain-containing proteins

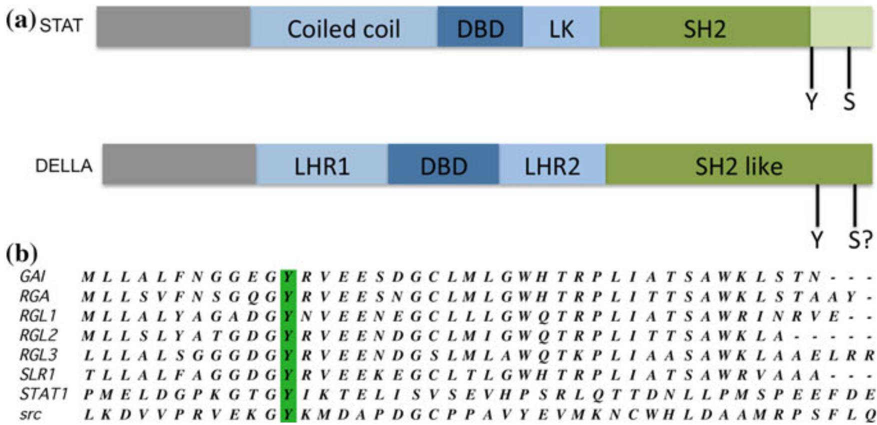

Fig. 2 Comparison of animal STAT and plant GARS. **a** GARS share similar domain structure to animal STAT. **b** Alignment of SH2 regions between STAT and GARS showing the conserved tyrosine kinases site among the STAT-like GRAS protein family

specific, although it is still unclear whether the SH2 domain contributes to this regulation.

Interestingly, a group of proteins called DELLA (Asp-Glu-Leu-Leu-Ala), which belongs to the subfamily of the plant-specific GRAS (named after GAI, RGA and SCR) regulatory protein family, also contain a highly conserved SH2-like domain in their C-termini (Richards et al. 2000; Hauvermale et al. 2012). The DELLAs proteins resemble STAT factors in that they contain two Leucine heptad repeat domains (LHR) and a SH2-like domain (Fig. 2). Recent studies have shown that these DELLA proteins regulate the activity of transcriptional regulators for gibberellin (GA) signaling (Zentella et al. 2007). The DELLA family in Arabidopsis comprises five homologs, including the repressor of ga1-3 (RGA), GA-insensitive (GAI), RGA-like1 (RGL1), RGL2, and RGL3 and one DELLA gene named SLR1 in rice (Hou et al. 2008). Although they share high homology in regard to their sequence and domain organization, genetic analyses have shown that these five homologs share overlapping and distinct functions during plant development. For example, both GAI and RGA are responsible for GA-induced vegetative growth and floral initiation, whereas RGL2 plays a more predominant role during GA-promoted seed germination (Hou et al. 2008; Lee et al. 2002; Tyler et al. 2004; Piskurewicz et al. 2008). In addition, it has been reported that DELLA proteins act as an integrator of multiple signaling pathways, such as auxin, abiotic stresses and plant pathogen responses (Yang et al. 2013; Gallego-Bartolome et al. 2011). These kinds of hormone and downstream effectors act in combination thus producing more varied responses than when acting individually.

3 SH3 Domain-Containing Proteins in Plants

The SH3 domain has been identified more than 30 years ago as a 60 amino acid segment shared among diverse signaling and cytoskeletal proteins of eukaryotes. The SH3 domain binds to its ligand via a proline-rich sequence, particularly those carrying the PxxP motif as well as other non-consensus ligands without the typical Pxxp motif (Saksela and Permi 2012). It is predicted that there are approximately 300 SH3 domain-containing proteins in animals, such the cytoskeleton proteins, the Ras proteins, and the Src kinase and many others, which regulate different cellular pathways (McPherson 1999).

Although the SH3 module has been extensively studied in yeast and animals and SH3 domain-containing proteins also exist in plants, relative little is known about the exact function of this scaffold adaptor in plant cells. Based on the known SH3 sequence blast, there are in total five proteins predicted to contain the SH3 domain in *Arabidopsis* and four in the rice genome (Fig. 3a, b). Interestingly, in plants, the

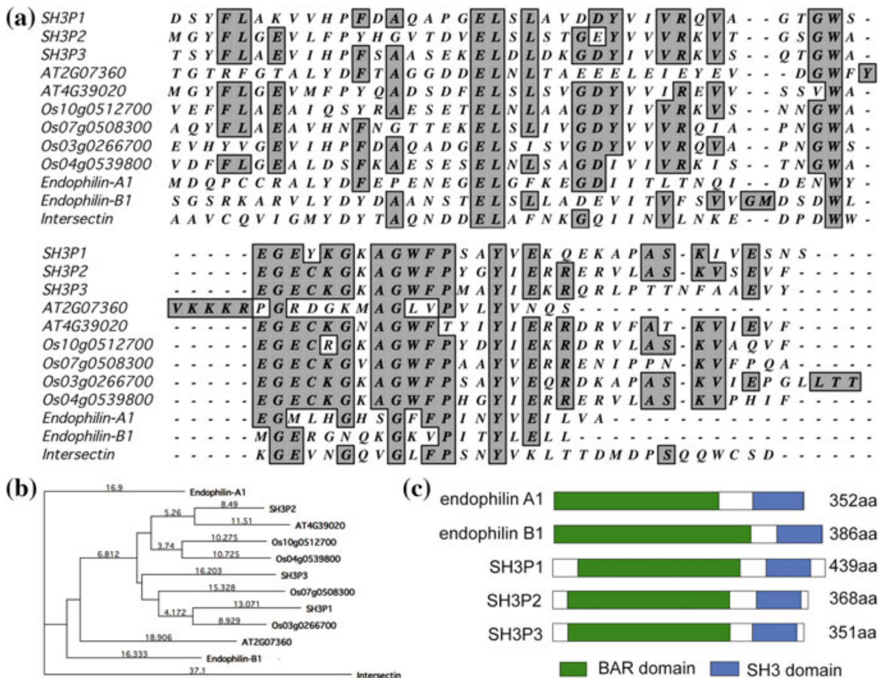

Fig. 3 Conserved SH3 domain-containing proteins in plants. **a** Arabidopsis and rice SH3-containing proteins were aligned with the SH3 domains of human endophilin A1, endophilin B1 and intersectin. Identical amino acids are indicated in the *gray* box. **b** Phylogenetic analysis of SH3 domain containing genes in plant and endophilin A1, endophilin B1, intersectin in animal. Results are predicted using Mac Vector. **c** AtSH3P proteins share similar domain organization to endophilin protein family with a N-terminus BAR domain and C-terminus SH3 domain

Table 1 Roles of AtSH3P protein family in plants

Protein	Domains	Membrane/ lipid binding ability	Interaction partner	Subcellular localization	Pathway	References
SH3P1	BAR, SH3	Yes	Auxilin-like protein; Actin	Clathrin-coated vesicles	Endocytosis	Lam et al. (2001)
SH3P2	BAR, SH3	Yes	ATG8; SH3P2	Autophagosome; Clathrin-coated vesicles?	Autophagy; Endocytosis	Lam et al. (2001), Zhuang et al. (2013), Zhuang and Jiang (2014)
SH3P3	BAR, SH3	Yes	ADL6	Clathrin-coated vesicles	Endocytosis, Vacuole degradation	Lam et al. (2001), (2002)

SH3 domain-containing proteins seem to belong to the same protein family that is involved in vesicle trafficking (Table 1). And notably, no SH3 domain-containing proteins have been found to be linked to the SH2 domain. One possible explanation is that during some conserved fundamental process such as vesicle trafficking that plant cells have also evolved evolutionally conserved molecules for regulation.

4 Roles of SH3 Domain-Containing Proteins in Clathrin-Coated Vesicle Formation

The SH3P subfamily constitutes the first reported SH3 domain-containing proteins in plants. In addition to the SH3 domain, they also contain a N-terminus coiled-coil domain that structurally resembles the N-BAR domain protein family (Fig. 3c), which are well known to function during membrane deformation events. Endophilins are one of the best-studied N-BAR subfamily in animal cells, and actively participate in vesicle trafficking pathways (Daumke et al. 2014; Frost et al. 2009). For example, during the clathrin coat mediated endocytosis pathway, endophilin A1 binds to the proline-rich domain of dynamin to promote vesicle scission and formation (Dawson et al. 2006; Frost et al. 2009).

SH3Ps contain three homologs, named as SH3P1, SH3P2, SH3P3 (Lam et al. 2001, 2002; Zhuang et al. 2013). Both SH3P1 and SH3P3 have been shown to be involved in clathrin-mediated pathways and subcellular studies have shown that SH3P1 is localized in the endomembrane system. Moreover, it is reported that SH3P1 interacts with auxilin, which may stimulate the uncoating of clathrin-coated vesicles (CCVs) together with Hsc70 (Krantz et al. 2013; Lemmon 2001; Ungewickell et al. 1995). SH3P1 may function as an inhibitor for the uncoating

event, whereas SH3P1 may function as a regulator or scaffold during the fission and the uncoating of CCVs (Lam et al. 2001). In addition, SH3P1 binds to actin via a non-SH3 region, implying its role in regulating cytoskeleton activity. In another study, it has been shown that SH3P3 cofractionated with a dynamin homolog ADL6 in plants (Lam et al. 2002). The dynamin ADL6 may participate in the CCV formation, as it has been shown to form a complex with several CCV components such as clathrin and γ-adaptin. Furthermore, other studies have shown that ADL6 is involved in protein trafficking from the *trans*-Golgi network to the vacuole but not to the plasma membrane (Jin et al. 2001; Lee et al. 2006). A potential PxxP motif has been identified in auxilin and ADL6, and deletion of the SH3 domain of either SH3P1 or SH3P3 abolishes the interaction, confirming that the SH3 domain is required for their binding. So far, it seems that these identified SH3 domain binding cargos are involved clathrin-mediated protein trafficking pathways. However, it appears that their preferential interacting partner(s) are involved in different subcellular events. For instance, SH3P1 interacts with auxilin to be involved in CCV-dependent endocytosis while SH3P3 associates with ADL6 and participates in vacuolar degradation. From sequencing alignment, they share very high identity (53 %) in the SH3 domain. Hence, it will be interesting for future studies to find out what causes their binding specificity, especially in terms of structural differences.

In addition to the biochemical data, information from mutant analysis is also needed to understand the specific roles of these SH3 domain-containing proteins during plant development and growth. During plant development, some conserved but fundamental machinery may be needed for some plant specific development process, such as seed germination and chloroplast development. It should be pointed out that ADL6 regulates gametophyte development in Arabidopsis, whereas mutation of ADL6 results in uncompleted cell plates and altered Golgi morphology (Backues et al. 2010). In regard to the association between ADL6 and SH3P3, genetic evidence for SH3 domain-containing proteins will be extremely useful for our understanding of the specific roles of the SH3 domain proteins in plants.

5 Roles of SH3P2 in Autophagosome Formation in Plants

Recently, our group has shown that one of the SH3 domain-containing proteins, SH3P2, participates in the autophagy pathway of plants (Zhuang and Jiang 2014; Zhuang et al. 2013). As a conserved cellular event, autophagy also occurs in plants cells to regulate the protein/organelle quality control and serve as an adaptive mechanism against unfavorable environmental conditions such as stress or pathogen infection (Li and Vierstra 2012; Liu and Bassham 2012; Hayward and Dinesh-Kumar 2011; Bassham et al. 2006; Floyd et al. 2012). Although the majority of autophagy related genes (*atg*) have been identified in the plant genome, the underlying mechanism as to how the autophagy pathway is regulated remains obscure. Based on sequence and domain comparison, we have found that SH3P2

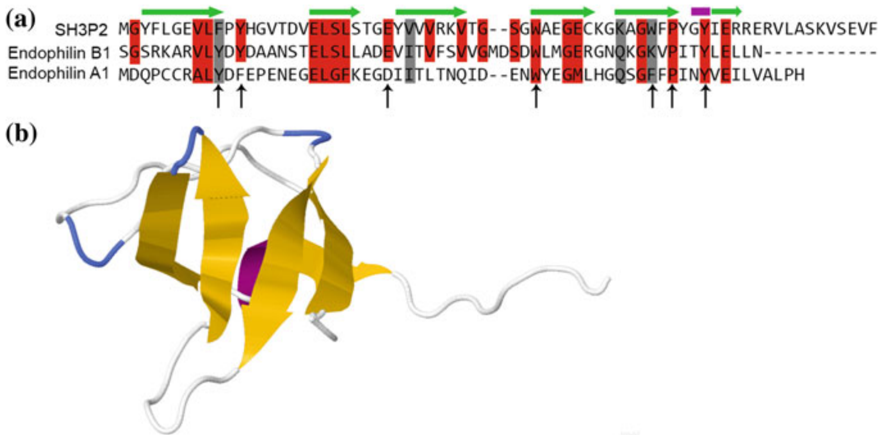

Fig. 4 Comparison of SH3 domain among SH3P2 and endophilin proteins. **a** Sequencing alignment of SH3 regions among SH3P2, endophilin B1 and endophilin A1. **b** A three-dimensional model of the SH3P2 SH3 domain based on endophilin B1

functions in a quite similar manner to endophilin B1 (also called Bif-1). In animal cells, Bif-1 was first found as an interactor of the Bax protein to control mitochondrial morphology (Etxebarria et al. 2009). Also, endophilin B1 is implicated to function during autophagy via an association with the PI3 K complex (Takahashi et al. 2007, 2008, 2009, 2011).

Sequencing alignment shows that SH3P2 shares high similarities to endophilin B1, but less to endophilin A1, especially in the SH3 domain region (Fig. 4a). SH3P2 and endophilin B1 share several conserved crucial functional residues (such as putative tyrosine phosphorylation site). Figure 4b shows a structural model for the SH3P2 SH3 domain predicted by using I-TASSER (Zhang 2008; Roy et al. 2010). The predicted SH3 secondary structural elements of SH3P2 fold into what might constitute a functional cargo-binding module. In animals, it has been shown that endophilin B1 interacts with UVRAG, which then forms a complex with the PI3 K complex. However, in plants a potential homolog has not been identified so far.

In our recent study, we have observed that SH3P2 is localized on the autophagosome membrane upon autophagy induction (Fig. 5). Immunoprecipitation studies demonstrated that SH3P2 forms a complex with the PI3 K components (Zhuang et al. 2013). In addition, we also found that SH3P2 interacts with the ATG8 members through its SH3 domain and the biological function of this interaction is still under investigation. It is possible that SH3P2 contains an ATG8 interacting motif (AIM) within the SH3 region. On the other hand, it should be pointed out that several studies have shown that the SH3 domain binds to ubiquitin, with an affinity comparable to the conventional SH3 ligand carrying the core PxxP sequence (Ortega Roldan et al. 2013; Stamenova et al. 2007; Trempe et al. 2009; Korzhnev et al. 2009). These subset of SH3 domain-containing proteins are involved in various pathways, and include the yeast endocytic protein Sla1, the

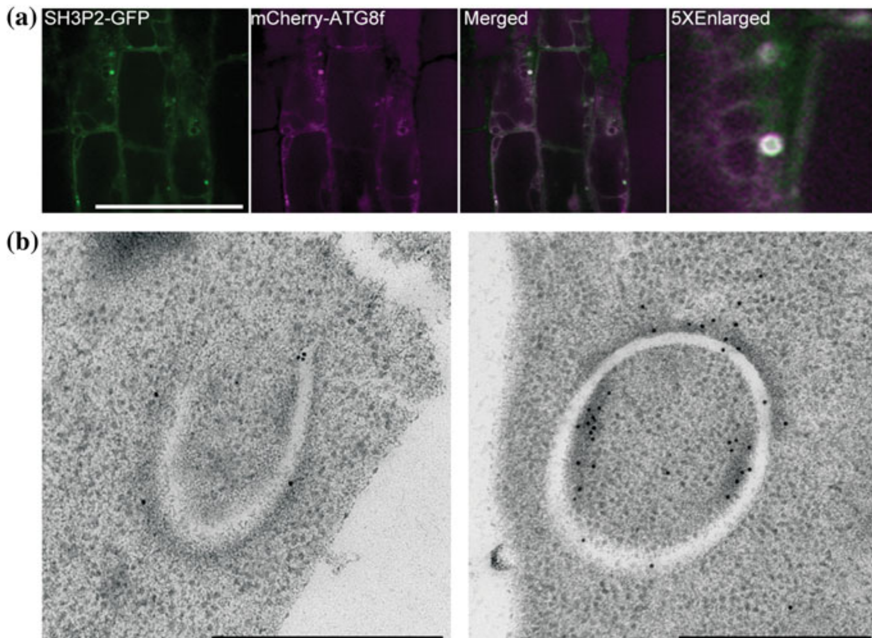

Fig. 5 Roles of SH3P2 in autophagosome formation in plants. **a** SH3P2-GFP translocated to autophagosome (mcherry-ATG8f) upon autophagy induction. Scale bar = 50 μm. **b** Electron microscopy analysis showing that SH3P2 is localized on autophagosome membrane indicated by anti-SH3P2 antibodies. Scale bar = 500 μm

mammalian amphiphysin protein and CIN85 protein. Ubiquitin and PxxP ligands may compete for binding to SH3 domains to regulate different protein complexes assembly in response to different signaling pathways (Stamenova et al. 2007). It will be interesting to test whether the SH3 domain of SH3P2 binds to ubiquitin, which would then be recognized by the ubiquitin receptor and finally targeted by ATG8 for degradation, as it is shown that several ubiquitin receptors contain an AIM motif to be recognized by ATG8 (Svenning et al. 2011; Zhou et al. 2013; Nakatogawa et al. 2012; Yamaguchi et al. 2010; Noda et al. 2010).

Interestingly, we have also observed that SH3P2 displays a mitochondria-like pattern under normal conditions (Fig. 6a, unpublished data). However, after autophagy induction, mitochondria are sometimes surrounded with the signals of SH3P2-GFP, while the majority of the SH3P2-positive punctae remain separate from mitochondria (Fig. 6b, unpublished data). It is possible that SH3P2 may play additional role(s) in linking mitochondria and autophagosomes. In animal cells, Bif-1 has been implicated to participate in apoptosis and is required for the maintenance of mitochondrial morphology and dynamics (Takahashi et al. 2005, 2013a, b; Etxebarria et al. 2009). Therefore, based on the connection between SH3P2 and mitochondria, we cannot exclude the possibility that SH3P2 may also function during plant apoptosis or mitophagy.

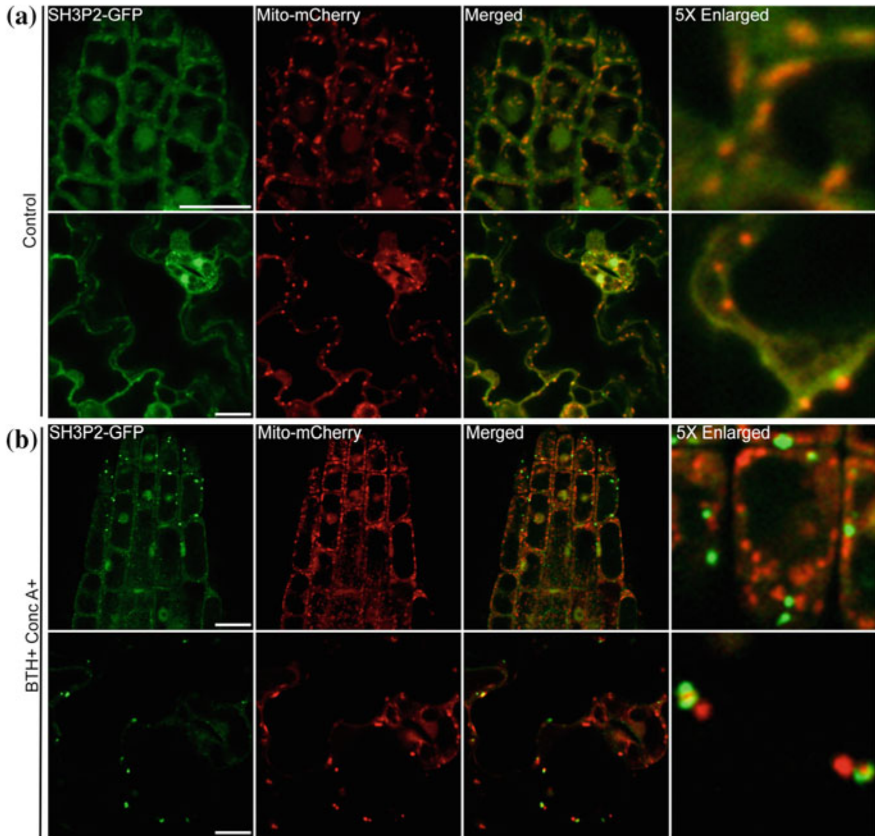

Fig. 6 Association of SH3P2 and mitochondria. **a** Transgenic Arabidopsis plants expressing SH3P2-GFP display a mitochondria pattern in root tip *top panel* and leaf *bottom panel* cells in normal condition. **b** SH3P2-GFP translocated to large punctae in root tip (*top panel*) and leaf (*bottom panel*) cells upon autophagy induction. Scale bar = 10 μm

6 Discussion and Perspective

Signaling transduction is a fundamental part of all eukaryotic cells that arose in evolution from the requirements to regulate cell division and proliferation, in which molecular modules are adapted to comprise multiple complexes for specific pathways. However, plants have a unique lifestyle and are dependent upon hormone regulation and light signaling transduction, which require fewer modules and may evolve plant specific molecules for signaling pathways. Therefore, it is not surprising to find that there are much fewer homologs of the signaling modules in plants.

Both SH2 and SH3 domains are well-known modules during signaling transduction and in animal cells a number of SH2 domain-containing proteins are

covalently linked to the SH3 domain and possess the protein tyrosine kinase activity. Only several SH2 domain-containing proteins are predicted in the plant genome and it seems that they only have a single repeat of the SH2 domain, while in animals most of the SH2 domain-containing proteins carry an additional SH2 domain repeat or are coupled to the SH3 domain. Although there is no typical tyrosine kinase present in plants, several studies have shown that some of the receptor-like kinases contain tyrosine phosphorylation sites which exert essential regulation roles on signaling transduction (Oh et al. 2009, 2010; Afzal et al. 2008; Lin et al. 2014; Hirayama and Oka 1992). However, no experimental evidence has been provided to show the direct interactions of these receptors and the SH2 domain-containing adaptors during a signaling process. Instead, plants have evolved another set of unique transcription regulators called DELLAs for similar regulation. These have a domain organization that is quite reminiscent to the STAT type SH2 domain-containing proteins in animals (Sun 2010; Richards et al. 2000; Yasumura et al. 2007). Although DELLA proteins share little sequence similarities to those SH2 domain-containing proteins in animals, they seem to be some conserved essential amino acids (e.g., tyrosine phosphorylation site) and functional domains (e.g., DNA binding domain) (Fig. 2a). This explains why the searching of a specific domain in a protein might be overlooked simply based on sequencing alignment, and during evolution some unconserved regions in the plant genome might have masked it. The DELLA protein family functions as a pivotal regulatory module for a wide range of core plant developmental process (Sun 2010; Pierik et al. 2007; Zentella et al. 2007). The plasticity of plant development and growth are largely dependent on hormone pathway regulation. In particular, different hormone pathways exhibit physiological redundancy and crosstalk with other(s). Evidence is accumulating which indicates that a transcription regulator is involved in multiple hormone pathways, thereby serving as integrators of multiple signals (Cui and Luan 2012; Depuydt and Hardtke 2011; Farquharson 2010; Peng 2009; Robert-Seilaniantz et al. 2011; Song et al. 2014). Therefore, this may also explain that why plants process fewer and less complicated transcription regulators. Also, these plant unique regulators may perform various roles in plant development. Recently, it has been reported that DELLA proteins direct bind to a tubulin-folding cochaperone called prefoldin in order to regulate the plant cytoskeleton (Dixit 2013; Locascio et al. 2013).

On the other hand, studies from the SH3Ps subfamily implies that in some fundamentally conserved cellular events such as vesicular trafficking and cytoskeleton movement, plants have acquired some conserved regulators for these basic activities (Fig. 7). The endomembrane system is conserved in plants and consists of compartments and trafficking components that are similar but also some which are specific to plants (Surpin and Raikhel 2004; Robinson et al. 2008). In yeast or animals, a number of SH3 domain-containing proteins have been reported and play essential roles in either signaling transduction or other cellular process. So far, only a few SH3-interacting proteins have been identified from pull down assays or yeast two hybrid analysis. These SH3 domain ligands are quite similar to those reported for the animal endophillin subfamily, which is also featured by the N-terminus

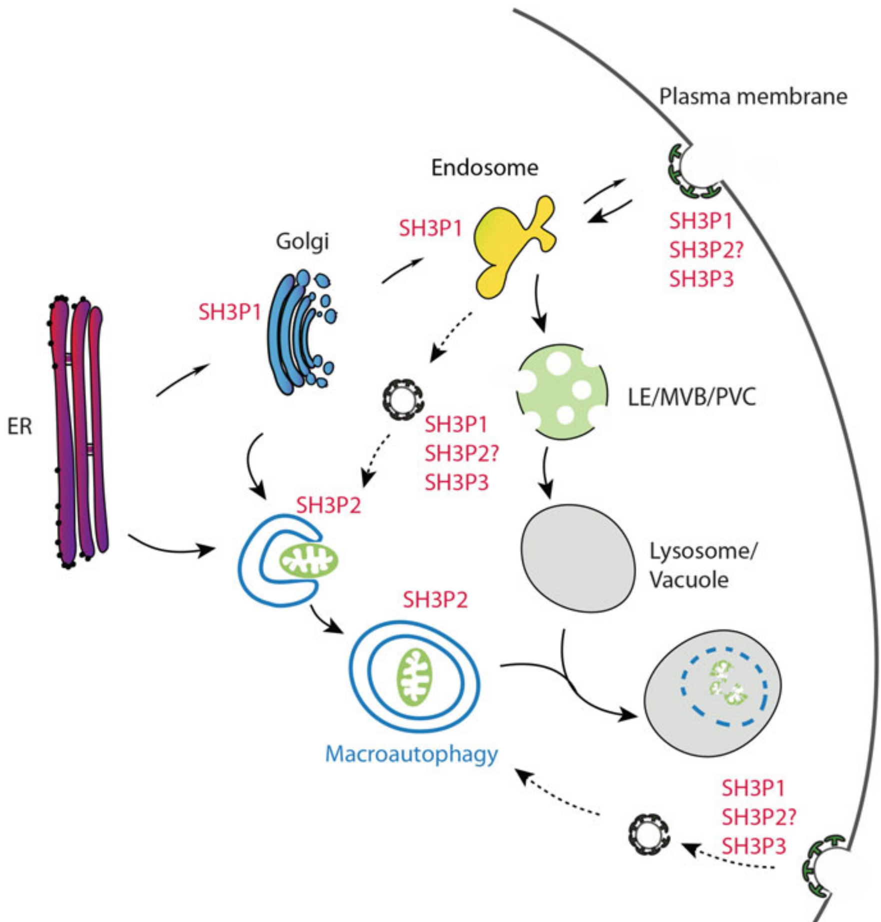

Fig. 7 Possible roles of SH3Ps in mediating membrane trafficking and autophagy in plants. Arabidopsis SH3Ps including SH3P1, SH3P2 and SH3P3 are involved in CCV formation. SH3P1 localizes in multiple endomembrane organelles/vesicles including Golgi, TGN, CCV and SH3P3 is related to CCV formation. Differently, SH3P2 is associated with mitochondria under normal condition and translocates to autophagosome membrane during autophagy

BAR domain. For example, SH3P1 binds to auxilin and actin, while SH3P3 may associate with dynamin in *Arabidopsis* (Lam et al. 2001, 2002). In animal cells there are various BAR and SH3 domain-containing proteins for different vesicle trafficking pathways. Our group has found that another SH3P homolog SH3P2 participates in autophagy in *Arabidopsis* (Zhuang et al. 2013). Although different SH3Ps are involved in distinct trafficking pathways, we also found that these SH3Ps may form homodimers or hetrodimers (unpublished data). These combinations of different homologs may facilitate the recruitment of downstream effectors for either temporal or spatial regulation. However, at the structural level, the detailed

interaction mechanism of these SH3 domains and their cargos have not yet been solved in plants, resulting in an unclear picture of the roles of these adaptor modules in the plant cell. Tools such as crystallography or nuclear magnetic resonance (NMR) will be needed in future to answer these questions, which may ultimately help us to better illustrate the specificity of these fundamental signal blocks in plant signaling pathways. Also, novel assays for the identification of the SH2/SH3 domain are needed to solve the complexity of protein sequencing during evolution. And more evidence at the experimental level is also needed for an understanding of domain-ligand interactions for plant development and growth.

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Versatility of SH3 Domains in the Cellular Machinery

Ana I. Azuaga and Salvador Casares Atienza

Abstract One of the main characteristics of SH3 domains is versatility. Cells make use of this versatility to create different adaptor proteins. SH3 domains reach this high versatility via: (i) The main SH3 recognition motif, proline-rich sequences, presents a geometric two-fold pseudo-symmetry and therefore ligands can bind in either of two possible orientations; (ii) In addition to the canonical sequence, SH3 domains can recognize other interaction motifs such as non-canonical proline-rich sequences; (iii) Recognition of their partners can be accomplished via tertiary interactions between two distinct SH3 domains (Vav n-SH3/Grb2 cSH3) or even with other proteins (Sla1/Ubq); (iv) SH3 domains can make use of different binding surface areas as well, in such a way that both canonical and non-canonical motifs can be recognized at the same time (Pex13p-Pex14p-Pex5p); (v) SH3 domains can also dimerize to form SuperSH3 structures (p47phox/p22phox) or even heterotrimers (CIN85/Cbl interaction) and (vi) SH3 domains can also participate in the autoregulation of their own containing proteins via intramolecular interactions, inducing conformational changes and subsequent exposition of part of these proteins to be recognized by their target (p47phox/p22phox; CIN85/Cbl). However, all these interactions in which SH3 domains take part feature just a moderate-to-low affinity and specificity. Cells, however, have been able to increase them using different procedures, but only when needed, to ensure the recognition of the right target at the right moment, since this intrinsically low affinity is necessary and guarantees a dynamic interaction between partners in the context of cellular plasticity.

Keywords Cell signaling · SH3 domain · Versatility · Proline rich · Interaction · Ligand · Motif · PRD · PPII

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1 Introduction

The full cellular machinery, i.e., all those processes that make the cell alive, such us cell growth, differentiation, motility, polarity, protein synthesis, metabolic pathways, signal transduction processes, DNA replication or even apoptosis are finely orchestrated by a complex network of protein-protein interactions. All these interactions take place in the cellular milieu and are finely regulated in time and space. Adaptor molecules are responsible for the spatial regulation, taking care of bringing other molecules to the correct place so that they can carry out their biological functions in a precise manner. These adaptor molecules are usually polypeptide chains arranged in one or more domains, able to bind to other proteic or non-proteic ligands and, in general, with no enzymatic activity. They also have the capability to connect proteins to other proteins, to the plasma membrane or to different intracellular organelles. This is how large signaling multimeric complexes are formed, gathering signaling molecules to specific subcellular localizations, and hence contributing to the specificity and the efficiency of cellular responses in a spatio-temporal manner (Csiszar 2006; Pawson et al. 2002). Moreover, the same adaptor molecules may operate at different levels in different signaling cascades, which implies that the cell requires of a lower number of molecules to give the same full response to a particular stimulus, therefore saving the metabolic cost associated to molecular delivery. More importantly, the multi-tasking capability of these adaptors allows these signaling networks to be highly dynamic and, therefore, independent signaling events are completely integrated just through a limited number of unique proteins.

Modular interaction domains are used in a combinatorial and repetitive manner within adaptor proteins. These domains are small (30–150 residues) protein modules; they are stable and display a compact structure with one or more binding sites. In addition, they are highly conserved and widely spread over the proteome (Pawson 1995, 2004; Pawson and Scott 1997; Pawson and Nash 2000, 2003). These domains are usually categorized according to homology in their structure and sequence. Each family of domains normally recognizes small sequences of amino acids (3–6 residues), quite specific and conserved on their target proteins (Jelen et al. 2003; Mayer 2001, 2006). However, it is now assumed that certain domain classes are able to recognize distinct peptide sequences, not necessarily related, and run multiple modes of binding. Moreover, different domain families can also identify a similar recognition pattern, providing these interactions with the complexity required to be the driving force of these signaling networks.

Among all modular domains, Proline Recognition Domains (PRDs) constitute the most abundant protein interaction modules in metazoan proteomes (Castagnoli et al. 2004). Since proline-rich sequences are also the most extensively spread sequences in all different genomes (Chandra et al. 2004; Rubin et al. 2000), it is reasonable to consider that both recognition partners have evolved together. These PRDs include seven known classes: Src homology 3 (SH3) (Mayer 2001), WW (named for a couple of highly conserved tryptophan residues) (Kato et al. 2004;

Kay et al. 2000), EVH1 (Enabled vasodilator stimulated-protein homology) (Ball et al. 2002; Prehoda et al. 1999; Volkman et al. 2002), GYF (which features a specific Gly-Tyr-Phe triad) (Freund et al. 1999), UEV (Ubiquitin E2 Variant) domains (Pornillos et al. 2002), Profilin (Mahoney et al. 1997, 1999) and the CAP (cytoskeleton-associated protein)-Gly domain (Saito et al. 2004). Generally speaking, each PRD family shows a unique preference for a specific consensus motif or canonical sequence in their target, which features at least one proline residue that forms the ligand core (Macias et al. 2002; Sudol 1998).

The SH3 domains are probably the most prevalent molecular recognition modules in the proteome (Li 2005; Mayer 2001). To date, more than 1500 different domains have been identified using database screening algorithms. They are part of proteins (such as kinases, lipases, GTPases, adaptor proteins, structural proteins and viral proteins) that participate in intracellular communication networks, in the organization of the cytoplasm skeleton and in membrane trafficking (McPherson 1999; Skorski et al. 1998; Stein 1998). Frequently, SH3 domains work as anchoring sites for substrate recruitment and the formation of supra-molecular complexes that often drive the enzymatic modification of some of its components. These modifications are then translated into the production of new interaction sites and the propagation and amplification of intercellular chemical signals (Kiyokawa et al. 1997; Klejman et al. 2002). Occasionally, these domains can also be essential pieces in the regulation of the enzymatic activity of the proteins they are part of, by means of intra-molecular interactions with other elements of the molecule (Arold et al. 2001; Barila and Superti-Furga 1998; Brabek et al. 2002; Brasher et al. 2001).

The general mechanism of action of PRDs was established in the early 90s (Feng et al. 1994, 1995; Lim et al. 1994; Musacchio et al. 1992b) using the SH3 domain as a model and, over the years, it has proved to be the basis of the ligand recognition shared by most of these PRDs. In fact, all peptide ligands recognized by most PRDs adopt a PPII helical conformation, despite the differences in peptide sequences and domain fold, which means that this PPII helix motif is a universal interaction scaffold for these recognition modules. It is however questionable that all these PRDs share a common evolution precursor, mainly because of the variances found in both sequence and structure between different domain families, but also because it has been demonstrated that whereas some folds exclusively interact with proline-rich sequences, some others can also bind to additional structural motifs. The binding surface of these PRDs generally comprises at least one hydrophobic cavity lined by highly conserved aromatic residues where an “xP” dipeptide accommodates, being “x” any hydrophobic residue. The unique chemical features of these aromatic residues make them especially suitable for binding sites, thus their abundance. On one hand, large side chains ensure strong van der Waals interactions with the ligand. Also, the planar structure of the side chains in this kind of residues seems to ideally fit in the ridges and grooves found on the PPII helix surface. Additionally, there is another pocket with much higher sequence variability that interacts with those residues of the ligand flanking the core recognition sequence, the “specificity pocket” (Chakrabarti and Janin 2002; Feng et al. 1994, 1995; Freund et al. 2002; Lim et al. 1994; Mahoney et al. 1997, 1999; Musacchio

et al. 1992b; Nguyen et al. 1998; Schleinkofer et al. 2004; Zarrinpar et al. 2003), which seems to modulate the selectivity of the interaction. In summary, even being different in both sequence and structure, the six families of PRDs described earlier exploit a common, conserved mechanism of binding to their target ligands and display remarkably similar binding-surfaces. All this could easily be the origin of the cross-reactivity within and between different PRD families. Moreover, the two-fold pseudo-symmetry of the PPII helix causes that many PRDs interact with ligands in two possible orientations (Lim et al. 1994; Zarrinpar et al. 2003), often called “forward” and “reverse”. In both orientations, the same hydrogen-bond donors and hydrophobic grooves of the domain are used for ligand recognition. This orientation flexibility may play an important role in domain function while expands their possible ligand pools.

The versatility of the SH3 domains relies on their special features. In spite of the fact that the shallow binding surface of any SH3 domain presents certain attributes for the accommodation of the PPII helical structure, there are no intricate qualities that allow this binding surface to distinguish subtle differences between two distinct proline-rich sequences. As a matter of fact, it has been shown that a given SH3 domain can interact with a few to several dozens of different peptide ligands (Li and Lawrence 2005). It is common in SH3-interacting proteins to contain multiple weak to moderate affinity binding sites, with K_d values ranging from high nM to low μM. Indeed, computational methods have been developed to identify these putative canonical SH3 domain binding sequences within proline-rich regions of such proteins to narrow down the size of peptide libraries used for high throughput analysis (i-Spot, (Brannetti and Helmer-Citterich 2003); SH3-Hunter, (Ferraro et al. 2007). The SH3 domain may dissociate quickly from one site and subsequently be recaptured by a neighboring site in the same molecule. Thus, the presence of multiple binding sites together with the capability of SH3 domains to recognize a collection of sequence motifs, effectively increases the local concentration of the SH3 domains and thereby promotes binding (Jia et al. 2005). Therefore, the incidence of various SH3 binding motifs in the same molecule may be a mechanism used by the cell to finely regulate SH3-mediated interactions (Li 2005).

Is this multiple and promiscuous recognition a problem for the cell? Also, how can the cell handle to respond precisely to stimuli with such a low specificity in these interactions? And moreover, how a given SH3 domain is able to unambiguously identify its natural targets among the hundreds of homologous PxxP motifs available in the cell pool?

A certain domain class must be able to recognize and therefore bind to a unique ligand to be useful in regulating defined cellular processes, nonetheless, a domain class exclusively dedicated to interact with just one or a few target sequences, unable to adapt to multiple binding events, would not be widespread in a proteome or even favored by evolution. Having multiple domain types that recognize similar targets under certain conditions, even with overlapping motifs, provides these signaling networks with additional modes of interaction. Therefore an apparent yet necessary contradiction exists in these protein-ligand interactions, which the cell has succeeded to circumvent.

There are essentially two main mechanisms by which a given protein can improve its ability to select for a particular target, depending on the source of this specificity. The "intrinsic" specificity resides in the domain itself. The "specificity pocket" of any SH3 domain comprises residues belonging to the RT and n-Src loops. The residues in these areas are quite variable and the loops are flexible in structure and, because of these features, these loops play important roles in modulating the specificity of the domain. Positively charged residues such as arginine and lysine have been known to provide additional "binding force" to the interaction between the SH3 domain and and target peptides through electrostatic interactions with residues in the specificity pocket and also to accurately orientate the ligand with respect to the binding groove on the SH3 domains (Feng et al. 1994, 1995; Wu et al. 1995). Moreover, regions outside the conserved binding surface of the SH3 domain can participate in ligand binding, which often leads to increased affinity and/or novel specificity. Thanks to these special characteristics, SH3 domains have addressed the promiscuity of binding in the cellular context by pursuing non-PxxP motifs, additional residues flanking the PxxP region, or extended binding surfaces to enhance the affinity and specificity of diverse interactions.

Another way to reach specificity is via "extrinsic" specificity. This specificity comes from the cellular context, co-operative events and subcellular localization. The formation of multimeric complexes can localize, orientate and facilitate the interaction between a specific domain and its natural target (Anton et al. 1998; Mayer 2001; Rohatgi et al. 2001). Cellular compartmentalization also contributes to specificity, as it has been shown for the interaction of the CD2BP2 protein and the proline-rich motifs present at the cytoplasmatic tail of the protein CD2. On the other hand, despite Fyn-SH3 domain can also interact in vitro with the same motif as CD2BP2 does, in vivo Fyn-SH3 has been detected inside lipid rafts which indeed hampers its interaction with CD2 (Freund et al. 2002). A change in the subcellular localization of a given domain or ligand will certainly alter the repertoire of possible interacting partners dramatically, owing to the change in their local concentration. This confers plasticity to the interaction and therefore allows a rapid remodeling of interactions in response to changes in the environment.

SH3 domains are small, typically 50–70 residues long, they are biologically relevant and usually, quite easy to produce in vitro, thus they have been extensively investigated both from a structural and a functional point of view. When searching the Protein Data Bank for "SH3" or "SH3 domain" more than 700 structure hits result, including NMR and X-ray structures of either isolated domains or bound to their cognate ligands as well as SH3 domains in the context of their full-length proteins (Camara-Artigas et al. 2009, 2010, 2011; Candel et al. 2007; Casares et al. 2007; Maignan et al. 1995; Musacchio et al. 1992a, 1994; Noble et al. 1993; Ortega Roldan et al. 2007). SH3 domains display a common beta barrel fold, with 5 antiparallel beta strands, β_1 to β_5, arranged in two orthogonal beta sheets of slightly different size, being the biggest one that formed by β_2- β_1- β_5 strands and the smallest one formed by β_2- β_3- β_4. In addition, three loops of different length keep these strands connected. The long RT-loop connects strands β_1 and β_2 and two

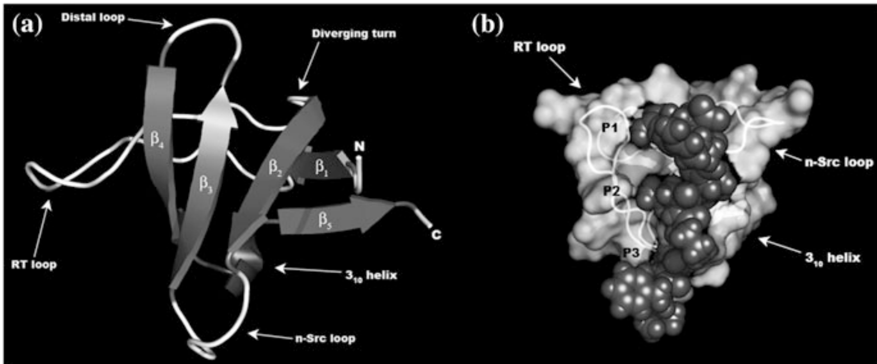

Fig. 1 Structure of an SH3 domain. **a** Tridimensional representation of the SH3 domain of α-spectrin (PDB entry: 1SHG) showing the main structural elements. **b** Representation of the 1:1 complex formed by the Src-SH3 domain and the PLR-1 nonapeptide, AFAPPLPRR, (PDB entry: 1PRM) showing the three binding pockets for canonical binding, P1 and P2 (the "affinity" pockets) and P3 (the "specificity" pocket). SH3 domain structural elements delimiting the binding crevice are also represented

shorter loops, n-Src and distal loops, connect β_2-β_3 and β_3-β_4 strands respectively. A short fragment of 3_{10} helix is also present in the SH3 domains, just placed between β_4 and β_5 strands (Ball et al. 2005; Dalgarno et al. 1997) (see Fig. 1).

2 Canonical Recognition Motifs

2.1 Polyproline Recognition Mediated by SH3 Domains: General Features

SH3 domains, like many other PRDs, interact with their cognate ligands with low affinity, with K_d's usually ranging from 1 to 200 µM (Mayer and Gupta 1998). This low affinity is a compulsory feature of these interactions since SH3 domains frequently play a central regulatory role in multiple signaling networks, where transient dynamic interactions are a must when cell responses to changes in its environment require a specific yet finely tuned adaptability. The canonic conserved sequence motif for most of the SH3 domains described to date is PxxP, where "x" is usually any hydrophobic residue (Ball et al. 2005; Cohen et al. 1995; Dalgarno et al. 1997; Kaneko et al. 2011; Zarrinpar et al. 2003). A more detailed description of this recognition pattern can be found, however, in Aasland et al. where the consensus sequence is described as ΦPpΦP, in which the "p" position is normally occupied by a proline residue and "Φ" usually represents a hydrophobic residue, regularly Leu, Pro or Val (Aasland et al. 2002). Most SH3 domains peptide ligands adopt a helical PPII conformation upon binding to their targets. The interaction

occurs onto a uniform and shallow hydrophobic crevice located at the surface of the domain, where two binding pockets are formed (P1 and P2). These two pockets are structurally chiseled to specifically recognize proline as well as N-substituted amino acids (Nguyen et al. 1998), and therefore they accommodate the ΦP dipeptides present in the core sequence. These two "affinity pockets" are formed by highly conserved residues located at the surface of the domain, such as the two tyrosine residues in the conserved ALYDY motif at the RT loop, the first tryptophan residue of the WW motif right at the edge of the β_3 strand, a proline residue at the β_4 strand and the tyrosine residue of the SNY motif of the 3_{10} helix (Zafra-Ruano and Luque 2012). Even though these two pockets specifically select for proline residues, the high abundance of proline-rich sequences in most proteomes (Chandra et al. 2004; Li 2005) prevents this selectivity landmark to be sufficient to discriminate between different SH3 domains or even different PRD families. Binding specificity arises however, from additional interactions established between residues flanking the core ΦPpΦP sequence in the peptide and a third pocket (P3), the "specificity pocket", defined by variable regions of the RT- and n-Src loops. In general, SH3 domains utilize the sequence and conformational variability found in these loops as a fine-tune mechanism to regulate both binding affinity and specificity for their cognate ligands (Arold et al. 1998; Hiipakka and Saksela 2007; Kaneko et al. 2011; Wu et al. 2007; Zafra-Ruano and Luque 2012).

Traditionally, SH3 domains have been classified according to the orientation of the peptide ligand upon binding. The PPII pseudo-symmetry only permits the SH3 ligands to bind in two possible orientations respect to the binding site (Feng et al. 1994): "forward" (N-to-C-terminal) or "reverse" (C-to-N-terminal). According to this classification, SH3 domains that bind peptide ligands in a "forward" orientation are referred as **class I** and those that bind their ligands in a "reverse" orientation as **class II**. Class I SH3 domains recognize targets with a positive charge at their N terminus, being the consensus sequence (R/K)xxPxxP whereas class II SH3 domains select peptides with a positively charged residue at their C terminus, with a consensus sequence PxxPx(R/K) (Kang et al. 2000; Kay et al. 2000; Mayer and Gupta 1998; Sparks et al. 1996). Peptide array screening experiments have suggested an overall natural preference for the class II ligands (Wu et al. 2007). However, the preferred orientation usually depends on the singularities of the SH3 domain binding site, being usually determined by (i) the presence of a basic residue located two residues N-terminal from the xPxxP motif in class I ligands and two residues C-terminal from that same motif in class II ligands, which forms a salt-bridge with an acidic residue located at the RT-loop of the domain (Feng et al. 1994; Lim et al. 1994) and (ii) the orientation of the side chain of a conserved tryptophan residue at the β_3 strand of the domain (Fernandez-Ballester et al. 2004). Along these lines, some studies have shown that, not only the orientation but also the consensus sequence selected can differ from one SH3 domain to another. SH3 domains from the Src family, like Src, Lyn and Fyn are prone to select peptides with similar consensus sequences: RPLPPLPXP, RXXRPLPPLPxP and RPLPP (I/L)P respectively (Cheadle et al. 1994; Rickles et al. 1994; Sparks et al. 1994; Yu et al. 1994). But, besides this "common" binding mechanism for most SH3

domains, some other domains within the SH3 family do not follow the exact same rules. The Abl-SH3 domain for example, interacts with ligands containing the consensus PPXΘXPPPΨP motif but not a positive charge in their sequences, and therefore represents a second specificity group (Zafra-Ruano and Luque 2012). Cortactin and p53bp2 SH3 domains however, recognize peptide ligands where conserved positively charged residues are flanking the PxxP core sequence; Cortactin SH3 selects +PPΨPxKPxWL and p53bp2 prefers RPXΨPΨR+SXP. PLCγ SH3 and Crk N SH3 both prefer peptides with a conserved basic residue located C terminal of the PxxP core, PLCγ SH3 prefers PPVPPRPxxTL and Crk N SH3 prefers ΨPΨLPΨK. Grb2 N SH3 domain selects a different consensus sequence, +ΘDXPLPXLPΦΦ; (Ψ, Θ and + represent aliphatic, aromatic and basic residues respectively in all model sequences provided here).

2.2 *Complexity in Polyproline Recognition Mediated by SH3 Domains*

Despite the wealth of structural and functional information collected over the last 20 years, the rational design of high affinity and specificity molecules, aimed to inhibit or modulate the poly-proline recognition process mediated by these domains, is still a quite challenging task. The reason may be found in the relatively low affinity for their naturally occurring ligands as well as in the delicate balance between specificity and promiscuity, frequently identified as a signature of these interactions (Cesareni et al. 2002; Landgraf et al. 2004; Tong et al. 2002). During the last two decades, multiple attempts have been made to identify and rationally design high-affinity ligands for SH3 domains (Dalgarno et al. 1997; Garbay et al. 2000; Lulf et al. 2011), employing a wide variety of methodologies such as screening of libraries of synthetic compounds (Li and Lawrence 2005, Oneyama et al. 2003, 2002), phage display strategies (Cheadle et al. 1994; Ferguson et al. 2004; Karkkainen et al. 2006; Panni et al. 2002; Rickles et al. 1995; Tong et al. 2002), structure-based peptide sequence optimization (Feng et al. 1994; Pisabarro et al. 1994; Pisabarro and Serrano 1996; Ren et al. 1993; Yu et al. 1994), stabilization of the PPII conformation using protein scaffolds (Cobos et al. 2004), insertion of non-peptidic components (Feng et al. 1996; Lawrence 2005; Mayer and Dimarchi 2005; Panni et al. 2002; Vidal et al. 2004) or D-amino acids (Schumacher et al. 1996) in the peptide ligands or even designing small molecule inhibitors (Inglis et al. 2005, 2004). However, the outcome resulting from all these efforts has been quite limited, with the only exceptions of the use of peptoid combinatorial libraries where key proline residues have been substituted by synthetic analogs (Aghazadeh and Rosen 1999; Nguyen et al. 1998, 2000), and the combinatorial modification of peptide scaffolds (Lawrence 2005; Li and Lawrence 2005), which have produce a few high-affinity ligands for SH3 domains, with K_d values in the nM range. In addition, Serrano and coworkers have applied rational design

strategies based on computational algorithms where merely structural information is considered to identify several high-affinity sequences for the Abl-SH3 domain (Pisabarro et al. 1994; Pisabarro and Serrano 1996). However, these satisfactory results are certainly not universal and thus these structure-based rational design methodologies cannot be seen as the panacea for the production of high-affinity ligands capable of modulating SH3 domains' activity.

Historically, design strategies intended to identify and optimize ligand sequences for these domains have been focused on the ultimate enhancement of the binding affinity, considering the Gibbs energy (ΔG) of binding exclusively. However, the notion of the ΔG of binding as the only discriminating parameter in a rational design strategy or a screening technique is indeed a pretty limited conception of the binding process, since ΔG does not provide information relative to the nature or the magnitude of the forces driving the interaction. This information is encrypted in the enthalpic and entropic contributions to the Gibbs energy of binding ($\Delta G = \Delta H - T\Delta S$) and represents the thermodynamic signature of the interaction, as they account for the relative magnitude of the intermolecular forces governing a particular interaction (Freire 2009; Luque 2010). In addition, since different combinations of enthalpic (ΔH) and entropic (ΔS) contributions can give rise to a similar binding affinity, a detailed knowledge of the energetics of the interaction is extremely valuable in the estimation of the driving forces of the binding process (Freire 2008; Lafont et al. 2007; Velazquez Campoy and Freire 2005). Moreover, it has been demonstrated that integrating these thermodynamic determinants into the rational design procedure can help identify and therefore select the best ligands for these domains in terms of binding affinity, selectivity (Chaires 2008; Holdgate and Ward 2005; Kawasaki and Freire 2011; Ladbury et al. 2010; Schon et al. 2011a; 2011b; Velazquez-Campoy et al. 2001) or even shelf life (Freire 2002; Ohtaka et al. 2002).

Over the last decades, a wide set of structural and thermodynamic studies has supported the idea of an SH3-mediated recognition of proline-rich sequences based almost exclusively on the insertion of the proline residues of the ligand into the two hydrophobic clefts present at the binding site of the domain, with very few polar interactions. Assuming this conception to be correct, one would expect a thermodynamic signature for this interaction dominated by the hydrophobic effect and, therefore, a positive entropic contribution as the main force driving the interaction. This favorable entropic term would be associated to a higher degree of conformational freedom of the solvating water molecules, which are released into the bulk of the solvent upon the interaction. Along these lines, the enthalpy associated to the interaction should be unfavorable (positive) or just slightly favorable (Velazquez Campoy and Freire 2005; Velazquez-Campoy et al. 2001). However, this hypothesis is far from reality, given that all thermodynamic studies of SH3 interactions carried out to date consistently show that the recognition of proline-rich sequences by these domains entails a remarkably negative (favorable) enthalpic contributions opposed by unfavorable (negative) binding entropy (Aitio et al. 2008; Arold et al. 1998; Arold and Baur 2001; Chan et al. 2003; Cobos et al. 2004; Ferreon and Hilser 2004; Ladbury and Arold 2011; McDonald et al. 2009; Palencia et al. 2004; Renzoni et al. 1996; Rubini et al. 2010; Seet et al. 2007; Wang et al.

2001; Wittekind et al. 1994). In summary, this observed thermodynamic behavior cannot be explained assuming that this interaction relies exclusively on a direct interaction between hydrophobic surfaces and, therefore, a more complex mechanism must be involved in this recognition function.

Investigating this apparent discrepancy between structure and thermodynamics is certainly not trivial. In fact, several studies have been carried out to try to decipher the thermodynamic determinants of this interaction and have identified multiple causes responsible for this striking behavior: (i) the redistribution of the conformational ensembles of the protein and the ligand when the interaction occurs, impacts on the binding energetics; (ii) when the ligand binds to the protein, it adopts a PPII conformation, reduces its conformational freedom, thus resulting in unfavorable entropic contributions, but also multiple interactions are established between the ligand and the domain and this results in favorable enthalpic effects (Ferreon and Hilser 2004); (iii) both the RT- and the n-Src loops become rigidified upon binding and, at the same time, the xP motifs of the ligand deeply insert into the domain's binding pockets, leading to a full reorganization of the hydrogen-bond network in the complex molecule (Arold et al. 1998; Ferreon and Hilser 2003; Ferreon et al. 2003), which is indeed cooperatively transmitted through the structure of the domain and significantly contributing to a negative (favorable) enthalpic effect (Cordier et al. 2000; Wang et al. 2001); (iv) the presence of interfacial water molecules at the binding site of the domain is responsible for additional effects and seems to play a crucial role in proline-rich ligand recognition by SH3 domains (Martin-Garcia et al. 2012; Zafra-Ruano and Luque 2012).

2.3 The Role of Water at the Binding Site of SH3 Domains

The abnormal thermodynamic signature associated to the interaction between the SH3 domains and their cognate ligands was initially identified for the Abl-SH3 domain and the de novo rationally designed peptide ligands p40 (APTYSPPPPP) and p41 (APSYSPPPPP). These interactions are characterized by high affinities, in the low μM range, and a high specificity for Abl-SH3 (Pisabarro and Serrano 1996). A detailed examination of the crystal structure of the Abl-SH3/p41 complex (Pisabarro et al. 1998) identified a full set of interfacial water molecules involved in a complex network of hydrogen bonds, mediating the interaction between the ligand and a series of residues in the domain: (i) waters mediating the interaction between residues at the n-Src loop and the specificity region of the ligand, (ii) waters bridging residues Asn114 and Ser113 at the 3_{10} helix in the domain and the PPII region of the ligand. These water molecules do not interact directly with the ligand. Instead, they form a highly polar extended surface and serve as adaptors, filling gaps and optimizing van der Waals interactions, therefore fulfilling the hydrogen bonding potential of both the ligand and the domain's binding site and helping to disperse charges. All these effects should favorably impact on the binding enthalpy (Luque and Freire 2002; Velazquez-Campoy et al. 2000) but, at

the same time, they would entail an unfavorable entropic cost as the water molecules get fixed at the binding interface, thus compensating the favorable enthalpic contribution. Thus, these results are consistent with this unexpected thermodynamic behavior for the Abl-SH3/p41 interaction and suggest that interfacial water plays a crucial role in it, contributing to the highly negative binding enthalpy observed.

Assuming all these effects as real, then the accepted model for the Abl-SH3/p41 interaction would be incomplete at its best, since this process would necessarily take place via two concurring mechanisms: (i) insertion of proline side chains of the ligand into the hydrophobic grooves at the binding site of the domain and (ii) establishment of a robust yet moldable network of hydrogen bonds mediated by water molecules, where residues flanking the canonical binding site are involved. This dual nature of the binding mechanism is in much better agreement with the observed thermodynamic signature for this interaction and, of course, should always be considered as a key factor for ligand rational design strategies (Zafra-Ruano and Luque 2012). However, this is not just a unique mechanism for the Abl-SH3/p41 interaction since a detailed analysis of the SH3 domains structural database has shown that a similar network of water molecules is present in many other SH3 domains (Martin-Garcia et al. 2012b). Remarkably, these conserved water molecules have been detected close to the poly-proline recognition region of the domain regardless the orientation of the peptide (class I or class II), conforming similar hydration patterns. Moreover, in addition to those water molecules located at the binding interface of the domain, several others have also been found in the close proximity of RT- and n-Src loops of SH3 domains, mediating interactions between these loops and the specificity regions of the ligand, with highly variable arrangements, mostly dependent on loops and ligand sequences. It has been demonstrated that all rational design and ligand identification strategies can greatly improve when detailed information regarding the role and the significance of each interfacial water molecule at the binding interface is integrated into the rationale (de Beer et al. 2010; Garcia-Sosa et al. 2005; Garcia-Sosa and Mancera 2006; Lie et al. 2011; Mancera 2002, 2007; Minke et al. 1999; Rarey et al. 1999; Roberts and Mancera 2008; Schnecke and Kuhn 2000; Thilagavathi and Mancera 2010; van Dijk and Bonvin 2006; Verdonk et al. 2005). In summary, the existence of interfacial water molecules is a common feature of all SH3-ligand interactions and therefore, that dual character of the interaction initially described after a close inspection of the Abl-SH3/p41 complex is a common feature of this binding process. This new conception of the interaction is now consistent with the unexpected thermodynamic behavior observed for these SH3 complexes and, therefore, this interfacial water network must be systematically included in any comprehensive study to fully unveil the molecular basis of the binding affinity and specificity and significantly improve any rational design approach.

Finally, it is important to mention that, even though there is substantially less structural information related to other PRD families, these water mediated interactions have also been found for WW domains, UEV domains and EVH-1 domains (Martin-Garcia et al. 2012b). Just like for SH3 domains, some interfacial water molecules are present at the binding interface of these domains, making it larger and

more polar. This would again imply an abnormal thermodynamic signature for those interactions where these domains are involved, with a large favorable enthalpic contribution. This is indeed the case for all the thermodynamic studies reported to date for poly-proline recognition mediated by non-SH3 PRDs and, therefore, this dual binding mechanism associated where a hydrophobic interaction is linked to a polar interaction built on a robust network of water-mediated hydrogen bonds, is possibly a general feature of most families of poly-proline recognition modules.

3 Non-canonical Recognition Surface on SH3 Domains

Kalirin (a RhoGEF protein) contains an SH3 domain (Kal-SH3) and several PxxP motifs (PLPP, PLSP and PKTP) that regulate GEF activity through both intra and intermolecular SH3 domain interactions (Schiller et al. 2006). Kalirin-SH3 has unique site(s) for binding PxxP peptides. Several hydrophobic residues that form the hydrophobic pockets in other SH3 domains, that accommodate the Pro residues in the PxxP polyproline type II helix, are not conserved in Kalirin. Gln24 in Kalirin should be Phe or Trp. In addition, several hydrophobic residues expected in the 3_{10} helix are not found in Kalirin. NMR experiments showed that different peptides bind in different sites on Kal-SH3 domain. Site 1 involves several residues from the RT and n-Src loops. Site 2 involves segments of the β_1, β_4, and β_6 sheets. A detailed analysis of chemical shift changes provided support for a two-site model. The PKTP peptide bound to clusters of residues in sites 1 and 2, whereas the PLSP peptide affected residues only in site 1. It is therefore clear, that PxxP peptides did not interact with Kal-SH3 at the canonical PxxP-binding sites.

One of the strongest SH3-ligand interactions found in nature is the interaction between IRTKS SH3 domain and $EspF_U$ $R47_5$ protein (Aitio et al. 2008). Chemical shift perturbation (CSP) analysis together with Isothermal Titration Calorimetry (ITC) experiments have shown an unusually tight SH3 binding with a dissociation constant close to 500 nM. The most unique feature of the IRTKS/$EspF_U$ complex is the presence of two authentic PxxP motifs in $R47_5$ both contributing to this interaction. The additional PxxP motif is accommodated by two hydrophobic slots in the extended specificity pocket of IRTKS SH3 in a manner that is virtually identical to the interaction of the canonical PxxP motif with the conserved SH3 proline-binding pockets of IRTKS. The N-terminal binding groove in IRTKS SH3, which accommodates another PxxP motif, harbors several hydrophobic residues in the n-Src loop and β_3 and β_4 strands as well in the specific pocket that render the specificity-determining region highly hydrophobic. This region in IRTKS is unique among known SH3 structures and accounts for its unusual ligand-binding properties. The recognition of a second PPII helical PxxP motif by the specificity-determining region observed in the IRTKS/$EspF_U$ complex is an extreme example of adaptability, which plays a decisive role in mediating host-pathogen interaction, resulting in seizure of host´s actin assembly machinery.

4 Non-canonical Recognition Motifs

SH3 domains may possess the most diverse specificity among interaction domains. In addition to recognizing the class I and II peptides with PxxP core, a number of SH3 domains have been shown to bind peptide sequences that lack such a motif.

4.1 RxxK

One of the first reported non-canonical interactions was the one between the UBPY peptide and STAM2-SH3 domain (Kato et al. 2004; Endo et al. 2000; Lohi and Lehto 2001; Takata et al. 2000), with the consensus sequence PX(V/I)(D/N) RXXKP. This interaction has been shown to play a regulatory role in endocytic trafficking of growth factor-receptor complexes through early endosomes and this association is essential for Hbp to exert its function. Structural determination by X-Ray indicated that in the structure of the complex between the SH3 domain and the peptide, the first moiety of the UBPY peptide is similar to a classical class II ligand while the second half adopts a right-handed 3_{10} helix conformation. This motif, whose consensus sequence was later established as RxxK, is a binding partner of several SH3 domains (Lewitzky et al. 2001; Liu et al. 2003; Li et al. 2006). The interaction between SLP-76 and Gads proteins is mediated by such motif. The structure of the Gads SH3-C domain/SLP-76 complex reveals a novel mode of peptide recognition. There are four, instead of three, binding pockets on the Gads SH3-C domain, and the SLP-76 peptide ligand assumes a unique structure that is characterized by a 3_{10} helix at the RSTK locus and is optimized for high-affinity binding. The structural reasons behind this alternative binding arise from the differences in the RT loop of the Gads SH3-C domain compared to other SH3 domains like the c-Src SH3 domain. The base of the RT loop in Gads SH3-C domain is shifted towards the n-Src loop while the tip of the loop swings away from the n-Src loop. This creates a binding groove that is narrower at the base and wider at the mouth than that present in the c-Src SH3 domain. On the other hand, the unusually high affinity seen in the binding of Gads SH3-C to SLP-76 may also have its origin in the extensive hydrophobic contacts of the peptide ligand with the Gads SH3-C domain. While interactions between the two xP units of the ligands with the hydrophophic pockets of conventional SH3 domains are rather superficial, which is also why most SH3-peptide interactions are weak; the corresponding pockets in the Gads SH3-C domain engage the peptide more tightly. Also, while in the case of Gads SH3-C, peptide's Ile4 is the residue that fits into the hydrophobic pocket and, together with Glu275 makes the interaction between the domain and the peptide quite tight, in the case of STAM2 SH3 this area is rather flat and there is not pocket (Kaneko et al. 2003). Those results demonstrate the highly specific ligand recognition ability of the Gads SH3 domain, whereas the STAM2 SH3 domain maintains lower recognition ability, much like that of ordinary SH3 domains.

A similar type of interaction has also been reported for the N-terminal SH3 domain of mouse PIX and the p21-activated kinase-interacting exchange factor. Just like in Gads SH3-C domain, there is a fourth binding pocket on the surface of the domain, thus explaining the higher affinity and specificity for its ligands (Li et al. 2006).

4.2 RKxxYxxY

Another example of SH3 binding motif devoid of proline residues is the consensus sequence RKxxYxxY, present in the SKAP55 protein, involved in the interaction with the SH3 domains of Fyn and Fyb (or SLAP130) proteins (Kang et al. 2000). This novel motif is similar to a class I proline-based motifs in which tyrosines might substitute for proline residues. Authors showed by surface plasmon resonance experiments that SH3 domains capable of recognizing class I proline motifs can recognize the RKxxYxxYY sequence as well, but this is not the case for those SH3 domains that recognize class II motif, which are indeed unable to bind to this anomalous sequence. On the other hand, NMR experiments demonstrated that this atypical motif uses the same binding surface as the canonical motif. In the case of the SH3 domains of Fyb and Fyn, the residues directly involved in the interaction form a contiguous surface area with a predominantly negative surface potential. The mode of interaction of the tyrosine motif is, however, different form the consensus rules established for the binding of standard proline-rich ligands to SH3 domains. The smaller contact surface for the tyrosine-based ligand may account for the lower affinity observed for this peptide.

Another protein that can bind this atypical motif is ADAP, by its C-terminal SH3 domain. Whereas the ADAP-SH3 domain binds to the RKxxYxxYY motif of SKAP-55, the SH3 domain of SKAP-55 can bind to a proline-rich region in ADAP. This interaction makes all cellular SKAP-55 present in the cell to be complexed to ADAP thus explaining their functional role (Duke-Cohan et al. 2006).

4.3 PxxDY

It has been shown that the SH3 domains present in the tyrosine kinase substrate Eps8 and related proteins selectively bind to the PxxDY motif (Aitio et al. 2008; Mongiovi et al. 1999). The authors studied the interaction between the SH3 domains of Eps8L1 family member with the PPVPNPDYEPIR sequence of the $CD3_{\varepsilon}$ cytomplasmatic tail protein. NM experiments revealed that the SH3 domain surface involved in the interaction with this peptide is the same used in the canonical interaction. Differences arise from pocket 1 in the domain. The presence of an isoleucine residue at position 531 instead of a tyrosine or a proline, typically found at this position in SH3 domains, renders the first hydrophobic specificity

pocket of Eps8 SH3 smaller and non-optimal for binding to the canonical motif and, therefore, no interaction between the first xP from the motif and the domain occurs. Another difference is the presence of a positive residue in the n-Src loop of Eps8 family SH3 domains, which forms a salt bridge to an aspartate residue in the motif, and may explain the specificity of this domain for the peptide.

4.4 R(S/T)(S/T)SL

Kim et al. carried out a study on the non-canonical interaction of Fus1p-SH3 domain with the consensus sequence R(S/T)(S/T)SL. These authors demonstrated that this sequence is necessary for binding but it is not sufficient (Kim et al. 2008). A deep structural analysis of the available structures of SH3 domains complexed with extended PxxP-containing peptides revealed two distinct binding surfaces. Surface I, which interacts with the PxxP motif, has been extensively described and is a narrow region. In contrast, surface II is much broader and residues found on this surface display considerably lower levels of conservation. These residues lie in the RT and n-Src loops as well as strands 3 and 4 of the domain and they interact with the Arg present in non-canonical sequences and residues flanking the core consensus peptide sequences. Residues in the n-Src loop participate in surface II, but the exact positions of these residues vary due to the significant structural heterogeneity seen in this region among different domains. Surface I and II lie at almost an oblique angle to one another, and they are delineated by the position of the highly conserved Trp36 residue, which forms part of both surfaces. By mutational analysis, authors established that surface I interact with the variable regions of the target peptides located N-terminal to the consensus sequence. It is surface II the one mediating the non-canonical interaction of Fus1p-SH3 domain and the peptide. Analysis of the alignment of SH3 domains from homologues of Fus1p show that several positions predicted to lie on surface II are highly conserved. This pattern of conservation implies that none of these domains recognizes a PxxP motif, and that they all likely utilize surface II to bind peptides in a manner similar to that of the yeast domain. Extending this observation to the interaction between non-canonical motifs and others SH3 domains, Kim and coworkers noticed that interactions with Surface II involving a key Arg residue can account for almost every "non-canonical" SH3 domain interaction that has been described in the literature. Also, these results suggest that any SH3 domain could display a high binding affinity if its target peptide interacts well with both binding surfaces. The ability of surfaces I and II to function together to bind to PxxP motifs, and the ability of surface II to function independently of surface I, fully explain how a single SH3 domain can recognize both canonical and non-canonical targets. This would also explain how these completely different targets can still show competitive binding behavior, as the Arg binding region would still be required to bind both targets.

4.5 PxxPR

CIN85 belongs to a small family of adapter proteins (CIN85/CD2AP) that function as docking partners for numerous signaling proteins. It is composed of three amino-terminal SH3 domains (SH3A, SH3B and SH3C) involved in the interaction with different partners. The interaction of this protein with its partner c-Cbl is mediated by a non-canonical PRD sequence, PxxxPR (Kowanetz et al. 2003, Kurakin et al. 2003), and the three SH3 domains bind to this motif acting as a platform bridging multiple Cbl molecules in mammalian cells. In addition to CIN85, PIX is also an SH3 containing protein able to interact with that same PxxxPR motif present in Cbl. The crystallographic structures of the complexes between a Cbl-b proline-arginine peptide and the SH3 domains of both PIX and CIN85(SH3A) show that the Cbl peptide induces the formation of an herotrimeric complex consisting of two SH3 domains and one peptide molecule (Jozic et al. 2005). The structures reveal as well that the interaction in both complexes is very similar to one another and also that the peptide binds simultaneously in class I and II orientations. This dual orientation has also been detected by NMR and ITC experiments and, it has been shown that it leads to an unusual curvature of the Trp36 indole conserved in all the SH3 domains and a strong broadening of NMR signals indicative of chemical exchange between multiple conformers, therefore supporting the formation of heterotrimeric complex (Ceregido et al. 2013). The authors in this study also investigated another member of the same protein family, CD2AP, with three SH3 domains arranged in a similar fashion as in CIN85. However, they could not identify trimeric species as a result of the interaction between the SH3A domain of CD2AP and Cbl-b. In fact, despite an unusual curvature of the Trp36 indole is observed as well, which is indicative of two possible orientations, no line broadening was detected that time, therefore suggesting that there are no significant conformational exchange phenomena for this CD2AP-SH3A/Cbl-b complex. Essentially, in solution, the predominant CD2AP-SH3A/Cbl-b complexes are dimers formed by the interaction of a single molecule of CD2AP-SH3A and one molecule of Cbl-b arranged either in type I or type II orientations instead of a trimeric state. In summary, this study shows that both SH3A domains in CIN85 and CD2AP proteins are able to interact with their targets in either type I or type II orientations depending on whether the atypical proline targets contains an arginine at the N-terminus (for type I) and/or at the C-terminus (for type II). The atypical PxxxPR recognition sequence thus needs to be extended to RxPxxxPR to include type I and type II interactions. The atypical PxxxPR recognition sequence thus needs to be extended to RxPxxxPR to include the type I interaction. In addition, CD2 protein is also capable of interact with the CIN85/CD2AP family via a PxxxPR motif but, in this case, the non-canonical sequence lacks an arginine residue at the N-terminal position, thus avoiding type I interactions. This differential mode of recognition provides the molecular basis for the distinct biological behavior observed between these two adaptors proteins and

point out again towards the versatility of SH3 domains and their role in the cellular machinery.

This type of interaction between SH3 domains and their targets where hetero-trimeric complexes are formed is by no means an odd case. Hashimoto and coworkers have showed that the interaction between the SH3 domain of cortactin protein and the proline rich peptide of AMAP1 protein (SKKRPPPPPPGHKRT) occurs in such a way where one single peptide molecule binds to two cortactin SH3 domains simultaneously (Hashimoto et al. 2006). In this complex, two amino acids of pocket 4 interact with both SH3 domains simultaneously. Although the interaction of this peptide is Type I respect to one of the SH3 domains of cortactin and Type II respect to the other one, this type II binding is atypical because the consensus basic amino acid, which is C-terminal to the PxxP motif, is not present. In addition, in Martín-García et al. the same type of interaction is analyzed as well. The authors found that the Fyn-SH3 domain interacts to a peptide from NS5A protein with an anomalous sequence (APPIPPPRRKR) following this same type of arrangement (Martin-Garcia et al. 2012a). In this case, the complex crystals showed four Fyn-SH3 domain chains (A-D) and two peptide chains (E-F) in the asymmetric unit. In Fyn-SH3 A the orientation of the peptide is N- to C-terminal, while in Fyn-SH3 D the orientation is the opposite. In the structure peptide E is bound to Fyn-SH3A and Fyn-SH3 B and peptide F bound to Fyn-SH3C and Fyn-SH3 D, simultaneously.

The N-terminal SH3 domain of the PIX protein and the PAK kinase also interact by the non-canonical PxxxPR motif already described. The SH3-binding segment of PAK contains the atypical consensus-binding motif PxxxPR, which is required for unusually high affinity binding (Hoelz et al. 2006). The arginine residue in this motif forms a salt-bridge and is tightly coordinated by a number of residues in the SH3 domain. This arginine-specific interaction appears to be the key determinant of the high affinity binding observed for PAK-derived peptides. Furthermore, C-terminal residues in the peptide are also engaged in additional contacts with the surface of the RT-loop in its partner PIX-SH3 domain, which significantly increases binding specificity. These two facts would explain the remarkably high affinity observed for the interaction between the PIX-SH3 domain and PAK sequences Another issue, pointed out by the authors, is the existence of two interacting PIX-SH3/PAK2 complexes in the crystal structure, in which dimerization was achieved by residues from a conserved portion of the PAK peptide, while ITC and fluorescence experiments showed a 1:1 stoichiometry. This dimerization issue becomes even more complicated when the structure of the PIX-SH3/Cbl-b complex is considered. In this last structure, two SH3 domains interact with a single peptide in a head-to-head fashion, rather than the head-to-tail conformation observed in PIX-SH3/PAK2 complexes. This seems to indicate that the interaction is completely peptide-dependent. This complex is akin to a SuperSH3 domain, allows for an additional level of regulation and illustrates de remarkable versatility of the PIX-SH3 domain.

Furthermore, there is another example of ternary SH3 domain-peptide complexes: the SuperSH3 domain described for the interaction between auto-inhibited

form of and the p22phox-bound state (see Sect. 8). However, in contrast to the interaction between the heterotrimers of CIN85 (SH3A) and PIX, the SH3 domains of p47phox are covalently linked and this link is required to allow the two SH3 domains to simultaneously bind the same peptide. The relative orientation of the SH3 domain in the heterotrimeric structures described above is different from that observed in p47phox as residues of the n-Src loop do not contribute to trimerization (Jozic et al. 2005). This type of interaction constitutes a novel mechanism by which SH3 domains can contribute to the formation of multiprotein complexes.

CIN85-SH3 domains can also bind the PxxxPR motifs present in ASAP1/AMAP1, Hip1R, SH1P1, and SH3KBP1 (Sato et al. 2013) proteins. Another important interaction via PxxxPR motif is the one between CD2AP, preferentially via its SH3B domain, with the cytoplasmatic p53 (the polymorphic variant P72R) (Panni et al. 2014). p53 is a crucial node for the regulation of cell physiology, in particular, the proline-rich region of the human p53. Since CD2AP is able to connect different partner proteins, it may also anchor p53, in particular the R isoform, in the cytosol, and therefore partially protect that p53 isoform from polyubiquitination and subsequent degradation.

Another atypical proline-rich motif CIN85-SH3 domain binds to is PxpxxRh. It was initially identified in the tumor suppressor protein lysyl oxidase precursor protein (LOX-PP) that functionally inhibits CIN85-mediated invasion by breast cancer cells. The binding of LOX-PP interfered with the CIN85 interaction with c-Cbl, and compromises those functions of CIN85 that are essential for the invasion process by tumor cells. LOX-PP appears to interact exclusively with the SH3B domain of CIN85. The main structural differences between the three SH3 domains of CIN85 lie in the n-Src and RT loops. The n-Src loop is the most divergent sequence region of the three. In SH3A domain, this loop is positioned further away from the peptide backbone in a distinct conformation and thus appears unable to form this interaction. The spatial architecture surrounding Arg116^{LOX-PP} is modulated by these loops, and may provide a precise geometry for effective binding (Sato et al. 2013).

Finally, it is important to remark that the SH3 domain surface involved in the interaction with these non-canonical (non-PxxP) motifs is not always the same. In Kami et al. (Kami et al. 2002) the authors showed that the three different SH3 domains studied; p67^{phox}-SH3C, Grb2-SH3C and Pex13p-SH3; accommodate the cognate non-PxxP peptides onto different regions on the molecular surface (see Fig. 2). This result clearly indicates that sequences with unconventional motifs occupy different binding sites on each SH3 domain. This versatility allows many different possibilities: An SH3 domain can interact with two different proteins simultaneously, using a conventional PxxP motif-binding site and another binding site for a non-PxxP sequence, leading to the assembly of a multiprotein complex, as seen in *S. cerevisiae* Pex13p-Pex14p-Pex5p. Otherwise, an SH3 domain can serve as a signaling switch by a competition mechanism between two possible interaction partners, for example the Grv2-SH3C domain, as the binding site of a non-PxxP motif overlaps the PxxP motif-binding site. Moreover, the presence of a PxxP motif and non-PxxP motif in the same polypeptide chain may generate a protein ligand

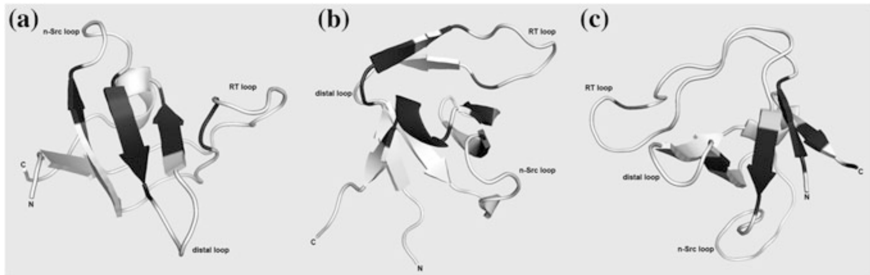

Fig. 2 Schematic representation of three anomalous binding surfaces for non-PxxP peptide recognition by SH3 domains. **a** p67^{phox} SH3C domain (PDB entry:1K4U), **b** Grb2 SH3C domain (PDB entry: 1GFC) and **c** Pex13p SH3 domain (PDB entry: 1NM7). Residues involved in ligand recognition are colored in *black*

with both high affinity and specificity via the multivalent binding mechanism, as seen in p67^{phox} – p47^{phox} and Csk-PEP (Ghose et al. 2001; Gregorieff et al. 1998) although in this last case the high affinity and specificity observed comes from the interaction mediated by residues distant from the PPII helix region on the peptide.

5 Tertiary Interactions

SH3 interaction via complementary surfaces has become a recurring theme in protein-protein interaction studies. Growing experimental evidences suggest that, in addition to peptide binding, SH3 domains can associate with other proteins via tertiary contacts that involve no defined motif.

5.1 Ubiquitin Interaction

The versatile nature of SH3 domains in ligand recognition and in regulating the formation and dissociation of protein complexes is illustrated by its interaction to ubiquitin protein.

The first article reporting an SH3-Ubiquitin (Ubq) interaction was published in 2007 by Stamenova and coworkers (Stamenova et al. 2007). In this work, the authors could establish that the third SH3 domain of Sla1 (Sla1 SH3-3; a yeast homolog of the human CIN85) was responsible of the Ubq binding. This was a surprising partner since Ubq does not carry a PxxP-like sequence at all. The interaction between Sla1-SH3-3 domain and Ubq occurs in the same hydrophobic groove on SH3 domains involved in the interaction with the canonical proline rich motif. and in the case of Ubq involve the Ile44 surface area, the same site of interaction with all ubiquitin binding domains (UBDs) described to date. Authors point out to a Phe at

position 75 of the SH3 domain as an essential residue for Ubq interaction. He et al. (He et al. 2007) determined the solution structure of the third SH3 domain of the yeast Sla1 protein in complex with monoubiquitin. Based on structural evidences authors explain why mutation of Phe75 to Tyr in Sla1-SH3-3 domain abolish binding between the domain and Ubq. The introduction of a hydroxyl group results in unfavorable steric clashes between SH3 moiety and Ile44 of Ubq probably due to the lack of a proper hydrogen bonding donor and acceptor groups near the hydroxyl group. This would explain the importance of Phe at position 75. However another feature(s) of the SH3 domain must also contribute to the ability to interact with Ubq. Indeed, some authors have also shown how some SH3 domains with Phe at position 75 do not bind Ubq as is the case for CD2BP1 while others with Tyr at this position do interact with Ubq like Nck2-SH3-3 (Kang et al. 2008).

Apart from those SH3 domains involved in the endocytotic pathway, SH3 domains participating in immune signaling bind ubiquitin with a variety of ubiquitin-recognition mechanisms. In Kang et al. six SH3 domains in this pathway (Kang et al. 2008) were investigated. Out of all those, two (Lck-SH3 and CD2BP1-SH3) have a Phe residue at position 73, and the other four (Nck2-SH3-1,2,3 and Fyn-SH3) have Tyr. NMR experiments showed that two of them, Lck-SH3 and Nck2-SH3-3, are able to bind ubiquitin. A closer examination of the data yielded clues to the underlying difference between the SH3 domains with Phe73 (Lck-SH3) and those with Tyr73 (Nck2-SH3-3). For Lck-SH3, the residues at the n-Src loop and the RT loop exhibited significant perturbations, whereas the residues in the 3_{10} helix showed only marginal perturbations. In contrast, for Nck2-SH3-3, the most affected residues were those in the RT loop or the 3_{10} helix, and the residues in the n-Src loop were much less affected. The nature of the residues affected upon binding to Ubq established that hydrophobic interactions in the RT loop and the 3_{10} helix regions might play more important roles in the SH3 domains with Tyr73 than those with Phe73. It is likely that the roles of each subregion involved in Ubq binding are different, even though the binding surfaces generally agree. Indeed, the observation that CD2BP1-SH3 does not bind Ubq corroborates that not only the Phe73 but also the individual primary structure should be considered in addressing the Ubq binding. Also the binding residues of Ubq involved in binding to the SH3 domains with Phe73 and Tyr73 seem to be different. There are three different sub-regions in Ubq involved in target protein interaction: region I centered around Leu6, region II around Ile44, and region III around Val70. Authors showed that Ubq binding by Lck-SH3 shows a large perturbation in region II of Ubq. By contrast, Nck2-SH3-3 showed much less perturbation in region II that in the other two sub-regions.

Therefore, the interaction mechanisms for Ubq biding could be quite different depending on the particular SH3 domains considered in each case. Another example in this direction is the binding between the third SH3 domain of CD2AP and CIN85 proteins and Ubq. Ortega-Roldan and coworkers, performed a structural and mutational analysis of the three SH3 domains of CD2AP and the third SH3 domain of CIN85 in their interaction with Ubq (Ortega Roldan et al. 2013). In this work, the authors found a distinct ubiquitin-binding mode for the third SH3

domains of both adaptor proteins, in which (i) the Phe73 does not play a key role and (ii) the interaction is characterized by a higher affinity for ubiquitin that is augmented by additional stretches of residues at the C-terminus of Ubq. Indeed, while the mutation of Phe73 to tyrosine in the first (SH3A) and second (SH3B) domains of CD2AP abolish binding similarly to what was observed for Sla1 SH3-3, the mutation of the corresponding Phe residue in CD2AP SH3-C has only a minor effect on ubiquitin binding as observed by both the NMR and ITC experiments. The distinct interactions between SH3 domains and Ubq can be rationalized by analyzing the surfaces of both proteins. Ubiquitin structures reveal that the main differences between both binding modes reside in the interactions between polar residues in the RT and n-Src loops and the Ubq C-terminus. Again these results indicate that the primary structure of the different SH3 domains should be considered in addressing their interaction with Ubq.

In terms of the role of SH3-Ubq and SH3-PRD interactions in cell machinery, the authors suggested that the competition for binding to SH3 domain by Ubq and peptide ligands acts as a regulation mechanism for the assembly of different protein complexes in a spatial and temporal manner. This is the case of the interaction of the SH3 domain of amphiphysins and CIN85 homologs that may bind to Ubq and proline containing ligands at different times in the assembly of primary endocytic vesicles or at different stages in the endocytic pathway (Stamenova et al. 2007). In this context, Bezsonova and collaborators (Bezsonova et al. 2008) proposed a simplified model in which equilibrium exists between CIN85-SH3 domains binding to Ubq and to Cbl. This equilibrium is shifted by changes in accessibility of PxxxPR sequence in Cbl. Without stimulation by EGF, the conformation Cbl adopts does not allow the interaction between CIN85-SH3 domains and its proline rich motif and, in this case CIN85-SH3/Ubq interaction is favored. However, upon EGF stimulation, the phosphorylation of Cbl and a conformational change in the molecule occurs, with an exposure or stabilization of PPII sequence, able to be recognized by CIN85-SH3 domains. Since the affinity of SH3-PPII interaction is higher than the one to Ubq, the interaction with Ubq is disrupted.

Another SH3 domain that binds Ubq is the SH3 domains of STAM2. This protein is member of the ESCRT complex (endosomal sorting complexes required for transport) with multiple modular motifs known to bind ubiquitin (VHS, UIM and SH3 domains). The SH3 domain of STAM has two natural partners: Ubq and the PxxK motifs of the UBPY molecule (Berlin et al. 2010; Niendorf et al. 2007); they both interact with the same region on the SH3 domain (Lange et al. 2012). Lange and collaborators studied these interactions and speculated with the hypothesis that there is a competitive binding between Ubq and UBPY to the SH3 domain depending on the concentration of ubiquitinated cargoes. At low concentration of ubiquitinated cargoes, UBPY would bind SH3, resulting in deubiquitination and subsequent recycling of the cargo protein. Inversely, at high cargo concentration, Ubq would replace UBPY and, therefore, prevent cargo proteins form recycling. Being this another example of regulation of macromolecular assembly in the cell.

The mammalian *parkin* gene encodes a 52 kDa protein that harbors a conserved N-terminal ubiquitin-like domain (Ubl). This domain binds SH3 domains within a subset of proteins containing a lipid-binding BAR domain, including endophilin-A, and are involved in vesicle trafficking (Trempe et al. 2009). As in the case of Ubq, Ubl domain binds to the same PRD-surface area of endophilin. In this case, the interaction is stronger than the one of Ubq and the SH3 domains of Sla1 and CIN85, but in the same order than that to a PRD peptide derived from the synaptojanin E2′binding site. Thus, the SH3 domain binds the Ubl and PRDs via a common site, with similar affinities in this case. Most of the Ubl residues interacting with the endophilin-A1 SH3 domain are identical to those found in Ubq. However, Asn8 and Arg74, which are conserved in all mammalian parkin Ubls and absent in Ubq are essential for SH3 binding. As in the case of Cbl phosphorylation and the exposition of its PRD domains to CIN85-SH3 domain, the regulation of the interaction between SH3 domain of endophilin and parkin Ubl domain is mediated by phosphorylation of a set of PRD proteins called dephosphins.

5.2 Other Tertiary Contacts

Plakins are a family of high molecular weight proteins that interconnect elements of the cytoskeleton and tether them to membrane associated structures. Plectin is a member of this family and has an SH3 domain inserted in the central repeat. The structure of this domain is not compatible with its binding to prolin rich motifs for two reasons (Ortega et al. 2011). First, three well-conserved aromatic residues that create the xP-pockets in canonical SH3 domains are substituted by amino acids with shorter side chains in plectin, namely Cys-840, His-865 and Cys-882. The presence of these three non-aromatic residues creates a flat surface in plectin instead of the two xP-pockets. Second, the RT-loop is three-residues shorter than that of a typical SH3 domain and the compass pocket of plectin is wider than that of other SH3 domains. The residues that form the putative binding site of the SH3 of plectin are also present in other plakins, suggesting that none of them recognize proline-rich sequences in a canonical manner. To date, no proline-rich ligand has been identified for any SH3 domain of this family. The results presented by the authors suggest that in plectin and other plakins members, the area of the xP-pockets has evolved to be engaged in an intramolecular interaction instead of in ligand recognition. It is possible, however, that this domain might mediate or contribute to the association with functional proteins via a non-PxxP binding mechanism.

SLAM-associated protein (SAP) is a free SH2 domain that regulates signal transduction events induced by at least six members of the SLAM family of receptors. This protein interacts with the Fyn-SH3 domain through a surface-surface interaction that does not involve proline-rich peptide sequences. SAP recruits Fyn-SH3 domain using a surface largely formed by the βF strand, the amino terminal end of the αB helix and the intervening turn. The complementary region of the Fyn-SH3 domain is formed primarily by the RT-loop and by strands β_2, β_3 y β_4.

The interaction surface on the SH3 domain overlaps with the binding site for the PxxP motif. A close examination of the distribution of charges on the surfaces of both domains reveals an electrostatically complementary interface. The SAP surface is positively charged, whereas the Fyn-SH3 surface is negatively charged. This constitutes an example of high level of diversity in the binding repertoire and function of modular signaling domains (Chan et al. 2003).

Another example of tertiary contacts between an SH3 domain and a protein surface is the interaction between the SH3 and mainly the Dbl homology (DH) domains of Asef (APC-stimulated guanine nucleotide exchange factor), a protein involved in actin cytoskeletal network reorganization (Murayama et al. 2007). The structure of Asef consists of the ABR (APC binding region), the SH3, the DH and the PH domains. The X-Ray structure of this protein reveals how the DH and PH domains interact with the SH3 domain. The last α-helix of the DH domain (α6), consisting of 37 residues, interact with the SH3 domain via the RT-loop and the C-terminal part. In the Asef structure, the interdomain interaction surface of the SH3 domain is perpendicular to its polyproline peptide binding groove and thus blocks one end of the groove. The Asef-SH3 domain competes with Rac for the binding site on the Asef-DH domain, and Rac only becomes able to interact with the DH domain once the SH3 domain is released from it. This Asef structure represents the autoinhibited form, with respect to the GEF (guanine nucleotide exchange factor) activity of Rac. Therefore, the SH3 domain plays the crucial role in autoinhibiting the Rac1 GEF activity of the DH domain in Asef.

6 SH3-SH3 Interaction

The Vav N-terminal SH3 domain (Vav n-SH3) does not bind to proline-rich molecules. Instead, it binds to the C-terminal SH3 domain of Grb2 (Grb2 cSH3) (Nguyen et al. 1998). There are two remarkable features of Vav n-SH3 as compared with known SH3 domain proteins. First, the RT loop of this SH3 domain is particularly notable for its unusual extension because there is a tretraproline sequence, which is in the PPII helical conformation, inserted in the RT-loop. In addition, this RT-loop is not fully exposed to the solvent but fixed to the region close to the putative proline-rich peptide binding surface on the SH3 domain. This tetraproline region occludes the interaction of a proline-rich peptide to the canonical binding site, due to steric hindrance. On the other hand and despite the tetraproline adopts a PPII helix conformation does not constitute the binding area to Grb2 cSH3 domain. Secondly, the hydrophobic residues present at the binding site of the domain are replaced by hydrophilic ones, therefore preventing the interactions between the conserved aromatic side chains and the proline, essential for peptide ligand recognition. The interaction between the N-terminal SH3 domain of Vav and the SH3 C-terminal domain of Grb-2 is however mediated by the surface of Vav n-SH3

opposite to the tetraproline region, as identified by chemical CSP studies (Nishida et al. 2001; Ogura et al. 2002). This represent a unique example of interaction since it requires the whole structure of both interacting SH3 domains.

7 Second Surface Binding Site in SH3 Domains (Different Than PRD Site)

There are several examples in the literature where an SH3 domain is capable of binding to different targets through different binding areas. This is the case of the Pex13p-SH3 domain (Douangamath et al. 2002) which is able to bind to Pex14p, involving a classical Type II PxxP-type interaction, and a second ligand, Pex5p, devoid of a recognizable PxxP motif, and hence, its interaction may be of a different nature. Indeed, Douangamath and collaborators, used NMR spectroscopy and X-Ray crystallography, and could show that Pex5p adopts an α-helical conformation and binds to a novel site on the Pex13p SH3 domain that is opposite to that of the Pex14p binding site. The Pex5p binding site is centered on a concave surface comprised of strands β_1 and β_2. As opposed to the conserved PxxP binding site for Pex14p, common to most SH3 domains, the Pex5p binding site is devoid of any conserved residues and, therefore, it is likely to be specific to this Pex13p-SH3 domain. Competition assays have demonstrated that both peptides can bind simultaneously to the SH3 domain (Pires et al. 2003). In the cell, Pex13p has been designated to play a pivotal role in perosixomal matrix protein import, where its function is required for both PTS1- and PTS2- signal-driven protein import complexes. This interaction is the first example identified of a distinct intermolecular ligand-binding site in an SH3 domain, structurally separated from the canonical PxxP binding site.

8 Tandem SH3 Domains

The incidence of various SH3 binding motifs in the same molecule may be a mechanism used by the cell to regulate SH3-mediated interactions (Li 2005). In this respect, modular interaction domains often occur in tandem in regulatory proteins and this allows affinity as well as specificity to be augmented through co-operative binding, involving multiple domains of the same or different kinds (Pawson 2004; Pawson and Nash 2003). Moreover, little is known about the possible effects of the presence of a second or even a third domain on the regulation in the affinity and the specificity of the binding to another domain within the same protein. To date, most efforts have been focused on the study of isolated single domains.

Multiple examples of proteins containing tandem domains exist in the genome like the CIN85/CMS family of adaptor proteins. In this family all three N-terminal SH3 domains are involved in a wide variety of different interactions to different

targets. Moreover, these three SH3 domains share higher similarity among themselves than to any other SH3 domains, suggesting that they may have overlapping specificities in binding. It is therefore of great interest to understand the effect of the presence of multiple SH3 domains in the mechanism of the interaction of this type of adaptor proteins as we described in Sect. 4

On the other hand, NADPH oxidase consists of six subunits that are partitioned between different subcellular locations in the resting state. In this state, the interaction of p47phox with p22phox, and thereby the translocation and activation of NADPH oxidase, is prevented by an auto-inhibited conformation of p47phox. This arises from an intramolecular interaction of the SH3 domains with a region in the C-terminal portion of the protein and involves non-canonical binding sequences. The C-terminal portion has been mapped to the polybasic region that does not contain a conventional SH3 binding motif. The structure of the complex, solved by multi wavelength anomalous diffraction (MAD), reveals an unexpected mode of target recognition by SH3 domains in which the conserved ligand-binding surfaces of both SH3 domains are juxtaposed so as to create a single binding groove that is occupied by the N-terminal portion of the polybasic region (Groemping et al. 2003). The sequence RGAPPRRSS of the polybasic region binds to this groove in an intramolecular interaction. Residues GAPPR adopt a PPII helix conformation and make contacts with both SH3 domains. This interaction is radically different form other SH3 domain/target complexes in which a single SH3 domain binds to a single target sequence. Two structural features are responsible of this particular domains arrangement. One is the covalent link between the SH3 domains that increase the local domains concentration and drives the extremely weak interaction. And the other is the presence of a conserved GWW-motif in the n-Src loops in the SH3 domains that allows the interface to be formed. In this motif, the first Trp is needed for ligand binding and is fully conserved in SH3 domains while the second Trp contributes to interdomain interaction. The Gly residue, however, does not interact with the targets at all but participates in van der Waals contacts with main chain atoms in the opposing n-Src loop. When checking the protein database, a few SH3 domains satisfy the GWW-motif. These include the CD2AP/CMS family of adaptor proteins, FISH, and a number of ORFs, encoding proteins of unknown function. It has been proposed that the formation of superSH3 domains might be a general phenomenon in cell signaling mediated by SH3 domains, however no direct evidences have been revealed so far. Hence, further studies are necessary to reevaluate the binding partners of multiple SH3 domain-containing proteins in order to establish a model for the mechanism of binding of these proteins to their targets as well as to confirm which of these uses such superSH3 domain arrangement.

Another example of a superSH3 domain is the splicing version Tks5 $SH3AB_{short}$ (Rufer et al. 2009). In this tandem, both SH3 domains bind synergistically to Sos1 protein. These two domains can, however, bind to dynamin individually. The observed binding for Tks5 cannot be explained by ligand-induced dimerization, as seen for complexes of PIX and CIN85 SH3 domains with a Cbl-b peptide (Hoelz et al. 2006; Jozic et al. 2005), because then the individual SH3A and SH3B domains of Tks5 would be expected to interact autonomously. The interactions

observed with $SH3AB_{short}$ cover all three classes of recognition sequences, either conventional or degenerate PxxP motifs or even entirely noncanonical sequences. The tandem SH3 domains in Tks5 employ two distinct types of binding modes: one class of peptides is recognized by single SH3 domains (e.g. dynamin), whereas a second class of peptides requires the presence of both domains to bind synergistically (e.g. Sos1). Therefore, the tandem constitutes a versatile module for the implementation of isoform-specific protein-protein interactions.

9 Conclusions

The fact that SH3 domains are involved in so many different functions within the cellular machinery makes them an important target to engineer proteins with specific signaling properties. A better knowledge of these domains and the principles governing recognition by modular domains will open doors to develop new therapeutic strategies to treat seriously impairing illnesses.

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Structure-Function Relationship of Bacterial SH3 Domains

Shigehiro Kamitori and Hiromi Yoshida

Abstract The Src Homology 3 domain (SH3 domain) is a protein domain that has been identified in the diverse signaling and cytoskeletal proteins of eukaryotes. A typical SH3 domain is composed of a β-structure consisting of five β-strands connected by three loops, called the RT loop, n-Src loop, and distal loop, and a short 3_{10} helix. A Pro-rich peptide ligand binds to a groove flanked by the RT loop and a short 3_{10} helix. SH3-like domains in prokaryotes have been predicted to act as the targeting domains involved in bacterial cell wall recognition and binding, and the bacterial SH3 domain has been identified in various bacteria with some variations. The most prominent difference in structure between the eukaryote and bacterial SH3 domains is in the RT loop. The bacterial SH3 domains have long insertions of amino acids in the RT loop, by which the corresponding region to the peptide ligand-binding site of the eukaryote SH3 domain is disrupted. This finding indicated that bacterial SH3 domains perform their functions in different manners from eukaryote SH3 domains. We herein provided an overview of the structure-function relationship of bacterial SH3 domains, on the domains targeting the bacterial cell wall, and the domains involved in metal-binding based on their X-ray structures.

Keywords Bacterial cell wall · Bacterial SH3 domain · Diphtheria toxin repressor · Endolysin · Endopeptidase · Ferrous iron-transport activating factor · Internalin · Metal-binding protein · X-ray structure

1 Introduction

The Src Homology 3 domain (SH3 domain) is a protein domain of approximately 60 amino acid residues, and has been identified in the diverse signaling and cytoskeletal proteins of eukaryotes. A typical SH3 domain is composed of a

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β-structure consisting of five β-strands connected by three loops, called the RT loop, n-Src loop, and distal loop, and a short 3_{10} helix (Saksela and Permi 2012). A peptide ligand binds to a groove flanked by the RT loop and short 3_{10} helix. The most conserved ligand binding residues in the eukaryote SH3 domains are Phe7, Phe9, Trp35, Pro49, and Tyr52, which form a hydrophobic groove to interact with Pro-rich peptide ligands (Fig. 1a).

SH3-like domains in prokaryotes have been predicted to act as targeting domains involved in bacterial cell wall recognition and binding (Ponting et al. 1999; Whisstock and Lesk 1999), and the bacterial SH3 domain has been identified in various bacteria with some variations. According to Pfam (Finn et al. 2014), a database of a large collection of protein families, the SH3 domain is classfied into nine member subfamilies; hSH3, SH3_1, SH3_2, SH3_3, SH3_4, SH3_5, SH3_6, SH3_8, and SH3_9, with bacterial SH3 domains being found in five subfamilies; SH3_3, SH3_4, SH3_5, SH3_6, and SH3_8. In this chapter, the eukaryote SH3 domain and bacterial SH3 domain have been designated as the SH3e domain and SH3b domain, respectively, for simplicity, and their secondary elements have been labeled as B1–B4, and H1 with capital letters, and b1–b7, and h1 with small letters, respectively.

X-ray structures of the bacterial SH3 domains (SH3b domains) found in PDB (Berman et al. 2003) are shown (Fig. 1b, c). In 1996, Qiu et al. firstly reported that the third domain of diphtheria toxin repressor (DtxR) had an SH3-like domain fold based on its X-ray structure (PDB ID: 2DTR/1C0W) (Qiu et al. 1996). In 2002, Marino et al. showed the X-ray structures of the three tandem repeated SH3b domains in *L. monocytogenes* invasion protein InlB (1M9S), as GW domains with conserved Gly-Trp residues (Mario et al. 2002). SH3b domains have since been detected in the X-ray structures of a glycylglycine endopeptidase (1R77) (Lu et al. 2006), γ-d-glutamyl-l-diamino acid endopeptidases (2HBW, 2FG0, 3H41, 3M1U, and 4R0K) (Xu et al. 2009; Xu et al. 2010), a ferrous iron transport activating factor (FeoA) (3MHX) (Su et al. 2010), and an endolysin (4KRT) (Tamai et al. 2014).

The amino acid sequence alignments of those with a SH3e domain (mouse c-Crk, 1CKA) (Wu et al. 1995) are shown (Fig. 2). The SH3b domains typically have additional amino acid residues to the SH3e domain. The five β-strands and short 3_{10} helix in the SH3e domain are conserved in the SH3b domain. The most prominent difference in structure between them is in the RT loop. SH3b domains have long insertions of amino acids in the RT loop, which form secondary structures, β-strands, and/or α-helices. Most SH3b domains have two β-strands in the RT loop, which form a β-sheet with five conserved β-strands, in which b2 and b6 are interacted by hydrogen bonds of the main chain atoms, thereby giving the closed conformation of the RT loop (Fig. 1b). In the SH3e domain, the loop region corresponding to b2 (b2 region) is far from B4, whereas that to b3 forms hydrogen bond interactions with B4 to give the open conformation in the RT loop for binding of the Pro-rich peptide ligand (Fig. 1a). The open conformation of the SH3e domain is caused by hydrophobic contacts between Phe9, Trp35, Pro49, and Tyr52, inhibiting the b2 region of the RT loop approaching B4. The set of Phe9, Trp35,

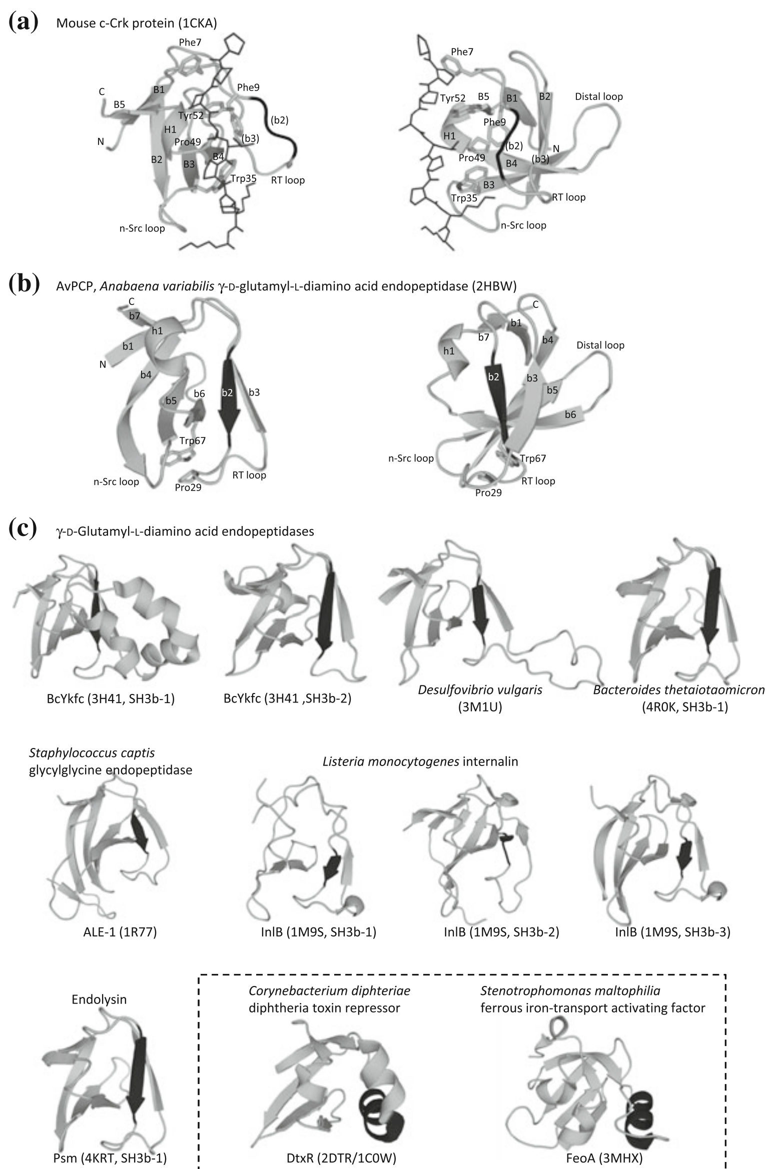
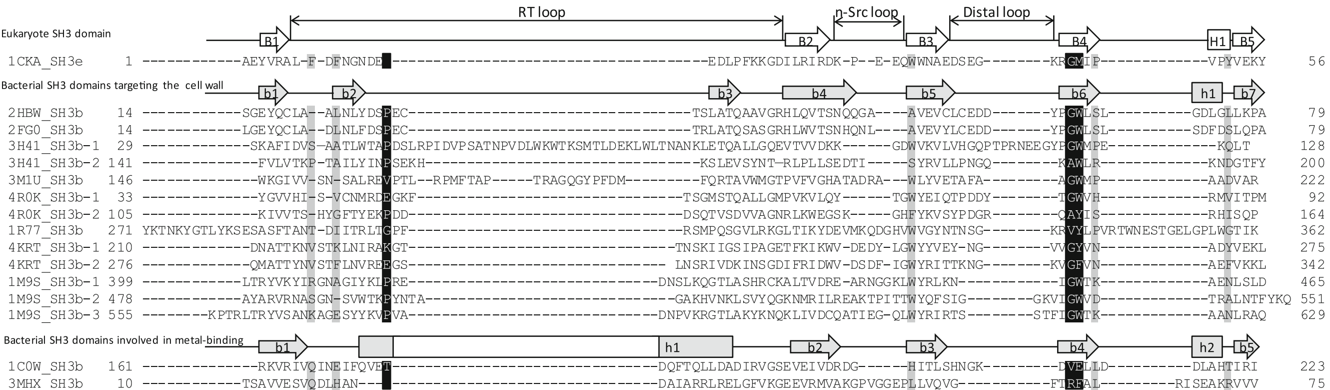


Fig. 1 X-ray structures of SH3 domains. **a** Structure of the SH3e domain of mouse c-Crk is shown with the bound Pro-rich peptide ligand in two directions. Secondary structure elements are labeled as B1–B4, and H1. The loop region corresponding to b2 in the SH3b domain is in black. **b** The structure of the SH3b domains of γ-d-glutamyl-l-diamino acid endopeptidase (2HBW) is shown in two directions. Secondary structure elements are labeled as b1–b7, and h1 in the SH3b domains, and b2 is shown in black. **c** Structures of other SH3b domains are shown in the same view direction as (**b**) with b2 in black. The structures of 2FGO, SH3b-2 in 4KRT, and SH3b-2 in 4R0 K, were omitted, because each had a pair domain with an almost equivalent structure. The SH3b domains of DtxR (2DTR/1C0W) and FeoA (3MHX) with different structures from the other SH3b domains are shown in a dashed line box



1CKA: Mouse c-Crk
2HBW: AvPCP, *Anabaena variabilis* γ-D-glutamyl-L-diamino acid endopeptidase
2FG0: NpPCP, *Nostoc punctiforme* γ-D-glutamyl-L-diamino acid endopeptidase
3H41: BcYkfc, *Bacillus cereus* γ-D-glutamyl-L-diamino acid endopeptidase
3M1U: *Desulfovibrio vulgaris* γ-D-glutamyl-L-diamino acid endopeptidase
4R0K: *Bacteroide thetaiotaomicrom* VPI-5482 γ-D-glutamyl-L-diamino acid endopeptidase
1R77: ALE-1, *Staphylococcus captis* EPK1 glycylglycine endopeptidase
4KRT: Psm, Endolysin by episomal phage phiSM101 of enterotoxigenic *Clostridium perfringens* type A strain SM101
1M9S: InlB, *Listeria monocytogenes* internalin
1C0W: DtxR, *Corynebacterium diphteriae* diphtheria toxin repressor
3MHX: FeoA, *Stenotrophomonas maltophilia* ferrous iron-transport activating factor

Fig. 2 Amino acid sequence alignment between SH3b domains with a eukaryote SH3 domain (c-Crk). The positions of β-strands and helices are shown by arrows and rectangles, respectively. The positions of conserved hydrophobic amino acid residues (Phe7, Phe9, Trp35, Pro49, and Tyr52) among the SH3e domains are shaded, and the positions of conserved amino acid residues (Pro29, Gly66, and Trp67 in 2HBW) among the SH3b domains are highlighted in black

Pro49 and Tyr52 is rarely conserved in SH3b domains. On the other hand, stacking interactions between Pro29 and Trp67 in the SH3b domain markedly contribute to its closed conformation. Pro29 is mostly conserved in SH3b domains, and Trp67 is conserved well in SH3b (GW) domains as the conserved sequence of Gly-Trp (Fig. 2). The SH3b domains of DtxR (2DTR/1C0W) and FeoA (3MHX) are structurally different from those of the other SH3b domains. They have α-helices in the RT loop instead of β-strands, and have no conserved Gly-Trp residues. However, the corresponding region to the peptide ligand-binding site of the SH3e domain is occupied by α-helices in an RT loop. Thus, the SH3b domains block and disrupt the peptide ligand-binding site in the SH3e domain by its own RT loop, indicating that SH3b domains perform their functions in different manners from SH3e domains.

In this chapter, we describe the three-dimensional structures of SH3b domains relating to their functions, the SH3b domains targeting the bacterial cell wall, and the SH3b domains involved in metal binding. Figures of molecular structures in this chapter were drawn by PyMol (DeLano 2002).

2 SH3b Domains Targeting Bacterial Cell Wall

2.1 Bacterial Cell Wall

Gram-positive bacteria possess a thick cell wall (∼250 Å) that surrounds their cytoplasmic membranes and provides physical protection. The bacterial cell wall consists of a mesh polymer of peptidoglycans and teichoic acids, bacterial polysaccharides with phosphate groups. In peptidoglycans, linear glycan backbones of alternating β(1-4) linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) are cross-linked by peptide side chains (Fig. 3a) (Vollmer et al. 2008; Popham 2013; Leyh-Bouille et al. 1970). Teichoic acids are covalently linked to peptidoglycans (wall teichoic acids), or to the lipids of the cytoplasmic membrane (lipoteichoic acids). The peptide side chains of peptidoglycans vary depending on the bacterial species. The chemical structure of the peptide glycan of *S. aureus* is shown (Fig. 3a). The lactic acid group of NAM forms an amide bond with a tetra peptide of l-Ala-γ-d-Glu-l-Lys-d-Ala, and a pentaglycine interpeptide bridge forms a crosslink between the ε-amino group of l-Lys and terminal carboxyl group of d-Ala in the neighboring peptideglycan chain. In *Bacillus subtilis*, the sequence of the stem peptide is l-Ala-γ-d-Glu-DAP (meso-diaminopimelic acid)-d-Ala-(d-Ala), and a direct cross-link is formed between DAP and d-Ala without an interpeptide bridge.

Three potential models for the bacterial cell wall have been proposed (Popham 2013); (i) a multiple layer model in which peptidoglycan chains parallel to the cell membrane are arranged circumferentially, forming a layer structure (Ghuysen 1968); (ii) a cable model in which peptidoglycan chains form 50 nm coiled cables arranged

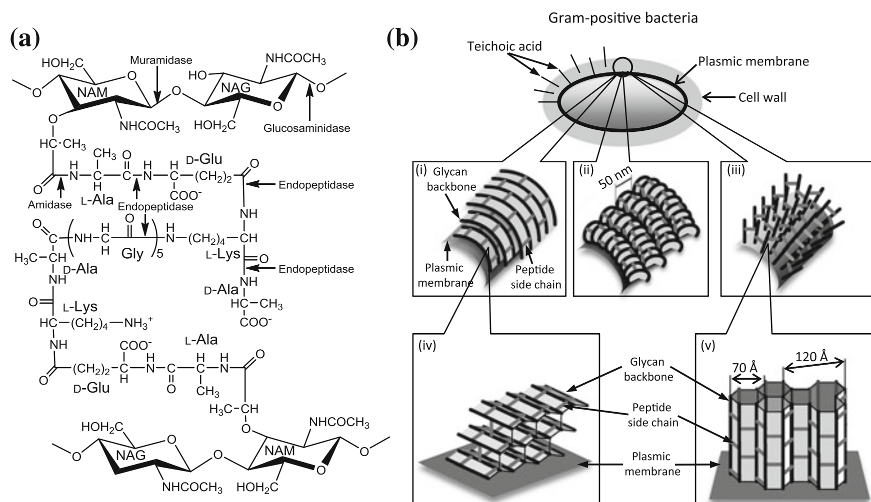


Fig. 3 Schematic diagrams of Gram-positive bacterial cell wall. **a** The chemical structure of the cell wall of *Staphylococcus aureus*. The hydrolyzing sites by muramidase, glucosaminidase, amidase, and endopeptidase are indicated by arrows. **b** Three potential Gram-positive bacterial cell wall models are shown. (i) A multiple layer model is shown, in which peptidoglycan backbones parallel to the cell membrane are arranged circumferentially, forming a layer structure. Black lines and gray lines show glycan backbones and crosslinks by peptide side chains, respectively. (ii) A cable model is shown, in which peptidoglycan strands formed 50 nm coiled cables arranged circumferentially. (iii) A scaffold model is shown, in which peptidoglycan backbones are arranged radially from the cell, perpendicular to the cell membrane. (iv) In the multiple layer model, peptidoglycans are expected to form not only intra-layer, but also inter-layer cross-links, giving a lattice structure in which glycan backbones and peptide side chains are vertical to each other. (v) The honeycomb-like scaffold model proposed by Meroueh et al. is shown. A hexagonal unit has an internal diameter of ~70 Å, and the absence of a peptidoglycan chain gives a pore size of ~120 Å

circumferentially (Hayhurst et al. 2008), (iii) a scaffold model in which peptidoglycan chains are arranged radially from the cell, perpendicular to the cell membrane (Fig. 3b) (Dmitriev et al. 2003; Meroueh et al. 2006). Each model has been supported by experimental data, and discussions on potential models still continue. The atomic three-dimensional structure of the Gram-positive bacterial cell wall remains elusive because it is not regularly repetitive enough to determine using biophysical methods. Meroueh et al. proposed a honeycomb-like cell wall model for Gram-positive *S. aureus* based on the NMR solution structure of the peptidoglycan fragment, in which a glycan backbone with a 3_{10} right-handed helical structure makes right angles to peptide side chains (Meroueh et al. 2006). This model is composed of hexagonal units with an internal diameter of ~70 Å, and the absence of peptidoglycan chains gives larger pores of ~120 Å, which is consistent with atomic force microscopic images showing pore sizes ranging between 50 and 120 Å (Touhami et al. 2004) (Fig. 3b, (v)). The lattice structure of the peptidoglycans in this model could also be applied to the multiple layer model (Fig. 3b, (iv)).

2.2 γ-d-Glutamyl-l-Diamino Acid Endopeptidase, AvPCP, and BcYkfc

A bacterial cell wall composed of peptidoglycans provides physical protection to the cell. The strong peptidoglycan must be lysed and reorganized during cell growth and cell division, which is known as peptidoglycan recycling process (Uehara and Park 2008). In this process, the glycan backbone of the peptidoglycan is hydrolyzed by muramidases and/or glucosaminidases, and amidases then hydrolyze the bond between the lactic acid group and l-Ala, giving murein peptides and short glycan fragments. Murein peptides are hydrolyzed by various endopeptidases into short peptides and/or amino acids, which are reused to reorganize the cell wall (Fig. 3a). The murein peptides include d-amino acids that are not in natural proteins and are not cleaved by typical peptidases.

Xu et al. showed the X-ray structures of γ-d-glutamyl-l-diamino acid endopeptidases (peptidoglycan cysteine endopeptidase, PCP) from cyanobacteria, *Anabaena variabilis* (AvPCP) and *Nostoc punctiforme* (NpPCP) (Xu et al. 2009), and *Bacillus cereus* (BcYkfc) (Xu et al. 2010), which can cleave the peptide bond between d-Glu and DAP in peptidoglycan recycling process. AvPCP and NpPCP had almost equivalent structures that were highly homologous (80 % sequence identity). The X-ray structures of AvPCP and BcYkfC are shown (Fig. 4).

AvPCP has the N-terminal SH3b domain and C-terminal catalytic domain (cysteine peptidase). The SH3b domain with 66 amino acids adopted a typical SH3b fold with seven β-strands. The SH3b domain made contact with the catalytic domain through b3 and b6, and the corresponding region to the peptide-ligand binding site in the SH3e located on the back of molecule (Fig. 4a). The SH3b

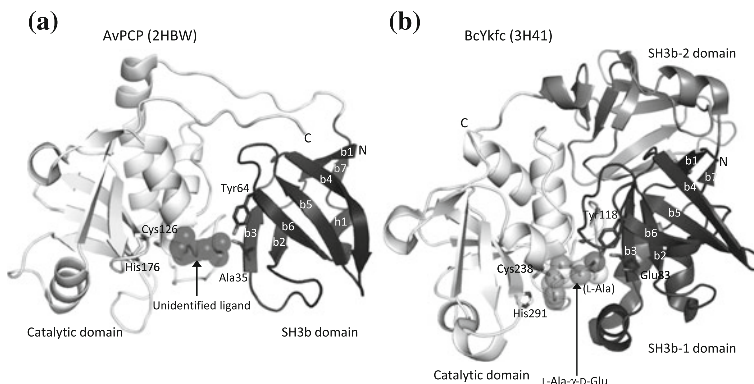


Fig. 4 Structures of γ-d-glutamyl-l-diamino acid endopeptidases. **a** The structure of AvPCP (2HBW) is shown with an unidentified ligand in the space-filling mode. The SH3b domain is shown in black. The active site residues of Ala35, Tyr64, Cys126, and His176 are labeled. **b** The structure of BcYkfc (3H41) is shown with the peptide ligand of l-Ala-γ-d-Glu. The active site residues of Glu83, Tyr118, Cys238, and His291 are labeled

domain and catalytic domain were connected by a long loop. The electron density of the unidentified ligand was detected at the interface between the SH3b domain and catalytic domain. Catalytic Cys126 and His176 were located proximate to one edge of the ligand, and, at the opposite edge of the ligand, the main chain carbonyl group of Ala35 and the side chain hydroxyl group of Tyr64 of the SH3b domain possibly interacted with the ligand.

BcYkfc had the N-terminal tandem-repeated SH3b domains and C-terminal catalytic domain. The SH3b-1 domain had the characteristic insertion of 30 amino acids forming three α-helices in the RT-loop, while the SH3b-2 domain with 60 amino acids did not have any significant insertion (Fig. 1c). The SH3b-1 domain was located at an equivalent position to the SH3b domain of AvPCP, and the SH3b-2 domain occupied the position of the long loop connecting the SH3b domain and catalytic domain in AvPCP. Xu et al. suggested that the nonessential SH3b-2 domain was evolutionarily lost over time (Xu et al. 2010). The X-ray structure of BcYkfc included the peptide ligand of l-Ala-γ-d-Glu, a product of this enzyme, which showed detailed enzyme-substrate interactions. Catalytic Cys238 and His291 were close to the carboxyl group of d-Glu, indicating that Cys could act as a nucleophile in the catalytic reaction. The interface between the SH3b domain and catalytic domain provided a favorable pocket for the amino group of l-Ala, in which the main chain carbonyl group of Glu83 and the side chain hydroxyl group of Tyr118 from the SH3b domain formed hydrogen bonds with the amino group of l-Ala. Tyr 118 was expected to be responsible for recognizing the amino groups of L-Ala, and is conserved well in the other γ-d-glutamyl-l-diamino acid endopeptidases (Xu et al. 2010). These finding showed that the SH3b domain could play an important role in defining substrate specificity by contributing to the formation of the active site pocket, which can efficiently recognize murein peptides with a free N-terminal l-Ala only.

The X-ray structures of γ-d-glutamyl-l-diamino acid endopeptidases from *Desulfovibrio vulgaris* (3M1U) and *Bacteroide thetaiotaomicrom* VPI-5482 (4R0K) have been found in PDB. The former structure has a SH3b domain with a long insertion in the RT-loop, and the latter structure has the tandem-repeated short SH3b domains (60 amino acids) without any significant insertions (Fig. 1c). The relative orientations and positions between the SH3b domains and catalytic domains are very similar to those of AvPCP and BcYkfc, respectively, which form the substrate-binding pocket between domains.

2.3 Glycylglycine Endopeptidase, ALE-1

Lysostaphin is a glycylglycine endopeptidase produced by *Staphylococcus simulans*, which can specifically lyse the interpeptide bridge of the *S. aureus* cell wall (Schindler and Schuhardt 1964). The lysostaphin proenzyme has three domains; an N-terminal domain of tandem repeats, a central zinc-containing metalloprotease catalytic domain, and a C-terminal bacterial cell wall targeting domain, and the

N-terminal domain is removed through maturation. ALE-1, which is produced by *Staphylococcus captis* EPK1, is a homologue of lysostaphin, and exhibits a lytic activity to *S. aureus* cell wall. ALE-1 does not undergo translational protein processing to have N-terminal tandem repeats, a central zinc-containing metalloprotease catalytic domain, and a C-terminal cell wall targeting domain in the mature form.

Lu et al. reported the X-ray structure of the bacterial cell wall targeting domain of ALE-1, which adopted a SH3-like fold (Fig. 5) (Lu et al. 2006). The targeting domain with 92 amino acid residues (SH3b domain) was larger than a typical SH3e domain with 60 amino acid residues. In the SH3b domain of ALE-1, an additional 20 residues at the N-terminus formed b0 and elongated b1, and the RT-loop had two β-strands (b2 and b3). In the n-Src loop, b6 was connected with b7 by a protruded loop, and the characteristic 3_{10} helix (H1) found in the SH3e domain was missing. An additional 20 residues at the N-terminus and a protruded loop between b6 and b7 covered the corresponding groove for the peptide-ligand binding in the SH3e domain, suggesting that the SH3b domain of ALE-1 accommodates another binding site for ligands.

Lu et al. also showed that the SH3b domain of ALE-1 had strong affinity to a pentaglycin, an interpeptide of the *S. aureus* cell wall, and that a mutant form truncating nine residues at the N-terminus markedly reduced binding to the *S. aureus* cell wall and/or a pentagylcine (Lu et al. 2006). Based on a computational method and site-directed mutagenesis analysis, Hayakawa et al. proposed that the cleft between the nine residues at the N-terminus and RT-loop acted as the binding site for a pentaglycine (Hayakawa et al. 2009). This cleft was located on the opposite side of the molecule to the peptide-ligand binding site of the SH3e domain. Asn274, Tyr276, and Thr278 from the N-terminal region, Thr298 from RT-loop, Glu320 from the n-Src-loop, and Tyr341 from b6 could interact with the peptide ligand (Fig. 5). This cleft was unique to the SH3b domain of ALE-1 with additional N-terminal residues. Since the X-ray structure of the full length of ALE-1 has not yet been determined,[1] the spatial arrangement of a catalytic domain and the SH3b domain currently remains unclear. However, the SH3b domain of ALE-1 may specifically recognize a pentaglycine, an interpeptide of the *S. aureus* cell wall, by its own cleft, in order to help the catalytic domain hydrolyzing a Gly-Gly peptide bond.

2.4 Endolysin, Psm, with Muramidase Activity

Endolysins, which are encoded by phages infecting bacteria, are expressed in the final stage of infection (Young 1992). These enzymes are transported from the cytoplasm through the cytoplasmic membrane by phage-encoded holins, and

[1]Recently, the X-ray structure of mature lysostaphin at 3.5 Å resolution (4LXC) was reported to show that catalytic domain and cell wall targeting domain are mobile to each other due to a highly flexible linker (Sabala et al. 2014).

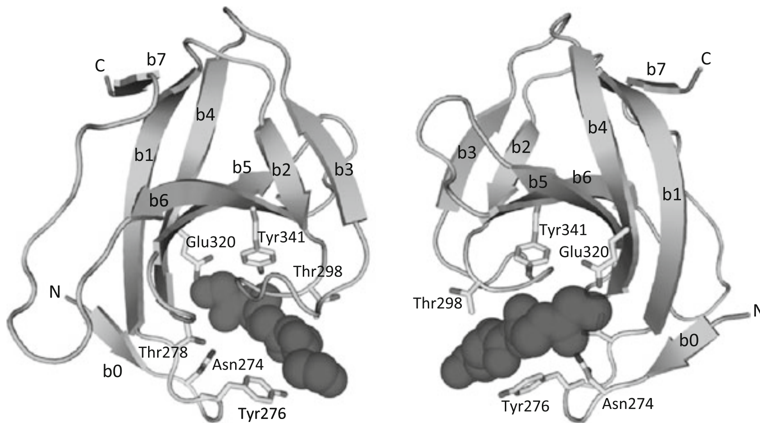


Fig. 5 Structures of the SH3b domains of the glycylglycine endopeptidase, ALE-1 (1R77) are shown in two directions. The modeled peptide ligand in the space-filling mode is superimposed at the predicted pentaglycine binding site. The amino acid residues possibly interacting with a pentaglycine are labeled

hydrolyze peptidoglycans in the bacterial cell wall, which allows for bacterial lysis and progeny phage release. Most endolysins are monomeric with an N-terminal catalytic domain and a C-terminal cell wall binding domain, which is indispensable for lytic activity (Croux et al. 1993). Since some purified endolysins previously showed to be able to kill host bacteria immediately in vitro (Loeffler and Fischetti 2003), endolysins are expected to be efficient antimicrobial reagents (O'Flaherty et al. 2009). The Gram-positive bacterium *Clostridium perfringens* (*C. perfringens*) is one of the pathogenic clostridial species. The endolysin, Psm, which is encoded by the episomal phage phiSM101 of the enterotoxigenic *C. perfringens* type A strain SM101 exhibited potent and species-specific lytic activity towards all tested strains of *C. perfringens*, but not the other *Clostridium* species tested (Nariya et al. 2011).

Tamai et al. reported the X-ray structure of Psm, which possesses an N-terminal catalytic domain that is highly homologous to *N*-acetylmuramidase, belonging to the glycoside hydrolase 25 family, and C-terminal tandem-repeated SH3b domains (Tamai et al. 2014). Three domains of Psm are arranged separately, and the SH3b domains are located on the opposite side to the catalytic site (Fig. 6a). SH3b-1 and SH3b-2 domains have 51 % amino acid sequence identity, and their structures are almost equivalent with the RMSD of the main chain atoms of 0.5 Å. Each of these has a β-sheet by seven β-strands and two short 3_{10} helices. The SH3b-1 and SH3b-2 domains are arranged in approximately 2-fold symmetry without a linker region. At both sides of the SH3b domains, an acetic acid and malonic acid from crystallization solutions were found in the deep positively charged cavity between b2 and b6, forming efficient salt-bridge interactions with Arg residues. Although the SH3b domains of Psm also closed the peptide-ligand binding site in the SH3e domain, a hydrophobic surface was formed between b2 and h2, to be extended to the deep

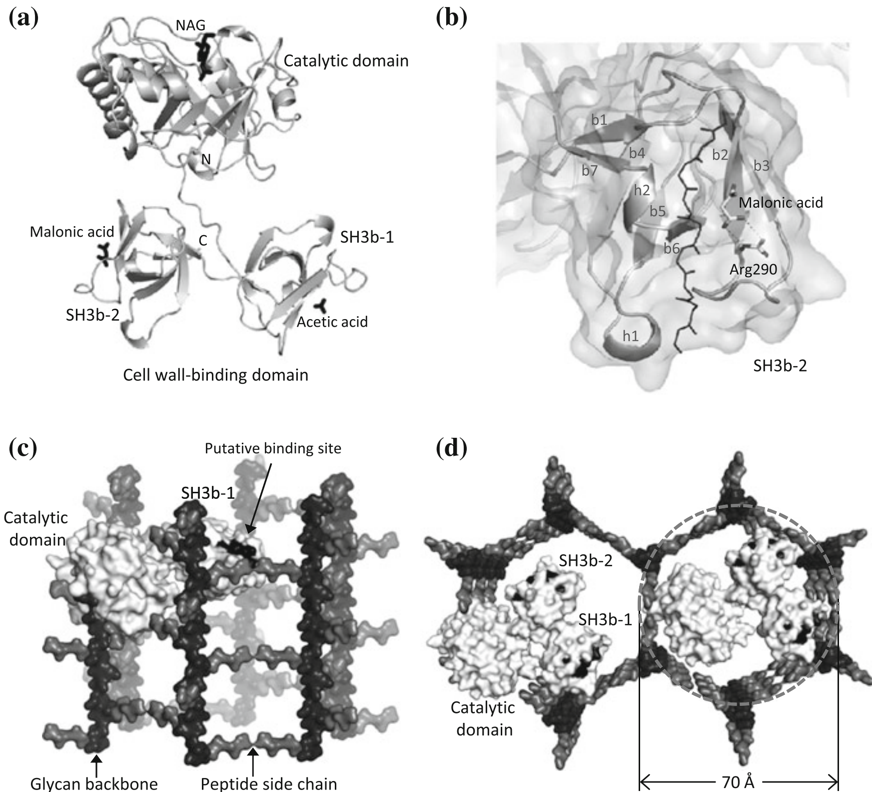


Fig. 6 Structure of the endolysin, Psm (4KRT). **a** The overall structure of Psm is shown with the binding *N*-acetylglucosamine (NAG) in the catalytic site, and the binding acetic acid and malonic acid in the SH3b domains. **b** A close-up view of the SH3b-2 domain is shown with the superimposed ligand (main chain) for the SH3e domain in Fig. 1a. Arg290 formed efficient salt-bridge interactions with malonic acid. **c** The cell wall recognition model of Psm is shown. Psm binds to a glycan backbone. The putative peptide-binding grooves of the SH3b-1 and SH3b-2 domains are shown in black. **d** As viewed from the glycan backbone axis, a Psm binding to the glycan backbone (*left*) and translated Psm to the center of a hexagonal unit (*right*) are shown. A circle with a diameter of 70 Å is drawn in a right hexagonal unit

positively charged cavity (Fig. 6b). This finding suggests that the SH3b domains of Psm may recognize the peptide ligand with a negatively charged carboxylate group. However, the inherent ligands for the SH3b domains of Psm have not yet been identified.

Tamai et al. proposed the cell wall recognition model of Psm using a honeycomb-like model, assuming that SH3 domains recognized the peptide bridges of peptidoglycans (Fig. 6c) (Tamai et al. 2014). The catalytic domain bound to a glycan backbone for hydrolysis. The SH3b domains were on the perpendicular plane to the glycan backbone axis, and the putative peptide binding surface of the SH3b-1 domain may have interacted with the peptide side chain. The SH3b-1

domain may have recognized the peptide side chains to assist catalytic domain binding to the glycan backbone for the catalytic reaction. The honeycomb-like model was composed of hexagonal units with an internal diameter of ~70 Å. Psm could enter the pores of hexagonal units with a size of 70 Å, maintaining binding orientation to the glycan backbone, and the SH3b domains could interact with peptide side chains (Fig. 6d). The peptide side chains of peptidoglycans run vertically to the glycan backbones to form a lattice structure. When Psm enters a pore in the cell wall, the SH3b domains presumably recognize peptide side chains to fix the catalytic domain in the proper orientation for binding to the glycan backbone. The spatial arrangement of the catalytic domain and SH3b domains of Psm may be favorable for recognizing the lattice structure, in which glycan backbones and peptide side chains cross at right angles.

2.5 Internalin, InlB

Listeria monocytogenes is a Gram-positive bacterium that causes severe infections leading to meningitis, abortions, gastroenteritis, and septicemia (Lorber 1997; Aureli et al. 2000). In invading host cells, *L. monocytogenes* uses internalins, bacterial surface proteins. Internalins exist in two forms, InlA and InlB, which have specific receptors for cell adhesion; E-cadherin for InlA, and tyrosine kinase receptor Met for InlB. The internalin family has an N-terminal domain with leucine-rich repeats (LRRs domain), which are required for binding to the receptors of host cells (Glaser et al. 2001). InlB consists of the N-terminal LRRs domain and C-terminal tandem-repeated SH3b domains. Three targeting molecules of the SH3 domains of InlB have been identified to date; lipoteichoic acid, heparine, and gC1q–R (a host cell surface protein) (Jonquières et al. 1999; Jonquières et al. 2001; Braun et al. 2000). InlB binds non-covalently and reversibly to the bacterial cell wall, and this dissociable attachment is achieved by interactions between the SH3b domains and the lipoteichoic acid molecules on the bacterial cell wall. InlB also binds to the glycosaminoglycan heparin of host cells through the SH3b domains to enhance InlB-mediated invasion. The third targeting molecules of gC1q–R were originally identified as a membrane protein that binds to the globular 'heads' of C1q (Ghebrehiwet et al. 1994).

Marino et al. reported the X-ray structure of InlB, revealing that InlB adopted an extended molecular structure with an 'L' shape (Marino et al. 2002) (Fig. 7). The N-terminal LRRs domain and SH3b domains are located 50 Å from each other, and the linker loop between them is invisible in the X-ray structure. Three tandem-repeated SH3b domains are aligned in a linear conformation, and each of them is in almost the same direction. The SH3b-1 had a highly flexible structure with the lack of secondary structures, while the SH3b-2 and SH3b-3 had relatively

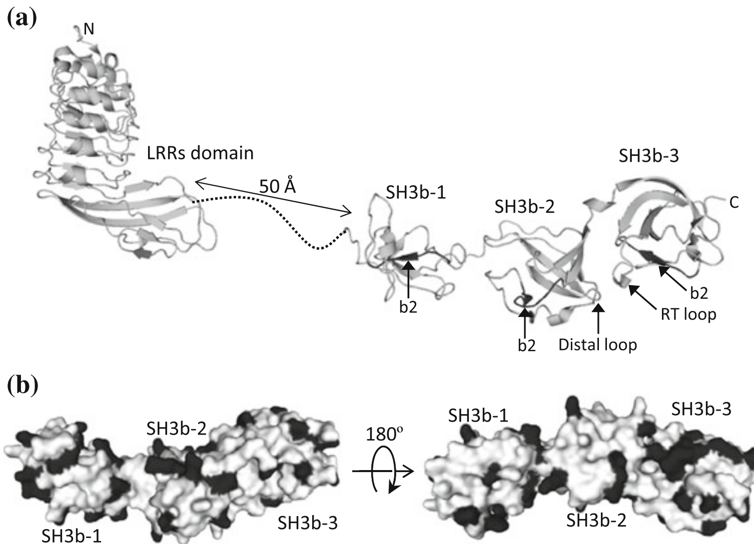


Fig. 7 Structure of the internalin, InlB (1M9S). **a** The overall structure of InlB is shown. The LRRs domain and SH3b domains are located 50 Å from each other, and the linker loop between them is invisible in the X-ray structure. The b2 strands of the SH3 domains are shown in black to highlight the orientations of the domains. **b** A surface representation of the SH3b domains is shown with the positively charged region in black

stable structures with seven β-strands forming a β-sheet, as found in a typical SH3b domain (Fig. 1c). This is because the SH3b-2 and SH3b-3 pair formed a stable structure unit through pairwise interactions between the distal loop of SH3b-2 and the RT loop of SH3b-3. On the other, SH3b-1 is isolated without a pairing domain. In all SH3b domains, the peptide ligand binding sites in an SH3e domain were occupied by their own RT-loop, indicating that this site did not act as the ligand-binding site. The surfaces of InlB SH3b domains are extremely basic, and the three targeting molecules of the SH3b domains of InlB, lipoteichoic acid, and heparin, and gC1q–R are acidic. Electrostatic interactions between the SH3b domains and targeting molecules are important for binding, which explained the dissociable attachment to the bacterial cell wall through lipoteichoic acid molecules. However, specific binding to gC1q-R could not be explained by electrostatic interactions alone, and the recognition mechanism for gC1q-R remains unclear.

Marino et al. showed that binding to gC1q-R and/or heparin required the release of InlB from the bacterial surface. Thus, they proposed that dissociable attachment to the bacterial surface via the SH3b domains permitted the localized release of InlB, and that the localized release of InlB may restrict the activation of Met to a small and localized area of the host cell, in order to couple InlB-induced host membrane dynamics with bacterial proximity during invasion (Marino et al. 2002).

3 SH3b Domains Involved in Metal Binding

3.1 Diphtheria Toxin Repressor, DtxR

The diphtheria toxin repressor (DtxR) belongs to a family of iron-dependent regulator (IdeR) proteins. Its activation depends on divalent metal iron and regulates the expression level of diphtheria toxin by interacting with the DNA of operator region to prevent downstream transcription (Pappenheimer 1977; Boyd et al. 1990; Schmitt and Holmes 1994; Tao and Murphy 1992).

Qiu et al. reported the X-ray structures of DtxR from *Corynebacterium diphteriae* in complexes with cobalt and manganese ions, and showed that DtxR consisted of three domains; the N-terminal DNA-binding domain with a helix-turn-helix (HTH) motif (residues 1–64), the second domain having two metal binding sites (residues 65–140), and the C-terminal SH3b domain (residues 147–226) (Qiu et al. 1996) (Fig. 8a). DtxR was found to be a dimer through the second domain (dimerization domain). The DNA binding domain and dimerization domain were associated strongly with each other, while the SH3b domain was isolated from the other domains. The SH3b domain had five conserved β strands, and two inserted α-helices in the RT loop, which blocked the corresponding groove to the peptide-ligand binding site in the SH3e domain (Fig. 1c).

The function of the SH3b domain of DtxR was revealed by determining the crystal structure of the DtxR-DNA complex, in which DtxR dimers bound DNA (Pohl et al. 1999) (Fig. 8b). Through binding to DNA, the three domains of DtxR

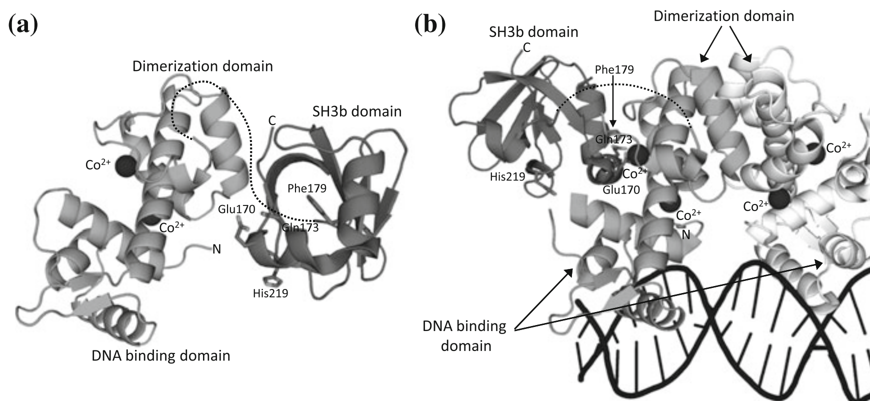


Fig. 8 Structures of the diphtheria toxin repressor, DtxR. **a** The overall structure of DtxR in its free form (2DTR) is shown with the binding metal ion. Glu170, Gln173, Phe179, and His219 are labeled. **b** DNA-binding structure of DtxR (1C0 W) is shown with Glu170 and Gln173 coordinating to the metal ion from the SH3b domain

were rearranged so that the SH3b domain could contact with both the DNA-binding domain and dimerization domain. The helices of the DNA-binding domains were shifted with a hinge motion against the dimerization domain to fit into the major groove of double strand DNA. This hinge motion of the DNA-binding domains was a key feature for activation. The metal-binding sites were close to the hinge region, and metal binding may have induced a hinge motion. The SH3b domain provided the amino acid residues (Glu170 and Gln173) in the RT loop in order to coordinate the metal ion at one of the metal binding sites with His79, Glu83, and His98 of dimerization domain. Phe179 formed hydrophobic contacts with Phe128 of the dimerization domain, and His219 interacted with Glu21 of the DNA-binding domain. The amino acid residues, Glu170, Gln173, Phe179, and His219 were conserved in the homologous members of the IdeR/DtxR family. The SH3b domains could stabilize the structure of DtxR by interacting with the DNA-binding domain and dimerization domain in order to strengthen binding to DNA. These findings suggested that metal-ion dependent DtxR may be partially regulated by the SH3b domain.

3.2 Bacterial Ferrous Iron-Transport Activating Factor, FeoA

A major route for ferrous iron uptake in bacteria was identified as the ferrous iron-transport (Feo) system. Three proteins are involved in the Feo system; FeoA with unknown function, FeoB containing an integral membrane domain to act as a ferrous permease and a GDP-dissociation inhibitor domain for stabilizing GDP binding, and FeoC containing a Fe-S cluster for serve as a transcriptional regulator for the *feo*ABC operon (Hantke 1987; Cartrön et al. 2006).

Su et al. described the structure of FeoA from *Stenotrophomonas maltophilia* (FeoA) (Su et al. 2010). The whole structure of FeoA adopted an SH3 domain fold with five anti-parallel β-strands, and additional α-helices at the N-terminal site, RT loop, and C-terminal β-strand (Fig. 9). A novel feature of FeoA was that it formed a unique dimer cross-linked by two zinc ions. The zinc ion was coordinated by His21 in the RT loop of a molecule and Glu52 in the n-Src loop of another molecule. His21 corresponds to Glu170 of DtxR, which also coordinated with the metal ion. Their positions, the center of the RT loop, may be favorable for interacting with metal ions. Su et al. proposed that FeoA may interact with FeoB between the SH3b domain and G-protein domain in order to regulate FeoB-dependent ferrous iron-uptake activity as a ferrous iron transport activating factor. However, the relationship between function and dimerization by the metal coordination of FeoA remains unclear.

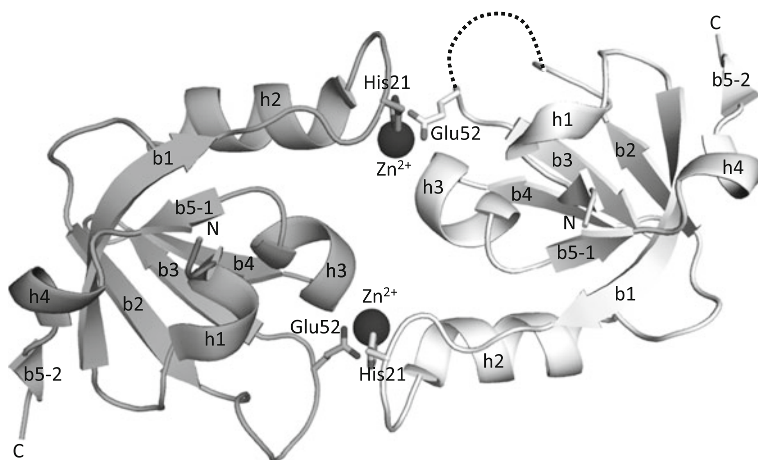


Fig. 9 The dimer structure of the ferrous iron-transport activating factor, FeoA (3MHX) is shown with zinc ions, which are coordinated by His21 and Glu52 from two molecules

4 Conclusion

The SH3b domains of γ-d-Glutamyl-l-diamino acid endopeptidases take part in the active site pocket to recognize the free N-terminal l-Ala of substrates. The SH3b domain of the glycylglycine endopeptidase, ALE-1, has a unique cleft, which recognizes a pentaglycine. The function of the SH3b domains of these enzymes may be to contribute to substrate recognition by forming a part of the active site or by being located close to the active site. The SH3b domains of the endolysin, Psm, are located away from the active site of the enzyme, suggesting that the catalytic domain and SH3b domains recognize different substrates/ligands, respectively. The SH3b domains have been proposed to recognize the peptide side chains of peptidoglycans in order to assist catalytic domain binding to a glycan backbone for the catalytic reaction, based on a honeycomb-like peptidoglycan model. The SH3b domains of internalin InlB have three targeting molecules; lipoteichoic acid, heparine, and gC1q–R. Although the molecular interactions between the SH3b domains and these target molecules have not yet been elucidated in detail, the dissociable attachment of the SH3b domains to the bacterial cell wall by electrostatic interactions is known to be important for sequential binding to heparin and gC1q–R. The function of the SH3b domain of the diphtheria toxin repressor, DtxR, is to stabilize the structure of DtxR binding to DNA by metal-coordinated interactions with another domain. Although the function of the bacterial ferrous iron-transport activating factor, FeoA, is currently unclear, FeoA was shown to form a unique dimer cross-linked by two zinc ions.

We provided an overview of the structure-function relationship of the SH3b domains based on their X-ray structures. SH3b domains have been increasingly identified from amino acid sequence and structural information. SH3b domains with novel functions are expected to be found in the future.

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Activation of PI3K by Thyroid Hormone Nuclear Receptors

Jeong Won Park and Sheue-yann Cheng

Abstract Thyroid hormone receptors (TRs) mediate the diverse biological activities of thyroid hormone T3 in growth, differentiation, and development and in maintaining metabolic homeostasis. Three major T3 binding TR isoforms (α1, β1, and β2), are encoded by the *THRA* and *THRB* genes. Increasing evidence has shown that besides the classical mode of nuclear actions, TRs could act via extra-nuclear signaling. One important extra-nuclear pathway is mediated via phosphatidylinositol-3-kinase (PI3K) signaling. PI3K consists of the p85α regulatory subunit and the p110 catalytic subunit. Among the p85α subunits's domains, there are three SH2 domains (NSH2, iSH2 and CSH2 domains). The activation of PI3K is consistently shown via physical interaction of TRα1 or TRβ1 with p85α in many cell types, leading to diverse physiological responses. Remarkably, in a mouse model of follicular thyroid cancer ($Thrb^{PV/PV}$ mouse) expressing a TRβ1 mutant PV, PV physically interacts with the CSH2 domain of p85α, resulting in an ~30-fold increase in PI3K activity. The PV/p85α complex is localized in both the nuclear and cytosolic compartments to activate the PI3K-AKT/PKB-mTOR-$p70^{SK6}$ and PI3K-ILK-MMP2 pathways to promote thyroid tumor growth and metastasis. Thus, these studies illuminate an important pathway initiated at protein–protein interaction of TR with the SH2 domains of p85α to relay signals through a phosphorylation cascade to affect various physiological and pathological outcomes.

Keywords Thyroid hormone receptors · Oncogenes · Protein–protein interactions · Signaling transduction · Thyroid hormone · Dominant negative actions · Mouse models

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1 Introduction

The SH2 domain was initially discovered through the observation that a stretch of amino acid sequence (~100 amino acids) in the central region of v-Fps/Fes oncoprotein is necessary for cellular transformation. Because the sequence of this domain is highly homologous to the corresponding region in Src family kinase, the name of SH2 (Src homology 2) was coined. So far, 111 proteins containing at least one SH2 domain encoded in the human genome have been found (Liu et al. 2011).

SH2 domains mediate selective protein–protein interactions with tyrosine phosphorylated (pTyr) proteins to coordinate and drive complex network signaling in cells. Signaling mediated through the use of pTyr regulates many key cellular and developmental processes including cell growth, proliferation, differentiation, and migration (Liu and Nash 2012). An important class of the pTyr-containing proteins is the receptor tyrosine kinases (RTKs). Upon binding to extracellular stimuli such as growth factors, RTKs dimerize and initiate signaling via phosphorylation of their cytoplasmic tails and scaffold proteins. Signaling effectors are recruited to these pTyr sites via SH2 domains and also p-Tyr-binding (PTB) domains. The specific interaction of signaling proteins with PTB motifs (e.g., SH2 domains) activates signaling pathways, such as the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K)-AKT pathways. These pathways are critical in the regulation of diverse cellular functions including cell proliferation, apoptosis, and differentiation. Aberrant activation of these pathways is involved in the development of various cancers (Polivka and Janku 2014; De Luca et al. 2012). Because these areas are being actively investigated, they will not be reviewed in the present article.

Another class of p-Tyr-containing proteins is made up of the members of the steroid/thyroid hormone nuclear receptor superfamily. The nuclear receptors are ligand-dependent transcription factors that regulate diverse cellular functions in growth, development, differentiation, reproduction, and metabolic homeostasis. The transcriptional activities of steroid/thyroid hormone nuclear receptors are regulated by their cognate ligands, hormone binding DNA response elements in the target genes, and the nuclear receptor co-regulators (e.g., corepressors and coactivators) (Perissi et al. 2010; York and O'Malley 2010). In addition, some members of this family are known to be subject to extensive post-translational modifications, such as phosphorylation, that further modulate the functions of the receptors (Anbalagan et al. 2012). Although the activities of the phosphorylated steroid hormone nuclear receptors such as the androgen receptor (Guo et al. 2006), progesterone receptors (Boonyaratanakornkit et al. 2001), and estrogen receptors (Castoria et al. 2001) have been reported to involve SH2 domains, the present article will focus on the new development in understanding the regulation of functions of thyroid hormone nuclear receptors (TRs) by SH2 domains.

2 Thyroid Hormone Nuclear Receptors

TRs belong to the superfamily of ligand-dependent transcription factors. They are encoded by *THRA* and *THRB* genes located on chromosomes 17 and 3, respectively (Cheng et al. 2010). Alternative splicing of the primary transcripts gives rise to three major thyroid hormone (T3) binding proteins: TRβ1, TRβ2, and TRα1 (Fig. 1). TRs consist of modular structures with the DNA-binding domain and the T3-binding domain at the carboxyl-terminus. These TRs are highly homologous in the DNA and T3-binding domains, except in the amino-terminal A/B domains (Fig. 1) (Cheng et al. 2010). The carboxyl-terminal region also contains multiple contact surfaces important for dimerization with its partner, the retinoid X receptor (RXR), and for interactions with corepressors and coactivators (Cheng et al. 2010; Millard et al. 2013; Dasgupta et al. 2014). The expression of TR isoforms is tissue-dependent and developmentally regulated (Cheng et al. 2010).

TRs occupy a central position in mediating the functions of T3 in growth, differentiation, and development and in maintaining metabolic homeostasis. The transcriptional activity of TRs is regulated at multiple levels (Cheng et al. 2010; Mullur et al. 2014). In addition to T3 regulation, the type of thyroid hormone response elements (TREs) located on the promoters of T3 target genes affects the magnitude and sensitivity of transcriptional response. A host of coregulatory proteins plays a further, critical role in modulating the gene regulatory functions of TRs (Cheng et al. 2010; Millard et al. 2013; Dasgupta et al. 2014). In the absence of T3, TRs repress basal transcription through association with a variety of corepressors (Perissi et al. 2010; Mullur et al. 2014). Binding of T3 induces structural changes to release the corepressors and to allow recruitment of coactivators (Perissi et al. 2010; Mullur et al. 2014). Corepressors harbor various enzymatic activities including arginine deamination, histone demethylation, and histone deacetylation that act to modify the chromatin structure so as to limit access by the basal transcription machinery (Perissi et al. 2010). Coactivator complexes, in contrast, harbor activities such as histone acetyltransferase, histone methylase, ubiquitination ligase, and

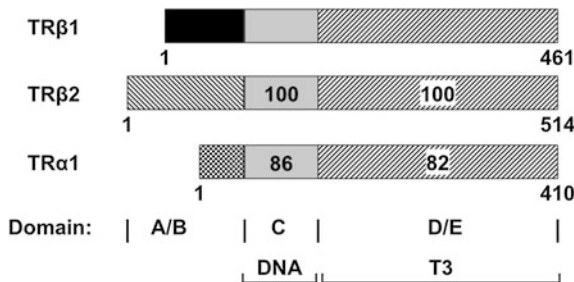

Fig. 1 Schematic comparison of thyroid hormone nuclear receptor (TR) isoforms. The amino terminal A/B domain is variable in length and sequences (marked by different *shading*). The extent of sequence homology in the DNA-binding domain C and T3-binding domain D/E is indicated

others that facilitate transcription by rendering chromatin more accessible to other transcription factors (Perissi et al. 2010; York and O'Malley 2010; Cheng et al. 2010). The tissue- and time-dependent expression of coregulatory proteins provides an additional level in the regulation of TR transcription activity (Cheng et al. 2010; Mullur et al. 2014).

Besides mediating the T3 action via transcription regulation, TR is also known to act via extra-nuclear sites to mediate nongenomic actions. These TR-involved nongenomic actions regulate growth, development, metabolism, and carcinogenesis via phosphorylation and activation of kinase pathways. One important nongenomic pathway has been shown to involve the interaction of TRs with PI3K in many cell types, resulting in diverse physiological and pathological outcomes, which are highlighted below.

3 Activation of PI3K by TRα1 in Cardiovascular System

T3 has profound effects in the cardiovascular system. It decreases systemic vascular resistance (SVR) and arterial blood pressure, enhances renal sodium reabsorption and blood volume, and augments cardiac inotropy and chronotropy (Klein and Ojamaa 2001). These effects result in a dramatic increase in cardiac output, as observed in hyperthyroidism. SVR, which is observed in thyroid hormone deficiency or hypothyroidism, is rapidly reversed with thyroid hormone replacement. Both TR isoforms are expressed in the heart, with TRα1 being the major isoform. While much is known about the actions of TR in the heart, the precise mechanism by which thyroid hormone regulates vascular tone and SVR is not completely understood.

Earlier, Simoncini et al. discovered that the estrogen receptor α (ERα), a member of the steroid hormone receptor superfamily, binds in a ligand-dependent manner to the p85α regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K) (Simoncini et al. 2000). PI3K is critical in regulating all aspects of cellular functions in signaling, membrane trafficking, and metabolic processes (Jean and Kiger 2014). PI3K functions as heterodimers consisting of a catalytic p110 subunit and a regulatory p85α subunit. The p85α regulatory subunit harbors three Src homology 2 (SH2) domains, NSH2, iSH2 and CSH2 domains. The iSH2 domain is an intervening p110-binding region (iSH2) between the NSH2 and CSH2 domains, constitutively interacts with the p110-binding catalytic subunit. Importantly, p85α recruits the protein complex to phosphorylated tyrosine commonly downstream of activated receptor tyrosine kinases to relay the signaling.

Stimulation with estrogen (E2) increases ERα-associated PI3K activity, leading to the activation of protein kinase B/AKT and endothelial nitric oxide synthase (eNOS). Recruitment and activation of PI3K by ligand-bound ERα are independent of gene transcription. Mice treated with E2 show increased eNOS activity and decreased vascular leukocyte accumulation after ischemia and reperfusion injury. This vascular protective effect of estrogen was abolished in the presence of PI3K or

eNOS inhibitors, thereby defining an important pathway involving the direct interaction of ERα with PI3K (Simoncini et al. 2000). That the SH2 domains of p85α could be involved in the physical interaction with ERα was supported by the observations that E2 treatment leads to an increase in eNOS activity, but not in p85α-deficient fibroblasts (p85α$^{-/-}$ fibroblasts). Moreover, fibroblasts co-transfected with p85α cDNA leads to elevated E2-stimulated eNOS activity, but in wild type fibroblasts co-transfected of a dominant-negative p85α mutant cDNA, markedly decreases E2-stimulated eNOS activity.

The observation of physical interaction of ERα with PI3K was extended to other members of the nuclear receptor superfamily such as TRs. PI3K activity was shown to be activated by T3 (Simoncini et al. 2000). Subsequently, how the interaction of TR with PI3K initiates rapid, nongenomic transcription effects in the cardiovascular system was further delineated. In vascular endothelial cells, the predominant TR isoform is TRα1. Interestingly, T3 increases the association of TRα1 with the p85α subunit of PI3K in endothelial cells, leading to the phosphorylation and activation of AKT and eNOS. Indeed, in the presence of the PI3K inhibitors LY294002 and wortmannin, but not the transcriptional inhibitor actinomycin D, the activation of AKT and eNOS by T3 is abolished. Administration of T3 to mice rapidly increases AKT activity in the brain, decreases mean blood pressure, reduces cerebral infarct volume, and improves neurological deficit score. These neuroprotective effects of T3 are greatly attenuated or absent in eNOS$^{-/-}$ and TRα1$^{-/-}$TRβ$^{-/-}$ mice. Moreover, T3-induced neuroprotective effects are completely abolished in wild type mice pretreated with LY294002 or the T3 antagonist NH-3. These findings indicate that the activation of the PI3K-AKT pathway can mediate some of the rapid, extra-nuclear effects of TR. Such rapid actions suggest that the activation of AKT and eNOS contributes to some of the acute vasodilatory and neuroprotective effects of thyroid hormone.

In these studies, while physical interaction of TRα1 with the p85α subunit of PI3K was demonstrated, whether the interaction was via the SH2 domain of p85α was not evaluated. However, the findings that T3 increases the association of TRα1 with the p85α subunit of PI3K suggests that T3-bound TRα1 is favored in its binding to the p85α subunit of PI3K for the activation of PI3K activity. These findings would suggest that the region in p85α with which TRα1 binds is amendable to the conformation of the liganded or unliganded TRα1, thereby invoking proper functional responses to relay the downstream signals.

4 Activation of PI3K by TR in Pancreatic Cells

The PI3K-AKT pathway was identified as a crucial regulator of cell proliferation, survival, and size in pancreatic β-cells (Elghazi et al. 2006). AKT targets cell regulators that control signaling cascades affecting insulin-mediated glucose transport, protein synthesis, cell proliferation, growth, differentiation, and survival. Overexpression of constitutively active AKT in β-cells in transgenic mice results in

augmented β-cell mass by activating β-cell proliferation and cell size (Bernal-Mizrachi et al. 2001). That TRs could play a role in the regulation of pancreatic β-cells via the PI3K pathway was first demonstrated by the finding that T3 protects pancreatic β-cells from pharmacologically induced apoptosis. In addition, T3 induces phosphorylation of AKT in the human insulinoma cell line CM (Verga Falzacappa et al. 2007). Subsequently, it was shown that T3-induced AKT phosphorylation is via TRβ1 in the rat pancreatic β-cell line (rRINm5F) and human pancreatic insulinoma cells (hCM cells) (Verga Falzacappa et al. 2007). Importantly, the p85α subunit of PI3K was shown to complex with TRβ1 in the cytoplasm by coimmunoprecipitation and colocalization experiments. The functional consequences of the physical interaction of TRβ1 with the p85α subunit of PI3K were further supported by the observations that the activated AKT targets β-catenin, $p70^{S6K}$, and mTOR to up-regulate protein synthesis and cell size (Verga Falzacappa et al. 2009). In addition, T3 was shown to induce the nuclear translocation of activated AKT (Verga Falzacappa et al. 2009). These observations suggest the possibility of TRβ1-dependent transduction of activation signals from cytoplasm to initiate transcription events to affect physiological outcomes in rRINm5F and hCM pancreatic β-cells.

However, the proliferation role of TR in pancreatic cells is not limited to the TRβ1 subtype. Recently, TRα1 was also shown to be involved in the regulation of proliferation of pancreatic β-cells (Furuya et al. 2010). TRα1 is over-expressed in rRIN5F cells via infection with TRα1 recombinant adenoviral vector (Ad TRα). Expression of TRα1 in RIN5F cells increases the expression of cyclin D1 at the mRNA and protein levels. Concurrently with the elevated cyclin D1, other cell cycle regulators such as cyclin-dependent kinases (CDK4 and CDK6), phosphorylated retinoblastoma protein (Rb), and E2F transcription factor are increased upon T3 stimulation. Knocking down of cyclin D1 by siRNA in AdTRα-infected cells leads to down-regulation of the cyclin D1/CDK/Rb/E2F pathway and inhibition of cell proliferation. The growth-promoting role of TRα1 was further documented in vivo in that in immunodeficient mice with streptozotocin-induced diabetes, intrapancreatic injection of AdTRα leads to the restoration of islet function accompanied by increased β-cell mass (Furuya et al. 2010).

Subsequent studies showed that one of the mechanisms by which TRα1 acts to increase cell proliferation is via T3-induced association of TRα1 with the p85α subunit of PI3K. The physical interaction of TRα1 with p85α leads to the phosphorylation and activation of AKT in pancreatic exocrine cells. Activation of AKT leads to the induction of transcription factors, pancreatic and duodenal homeobox 1 (Pdx1), basic helix-loop-helix factor neurogenin-3 (Ngn3), and transcription factor MafA (MafA), critical for pancreatic cell development. Pdx1 controls the growth and development of the pancreatic bud; Ngn3 is required for formation of endocrine progenitors; and MafA and Pdx1 are required for the maturation of β-cells (Murtaugh and Melton 2003). That association of T3-bound TRα1 with the p85α subunit of PI3K results in the activation of AKT to affect the downstream transcription factors for β-cell reprogramming for maturation. This observation was further confirmed by treatment of pancreatic exocrine cells with LY294002, a

specific inhibitor of PI3K. No induction of Pdx1, Ngn3, or MafA was found in the T3-bound TRα1-activated pancreatic exocrine cells treated with LY294002 (Furuya et al. 2013). These studies showed the critical role of the physical interaction of TRα1 with p85α in the activation of AKT and in the induction of pancreatic transcription factors for the development of islets in the pancreatic exocrine cells. However, these studies did not define whether SH2 domains of p85α are involved in the physical interaction with TRα1.

5 Activation of PI3K by TRβ1 in Human Skin Fibroblasts

In addition to vascular endothelial cells and pancreatic beta cells, TRβ1 was also shown to interact with PI3K in the cytosol of normal human fibroblasts (Cao et al. 2005). This interaction leads to the activation of PI3K and its downstream signaling cascade via sequential phosphorylation and activation of AKT, mammalian target of rapamycin (mTOR), and $p70^{SK6}$. Phosphorylation of mTOR is rapid, within minutes after T3 treatment. The rapid phosphorylation is insensitive to cycloheximide treatment, indicating that the T3-induced effect is independent of the requirement of new protein synthesis. Coimmunoprecipitation assays indicated that TRβ1 physically interacts with the p85α subunit and requires T3. Interestingly, within minutes after T3-induced activation of PI3K, phosphorylated cytosolic AKT is translocated into the nuclei as visualized by confocal scanning images (Cao et al. 2005). The AKT downstream effectors, mTOR and phosphorylated mTOR, are only detected in the nucleus in T3-stimulated cells. The expression of a target gene, ZAKI-4α, an endogenous calcineurin inhibitor, was found to be activated via this T3-dependent signaling cascade (Cao et al. 2005).

However, ZAKI-4α is not the only target gene that is activated via this T3-dependent PI3K-AKT-mTOR signaling cascade. Further studies showed that 91 up-regulated and 5 down-regulated T3-target genes were identified in normal human skin fibroblasts by quantitative fluorescence cDNA microarrays (Moeller et al. 2005). Additional validation and functional analyses uncovered new genes either positively regulated by T3, such as AKR1C1-3, PFPK, RAB3B, HIF-1α, COLVIA3, and STC1, or negatively regulated by T3, such as FGF7 and ADH1B. Genes found to be induced by T3 in other species, such as BTEB1, GLUT1, and MCT4, were also identified in human skin fibroblasts (Moeller et al. 2005, 2011). Among the up-regulated genes, the transcription factor subunit hypoxia-inducible factor (HIF)-1α gene is of great interest. Concurrent with the T3-induction of HIF-1α, its functionally related genes, the glucose transporter (GLUT)1, platelet-type phosphofructosekinase (PFKP), and monocarboxylate transporter (MCT) 4 genes are also up-regulated by T3 in human skin fibroblasts. These genes play an important role in glucose metabolism. GLUT1 mediates glucose uptake, PFKP is important in glycolysis, and MCT4 is key in lactate export. The identification of these genes is consistent with the known cellular role of T3/TRβ1 in glucose metabolism (Moeller et al. 2005, 2006). However, it is important to point

out that the activation of these glycolytic genes is initiated in the cytosol via direct interaction of T3-bound TRβ1 with the p85α subunit of PI3K and the subsequent phosphorylation cascade signaling. Therefore, this mode of the TR-PI3K actions further expands the versatile ways by which T3 acts to attain its diverse physiological activities.

6 In Vivo Oncogenic Activation of PI3K by TRβ Mutants in Thyrocytes

6.1 Discovery of Oncogenic Actions of a TRβ1 Mutant

Shortly after the cloning of the *THRB* gene (Weinberger et al. 1986), TRβ mutations were discovered to cause the genetic syndrome of resistance to thyroid hormone (RTH) (Weiss and Ramos 2004). RTH is a syndrome characterized by reduced sensitivity of tissues to the action of thyroid hormones. This condition is characterized by elevated levels of circulating thyroid hormones associated with normal or high levels of serum thyroid stimulating hormone (TSH). The most common form of RTH is familial with autosomal dominant inheritance (Dumitrescu 1830; Olateju and Vanderpump 2006). Patients are usually heterozygotes with only one mutant TRβ gene with mild abnormalities. Clinical features include goiter, short stature, decreased weight, tachycardia, hearing loss, attention deficit–hyperactivity disorder, decreased IQ, and dyslexia (Dumitrescu 1830; Olateju and Vanderpump 2006). Three patients homozygous for mutant TRβ have been reported so far (Usala et al. 1991; Ferrara et al. 2012). These patients display a complex phenotype of extreme RTH with markedly higher levels of thyroid hormone and TSH than those described for all heterozygous patients reported. Most TRβ mutants derived from RTH patients have reduced T3-binding affinities and transcriptional capacities. These TRβ mutants exhibit a dominant-negative effect. About 122 different mutations have now been identified belonging to 300 families (Olateju and Vanderpump 2006).

The discovery that mice harboring a homozygous mutation of an RTH mutant, PV, spontaneously develop follicular thyroid cancer indicates that mutations of the *Thrb* gene can cause diseases other than RTH (*Thrb*$^{PV/PV}$ mice; (Suzuki et al. 2002)). PV was derived from a patient with RTH characterized by elevated thyroid hormone levels accompanied by normal TSH, short stature, goiter, and tachycardia (Parrilla et al. 1991). PV has an unusual mutation in exon 10, a C-insertion at codon 448, which produces a frameshift of the carboxyl-terminal 14 amino acids of TRβ1 (Fig. 2) (Parrilla et al. 1991). PV has completely lost T3 binding and transcriptional activity and exhibits potent dominant negativity.

Similar to RTH patients with two mutated *THRB* alleles, *Thrb*$^{PV/PV}$ mice exhibit highly increased thyroid hormones accompanied by markedly elevated serum TSH levels (Suzuki et al. 2002; Kaneshige et al. 2000). As *Thrb*$^{PV/PV}$ mice age, they spontaneously develop follicular thyroid carcinoma with pathological progression

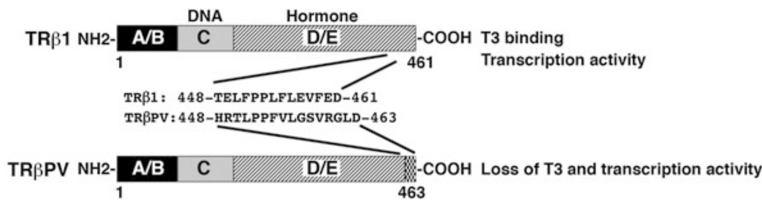

Fig. 2 Schematic comparison of TRβ1 and TRβPV structure. The TRβPV mutation was identified in a patient with resistance to thyroid hormone. The TRβPV mutation is a frame-shift mutation due to a C-insertion at codon 448 of TRβ1. The carboxyl-terminal sequences of the wild type TRβ1 and the TRβPV mutant are indicated. As a result of the frame-shift mutation, PV does not bind T3

and frequency of distant metastasis similar to human thyroid cancer (Suzuki et al. 2002). The altered signaling pathways during thyroid carcinogenesis of $Thrb^{PV/PV}$ mice have been extensively characterized. Over-activation of tumor promoters such as cyclin D1 (Ying et al. 2003), β-catenin (Guigon et al. 2008), and the pituitary tumor-transforming gene (Ying et al. 2006; Kim et al. 2007) drives thyroid cancer progression of $Thrb^{PV/PV}$ mice. Thyroid cancer progression is accelerated by repression of tumor suppressors such as peroxisome proliferator-activated receptor γ (PPARγ) (Kato et al. 2006). These altered signaling pathways during thyroid carcinogenesis of $Thrb^{PV/PV}$ mice are consistent with the changes reported for the carcinogenesis in the human thyroid. The potential molecular targets identified in these altered signaling pathways were tested in $Thrb^{PV/PV}$ mice. $Thrb^{PV/PV}$ mice treated with an agonist to activate the tumor-suppressing activity of PPARγ exhibit marked delay in tumor progression, increased survival, and no apparent metastasis (Kato et al. 2006), thereby firmly establishing that the $Thrb^{PV/PV}$ mouse is an ideal model not only to understand the molecular genetics of thyroid cancer, but also to elucidate the molecular basis of oncogenic actions of TRβ1 mutants in vivo.

6.2 Over-Activation of PI3K Activity in Thyrocytes by PV in Vivo

Studies of primary human thyroid cancer specimens by several groups showed AKT overexpression and overactivation, particularly in follicular thyroid cancer (Ringel et al. 2001; Miyakawa et al. 2003). Consistent with human thyroid cancer, AKT is activated in thyroid tumors of $Thrb^{PV/PV}$ mice by over-expression of phosphorylated AKT (pAKT) as carcinogenesis progresses (Kim et al. 2005). To elucidate the underlying molecular mechanisms by which the mutant PV activates AKT activity, the activity of its upstream kinase, PI3K, was examined and found to be 40- to 50-fold higher in the thyroid of $Thrb^{PV/PV}$ mice than in wild type mice (Furuya et al. 2006). The possibility that the increased kinase activity of PI3K results from physical interaction of PV with the p85α regulatory subunit was evaluated by co-immunoprecipitation analysis of thyroid extracts of $Thrb^{PV/PV}$ mice. Indeed, the

p85α regulatory subunit of PI3K was detected in a concentration-dependent manner in of *Thrb*$^{PV/PV}$ mice (lanes 5–7, Fig. 3a), but very weakly in wild type mice (lanes 2–4; Fig. 3a). These results indicate that more PV in the thyroid of *Thrb*$^{PV/PV}$ mice is bound to p85α than TRβ in wild type mice. The increase in the binding of PV to PI3K shown in Fig. 3 is not due to increased PV protein abundance in the thyroid of *Thrb*$^{PV/PV}$ mice because Western blot analysis indicates a similar abundance of TRβ protein in the thyroid of wild type mice and of PV protein in the thyroid of *Thrb*$^{PV/PV}$ mice.

The sites of TRβ with which p85α interacts were mapped by glutathione S-transferase (GST)-pull down assays. Using a series of sequentially truncated TRβ1, we mapped sites to the ligand-binding domain of TRβ1 or PV (Furuya et al. 2006). Figure 3b-I shows the schematic representation of the domain structures of p85α. As shown in Fig. 3b, the region of p85α with which TRβ1 (or PV) interacts is localized to the C-terminal SH2 (CSH2) domain of p85α (Fig. 3b-II-a and II-b) (Furuya et al. 2007). Taken together, these results indicate that physical interaction of PV with the CSH2 domain of p85α leads to marked increases in PI3K activity.

The functional consequences of interaction of PV with PI3K were evaluated by analyzing two downstream signaling pathways: the AKT-mTOR-p70^{S6K} and the integrin-linked kinase (ILK) pathways. The former is known to mediate cell growth and proliferation (Carnero and Paramio 2014), and the latter is involved in cell migration, invasion, and inhibition of apoptosis (Persad and Dedhar 2003). Indeed, consistent with the activation of PI3K, the phosphorylated AKT, mTOR, and p70^{S6K} are increased in both the nuclear and cytoplasmic compartments without significant alteration of the respective total protein abundance, thereby indicating the activation of the signaling pathway via phosphorylation cascades (Furuya et al. 2006). The increased PI3K activity also activates the ILK-matrix metalloproteinase-2 (MMP2) pathway in the extracellular compartment (Furuya et al. 2006). Increased expression of MMP2 is known to increase the degradation of the extracellular matrix that affects cancer cell invasion and metastasis (Li et al. 2013). The activation of the AKT-mTOR-p70^{S6K} and the ILK pathways propels thyroid cancer progression, indicating that PV could act via nongenomic signaling to exert its oncogenic effects.

In addition to the biochemical evidence shown in Fig. 3, the activation of PI3K by PV due to its physical interaction with the CSH2 domain of p85α was further supported by studies that treated *Thrb*$^{PV/PV}$ mice with LY294002 (LY), a potent and specific PI3K inhibitor. The effect of LY on the spontaneous development of thyroid cancer of *Thrb*$^{PV/PV}$ mice was evaluated (Furuya et al. 2007). Analysis of Kaplan-Meier cumulative survival curves showed that LY-treated *Thrb*$^{PV/PV}$ mice survive significantly longer, suggesting that it is effective in prolonging the survival of *Thrb*$^{PV/PV}$ mice and reducing thyroid tumor growth. Importantly, histopathological evaluation showed that whereas the untreated mice exhibit advanced hyperplasia, the treated mice show only early hyperplasia. While vascular invasion is frequent in untreated mice ($\sim$40 %), it is rare in treated mice ($\sim$5 %) with frequent appearance of apoptotic bodies. In contrast to the untreated mice in which the occurrence of lung metastasis is frequent (25 %), no metastasis occurs in the lung of

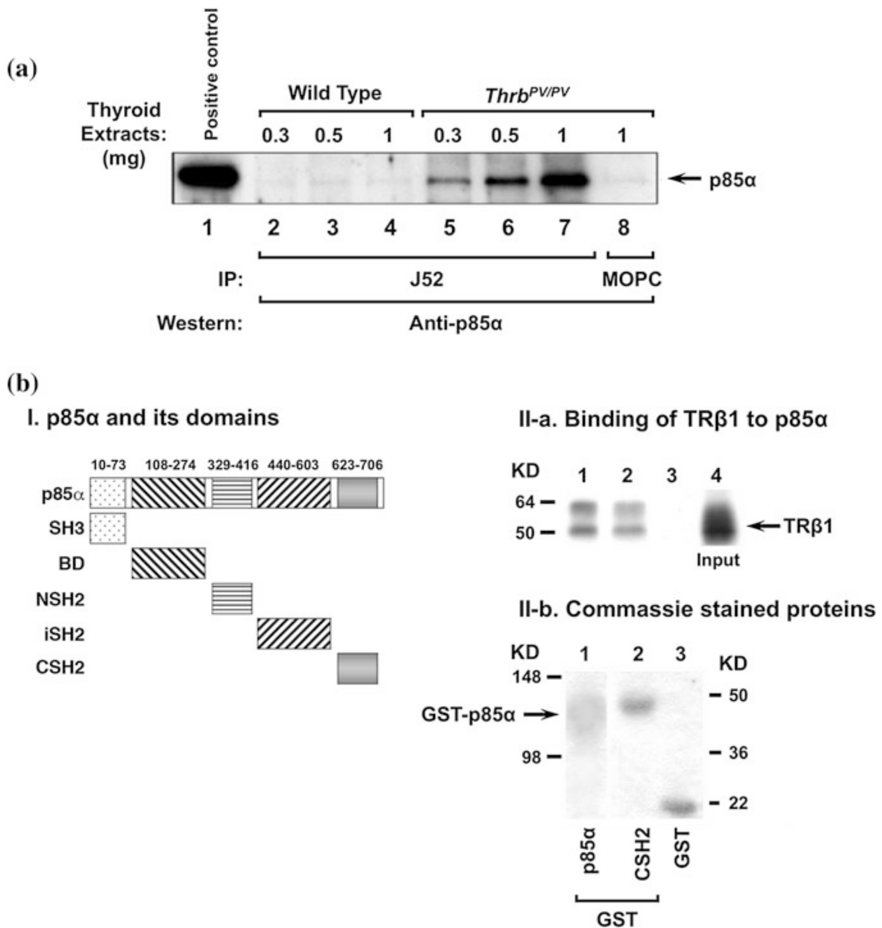

Fig. 3 Physical interaction of PV with PI3K in thyroids of *Thrb*$^{PV/PV}$ mice. **a**. PV is associated with PI3K in vivo as shown by coimmunoprecipitation. Three hundred, 500, or 1000 μg of pooled protein lysates from thyroid extracts of six wild type mice or three *Thrb*$^{PV/PV}$ mice, respectively, were immunoprecipitated with J52 anti-TR antibody and subjected to immunoblot analysis probed with anti-p85α antibody. **b**. TRβ1 interacts with the CSH2 domain of p85α by GST-pull down assays. *b-I* Schematic representation of p85α and its domains. The number of amino acid indicate the beginning and ending amino acids in the domains used in the conjugation of GST to each domain for analysis of GSP-pull down assays. *bII-a* Binding of TRβ1 with the CSH2 subunit of p85α. Equal amounts of GST-p85α fusion proteins (full-length and truncated domains as marked) were each incubated with 10 μL of ^{35}S-labeled TRβ1 (lanes 1–3) synthesized by *in vitro* transcription/translation. Lane 4 shows the input. After electrophoresis, the gel was dried and autoradiographed. *bII-a* Coomassie blue staining of the SDS/PAGE gel shows that similar amounts of GST-fused proteins (lanes 1 and 2) and GST proteins (lane 3) were used (*3b-II-b*). The molecular size markers for lane 1 is shown on the left of 3b-II-b and for lanes 2 and 3 are shown on the right. No binding of TRβ1 with GST-SH3 or GST-NSH2 was detected (not shown)

treated mice. These observations clearly show that treatment of *Thrb*$^{PV/PV}$ mice with LY delays thyroid tumor progression and blocks metastatic spread to the lung (Furuya et al. 2007).

Molecular analyses have provided additional evidence that LY-induced delay in tumor progression acts through the PI3K-AKT signaling. Comparing the extent of phosphorylated effectors in the PI3K-AKT-mTOR-p70^{S6K} pathway showed that the protein abundances of p-AKT, p-mTOR, and p-p70^{S6K} in thyroid tumors of LY-treated *Thrb*$^{PV/PV}$ mice are markedly lower than those effectors of untreated mice. Importantly, LY treatment does not significantly alter the cellular levels of total AKT, total mTOR, and p70^{S6K} proteins (Furuya et al. 2007), indicating the activation is via a phosphorylation cascade of these effectors. Thus, these results further illustrate that the delay in thyroid tumor progression of *Thrb*$^{PV/PV}$ mice by LY treatment is initiated via attenuating the PI3K activity. These findings further illuminate the critical role of activated PI3K in the thyroid carcinogenesis of *Thrb*$^{PV/PV}$ mice via direct PV-p85α interaction.

6.3 Regulation of PV-Activated PI3K Signaling by Nuclear Receptor Corepressor 1

The transcriptional activity of TR is regulated by a host of nuclear coactivators and corepressors (Perissi et al. 2010; York and O'Malley 2010; Lonard and O'Malley 2007). In the absence of T3, the unliganded TR recruits the nuclear receptor corepressor 1 (NCOR1) and the nuclear receptor corepressor 2/silencing mediator for retinoid and thyroid hormone receptors (NCOR2/SMRT) for transcriptional repression. Binding of T3 leads to a conformational change in the TR that releases the NCOR1/NCOR2 complex and allows for the recruitment of a multiprotein coactivator complex for transcriptional activation (Glass and Rosenfeld 2000). Via modulation of receptor transcriptional activities, coregulators serve as master regulators of cell functions and integrate a variety of signal transduction pathways (Lonard and O'Malley 2007).

The importance of the regulatory role of NCOR1 in TR functions is evident in that its aberrant association with TRβ underlies RTH (Love et al. 2000; Privalsky 2004). Recently, Astapova et al. created a mutant mouse (*Ncor1*$^{\Delta ID}$ mice) expressing an NCOR1 mutant protein (NCOR1ΔID) in which the receptor interaction domains have been modified so that it cannot interact with the TR or PV (Astapova et al. 2008). Crossing *Thrb*^{PV} with *Ncor1*$^{\Delta ID}$ mice yielded progeny exhibiting a modest but significant correction of the abnormally elevated TSH and thyroid hormone levels found in *Thrb*^{PV} mice. Moreover, thyroid hyperplasia, weight loss, and other hallmarks of RTH were also partially reverted in mice expressing NCOR1ΔID (Fozzatti et al. 2011). These studies suggest that the aberrant recruitment of NCOR1 by RTH TRβ mutants leads to clinical RTH in humans, and thus underscores the important regulatory role of NCOR1 in TR transcriptional activity.

While co-repressors are mainly localized in the nuclei, increasing evidence has been presented that they can translocate to cytoplasm (Espinosa et al. 2002; Hermanson et al. 2002; Rosenfeld et al. 2006). The redistribution of nuclear NCOR1 to the cytoplasm provides a mechanism to control differentiation of neural stem cells into astrocytes (Hermanson et al. 2002). Moreover, Sardi et al. also showed that cytoplasm NCOR1 forms complexes with a cleaved product of ErbB4 (a member of the EGF receptor family) and the signaling protein TAB2 and translocates into the nucleus to regulate astrogenesis in the developing brain (Sardi et al. 2006). NCOR1 was also found to express in the cytoplasm of epithelial cells and myofibroblasts of colorectal carcinoma to potentially contribute to initiation and progression of colorectal carcinoma (Tzelepi et al. 2009). These studies clearly show that the NCOR1 could act at the extra-nuclear sites.

NCOR1 is known to modulate the actions of TRβ mutants in vivo and in vitro (Love et al. 2000; Fozzatti et al. 2011). That NCOR1 could alter the oncogenic action of PV by modulating the interaction of PV with PI3K was ascertained in the thyroid of $Thrb^{PV/PV}$ mice (Furuya et al. 2007). Confocal fluorescence microscopy showed that both NCOR1 and p85α are localized in the nuclear as well as in the cytoplasmic compartments (Fig. 4a). Interestingly, NCOR1 was found to physically associate with p85α in the thyroid of $Thrb^{PV/PV}$ mice (Fig. 4b-II). The amino terminal R1 and the C-terminal R4 and receptor interaction domain (RID) are the regions of NCOR1 to interact with p85α The CSH2 was identified as the region that interacts with the RID of NCOR1 (Figs. 4b-I, b-II). Since both NCOR1 and TR interact with the same region of the CSH2 domain of p85α, whether NCOR1 competes with TRβ or PV for binding to p85α was further evaluated. GST-pull down assays showed that in the presence of increasing concentrations of NCOR1 (RID), the binding of TRβ to p85α is decreased in a concentration-dependent manner (compare lanes 2–4 with lane 1; Fig. 4c-I). NCOR1 also competes with PV for association with p85α in a concentration-dependent manner (Fig. 4c-II). In addition, comparison of the binding of p85α with TRβ1, PV, or NCOR1 (RID) shows that PV interacts with p85α with the relatively highest affinity in the rank order of PV > TRβ > NCOR1 (Furuya et al. 2007).

The finding that PV and NCOR1 compete for binding to the same site of p85α suggests that a decrease in the cellular NCOR1 would lead to an activation of PI3K activity and its downstream signaling. Overexpression of NCOR1 in thyroid tumor cells of $Thrb^{PV/PV}$ mice reduces PI3K signaling as indicated by the decrease in the phosphorylation of its immediate downstream effector, p-AKT. Conversely, lowering cellular NCOR1 by siRNA knockdown in tumor cells leads to over-activated p-AKT and increased cell proliferation and motility. Furthermore, NCOR1 protein levels are significantly lower in thyroid tumor cells than in wild type thyrocytes, allowing more effective binding of PV to p85α to activate PI3K signaling and thus contributing to tumor progression. Taken together, these results indicate that NCOR1, via protein–protein interaction, is a novel regulator of the PI3K signaling via competitive binding to the CSH2 domain of p85α. These findings illustrate the importance of the CSH2 domain in mediating the cellular functions of NCOR1 and TRβ mutants via extra-nuclear signaling.

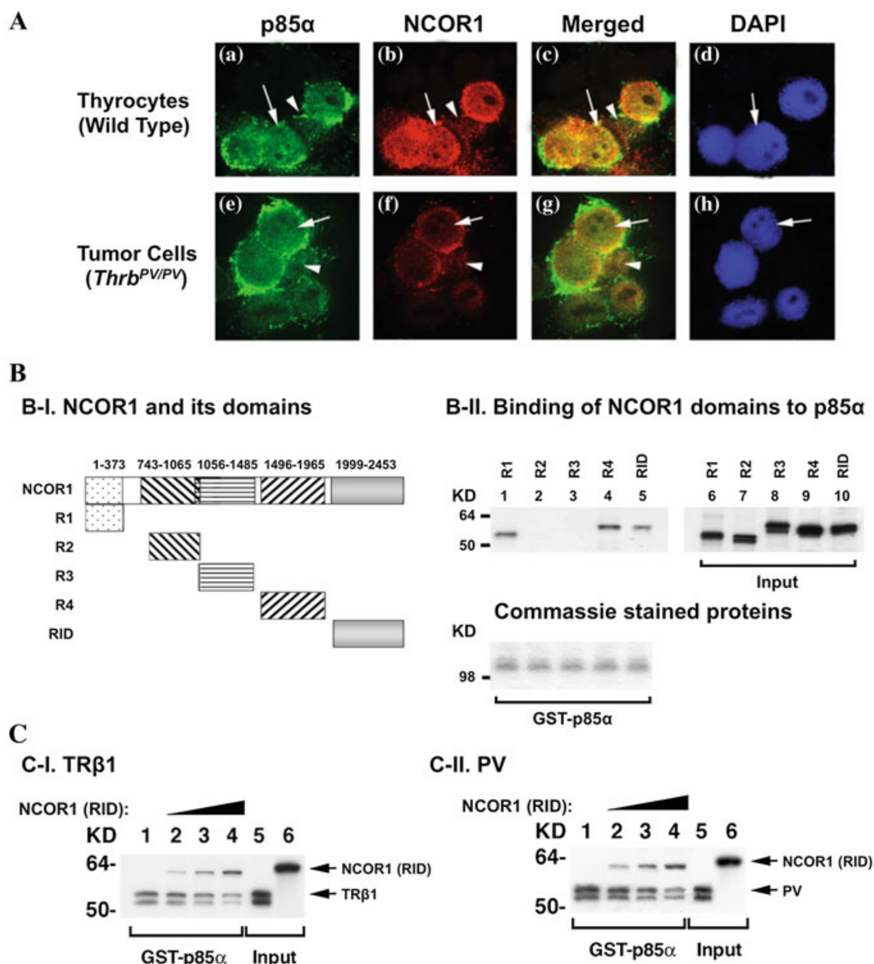

Fig. 4 Physical interaction of NCOR1 with PI3K in thyroids of $Thrb^{PV/PV}$ mice. **a**. Localization of endogenous NCOR1 with transfected Flag-p85α in primary thyroid cultured cells of wild type mice (panels a–d) and thyroid tumor cells of $Thrb^{PV/PV}$ mice (panels e-h) was visualized by confocal fluorescence microscopy. Flag-p85α (panels a and e; *green*) and NCOR1 (panels b and f; *red*) were first stained with anti-Flag (M2) and anti-NCOR1 antibodies and then with secondary antibodies for visualization. Panels c and g show the merged images of p85α and NCOR1 for thyroid cells of wild-type mice and tumor cells of $Thrb^{PV/PV}$ mice, respectively. Panels d and h show nuclear staining with DAPI. **b**. Binding of NCOR1 domains to p85α. *b-I* Schematic representation of NCOR1 and its domains. *b-II*. Binding of p85α to NCOR1 determined by GST-pull down assays. GST-p85α fusion proteins were incubated with 10 μL of [^{35}S]-labeled domains of NCOR1 proteins as indicated (lanes 1–5) prepared by in vitro transcription/translation. Lanes 6–10 are the 5 % input of the protein lysates used in the GST-pull down assay. Upper panel: autoradiography; lower panel: Coomassie blue staining. **c**. NCOR1 (RID) competes with TRβ1 (panel I) or PV (panel II) for binding to p85α. GST-p85α fusion protein was incubated with 2 μL of [^{35}S]-labeled TRβ1 (I) or [^{35}S]-labeled PV (II) in the absence (lane 1) or presence of 1, 2, or 4 μL of NCOR1 (RID) (lanes 2–4, respectively). After electrophoresis, the gel was dried and autoradiographed

7 Conclusion and Perspective

This chapter briefly reviews what is currently known about an extra-nuclear signaling pathway via the activation of PI3K by TRs. The importance of this TR-PI3K activated pathway has been documented in many target tissues such as vascular endothelial cells (see Sect. 3), pancreatic cells (see Sect. 4), human skin fibroblasts (see Sect. 5), and thyrocytes (see Sect. 6). The activation of PI3K in these different cell types is consistently shown via direct physical interaction of TRα1, TRβ1, or a TRβ1 mutant, PV, with the p85α subunit of PI3K. However, such interaction leads to diverse functional consequences including vasodilatory cardiac and neuroprotective effects; increased pancreatic β-cell size, survival, and functions; induction of HIF-1α and glucose metabolism; and promoting thyroid carcinogenesis. These findings illustrate an important signaling initiated via protein–protein interaction of TR with p85α relaying through a phosphorylation cascade of AKT-mTOR and $p70^{S6K}$ to affect target gene expression that has an impact on various physiological and pathological outcomes.

The uncovering of the extra-nuclear mode of action of TRs in the activation of PI3K has expanded our current understanding of TR biology and the molecular mechanism of TR actions. TR-regulated gene expression was once thought to be mainly nuclear, through binding of TR to the specific DNA sequences in the promoter of affected target genes. However, the work summarized in this chapter provides compelling evidence that TR could act via extra-nuclear sites to exert diverse cellular functions. By acting at the extra-nuclear sites, TR could act independent of the requirement of new protein synthesis: the signals could be amplified via a phosphorylation cascade and could respond rapidly to T3 to meet the cellular needs. Moeller et al. (Moeller et al. 2005, 2006, 2011) have identified several T3-induced genes in glycolytic pathways initiated via PI3K-AKT signaling, but it is reasonable to expect that additional genes involved in other cellular functions in other target tissue are yet to be discovered. Therefore, one challenge is to identify comprehensively the target genes downstream of the T3/TR-PI3K-AKT signaling in different target tissues. Such research in this area certainly could advance our understanding of the extent of the network and the pathways affected by the direct interaction of TR with PI3K.

Another challenge is to elucidate how TR interacts with the p85α subunit of PI3K at the structural level. Most studies have provided the evidence to demonstrate the physical interaction of TR with p85α. However, p85α protein consists of five domains: the amino terminal SH3 (NSH3), BH, NSH2, iSH2 and CSH2 domains (See Fig. 3b-I). The crystallographic structure of each domain has been determined and analyzed (Liang et al. 1996; Musacchio et al. 1996; Nolte et al. 1996; Mandelker et al. 2009; Pauptit et al. 2001). Furuya et al. has shown that the ligand-binding domain (LBD) of TRβ1 or PV is the region that interacts with the CSH2 domain of p85α (Furuya et al. 2006, 2007). The x-ray crystallographic structure of the TRβ1 LBD has been reported (Wagner et al. 1995). In view of the availability of structural information about CSH2 and the LBD of TRβ1, it would

be useful to map out how these two domains interact structurally and to identify the amino acids involved in the direct interactions of these two proteins. Importantly, the elucidation of how LBD binding affects the CSH2 structure would provide a fundamental understanding of TR-p85α complex-induced activation of the catalytic p110 subunit of PI3K. Such information is critically important in view of the findings that oncogenic action of PV is via over-activation of the PI3K-AKT. If detailed structural information about the interaction of LBD with p85α should become available, then strategic design of specific inhibitors to block activation when PV is associated with p85α could become feasible for the development of novel treatment modalities for thyroid cancer.

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SH Domains' Interaction with SLiMs: Maximizing Adaptivity of Signaling Networks

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Abstract Protein interactions mediated by SH2 and SH3 domains that appear in a large number of proteins are of particular interest since they are expected to have an impact on diversities of cellular processes such as cell growth, cell migration, immune response, etc. SH2 and SH3 domains recognize and bind to specific primary sequences less than 10 amino acids in length called Short Linear Motifs (SLiMs). By systematically studying the conservation of SLiMs recognized by SH2 and SH3 domains, we found that the conservation of SLiMs is highly correlated with function. We hypothesized that studying specificity of this conservation would shed light on where, when, how and with whom the corresponding domain arose, thrived and contributed to signaling networks' adaptivity to environmental changes. Conservation specificity was examined in different protein functional groups, in submolecular regions, in subcellular locations and under the condition of domain coupling. We observed that domains and their specific major binding partners flourished in approximately the same period of time, or "Specificity recapitulates phylogeny". The fact that new domains rarely participate in old functions is justified using a multi-level adaptive evolution model. Furthermore, we found that SH domain binding SLiMs occur mostly in disordered protein regions that lack a rigid 3D structure. A "Shuttle Bus" model was proposed that explains the fact that SH2 and SH3 domains are frequently found within a single protein. Methods, models and hypotheses proposed here can be applicable to the research of other domains, domain combinations, and more general protein-protein, protein-nucleotide interactions in the future.

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1 Background

Selective protein-protein interactions are important for cellular functions and are often mediated by protein domains that recognize specific primary sequences within target proteins (Pawson 2004) called Short Linear Motifs (SLiMs) as exemplified in the case of phosphotyrosine (Eckhart et al. 1979) and SH2 domain binding (Sadowski et al. 1986; Waksman et al. 1993). Accurate prediction of SLiMs has been difficult because they are short (often <10 amino acids) and highly degenerate. A major advance in SLiM identification came with a peptide library-based technique that can map the sequence motif recognized by an SH2 domain without prior knowledge of in vivo interaction sites (Songyang et al. 1993). Similar peptide library experiments have been performed to map the motifs recognized by other domains (Pawson and Nash 2000). Motifs discovered through polypeptide library screening have shown high levels of agreement with reported domain interaction sites (Songyang et al. 1993). This became the basis for Scansite (Yaffe et al. 2001; Obenauer et al. 2003), a bioinformatics program developed to predict SLiMs in query proteins that are recognized by specific protein domains. Other bioinformatic approaches, such as those available in Minimotif-Miner (Balla et al. 2006), QuasiMotifFinder (Gutman et al. 2005), MCS (Dinkel and Sticht 2007) and a tree-based scoring (Chica et al. 2008) applied evolutionary conservation as well as other sequence filters to assess the functional relevance of a hit.

Both peptide library screening and evolutionary conservation proved to be useful in predicting motifs, and we hypothesized that combining them would discriminate between classes of proteins that have functional SLiMs and those that do not. To address this issue, we conducted a global statistical analysis on the conservation of SLiMs recognized by SH2 and SH3 domains (Ren et al. 2008a, b), with comparison to PDZ and S/T kinase domains in different functional classes of proteins. Naturally we would like to ask these questions: How did domain mediated interactions arise? Is there any correlation between the emergence of certain functions and certain domains? How do new domains and new functions fit into existing frameworks? Here, we tried to answer these questions based on the specificity of SLiMs conservation. We presented a model of how this domain-mediated cellular function could have possibly evolved to maximize adaptation to different levels of environmental changes.

Traditionally proteins are believed to function in some form of three-dimensional (3D) structure represented by the "lock and key" or by the "induced fit" theory. More and more examples show that some biological functions of proteins require that the protein structure be more flexible. Disordered protein regions are those sequences in protein that do not have rigid three-dimensional structures. In plots of disorder

prediction versus residue number, several sharp dips flanked by regions strongly predicted to be disordered in several different proteins were associated with sites that bind to respective protein partners (Garner et al. 1999). This observation was independently made somewhat later (Callaghan et al. 2004). Further analysis on such complexes was carried out (Mohan et al. 2006; Vacic et al. 2007), predictors were developed (Oldfield et al. 2005; Cheng et al. 2007). In order to better understand SH domain mediated binding within submolecular regions, we studied the specificity of SLiM conservation in ordered and disordered segments of possible interacting proteins.

While most SH domain mediated cellular signaling occurs in the cytoplasm and the plasma membrane, there are reports that these signaling proteins tend to translocate from one place to another upon stimulation. Furthermore, it's interesting that many Tyr-Kinases that are supposed to be at the upstream of SH2 domain signaling contain both SH2 and SH3 domains. How is this domain coupling related to function and subcellular localization? We hypothesized that conservation specificities of SLiMs would provide useful information on those signaling mechanisms.

SLiMs are known to interact with corresponding functional domains, which might be found in a number of unrelated proteins. These interactions are of particular interest as they might produce a widespread impact on diversities of cellular processes. As this article is mainly on SLiMs recognized by SH2 and SH3 domains, these functional modules are briefly introduced below.

The Src homology 2 (SH2) domain is a prototypical functional module of ~ 100 amino acids that contains a central anti-parallel β-sheet surrounded by two α-helices (Waksman et al. 1993). SH2 domains represent the largest class of known phosphotyrosine (pTyr)-recognition domains (Pawson et al. 2001). These domains bind specific pTyr-containing motifs, which are typically found in complexes as an extended β-strand that lies at right angles to the SH2 β-sheet (Liu et al. 2006). The SLiM-SH2 interactions typically couple activated protein tyrosine kinases (PTKs) to a number of intracellular pathways regulating various aspects of cellular communication (Pawson and Nash 2000). Overall, the SH2 domain is an important functional module found in a great variety of proteins regulating functionally diverse processes. Recently, these SH2-containing proteins were classified into 11 functional categories (Liu et al. 2006). Evolutionary study revealed that SH2-like domains may predate dedicated protein tyrosine kinases, appear in both divisions of Eukaryota (the bikonts and unikonts) (Liu and Nash 2012). However, there are no pTyr-binding SH2 domains in fungi, although there is one clearly homologous domain found in the yeast protein SPT6. It was not until the emergence of modern tyrosine kinases just prior to the evolution of the metazoans, that SH2 domain representation in the genome became rapidly expanded (Pincus et al. 2008).

Src-homology 3 (SH3) domains are small protein modules of ~ 60 amino acid residues that typically contain five or six β-strands arranged as two tightly packed anti-parallel β-sheets (Bar-Sagi et al. 1993). The linker regions may contain short helices. Two SH3 variable loops, the RT and n-Src loops, flank a SLiM-binding site that consists of a hydrophobic patch that contains a cluster of conserved aromatic residues (Nguyen et al. 1998). Two classes of SH3 domains have been defined, Class

1 and Class 2, which recognize RKXXPXXP and PXXPXR motifs, respectively (Mayer 2001). An interesting feature of SH3 domains is the palindromic nature of their ligands; i.e. these domains can bind the SLiMs in either orientation (Feng et al. 1994). SH3 domains were found in a great variety of proteins, e.g., in a number of proteins with enzymatic activity, in adaptor proteins that lack catalytic sequences and in cytoskeletal proteins, such as fodrin and yeast actin binding protein ABP-1. SH3 domains mediate assembly of specific protein complexes via binding to proline-rich peptides in their respective binding partner. They are involved in cell-cell communication and signal transduction from the cell surface to the nucleus (Pawson 1995). SH3 domains are believed to have emerged earlier than SH2 domains with SH3 domain mediated binding already adopted the classical PPII conformation in yeast (Douangamath et al. 2002). SH3-like domains were found in some prokaryotes (Whisstock and Lesk 1999). However, they lack certain key conserved residues, and the structure and function of these domains are unknown (Zarrinpar et al. 2003).

Interestingly, SH2 and SH3 domains are frequently found together in the same protein. However, certain proteins contain a single SH2 or SH3 domain, while others contain several copies of either domain (Bar-Sagi et al. 1993; Cohen et al. 1995). Some SH2 domains (e.g., Crk SH2 domain) contain specific SH3 domain-binding sites (Anafi et al. 1996), thus linking together SH2- and SH3-mediated regulatory networks.

2 Results

2.1 Relative Conservation (C_R) of SLiMs

Traditional methods measure sequence conservation without considering the conservation background of the protein. Here, we took background conservation into consideration by measuring the relative conservation score (Ren et al. 2008a, b). Our central hypothesis was that SLiMs should be subject to two kinds of evolutionary selection. The first is background selection, which is imposed upon the entire length of the protein sequence, due to the integral function of the protein. The second is SLiM-specific selection superimposed on the background, due to the special function mediated by the SLiM.

Therefore, a well-conserved SLiM in an overall highly conserved protein does not guarantee independent importance. In this case, the high sequence matching probably results because the SLiM is an integral part of the conserved protein structure. For example, although the putative SH2 binding Tyr-SLiM in Histone H3.1 is conserved among sequences from all selected species, their relative conservation was low because of the highly conserved background (Fig. 1a). Conversely, a high relative conservation is an indication that the given SLiM motif may play an important physiological role. As shown in Fig. 1b, the Tyr-SLiMs in the C-terminal of IL4R are well conserved while the full-length protein is not so

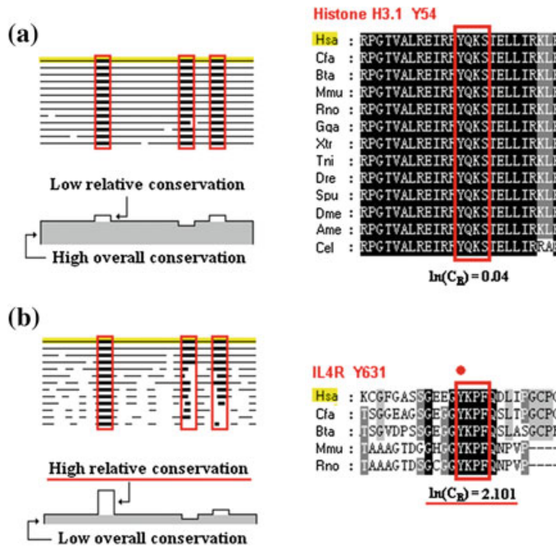

Fig. 1 Relative conservation of SLiMs. **a** Low relative conservation of conserved SLiM in overall conserved protein. Schematic illustration (*left panel*) and alignment (*right panel*) around Y54 of Histone H3.1. **b** High relative conservation of conserved SLiM in overall less conserved protein. Schematic illustration of relative conservation (*left panel*) and alignment (*right panel*) around Y631 of IL4R

well conserved, and thus these SLiMs exhibit a high relative conservation score. In fact, this tyrosine motif is reported to bind to SH2 domains (Kashiwada et al. 2001). Thus, the advantage of the relative conservation method is the capability to discriminate SLiMs conserved under constraints of the integral protein from those conserved to serve as functional motifs. Conserved motifs in conserved proteins might or might not be important; when the SLiM and its protein environment exhibit similar degrees of conservation there is simply no information regarding potential importance. Such SLiMs are reasonably considered to be less likely to function independently compared with those SLiMs that are more conserved than their surrounding sequences.

2.2 *"Specificity Recapitulates Phylogeny": System Level Conservation Analysis of SLiMs Recognized by SH Domains with Comparison to PDZ and S/T Kinase Domains*

In this section and the sections that follow, we use "SLiM conservation" to indicate relative conservation unless specified otherwise.

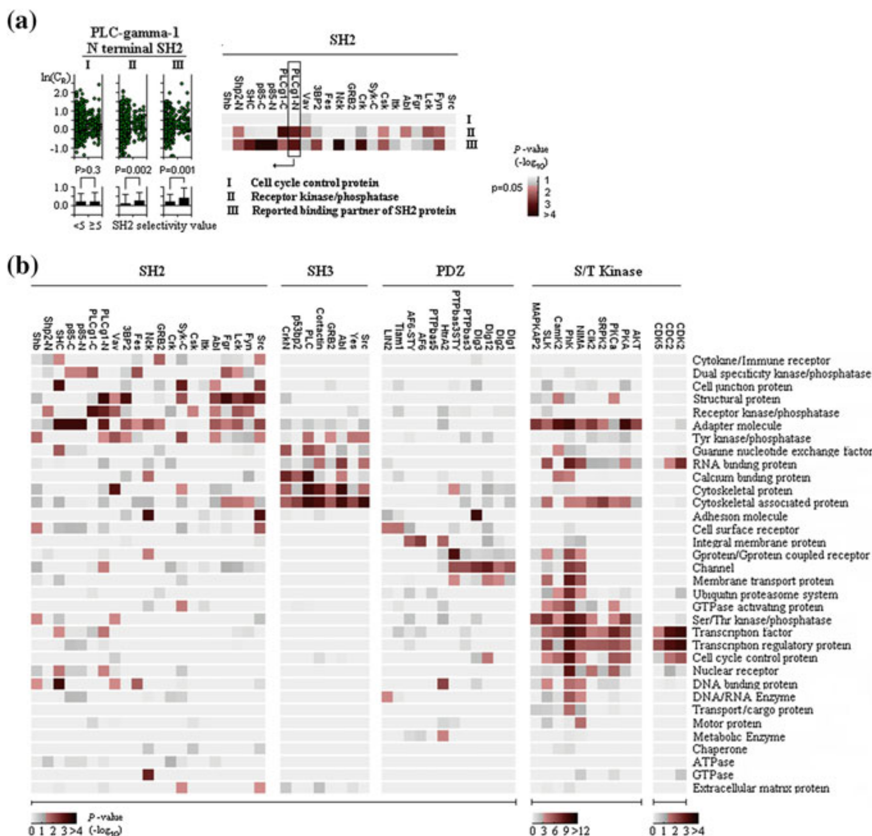

Fig. 2 Conservation analysis of SLiMs recognized by SH2, SH3, PDZ and S/T kinase domains in different protein functional classes. **a** Conservation analysis of potential SH2 binding Tyr-SLiMs in cell cycle control proteins, receptor kinases/phosphatases and reported SH2 binding partners. The PLC-gamma-1 N terminal SH2 domain is shown as an example (*left panel*). The x-axis represents the selectivity of the PLC-gamma-1 N terminal SH2 domain, and the y-axis indicates the logarithm of C_R. The Mann-Whitney test was performed to calculate the significance of the increase of conservation between SH2 non-selected (selectivity value <5) and selected (selectivity value ≥5) Tyr-SLiMs. A color-coded map of p-values is shown on the *right*. **b** Conservation analysis of SLiMs recognized by SH2, SH3, PDZ and S/T kinase domains in different protein functional groups. Color-coded maps of p-values are shown below

Using the PLC-γ1 N-terminal SH2 domain as a model to study the relationship between conservation and function of SLiMs, we found that Tyr-SLiMs predicted to bind to the PLC-γ1 N-terminal SH2 are significantly more conserved in PLC-γ1 binding proteins and receptor kinase/phosphatases. No significant increase in $\ln(C_R)$ score was observed in cell cycle control proteins(Fig. 2a).

We then systematically examined the conservation of SLiMs recognized by SH2 and SH3 domains and compared with PDZ and S/T Kinase domains in representative protein functional classes taken from the Hprd database (Peri et al. 2003) (Fig. 2b).

Table 1 Molecular functional classes frequently reported to interact with SH2 and SH3 compared with PDZ domains and S/T kinases

Domain	Molecular function	Binding ratio[a]
SH2	Receptor kinase/phosphatase	0.53
	Tyrosine kinase/phosphatase	0.51
	Cytokine/immune receptor	0.36
	Adapter molecule	0.20
	Cell surface receptor	0.14
SH3	Tyrosinekinase/phosphatase	0.32
	Adapter molecule	0.18
	Guanine nucleotide exchange factor	0.12
	Cytoskeletal protein	0.11
	GTPase activating protein	0.11
PDZ	Channel	0.214
	Adhesion molecule	0.075
	Cell surface receptor	0.052
Kinase		*Phospho ratio*[b]
S/T Kinase	Serine/threonine kinase/phosphatase	0.00442
	Cell cycle control protein	0.00397
	RNA-binding protein	0.00334
	Transcription factor	0.00320
	Adapter molecule	0.00296
	Structural protein	0.00259
	Transcription regulatory protein	0.00255

[a]The binding ratio is calculated as the percentage of proteins that interact with proteins containing SH2, SH3, or PDZ domains

[b]The phosphorylation ratio is calculated as the ratio of serine residues that are phosphorylated

We randomly picked a standard number (2000 for SH2 and SH3; 500 for PDZ and 6000 for S/T Kinases) of SLiMs from each molecular functional group for statistical analysis. The sampling process was repeated 1000 times and the final p-value was obtained from the average Mann-Whitney test Z score (Ren et al. 2008).

Those functional groups that show significant increase of conservation highly correlated with those that frequently interact with respective domains according to HPRD database (Table 1). We observed that SH2-recognized SLiMs are most highly conserved in receptor kinases/phosphatases, adaptor molecules, tyrosine kinases/phosphatases and structural proteins; conservation was occasionally found in cytokine/immune receptors, cell junction proteins and cytoskeletal-associated proteins (conservation profiles of individual functional groups can be found in suppl. of (Ren et al. 2008)). Most other protein functional classes have little conservation signal.

For SH3-recognized SLiMs, conservation was significant in cytoskeletal and cytoskeletal-associated proteins. calcium binding proteins, RNA binding proteins, Tyr-kinases/phosphatases and guanine nucleotide exchange factors.

The conservation signal was almost absent in other functional classes. This is largely consistent with those frequently reported SH3 interacting protein groups.

Consistent with biochemical evidences that PDZ domains frequently interact with membrane proteins, we found that PDZ domain-recognized SLiMs are specifically conserved in membrane proteins including channels, integral membrane proteins, cell surface receptors, G protein/G protein coupled receptors and membrane transport proteins.

Proteins containing SLiMs recognized by S/T kinases in the basophilic group seem to be involved in a wider variety of cellular functions such as signal transduction (adaptor proteins and Ser/Thr kinase/phosphatases), cytoskeletal-associated proteins, transcription and cell-cycle control, and also in some membrane proteins. However, the proteins containing conserved SLiMs recognized by proline-dependent Ser/Thr kinases (including CDK2, CDC2 and CDK5) were specifically involved in transcription and cell-cycle control, with almost no conservation signal from other functional categories.

Remarkably, most functional classes of proteins with a significant conservation signal were highly specific for the signal within one group of domains, but not in other groups. For example, receptor kinase/phosphatase group show conservation signal only in SH2 domain group and transcription factors only in Ser/Thr kinase domain group. Nevertheless, a few protein functional classes exhibited a significant conservation signal in multiple groups of domains, such as adaptor molecules and cytoskeletal-associated protein groups; this corresponds to the fact that these proteins participate in multiple signaling pathways involving interactions with more than one domain.

In the process of evolution, from bacteria to animals, more sophisticated molecular systems were employed to handle additional complexities of the organism. We observed that those molecular machineries that trace back to prokaryotic cells such as cell cycle control protein, transcription factor, transcription regulatory protein, Ser/Thr kinases/phosphatase have interactions mainly with protein domains originated from prokaryotes such as S/T kinase domains. Those protein functions that developed later in animals such as Tyr kinase/phosphatase, receptor kinase/phosphatase, cytokine/immune receptor and cell junction proteins mainly interact with SH2 domains which is characteristic of animals. The similar trend was observed in SH3 and PDZ domains. It seems that evolutionarily old functions mainly interact with old domains and newly evolved functions call for new domains (Estimated origin of domains are listed in Table 2. Estimated origin of representative functions that frequently bind to respective domains are listed in Table 3.).

Table 2 Estimated origin of domains

Domain	Approx. originated from
SH2	Animal
SH3	Fungi
PDZ	Bacteria
S/T kinase	Bacteria

Table 3 Estimated origin of representative protein functions that frequently bind to respective domains

Domain	Function	Approx. originated from
SH2	Tyr kinase/phosphatase	Animal
	Receptor kinase/phosphatase	Animal
	Cytokine/immune receptor	Animal
	Cell junction protein	Animal
SH3	Guanine nucleotide exchange factor	Fungi
	Calcium binding protein	Fungi
	Cytoskeletal protein	Fungi
	Cytoskeletal associated protein	Fungi
PDZ	Gprotein/Gprotein coupled receptor	Fungi
	Integral membrane protein	Bacteria
	Channel	Bacteria
	Membrane transport protein	Bacteria
S/T kinase	Ser/Thr kinase/phosphatase	Bacteria
	Transcription factor	Bacteria
	Transcription regulatory protein	Bacteria
	Cell cycle control protein	Bacteria

We hypothesized that in most cases, new functions arose with participating protein domains and corresponding binding partners flourished in approximately the same period of time. In other words, the phylogeny of a domain is mirrored in its binding specificity, or "Specificity recapitulates phylogeny" (Fig. 3). Occasionally, old domains also participate in binding proteins in new functions like S/T Kinase domain interacting with cytoskeletal associated proteins and adapter molecules. The rationale for this is either proteins in new functions contain ancient sequences that participate in classical pathways or new roles have been assigned to the old domain. While old domains occasionally bind to proteins with new functions, the reverse is relatively rare, which is probably because that old functions are more fundamental and usually well optimized. Small interference of an old function would have a diversity of impacts on the system, thus not favored in evolution. The association between domain specificity and phylogenetic development was justified using a multi-level adaptive evolution model as described below.

As shown in Fig. 4, the environment constantly changes with time. There are drastic changes (from E_1 to E_2) and mild changes (from E_{111} to E_{112}). In order to maximize the adaptation of the cellular signaling network to different levels of environmental changes, different set of protein domains were developed accordingly. Some ancient domains participate in fundamental functions that are required under all environmental changes, while some recently developed domains are for the best adaptation to milder level changes. When the environment changes from E_{111} to E_{112}, in order to adapt to the new environment, the cell has to suppress domain D_{111} and its associated function F_{111} which has lost adaptive advantage,

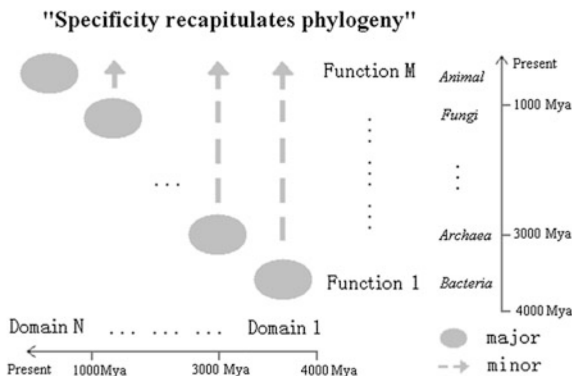

Fig. 3 "Specificity recapitulates phylogeny" Shown is a possible relationship between domains and protein functional groups. *Dark* olive areas are postulated major protein function groups that the domain selectively binds to; dashed arrows are possible minor ones. Evolutionarily old functions mainly interact with old domains and newly evolved functions call for new domains. Occasionally, old domains also participate in binding proteins in new functions. However, the reverse is relatively rare suggesting that it is strongly disfavored in evolution for new domains to regulate old functions. According to this theory, a domain got expanded in the genome only when it helps with some newly evolved functions. There are mechanisms that make sure the new domains do not interfere with old functions

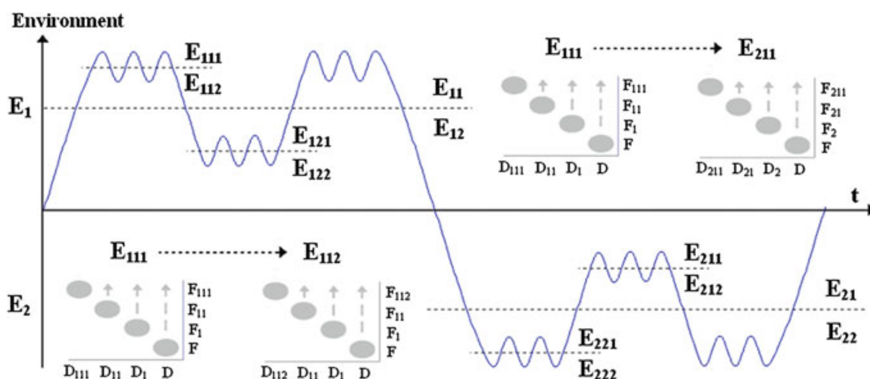

Fig. 4 A multi-level adaptive evolution model of domain mediated signaling networks. Old domain (D) and its associated function (F) are required over high level of environmental changes (E). On the other hand, recently emerged domain (D_{111} for example) is optimized for adaptation to recently low level environmental fluctuations (E_{111}). In general, Domain D_i and its associated function F_i is developed and optimized under environment E_i. It's usually not optimal for a new domain such as D_{111} to regulate old functions (F, F_1 and F_{11}) since this would compromise the fundamental functions of the cell when environment changes (to E_{112} for example) and D_{111} lost adaptive advantage and is replaced by another domain (D_{112})

and develop new domain D_{112} and its associated function F_{112}. If domain D_{111} had played influential roles in old functions such as F, F_1 and F_{11}, suppressing of D_{111} would compromise these functions which are also needed under environment E_{112}. Therefore, it's usually not optimal for a domain (D_{111}) that was adapted to recently mild level environmental change (E_{111}) to regulate functions adapted to higher level changes (F, F_1 and F_{11}). According to this model, some domains adapt to major changes in history and some adapt to recent relatively mild fluctuations. However, information about mild changes that happened in ancient history was largely lost. For example, as the environment changes from E_{111} to E_{211}, Domains D_{111}, D_{11} and D_1 that adapted under E_1 no longer fit the new environment E_2 and have to be replaced. This model also suggested that expression of some domains may be temporarily suppressed under certain environment, but can be reactivated when the environment changes back again soon, allowing quick adaptation. With domains mediate specific functions at different adaptation levels, combinations of different domains yield more complicated functions that are ready to get expanded when the time and conditions are right.

Although there are ample reports on the coevolution of proteins that interact with each other such as enzyme and its ligands, there are few reports that study their evolutionary relationships on a genomic scale. From system level analysis of conservation of SLiMs recognized by four representative domains that mediate protein-protein interactions, we found a correlation of conserved SLiMs and function. Furthermore, we found the functional specificity of each domain reflects its phylogenetic development, which was justified using a multi-level adaptive evolution model. To our knowledge, this is the first time the association between domain specificity and phylogenetic development has been clearly demonstrated at genomic level. While this association is manifest at the domain level, the situation would be more complicated at the protein level, since proteins are usually formed by a combination of domains and disordered/unstructured regions.

2.3 "Intrinsic Disorder" is Important for Short Linear Motifs to be Recognized by SH Domains

To investigate the functional importance of domain-recognized SLiMs in ordered and disordered regions of proteins, we performed a systematic analysis on the evolutionary conservation of SLiMs in predicted ordered and disordered protein sequences from different protein functional groups. For a given domain under study, proteins were first grouped according to their molecular functions then further grouped into three categories according to the involvement of interaction with that domain (frequent, occasional or rare). In each of the categories obtained from the last step, proteins sequences were sorted into ordered and disordered regions according to disorder predictor VL3 (see Ren et al. 2008 for detailed methods). The SLiMs in both ordered and disordered protein regions were further grouped into low, lower medium, upper medium and high domain selectivity

values. Conservation profiles were calculated for SLiMs in each group. The final output was the difference of $\ln(C_R)$ values between SLiMs with lower medium, upper medium and high selectivity values as compared to those SLiMs with low selectivity values. The conservation profiles were first averaged within each protein functional group, and then over the different functional groups within frequent, occasional and rare domain binding categories to avoid over-representation of any particular functional groups.

As expected, in those functional groups that are frequently reported to interact with respective domains, the conservation signal is the highest in the motif region that mediates the interaction (Fig. 5). Furthermore, conservation signal is the highest in frequent binding partners while progressively lowered from occasional to rare binding partners.

The conservation of the SLiMs is more manifest in disordered than ordered protein regions in all three domains examined. However, there are still some differences among the three domains. Tyr-SLiMs recognized by SH2 domains are conserved in disordered but not in ordered protein regions. The same pattern was observed for PXXP containing SLiMs recognized by SH3 domains. Interestingly, the sequences nearby the PXXP motifs recognized by SH3 have high conservation score. One possible explanation is that the proline residue is strongly disorder-promoting, and so a structured sequence

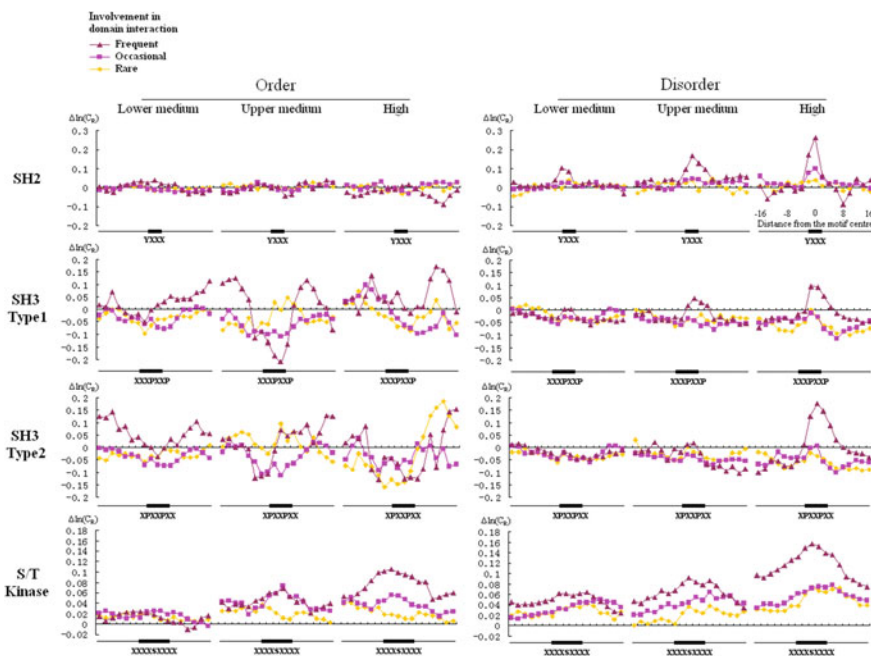

Fig. 5 Conservation profiles of Short Linear Motifs (SLiM) in ordered and disordered protein regions. Conservation profiles of SLiMs with lower medium, upper medium and high selectivity values for SH2, SH3 and S/T kinase domains in functional groups that are frequent, occasional or rare interaction partners of each domain

containing a PXXP motif would be expected to be an unstable element in the rigid structure. In order to compensate for the loss of structural stability brought about by the PXXP motif, the neighboring residues would become more important for the maintenance of the stability, which may explain their evolutionary conservation. In comparison, SLiMs recognized by Ser/Thr kinases are conserved in both ordered and disordered protein regions but are more conserved in disordered regions.

2.4 The "Shuttle Bus" Mechanism: SH Domains Mediated Interactions in Subcellular Localization and in Multi-domain Signaling

Using SH2 domain-interacting SLiMs as a model, we applied our method of conservation analysis to study additional aspects of SLiM conservation. Specifically, we investigated the conservation of SLiMs in proteins that interact with two different protein domains in a signaling pathway, and we studied the relationship between conservation of SLiMs and cellular localization.

Consistent with the observation that SH2-mediated signaling mainly occurs in the cytoplasm, we found a conservation signal for SH2-recognized SLiMs in cytoplasmic but not extra-cellular regions in both Type I and II membrane proteins. (For Type I membrane proteins, the cytoplasmic side is C-terminal, while for Type II membrane proteins it is N-terminal.) Since the majority of membrane proteins are Type I, we further classified this group by protein function. The conservation signal is significant for SLiMs on the cytoplasmic side of receptor kinases/phosphatases, cell surface receptors, cytokine/immune receptors and adhesion molecules, but weak for SLiMs in channels and metabolic enzymes (not shown, see Ren et al. (2008) for details).

SH2-domain binding is dependent on tyrosine phosphorylation, which is catalyzed by Tyr kinases. Accordingly, SLiMs recognized by Tyr kinases should be more likely to interact with SH2 domains. We found that SH2-recognized SLiMs that were selected for based on the presence of a common tyrosine kinase motif (containing E/D up to four amino acids from the tyrosine on the N-terminal side) are more conserved than those without this selection, especially in cytoplasm and plasma membrane signaling proteins (Fig. 6).

On the other hand, many tyrosine kinases (including the well-known Src family kinases) and adaptor molecules have both SH2 and SH3 domains, and it has been suggested that proteins containing multiple SH3 binding sites are more likely to be tyrosine phosphorylated and bind to SH2 domains as supported by biochemical studies (Pellicena and Miller 2001; Nakamoto et al. 1996). Consistent with this reasoning, SH2-recognized Tyr-SLiMs in signal transduction proteins that have more than ten PXXP SH3 binding motifs are significantly more conserved than SLiMs without this selection. This increased conservation is especially manifest for signal transduction proteins in the cytoplasm (which is not observed in other functional classes (Ren et al. 2008)). However, lack of conservation in the plasma

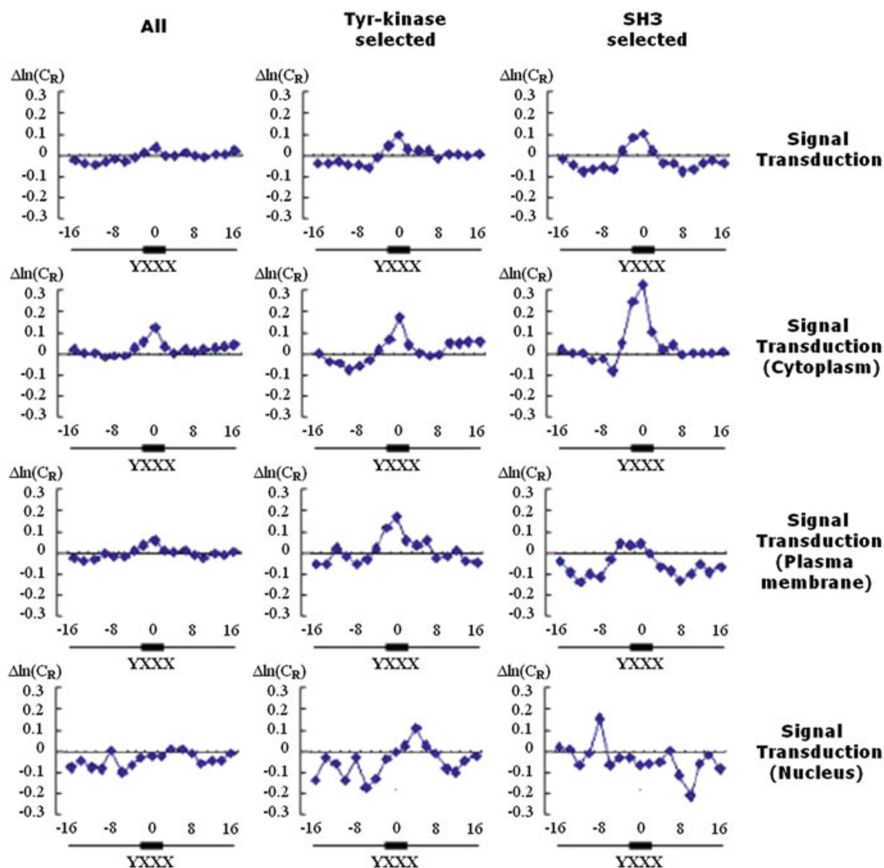

Fig. 6 Conservation profile of Tyr-SLiMs after tyrosine kinase or SH3 domain selection

membrane counterpart, suggesting signal proteins that attract SH3 domains at the plasma membrane are not favored for their SH2 binding. This gave us hints that there is some intricate relationship between the two domains.

From analysis of functional groups we knew SH3 domains' major binding partners are cytoskeletal and cytoskeletal associated proteins. SH3 domains also bind to some cytoplasmic signaling proteins such as guanine nucleotide exchange factors and Calcium binding proteins. Although there are reports about SH3 binding to plasma membrane proteins, our conservation analysis suggested most SH3 binding sites at plasma membrane may not be functionally important, suggesting SH3 domains tend to have subcellular preferences in cytoskeleton or cytoplasm but not in plasma membrane. This explained why plasma membrane proteins that contain putative SH3 binding sites don't show any increase of conservation signal in their SH2 binding SLiMs. Combined with the fact that SH2 domain and pTyr mediated signaling initiates at the plasma membrane, we hypothesized that these multi-domain proteins apply a common "Shuttle Bus" mechanism to commute

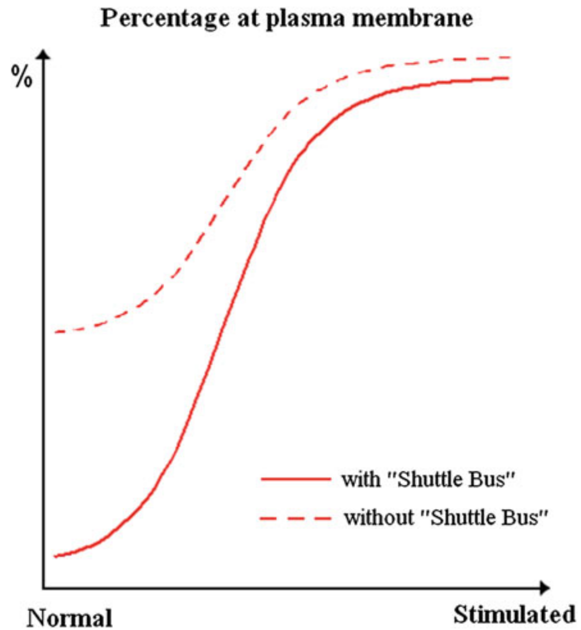

Fig. 7 Schematic illustration of the change of protein concentration at plasma membrane with and without the "Shuttle Bus" mechanism

among different subcellular locations. Under normal conditions, since most of these proteins bind to cytoskeleton and its associated proteins through their SH3 domains, their concentration at the plasma membrane is much lower (Fig. 7). This is advantageous to greatly increase the signal-to-noise ratio compared to systems without this mechanism. When the cell is stimulated, certain receptors at the plasma membrane become Tyr phosphorylated which attract SH2 domains and relocate these proteins to plasma membrane. After the stimulation, as more cytoplasmic proteins become Tyr phosphorylated, some of these SH2-SH3 proteins relocate to the cytoplasm, binding to cytoplasmic signaling proteins through both domains. After the cell goes back to normal, they too go back to their original positions. This model is supported by biochemical evidences on these multi-domain proteins (Bar-Sagi et al. 1993). While other regulatory mechanism also exist such as Src SH2 and SH3 domains binding to its own sequence to form a closed, inactive state (Xu et al. 1997), the "Shuttle Bus" model may be more universal and widely applied by proteins of radically different functions. This also explains why there are so many proteins in higher eukaryotic genomes contain both SH2 and SH3 domains, including Tyr-kinases, adapter proteins, phospholipases and GEFs etc. By inserting both SH2 and SH3 domains, the original protein has bought a ticket to a cellular "Shuttle Bus" which not only relocates the protein but also greatly improves signal-to-noise ratio without losing much sensitivity.

Other multi-domain proteins such as those containing both SH3 and PDZ domains, which are also well represented in the human genome, could have applied the same strategy since the subcellular location of these two domains are also

cytoskeleton vs. plasma membrane. Further evidence supporting this model comes from the fact that not a single protein contains both SH2 and PDZ domains in the human genome. This can be explained that both domains have plasma membrane localization but PDZ domains' constant binding somehow interfered with SH2 domains' pTyr dependent dynamic signaling, thus not favored in evolution.

3 Discussion

Protein-protein interactions mediated by SLiMs have a widespread influence on cellular functions (Pawson 2004; Neduva et al. 2005). We show here that studying the conservation specificity of SLiMs recognized by different domains would shed light on where, when, how and with whom the corresponding domain arose, thrived and contributed to maximizing the adaptivity of signaling networks (Fig. 8).

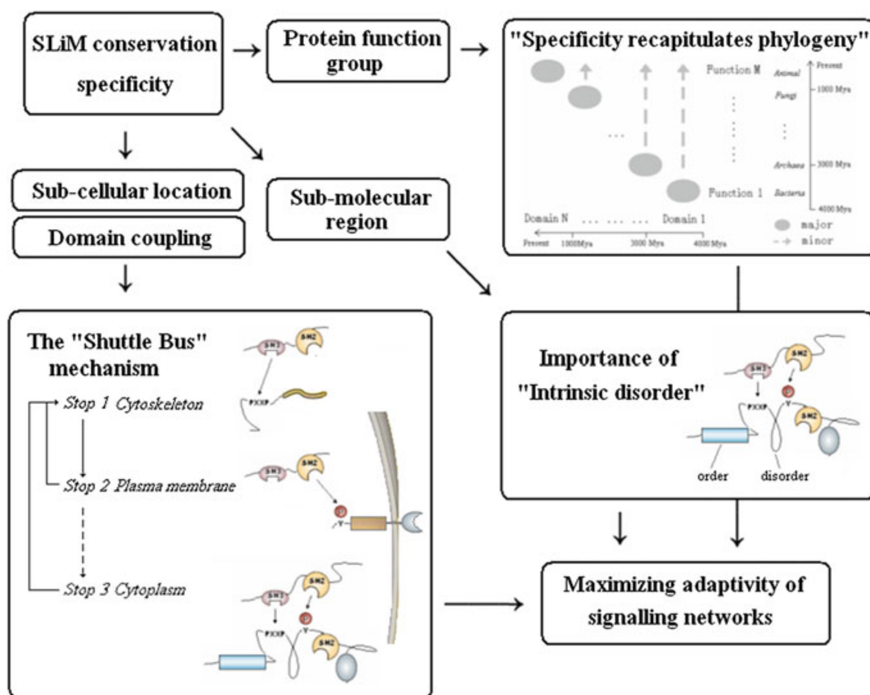

Fig. 8 From SLiM conservation specificity to how signaling networks maximize adaptivity. Conservation specificity of SLiMs examined in different protein functional groups, in submolecular regions, in subcellular locations and under the condition of domain coupling gave us functional, structural, and phylogenetic implications on how signaling networks maximize adapativity to environmental changes

System level studies indicated SLiMs are specifically conserved in proteins from certain functional classes that the corresponding domains are supposed to play a significant role, which is supported by data from biochemical and functional studies. This specificity is unique with very little overlap among four representative domains, suggesting of highly disciplined occurrence of functional SLiMs in spite of the appearance that they could have randomly emerged. We observed that domains and their specific major binding partners flourished in approximately the same period of time, or "Specificity recapitulates phylogeny". Thus, just as the body bears the traces of phylogenetic development, so also does the protein domain. Only with system level evolutionary pressure keeping active SLiMs in certain restricted functions, and some domains adapt to major changes in history while some adapt to recent relatively mild fluctuations, interacting domain sequences such as SH domains can be added to or deleted from proteins to open or close a gate to interactions with a highly specific set of functions, without compromising fundamental functions of the cell, and help to quickly maximize adaptivity of the signaling network to different levels of environmental changes. This multi-level adaptive evolution model explains the association between domain specificity and phylogenetic development.

The correlation between SLiM conservation with disorder prediction demonstrates that functional SLiMs recognized by each domain occur more often in disordered as compared with structured regions of proteins. From an evolutionary perspective, ordered or structural regions are generally more conserved than disordered regions (Brown et al. 2002). Disordered regions are like regulatory parts that are preferred by domains like SH domains during evolution to expedite adaptation for their structural accessibility, and more importantly, relatively less interference with the function of structural parts within the target protein.

The specificity and maximization of adaptivity of the domain was not only observed in protein functional groups and submolecular regions, but also in subcellular locations. The fact that SH2 mediated signaling starts at the plasma membrane which is not the preferred localization for SH3 domains suggests that proteins containing both domains may utilize a "Shuttle Bus" mechanism to commute between different locations when the cell is under different conditions. This mechanism can be implemented by other multi-domain proteins and it gives us a glimpse of the complexity of behaviour that could have brought about by combinations of domains, which offers an efficient way of linking different functions and subcellular locations for better adaptation.

It was recently reported that several bacterially secreted cytotoxins contain multiple repeated Tyr-SLiMs with high affinity for both tyrosine kinases and SH2 domains (Campellone and Leong 2005; Backert and Selbach 2005; Clifton et al. 2004; Schulein et al. 2005; Selbach et al. 2009; Pagano et al. 2013). Many of these cytotoxins are phosphorylated upon entry into host cells and bind to a variety of SH2 proteins. For example, the CagA protein secreted by *Helicobacter pylori* can be phosphorylated by Src and associates with Shp2 (Tsutsumi et al. 2003) and Csk (Tsutsumi et al. 2003) SH2 domains, which is essential for cellular changes induced by the bacteria. Similar events on phospho-Thr and FHA domain in viral hijacking

of cellular processes were also reported (Chaurushiya et al. 2012). The strong cellular response initiated by these SH2 or FHA domains binding pTyr/pThr-SLiMs further supports our assumption that SLiMs are under continuous evolutionary selection to preserve functional sites and eliminate harmful mutations. Recent work on the negative selection of SH3 domain-recognized sequences (Zarrinpar et al. 2003) also suggests that SLiMs may undergo strong evolutionary selection.

Although our results from conservation analysis correlated well with biochemical data in general, our method is still prone to error. First, our motif prediction is based on in vitro peptide scanning techniques, which may be biased due to differences between in vitro and in vivo conditions. Second, we assumed that each position of the SLiM contributed equally to binding, and only SLiMs that were conserved at each position were assumed to be conserved. To improve this method in the future, different weights could be assigned to each position, and amino acid similarity could be considered. Finally, evolutionary conservation can only provide indirect clues regarding function. For example, some SLiMs may only be important for a few species, and these would not have been detected in our analysis.

Domains such as SH domains mediated interaction with SLiMs give us a good opportunity to study protein-protein interactions at system level. As motifs recognized by other domains are better defined, conservation analysis will be able to provide valuable clues as to their functional roles, phylogenetic positions, subcellular localization, as well as relation with other domains. Recently, novel peptide array based technology has been developed and is becoming increasingly available (Uttamchandani et al. 2003; Tinti et al. 2013). New technologies combined with genomic and proteomic data (Peri et al. 2003) are expected to make motif discoveries easier and potentially more accurate. Methods, models and hypotheses proposed here are not restricted to SH Domains, but could be applicable to the research of other domains, domain combinations, and more general protein-protein, protein-nucleotide interactions in the future to elucidate their roles in cellular signaling networks and in the process of evolution.

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SH Domains and Epidermal Growth Factor Receptors

Natalya Kurochkina, Udayan Guha and Zhong Lu

Abstract Expression and increased activity of receptor and nonreceptor tyrosine kinases is a characteristic of many cancers. Receptor tyrosine kinase epidermal growth factor (EGFR) is one of the key molecules associated with cancer. Mutant alleles of EGFR L858R and Del E746-A750, which are common in human lung adenocarcinomas, result in increased phosphorylation of signaling molecules. Receptor tyrosine kinases and EGFR inhibitor MIG6 are more phosphorylated in human bronchial epithelial cells expressing mutant compared to those expressing wild type EGFR. Activation of EGFR results from the formation of a homodimer or heterodimer. EGFR and its active form resembles inactive conformations of Src and Hck. In this chapter, active and inactive conformations of EGFR and its oncogenic and drug-resistant mutant forms are described together with various modes of EGFR interactions with substrates and inhibitors.

Keywords SH2 domain · SH3 domain · Growth factor signal transduction · Protein conformation · Src-family

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1 Introduction

Protein tyrosine kinases (PTKs) proved important role in transduction and amplification of exogenous signals received by cells. Since the discovery of *src* family of genes, the product of *Rous sarcoma* virus oncogene, *v-src,* and the product of the cellular proto-oncogene, *c-src,* PTKs were shown to be essential components of many cell signaling pathways (Hunter and Sefton 1980; Kefalas et al. 1995; Parsons and Parsons 2004).

Receptor and non-receptor protein tyrosine kinases form two large groups. Among non-receptor PTKs, the most abundant are *src* and *tec* protein tyrosine kinases which together comprise approximately 40–45 % of cytoplasmic PTKs (Nore et al. 2003). Ten members of *c-src* family (*blk, c-fgr, fyn, hck, lck, Lyn, c-src, c-yes*, *yak, and frk*) (Amata et al. 2014) encode cytoplasmic proteins that function in T-lymphocyte maturation and activation, bone maintenance, learning and memory. Five members of *tec* family include Bruton's tyrosine kinase, *btk*, *itk*, *tec*, *bmx*, and *txk/rlk* (Nore et al. 2003). PTK contains six domains: (1) N-terminal domain that contains sites of lipid modifications important for PTK targeting to the plasma or intracellular membrane (SH4) (2) unique domain (UD), (3) catalytic domain (SH1), (4) phosphotyrosine recognition domain (SH2), (5) polyproline sequence specific (SH3) domain, and (6) C-terminal tail with a regulatory tyrosine (Gmeiner and Horita 2001). SH2 and SH3 domains can also be found in adaptor proteins that lack enzymatic activity, such as Grb-2 and α-spectrin, and in proteins that possess other than tyrosine kinase activity: phospholipase C-γ1 (PLC-γ1), *ras* GTPase-activating protein (*ras* GAP), phosphatidyl-inositol 3-kinase (PI3-kinase) (Kefalas et al. 1995). Depending on definition of a domain we can also say that there are three domains and three additional regions. We will call all of them domains throughout this chapter.

Some PTKs (Fyn, Src, and Yes) are expressed in most cell types. Others exhibit specific expression: Hck in myeloid cells, Blk in B cells, Lck in T lymphocytes (Arold et al. 2001).

Large group of receptor protein tyrosine kinases (RTKs) include epidermal growth factor, nerve growth factor, fibroblast growth factor and other receptors that contain extracellular ligand binding domain, membrane anchoring domain, cytoplasmic protein tyrosine kinase domain, regulatory domains, and C-terminal tail. Growth factor binds to the extracellular portion of the receptor and induces dimerization that triggers formation of signaling complexes and turns on signaling cascades on the cytoplasmic side of the membrane. Receptors are specifically regulated by multiple adaptor proteins (Kalman et al. 2013).

2 Growth Factors and Receptors

Nerve growth factors (NGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), stem cell factor (SCF), transforming growth factors (TGFs), vascular endothelial growth factors (VEGFs), and epidermal growth factors (EGFs) each specifically bind to their receptors located at the cell surface and activate the receptor.

Each type of neurotrophins, NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3/7 (NT-3–NT-7) and other, promote the survival of certain sets of neurons. Two classes of cell surface receptors, the Trk receptors and the p75 neurotrophin receptors, are activated by the specific ligand. TrkA/NGF, TrkB/(BDNF or NT-4/5), TrkC/NT-3 and p75NTR/NGF complexes are main components that regulate development and support of the nervous system (Ultsch et al. 1999).

Target cell derived neurotrophic factor, NGF, is essential for survival of developing sympathetic and cutaneous sensory neurons; tyrosine kinase TrkA complex with NGF travels retrogradely from the axon to the cell body and induces transcriptional and signaling events necessary for survival (Deppmann et al. 2008).

In response to neurotrophins, β-actin mRNA is both targeted to axons and locally translated there. Local protein synthesis of β-actin allows rapid response that is independent of cell body. Impa1 mRNA is targeted to sympathetic neuron axons and locally translated in response to nerve growth factor (NGF) stimulation of distal axons.

Neurotrophin stimulation of distal axons elevates production of proteins responsible for axon growth and maintenance and depends on retrograde pathways. For example, neurotrophin induces transcription of bclw mRNA in cell bodies that involves Trk-Erk5 pathway. Neurotrophins regulate Bclw at various stages: transcription, transport, and local translation. Neurotrophic regulation of local, axonal synthesis of Bclw is necessary for supporting axonal survival. Bclw/Bax suppresses cascade 6 apoptotic activity. A bidirectional mechanism is employed: retrograde neurotrophin signaling from the axon activates transcription of response genes and leads to increased expression of axon-targeted mRNA and protein necessary for axon survival. These events contribute to our understanding of regulatory mechanisms of normal development of neuronal circuitry and axonal degeneration (Cosker et al. 2013).

FGFs activate intracellular signaling by binding to cell surface tyrosine kinase receptors FGFR1-4. FGFRL1, member of FGFR family, lacks a kinase domain and was proposed to act as a decoy receptor to inhibit FGFR ligand-induced signaling. Expressed in pancreatic islet β-cells, FGFR1 regulates insulin processing via canonical ligand binding. FGFRL1 is expressed in plasma membrane and insulin secretory granules of β-cells. Its intracellular domain contains a pY SH2-binding motif that binds phosphatase SHP-1 and stimulates ERK1/2 pathway. SH2-domain of SHP-1 when not bound to FGFRL1, autoinhibits the phosphatase. Its unique intracellular domain contains a tandem tyrosine-based motif involved in endocytosis and a histidine-rich region, site of metals binding. FGF/FGFR signaling is

known to regulate development of the embryonic and neonatal pancreas (Silva et al. 2013). Many tyrosine phosphatases act as tumor suppressors. Shp2, was found to act as tumor suppressor but also as a positive signal transducer (Li et al. 2012).

PDGF and EGF are signaling molecules that bind to their cell surface receptors, PDGFR and EGFR, and induce receptor activation. Functionally significant differences in mitogenic signaling for these growth factors involves stimulation by PDGF of sphingosine kinase activity in fibroblasts and increase intracellular levels of sphingolipid metabolites that lead to cell proliferation (Rani et al. 1997). Cancer associated fibroblasts secreted soluble factors affect not only cancer cells but also many other types of cells (Räsänen and Vaheri 2010). For both growth factors, receptor and MAPK activation, mediated by adaptor proteins, leads to the activation of transcription factors. Networks of transcription factors in healthy and transformed cells play key role in cell growth, differentiation and development being determinants of cell states (Sive and Göttgens 2014).

Expression and increased activity of receptor and nonreceptor tyrosine kinases is characteristic of many cancers (Brueggemeier et al. 2005). EGFR is one of the key molecules associated with cancer. Approximately 60–65 % of all adenocarcinomas are linked to mutations in one of oncogenes *KRAS, EGFR, ALK* fusion, *BRAF, HER2, NRAS,* or *MEK1* (Suehara et al. 2014) and many inhibitors of pathways controlled by these genes are protein protein interactions modules carrying SH2, SH3, and proline rich domains (Fiorentino et al. 2000; Frosi et al. 2010; Guvakova et al. 2014; Xu et al. 2005; Wendt et al. 2015). Mutant alleles of EGFR L858R and Del E746-A750, which are common in human lung adenocarcinomas, result in increased phosphorylation of signaling molecules. Receptor tyrosine kinases and EGFR inhibitor MIG6 are more phosphorylated in human bronchial epithelial cells expressing mutant compared to those expressing wild type EGFR (Guha et al. 2008). New data suggest that Mig6, phosphorylated at Y394/395 in EGFR-mutant human lung adenocarcinoma cell lines, stabilizes EGFR since its interaction with EGFR increases; MIG6 also does not promote degradation of mutant EGFR (Maity et al. 2015). EGF, TGFα, and amphiregulin are EFFR ligands. Activation of EGFR results from the formation of a homodimer or heterodimer paired with another family member such as HER2-4 (Kalman et al. 2013). EGFR asymmetric dimer activation (Zhang et al. 2006, 2007) does not require autophosphorylation of the activation loop (A-loop). Some mutations such as L858R can activate the receptor (Yoshikawa et al. 2012). As a result, C-terminal tyrosines are autophosphorylated and via involvement of SH2 domain bind downstream signaling molecules initiating signal transduction, i.e. MAPK, PI3 K/Akt, PLC, STAT, and SRC/FAK pathways (Kalman et al. 2013). Crystallographic structures of the active conformation of unphosphorylated EGFR alone and in complex with the inhibitor erlotinib show that EGFR kinase activation loop adopts a conformation similar to that of the phosphorylated form of insulin receptor kinase (Stamos et al. 2002). Recently determined structures of EGFR mutant forms alone and in complexes with MIG6, gefitinib, and dacomitinib demonstrate various conformational states of the molecule (Gajiwala et al. 2013). Activity in Src and Hck enzymes is regulated by phosphorylation of a specific tyrosine at the C-terminus. Intramolecular binding of

SH2 domain to phosphotyrosine keeps the receptor in the inactive state. The regulatory mechanism involves SH2 and SH3 domains that affect relative orientation of the N-lobe and C-lobe of the kinase. SH3 domains of tyrosine kinases Src, c-Abl, and Bcr-Abl autoinhibit their kinase activities (Review: Kristensen et al. 2006). EGFR bound to Lapatinib (GW572016) exhibits conformation similar to that of Src or Hck in inactive state. EGFR active form resembles inactive conformations of Src and Hck (Gajiwala et al. 2013). Activation mutant L858R and double mutant L858R/T790M (Fig. 1) retain conformation similar to inactive state and were proposed to possess greater propensity to form asymmetric diners that explains their unregulated activity. Inhibitor gefitinib binds to the inactive kinase V948R/L858R/T790M mutant (Fig. 2).

Phosphotyrosine residues that are required for signaling mediated by receptor tyrosine kinases, such as EGFR, are unique binding sites of proteins containing SH2 domains (Koch et al. 1991). SH2 domain of the Src protein binds EGFR. Several phosphopeptides corresponding to five major autophosphorylation sites of EGFR Y1173, Y1148, Y1086, Y1068, and Y992 and putative phosphorylation

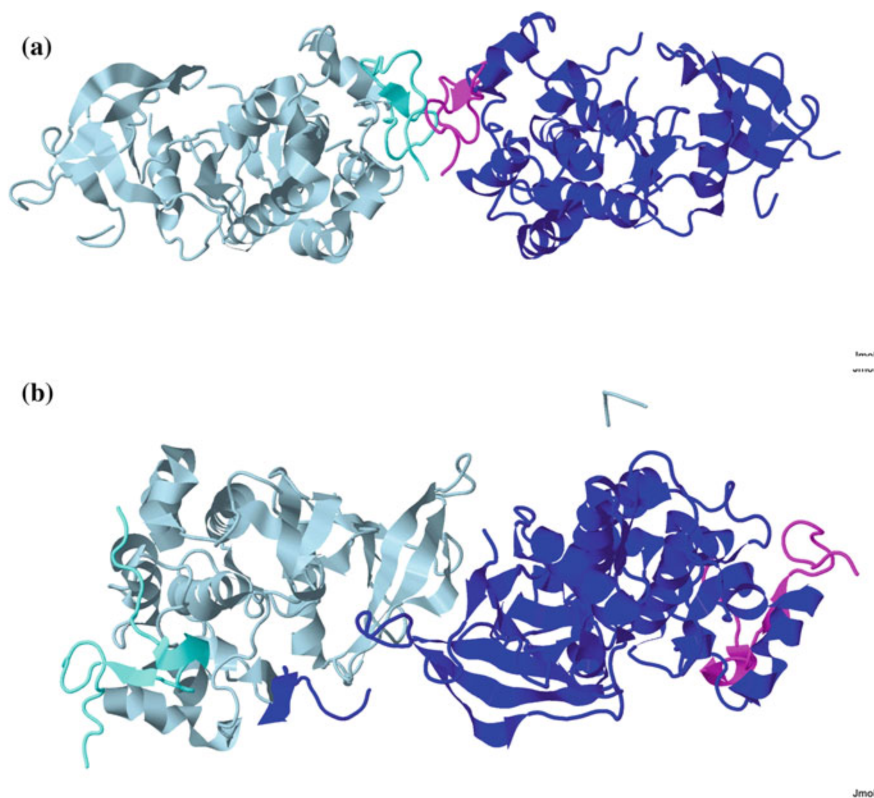

Fig. 1 EGFR structure. Kinase domain (*blue, green*) in complex with inhibitor MIG6 (*magenta* and *cyan*) (**a**) Wild type /2rf9/ (**b**) Drug-resistant mutant L858R/T790M /4i21/

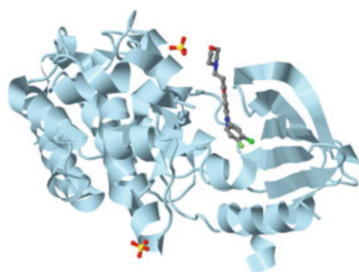

Fig. 2 EGFR complex with gefitinib. Kinase domain (*ribbon*); inhibitor (cpk). Structure of gefitinib/erlotinib resistant mutant V948R/L858R/T790M /4i22/

sites Y1114, Y1101, and Y1045 were used to show Src SH2/SH3 interaction with EGFR. SH2/SH3 construct is better compared to SH2 alone. Therefore, SH3 facilitates association of SH2 with EGFR phosphotyrosine sites (Luttrell et al. 1994). Role of phosphorylation within SH3 domain was studied for CAS, a tyrosine phosphorylated protein in cells transformed by v-*crk* and v-*src* oncogenes that are responsible for invasiveness of Src-transformed cells. Using phosphomimicking Y12E and non-phosphorylatable Y12F mutants (Janoštiak et al. 2011) it was shown that the Y12E mutation leads to decreased interaction of CAS SH3 with FAK and PTP-PEST and reduced tyrosine phosphorylation of FAK whereas Y12F mutation results in hyperphosphorylation of CAS substrate domain, slower turnover of focal adhesions, decreased cell migration, and decreased invasiveness. GEF for Rap1 and R-Ras, C3G, transduces signal to c-Jun kinase (JNK). R-Ras activates JNK-dependent transcription and cell transformation (Mochizuki et al. 2010). Cas contains multiple sites of interactions with SH2 and SH3 domains: polyproline regions, NxxY motifs, and SH3 domain. Tyrosine phosphorylation mediates Cas binding to SH2/SH3 of Lck (Nasertorabi et al. 2006).

Signaling pathways of wild type EGFR and its mutant forms differ in regulation and trafficking (Hampton and Craven 2014). Tyrosine phosphorylation very often abolishes binding and results in dissociation of the modified molecule from the enzyme (Zhao et al. 1998; Wang et al. 2013; Tatarova et al. 2013).

Epithelial lumen surface glycoproteins, mucins, which function to protect mucous epithelium, contain several domains including protein tyrosine kinase domain homologous to EGFR, transmembrane domain, MIG6 domain, and multiple O-glycosylation sites (Nollet et al. 1998). Some mucins were shown to mediate breast cancer cell migration through interaction with intracellular adhesion molecule 1 that depends on mucin cytoplasmic domain activation by c-src with involvement of competitive SH3 binding (Gunasekara et al. 2012).

3 Cytoplasmic PTK

Cytoplasmic PTKs exhibit many similarities to RTKs. Their kinase domains are regulated by SH2/SH3 and the C-terminal domains. Kinase domains of EGFR and Lck, for instance, and their mode of regulation carry many important conserved features. C-terminus of EGFR contains several tyrosine phosphorylation sites that regulate binding of SH2-containing molecules to EGFR upon phosphorylation. Peptides with phosphotyrosine bind to EGFR interdomain lobe (Stamos et al. 2002; Wood et al. 2004).

Src family kinases, including Lck, are regulated by phosphorylation of its C-terminal tyrosine in a 'tail' peptide that binds to SH2/SH3 domains and inhibits the kinase activity. If the 'tail' peptide is dephosphorylated or SH3/SH2 domains bind to competitive ligands, kinase is activated by autophosphorylation of a tyrosine in its activation loop (Eck et al. 1994; Pisabarro et al. 1998; Sicheri et al. 1997; Schweimer et al. 2002; Cowan-Jacob et al. 2005). SH3 and SH2 domains are connected by a linker that contains polyproline sequences involved in regulation process. In some kinases, such as C-terminal Src kinase, Csk, not activation loop phosphorylation but peripheral motifs and SH2/SH3 are required for its activation/deactivation. Mutational studies in the regions of the interface between the kinase domain and regulatory domains help to identify key residues and understand how peripheral regions relay signals to the active site. SH2-kinase linker, disulfide bridge, and SH2 loops located at a distance more than 45 Angstrom from the active site, particularly CD and DE loops, are important allosteric sites (Barkho et al. 2009).

4 SH4 Domain

Attachment of the molecule to the membrane utilizes SH4 domain (Silverman and Resh 1992). These domains are subject to posttranslational modifications: myristoylation and/or palmitoylation. Fyn SH4 unique sequence dual acylation controls intracellular localization (Yamada and Bastie 2014).

5 Unique Domain

Domain between the N-terminal SH4 and regulatory SH3 domains is named Unique (UD) since it is less conserved in its amino sequence within src family tyrosine kinases. However, strong conservation of the UD of each SFK member among different organisms suggests some important function. Conservation of residues of the UD in Src, Fyn, and Yes is associated with its binding to various targets: acidic lipids, SH3, and calmodulin. This region is phosphorylated in Lck (Ser 59 in proline

rich region), Lyn (Y32), Yes, Fgr, Fyn (S21 in RxxS site of PKA), Src (S17, T37, S75), and autophosphorylated in Hck (Y29). For Hck, autophosphorylation of the UD domain along with autophosphorylation in the activation loop of the catalytic domain contributes to its activation (Amata et al. 2014).

6 SH3 Domain-Containing Proteins

SH3 domains mediate protein-protein interactions (Gmeiner and Horita 2001; Kurochkina and Guha 2013). They bind proline-rich sequences, particularly those carrying PxxP motif, in left-handed polyproline 2 (PPII) conformation (Musi et al. 2006). For some, important function in recognition and signaling was assigned, but for others it has to be determined (Foth et al. 2005). Posttranslational modifications of SH3 s play important role in regulation of cellular processes. Synapsin I, through SH3- or SH2-mediated interactions, activates Src. Src-mediated tyrosine phosphorylation of synapsin I increases binding to synaptic vesicles/actin and formation of synapsin dimers, whereas serine phosphorylation increases synaptic vesicles availability for exocytosis by impairing synapsin association with synaptic vesicles and/or actin (Messa et al. 2010). Adaptor protein Crk (Matsuda et al. 1996; Kobashigawa et al. 2007) for which two isoforms are known, CrkI (SH2-SH3) and CrkII (SH2-SH3-SH3), regulates cytoskeletal reorganization and motility involved in cell growth by facilitating protein-protein interactions. CrkII regulates NWASP and Cdc42 activation, actin polymerization, and development of tension in muscle. CrkII SH3 N mutants inhibit tension development upon stimulation of smooth muscle with acetylcholine possibly due to disruption of Ca^{2+} signaling pathways (Tang et al. 2005). The linker region between two SH3 domains contains a regulatory tyrosine Y222. Regulation of Abl kinase by Crk is proposed to occur via intermolecular and intramolecular PxxY sequences (Reichman et al. 2005). Some SH3 binding sequences are phosphorylated such as $RKXXY^{294}XXY^{297}$ motif in SKAP-55 bound to ADAP SH3c. Interestingly, SH3 domain of SKAP-55 binds a proline-rich region of ADAP (Duke-Cohan et al. 2006). This sequence, represented as RxxYxxY or RxxYxxF, is reminiscent of Class I motif RxxPxxP (Kang et al. 2000). There can be drawn a parallel with SKAP-55 and ADAP mode of binding: whereas the FYB SH3 binds tyrosine-based site in SKAP-55, the SKAP-55 recognizes proline-rich site in FYB. Adaptor protein Nck associates with a number of target proteins through a preferred motif. The phosphorylatable serine in the motif is a regulatory site that mediates binding to PAK (Zhao et al. 2000). Nck interaction with CD3ε regulates T cell receptor activity with involvement of noncanonical binding of Nck2 first SH3 motif to PxxDY sequence (Takeuchi et al. 2008). Phosphorylation of tyrosine in this motif abolishes binding of Nck and EGFR substrate, Eps8 (Kesti et al. 2007). Recently, synergetic binding of Cdc42 and EPS8 SH3 to Crib domain and adjacent proline-rich region of IRSp53 have shown possible mechanisms of regulation of membrane and GTPase function (Kast et al. 2014).

Regulation of GTP-binding proteins is important function of SH3 domains that extend their role as mediators of protein-protein interactions. Dynamin binds to microtubules via its C-terminus. This binding induces GTPase activity. For example, GST-Grb2 protein and GST-SH3 domains of c-src, fgr, and fyn stimulate dynamin GTPase activity, whereas C-terminal Grb2 SH3 domain, PLCγ, and p85α bind efficiently but do not stimulate GTPase activity (Gout et al. 1993).

Hydrophobic and electrostatic interactions are important for recognition of polyproline sequences by SH3 domains. Class I sequence RXLPPXP and class II sequence XPPLPXR include PxxP hydrophobic motif that adopts polyproline type II conformation. These sequences are flanked by charged residues. Although class I and class II motifs contain charged residues, flanking polyprolines from one side, and this arrangement is linked to ability of these motifs to bind SH3 domain in two different orientations, sequences with polyprolines only were identified as class III ligands. Besides, noncanonical sequences were identified: PxxDY in EPS8, RKxxYxxY in SKAP55, RxxK in GADS, PXXXPR in βPIX and CIN85/CNS, RxxPxxxP in BK channels. Hydrophobic and charged residues flanking PxxP motif were shown to enhance interaction. Arginines and lysines, in addition to charge, contain large hydrophobic chain that very often is important for association. Some sequences bind without proline (Mayer 2001; Kang et al. 2000; Gushchina et al. 2011; He et al. 2014; Teyra et al. 2012; Kim et al. 2008; Pires et al. 2003; Tian et al. 2006; Tonikian et al. 2007).

Regulation by association of SH3 domains also occurs via formation of homodimers (IB1 protein; Kristensen et al. 2006) or heterodimers (VAV N-terminal and GRB2 C-terminal SH3 domains complex; Nishida et al. 2001).

Very important functions of SH3 are emphasized in giving rise to emerging actin filaments controlled through specific assembly of SH3 regulated multiprotein complexes by phosphorylation and guanine exchange, regulation of cytoskeletal organization and gene expression during development (Mayer 2001; Kawauchia et al. 2001).

SH3 domains are also regulated by phosphorylation. Tyrosine phosphorylation in SH2, SH3, and WW domains of adaptor proteins is more frequently observed than serine/threonine phosphorylation indicating its important function. Tyrosine phosphorylated SH3 domains can reduce, disrupt, or increase binding, affect protein localization, stimulate or inhibit signaling, regulate gene expression. The most frequently phosphorylated position within SH3 domain corresponds to c-Src Tyr-90 and is characterized by a consensus sequence ALYD(Y/F) (Nore et al. 2003; Tatarova et al. 2013). In tertiary structure, this sequence belongs to the loop between the first two β-strands named RT that is involved in recognition of binding partners. Second frequently phosphorylated site corresponds to Tyr-131 and is located in the distal loop, also peptide binding pocket (Review: Kurochkina and Guha 2013). Autophosphorylation of the RT loop of SH3 domain in Itk, Btk, and other TEC family members of non-receptor tyrosine kinases results in the kinase activation (Joseph et al. 2007; Park et al. 1996). Activation of Btk involves phosphorylation of one tyrosine in activation loop followed by autophosphorylation of another tyrosine in SH3 domain. Tec family PTKs exhibit preference for

Table 1 Phosphorylation and mutations of tyrosine residues in SH3 domains

Modification	Effect	Reference	Protein/Pdb code/
Y209F	Site of phosphorylation by Bcr/Abl or EGFR in vivo. Enhances BCR/Abl-induced ERK activation and fibroblast transformation; potentiates and prolongs Grb2-mediated activation of Ras, mitogen-activated protein kinase and c-Jun N-terminal kinase in response to EGF stimulation; this confirms that role of phosphorylation is to diminish Bcr/Abl-induced fibroblast transformation	Li et al. (2001)	CGrb2 /2vwf/ Y209: 3_{10} helix between β4 and β5 NGrb2 /1aze/ Y7: RT loop
pY209	Negatively regulates EGF-induced ERK and JNK activation; inhibits the binding of Sos	Li et al. (2001)	
	c-Abl, activated by the VEGFR-2/PI3 K pathway, phosphorylates Grb2 Y209 and Nck1 Y105 to down-regulate the MAPK activity	Anselmi et al. (2012)	
Y7F/Y209F	Impairs Sos binding by Grb2	Li et al. (2001)	
Y52F	In contrast to Y209F, does not impair Sos binding by Grb2	Li et al. (2001)	NGrb2 /1gbq/ 3_{10} helix between β4 and β5
pY138	Site of phosphorylation in vivo by PDGF receptor. Reduces binding of peptide ligands in vitro	Broome and Hunter (1997)	CSrc /1qwe/ 3_{10} helix between β4 and β5
Y138F	No effect on PDGF-induced c-Src activation in Src^{-} mouse fibroblast cell line	Broome and Hunter (1997)	
Y546A	Destabilization of dimer	Kristensen et al. (2006)	Abi /2fpd/ 3_{10} helix between β4 and β5
Y355A, F, I, or M	Decreases interaction with PBS2 and leads to decreased HOG pathway response	Marles et al. (2004)	SHO1 /2vkn/ 3_{10} helix between β4 and β5
Y180F	Decrease of kinase activity of the full-length enzyme by increasing km of peptide substrate	Joseph et al. (2007)	Itk /1awj/ RT-loop autophosphorylation
Y180E	Alters binding capability		

(continued)

Table 1 (continued)

Modification	Effect	Reference	Protein/Pdb code/
Y223F	Partially restores calcium mobilization and Plcγ2 phosphorylation in a Btk deficient cell line; enhances the oncogenic ability of a gain-of-function Btk mutant; alters binding activity	Kurosaki et al. (1997), Yang and Desiderio (1997) Park et al. (1996)	Btk /1aww/ autophosphorylation RT-loop
Y309A	Decreases interaction with PBS2 and leads to decreased HOG pathway response	Marles et al. (2004)	SHO1/2vkn/ RT loop
Y206	Autophosphorylation, but not transphosphorylation.	Nore et al. (2003)	Tec-SH3
PY251	Binds Abl SH2 and promotes its transactivation	Sriram et al. (2011)	Crk SH3 RT loop

phosphorylation of their own SH3 domains. Transphosphorylation can be specific not only for SH3 domains but for joint SH3/SH2 domain. One of the proposed regulation mechanisms suggests that when coexpressed in a single cell type (Btk and Tec in B-cells or Itk and Txk in T-cells) these kinases may use transphosphorylation to mediate activity of each other (Nore et al. 2003). Phosphorylation of key residues in SH3 domains affects their function in many ways (Table 1).

SH3 domain has been implicated in important processes in cell biology. SH3 domains play critical roles in migration and invasiveness (Yamada et al. 2011), actin reorganization induced by extracellular signals (Antoku and Mayer 2009), and shaping spines in neurons (Sheng and Kim 2000; Ehlers 2002). The role of SH3 domain in these biological processes has implication in cellular homeostasis, as well as disease states.

7 SH2

SH2 domains bind phosphotyrosine sequences and contribute to the assembly of signaling complexes that are formed as a result of the growth receptor stimulation. EGF, PDGF or other receptors contain tyrosines that are phosphorylated and bind to SH2 domains of the downstream signaling molecule such as p85 subunit of PI3 kinase, the GTPase-activating protein, growth factor receptor-bound protein 2, and PLCγ. Specificity of binding is determined by several amino acids surrounding phosphotyrosine. Several regulatory events are important for the signaling: (1) kinases are phosphorylated at their C-terminal tyrosine and phosphotyrosine binding to SH2 domain inhibits kinase; (2) the kinase binds to the activated receptor via SH2 domain (Bibbins et al. 1993).

Downstream molecules signaling also involves specific interactions of their SH2 and SH3 domains. One of the important signaling molecules, PLCγ, activates receptor tyrosine kinase receptors (EGFR, PDGFR). SH2 domain of PLCγ also interacts with adaptor proteins (GRB2, SOS1, AP180). When cells are stimulated by growth factors, PLCγ is recruited to the plasma membrane where its substrates are located. Its SH3 domain interacts with βPix-a, GEF for small G-proteins RAC1/Cdc42 important for actin cytoskeleton reorganization. This interaction contributes to tumor growth in breast cancer cells. PLCγ expression was also increased in colorectal cancer. Polyproline region at the C-terminus of PLCγ can bind SH3 domains of PLCγ, PAK, and GIT1. PLCγ induces RAC activation and Iba1-dependent membrane ruffling (Bae et al. 2005). Competition takes place between PLCγ and Grb2 for binding to FGFR. Low concentrations of Grb2 elevates metastatic potential of FGFR–expressing cells (Timsah et al. 2014). PLCγ/Grb2 dimer binding to 2 molecules of FGFR occurs via Grb2 SH3 domain and results in the formation of an active heterotetramer even without a growth factor stimulation. The two SH2 domains of PLCγ have distinct roles: the N-terminal SH2 domain regulates binding of PLCγ to receptor protein tyrosine kinases which then phosphorylate PLCγ; The C-terminal SH2 domain binds this phosphorylated tyrosine and activates phospholipase activity (Poulin et al. 2005).

Although SH2 domain mainly binds phosphorylated tyrosine, binding can also occur to phosphorylated serine/threonine as demonstrated by binding of Abl SH2 to BCR protein or binding of Fyn/Src SH2 to Raf1 (Pendergast et al. 1991). Raf1 also was found to interact with the SH2 domain of Src and this intéractíon depends on arginine. Interaction of Raf1 with Src SH2 is weaker but can be enhanced by the presence of SH3 and N-terminus. Another example of deviation from the specific pattern is binding of Src SH2 to nonphosphorylated PDGFR peptide (Cleghon and Morrison 1994). In some proteins, high affinity binding can be achieved with contribution of two SH2 domains as demonstrated by GAP or p85 binding to PDGF receptor, whereas binding of one SH2 domain does not result in stable complex (Cooper and Kashishian 1993). SH2 domains are also regulated by phosphorylation (Jin et al. 2015).

8 SH1

The first tyrosine kinase discovered, a product of the viral gene v-src, lacks autoinhibition and is constitutively active resulting in cell-transforming activity, whereas its cellular counterpart, c-src, is subject to regulation (Hunter and Sefton 1980; Brown and Cooper 1996; Sicheri and Kuriyan 1997).

The structure of the catalytic domain, SH1, of many tyrosine kinases was extensively studied by crystallography, NMR, and other methods in active and inactive states and bears common architecture of other kinases, such as protein kinase A (PKA). Two lobes, N-terminal (N-lobe) and C-terminal (C-lobe) form a cleft at the interface that hosts ATP molecule. N-lobe comprises a five-stranded

β-sheet and regulatory α-helix (αC). C-lobe is predominantly α-helical. Important segments include ATP-binding glycine-rich strand-beta-strand motif, metal-binding catalytic segment, and subject to phosphorylation activation segment. Complexes of the kinase with nonhydrolizable ATP analogs and peptides substrate analogs reveal protein groups responsible for interactions with nucleotide, phosphate groups and substrate. Comparison of the crystal structures of human c-Src and human Hck in inactive state shows high similarity of the two kinases. SH2 and SH3 domains are bound intramolecularly: SH2 to the regulatory C-terminal tyrosine, SH3 to the polyproline type II helix of the linker between the SH2 and catalytic domains. These interactions can be replaced by intermolecular upon kinase involvement in interactions with other proteins. SH2 and SH3 domains do not impede substrate access to the active site or change orientation of the kinase lobes. The regulatory role of SH2 and SH3 domains to inhibit kinase activity is linked to the displacement of helix αC and influence of flexibility of the kinase lobes. In the inactive state, αC is displaced and essential catalytic residues are removed from the active site. Phosphorylation of the activation segment results in active state in which catalytic residues are positioned in conformation required for the catalysis.

9 Adaptor Proteins

Adaptor and scaffold proteins lack enzymatic activity. Their major role is to bring together numerous components of the signaling pathway. This role is critical for the function of signaling complexes. Disregulation of this processes results in many pathologies including cancer development. Multidomain CAS (SH3-containing) and NSP (SH2-containing) families that interact with each other, contain regulatory phosphorylation sites and are important for transmembrane receptors signaling including integrin receptor for extracellular matrix proteins, growth factor receptor tyrosine kinases, and cytokine receptors (Wallez et al. 2012). Adaptor proteins of the GAB family are essential for embryo survival (Wang et al. 2015). Gab1/SHP complex is associated with linking stimulation to proliferation and was recognized as a key participant of the liver regeneration process (Pagano et al. 2012). SH2B1 is an adaptor protein that contains SH2 and PH domains. It enhances insulin regulation of glucose metabolism. Impaired function of SH2B1 results in obesity and type II diabetes. SH2B1 regulates TAG biosynthesis, lipolysis, and VLDL secretion (Sheng et al. 2013).

Adaptor protein Grb2 (Seem-5 in *C. elegance,* DRK in *Drosophila*) (Mayer 2001) links RTKs to *ras* pathway. Grb2 SH2 binds to phophorylated tyrosines of EGFR, Erbb2, PDGFR, Shc, Insulin receptor substrate 1, and focal adhesion kinase. Grb2 SH3 binds to *ras*-guanine nucleotide exchange factor Sos1, mainly its C-terminal proline-rich regions, that leads to activation of Ras/MEK/MAPK (extracellular signal-regulated kinase, ERK) (Sastry et al. 1997; Qu et al. 2014a). Grb2 SH2/SH3 roles were established as mediator of actin polymerization (Bisson et al. 2012), proangiogenic events (Soriano et al. 2004) and coupling of PAK1 to

activated Grb2 (Puto et al. 2003). Grb2 carries out another important function—negative regulation of cell signaling through receptor degradation that involves ubiquitin ligases and inhibitory molecules. The assembly of kinases, such as Cbl, and inhibitory molecules, such as Sprouty, into multiprogein complex lines out the following events: Grb2-SH2 binds RTK phosphotyrosine, Grb2-SH3 binds RTK proline rich region, and Cbl binds inhibitory molecules, such as SHIP. Formed complex binds Ubc E2 enzymes initiating RTK degradation (Reebye et al. 2012). Some SH2 domains bind pY-containing peptides in extended conformation; other, such as GRB2 and GRB7, in β-turn conformation. The SH2 domain of Grb7 specifically binds phosphotyrosine 1139 of the ERB2 receptor; the SH2 domain of GRB14 binds phosphotyrosine 766 of the FGFR. The SH2–GRB10 binds many different proteins: insulin, IGF1 and PDGFb receptors, RET, KIT, Raf1, NED4, and MEK1. Peptides inhibitors that bind to pY binding site were developed and used as combinations treatments against cancer. Phosphotyrosine as part of a drug peptide has drawbacks such as strong charge of the phosphogroup that impairs its ability to cross cellular membrane (Spuches et al. 2007). Some peptides are designed to bind pY + 1 position and exhibit high affinity toward Grb2 (Kang et al. 2007). GRB2 SH2 phosphotyrosine binding site is also used to inhibit GRB2 action by pharmacological agents, such as bicyclic peptide BC1 and cyclic peptide HT1, mimicking phosphorylation. Constrained peptides despite net negative charge have better penetration, metabolic stability, affinity and selectivity (Quartararo et al. 2014).

Grb2 C-terminal SH3 (CSH3) domain binds the N-terminal SH3 domain of VaV, a nucleotide exchange factor for the Rho/Rac family of proteins expressed in hematopoietic cells. VaV SH3 binding site for proline sequence is blocked by its own RT loop and has a regulatory role. VaV forms multiprotein complexes with involvement of Grb2 and PI3 k, Slp76, and SBC in T cells, Slp65 in B cells, and Rac1, and mitogen activated kinase in mast cells (Nishida and Hirano 2003). N-terminal and C-terminal SH3 GRB2 domains possess different specificity toward their ligands (Paster et al. 2013). Grb7-10-14 proteins exhibit common architecture: N-terminus polyproline region binding SH3 domains followed by RA, PH, and SH2 domains (Holt and Siddle 2005). RA-PH domains bind small GTPases and phosphoinositide lipids (Qamra and Hubbard 2013).

Linker for activation of T-cells (LAT), integral membrane adaptor protein upon TCR stimulation and phosphorylation on several tyrosines binds to SH2 of PLCγ, Grb2, or Gads. Association of Grb2 with SOS and GADS with SLP76 brings Sos, Cbl, SLP76 and LAT together (Houtman et al. 2004).

Adaptor proteins regulation of RTKs that involves ubiquitination is important for downregulation of their activity. Many SH3 domains are identified as ubiquitin-binding domains that can direct proteins to degradation/recycling pathways. C-terminal (third) domain, one of three SH3 domains, of both adaptor proteins CIN85 and CD2AP, exhibit various modes of interactions with ubiquitin. Mechanism of EGF-dependent CD2AP/CIN85 monoubiquination allows selective recognition by ubiquitin of various molecules (Roldan et al. 2013).

CRM-like (CrkL), Nck (Ngoenkam et al. 2014; Li et al. 2014), Stap -2 (Sekine 2014), Gab (Wang et al. 2015), Kindlin (Qu et al. 2014b), APS (Xu et al. 2003) and many other adaptor molecules are regulated by phosphorylation and influence cellular pathways by extensive network of effector molecules.

10 Multiprotein Complexes and Signaling Pathways

Multiprotein complexes that are formed with receptors and adapter proteins are important for activation of signaling pathways. Cell surface receptor signaling, initiated with activation of the receptor in the cellular membrane, and intracellular receptor signaling that involves molecules in the cytoplasm, the nuclear membrane, and the nucleus, are two major routes of signal transduction. More and more data confirm that intracellular adaptor proteins play important role to relay growth factor signals for subsequent transcriptional and translational regulation and cell fate determination. They are able to interact simultaneously with several other molecules bringing them together in a multiprotein complex and amplify signaling cascades (Kebache et al. 2002; Semela and Dufour 2004; Huizing et al. 2008; Reebye et al. 2012; Au et al. 2013; Di Fiore et al. 2002; Shelby et al. 2013).

Fyn-mediated signaling pathway in bone marrow-derived mast cells induced in response to allergic inflammation involves binding of Fyn/SH2 to vimentin, pyruvate kinase, p62 ras-GAP associated phosphoprotein, SLP-76, HS-1, and FYB (Nahm et al. 2003). Fyn SH3 interacts with Liver kinase β1 (LKB1) proline rich domain and this interaction affects LKB1 subcellular localization and ability of LKB1 regulate phosphorylation of AMPK, its downstream signaling molecule. Fyn/peptide interaction shows to reproduce features of kinase inhibition and AMPK activation (Yamada and Bastie 2014). Neutrophils, responsible for host defense against microorganisms, are mediated by various cell surface receptors, such as G-protein-coupled receptors, Fc receptors, cytokine receptors, lectins, NOD-like receptors, and many others. Signal transduction pathways activated by the receptors involve Src-family kinase signaling. G-protein-coupled receptors signaling, for instance, occurs via several pathways: PLCβ2/3, PI3 Kγ, and Src-family kinase (Futosi et al. 2013). B cell receptor (BCR) pathway regulation involves interaction between the phosphorylated ITIM of the IgG receptor FcγRIIB and SH2 of inositol 5’-phosphatase (SHIP), proline rich motifs binding to SH3 domains of Grb2 and PLCγ, and NPXY motif of SHIP interaction with phosphotyrosine base domain of Shc (Tridandapani et al. 1999; Leung et al. 2008).

Signaling complexes assemble via specific interactions of proteins with multiple ligands. Membrane associated guanylate kinases (MAGUK) cluster and anchor glutamate receptors and other proteins at synapses. The MAGUK family of proteins includes PSD95, PSD93, SAP102, SAP97, essential components of postsynaptic density, PSD. Stabilization of SAP102 at the PSD depends on SH3/GK domain. Actin, a core skeletal component in spines, interacts with multiple proteins of PSD (Zheng et al. 2010). MAGUKs indirectly bind to actin via complexes

SAP97—MyosinVI—actin, PSD-95—SPAR—actin, PSD-95——GKAP—Shank—cortactin—actin, and PSD-95—NMDAR—actinin—actin (Petralia et al. 2008).

SH2 and SH3 domains are important components of assembly of signaling complexes. These and other protein interactions domains, such as helical toroids and beta-helices, are highly selective toward their ligands. Protein-protein and protein-ligand interactions to a large extent depend on specific interactions of amino acids at secondary structure interfaces that determine parameters characterizing angles, distances, chirality and shape of the assemblies (Kurochkina 2008; Kurochkina and Iadarola 2015).

11 Modes of Regulation

SH2 and SH3 were shown to exhibit various ways of regulation of the kinase activity. In ARMS/Kidins220, for instance, mutually exclusive events are observed: binding of polyproline sequence to CrkL/SH3 domain or binding of the adjacent phosphorylated tyrosine sequence to CrkL/SH2 domain (Arevalo et al. 2006; Akiva et al. 2012). Similarly, coupling of phosphorylation and binding with alternation of binding events happens in CD3e-Eps8L1/SH3-Zap70/SH2, growth hormone receptor-Nck1/SH3-STATS-SH2, and Cbl-Src/SH3-Fyn/SH2 complexes. Some alternatively binding pairs involve PDZ/PDZ and WW/SH2 and there exist many potential double switches in Grb2, Ptp2, Stat3/5, Crk and other proteins.

SH2/SH3 regulation of the kinase activity also involves an interdomain linker regions SH2/SH3 and SH2-kinase. In inactive (or closed) conformation of the kinase demonstrated by crystallographic structures of Src and Hck, SH2 domain binds to the phosphotyrosine pY527 in the C-terminal tale of the kinase and SH3 domain binds to the N-terminal lobe via polyproline sequence of the SH2/kinase linker leading to movement of the regulatory helix C. Dephosphoryation of Y527 or binding of ligand, such as viral NEF/SH3, activates the kinase. Intramolecular interaction can be replaced by intermolecular interaction as is observed upon NEF, HIV accessory protein, binding to Hck SH3 via polyproline region and displacing Hck PxxP site (Jung et al. 2011). Another example is provided by the focal adhesion kinase (FAK) activation and deactivation via SH2/SH3 interplay without requirement for phosphorylation of corresponding Y416. Fyn, Abl, Grb2, and Lck contain tandem SH2/SH3 domains. SH2/SH3 domains of Fyn retain conformation reminiscent of that in complex with inactive kinase even in the absence of the kinase domain. This conformation (3_{10} helix) is stabilized by SH2/SH3 polyproline sequence and represents an independent fragment (Arold et al. 2001).

Regulation by tyrosine phosphorylation is not unique to animals. For example, genome of unicellular protist *Monosiga brevicollis* contains tyrosine phosphatase (PTP) with two SH2 domains just like in animals (Zhao and Zhao 2014).

12 Diseases and Therapies

Field of drug development has tremendous advancements as more and more new inhibitors and mediators of catalytic activity of PTKs and protein-protein interactions become available for treatments. Many more emerging therapies come from new area of stem cell—based therapies. PTKs, protein tyrosine phosphatases, and SH2 domains comprise a module that coordinates intracellular phosphotyrosine-based signals as a response to extracellular ligands. Intracellular pathways regulated by tyrosine phosphorylation play a critical role in biological processes that maintain and restore cell life functions. One example is the process of liver regeneration. Involvement of not only reserve progenitor cell population but to a large extent mature hepatocytes which are stimulated to re-enter the cell cycle and to replicate as a result of complex regulation of metabolic pathways is remarkable demonstration of new applications (Pagano et al. 2012).

Stem cell factor receptor, c-Kit, which is mainly expressed in early hematopoetic stem cells and detected after differentiation only in mast cells and dendritic cells, represents attractive source of new drug development. Its mutant forms are associated with small cell lung carcinoma, malignant melanomas, colorectal cancer, and gastrointestinal stromal tumors. Imatinib, tyrosine kinase inhibitor of BCR/Abl and platelet-derived growth factor, is also effective against c-Kit in GIST treatment. Since c-Kit is involved in asthma and allergy progression, its inhibitor masitinib is used in treatment of asthmatics (Lennartsson and Rönnstrand 2012).

Antibodies against RTKs are good for treatments that handle overexpressed receptors. Both selective inhibitors that has fewer side effects and broad selectivity inhibitors that target multiple pathways were designed that act in tyrosine kinase pathways (Lennartsson and Rönnstrand 2012). Significant progress was made in applying tyrosine kinase inhibitors in treatment of cancers including non-small-cell lung cancer (Nguyen and Neal 2012).

Ephrin-mediated Eph receptors signaling undergoes modifications in epithelial cancers and forms another important group of pharmacological targets, particularly regarding nonmelanoma skin cancer and psoriasis. Abnormal EphB receptors and Ephrin B function is linked to malignancies in the gut. EphA receptors are known as tumor suppressors in the skin (White and Getsios 2015).

SH2 regulated pathways play an important role in most cancers including breast and lung cancer. Targeting SH2 domains presents problems for some groups of compounds since phosphotyrosine negative charge is an obstacle that prevents molecules from crossing the membrane and being delivered. Nevertheless, peptidomimetic substances, hydroxysalycilates, overcome this problem and inhibit STAT3 SH2. GRB2 peptidomimetic inhibitors based on phosphanates are less charged, are able to cross cell membrane and can be used for treatments. GRB7 cyclic peptides represent another successful alternative. STAT3 pathway inhibitors, STA-21 and cryptotanshinone, act not directly on SH2 domain of STAT3 but via off-target sites (Brábek et al. 2005; Morlacchi et al. 2014).

Receptor Patched 1 contains polyproline sequences at its C-terminus that bind SH3 or WW domains. This part of the molecule is important for its interaction with c-src and signaling via Hh pathway critical for mammary gland development in verterbrates: the results produced by studies of *mesenchymal dysplasia* gene variant (Harvey et al. 2014).

Chonic myeloic leukemia is associated with BCR-ABL tyrosine kinase activity that affects Ras, PI3 K, Jak/Stat, and NFkB pathways stimulating proliferation and inhibiting apoptosis. Existing therapies using tyrosine kinase inhibitors imatinib, dasatinib, and nilotinib face resistance due to mutations in BCR-ABL gene and stem/progenitor cells unresponsiveness. Drug resistance problem can be addressed by introducing other candidate molecules such as adaptor protein Abi1 that contains SH3 and WW40 domains which can be targeted for the disruption of AH1 interaction with BCR-ABL and JAK2 (Liu et al. 2012).

Stem cell based therapies find more and more applications in the development of new treatments. Human umbilical cord perivascular cells (HUCPV), for example, are used to produce morphologically homogenious population of fibroblastic cells that expresses α-actin, desmin, vimentin, and 3G5 for cardiovascular tissue engineering. Subpopulations of these cells do not express class I/II major histocompatibility antigens and can be valuable source of compatible tissues (Sarugaser et al. 2005). Hematopoietic stem cell transcription regulation and understanding changes associated with aging or disease provide basis for possible new treatments (Sive and Göttgens 2014; Babovic and Eaves 2014).

Stem cell therapies have wide range of promising applications to treatments of neuronal injuries and orthopedics medicine (Law and Chaudhuri 2013). New cell lines are explored for their potential in promoting adult neurogenesis, particularly transcriptional events that accompany signaling by growth factors and cytokines leading to cell proliferation and migration (Williams et al. 2013).

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SH2 Domain Structures and Interactions

Piraveen Gopalasingam, Lee Quill, Mark Jeeves and Michael Overduin

Abstract Src homology 2 (SH2) domains are signalling modules consisting of 100 amino acid residues which typically recognize phosphotyrosine-containing protein sequences. As such, they mediate protein–protein interactions in order to assemble signalling complexes and transduce information to alter cellular behaviour and physiology. Here we review the progress in understanding the molecular functions and structures of SH2 domains since their discovery in 1986. Diverse binding modes are revealed by studies of a variety of ligand complexes, suggesting that their mechanisms have adapted throughout the evolution in tandem with tyrosine kinases and phosphatases. Together they form the information networks responsible for development of the cellular phosphoproteome and the ensuing multicellular complexity found in higher eukaryotes. Understanding the structures, dynamics and interactions of these remarkable domains at a more predictive level is providing a rational basis for designing targeted interventions into the many disorders that result from deregulated phosphotyrosine signalling.

Keywords Phosphopeptide recognition · Phosphotyrosine binding · Protein phosphorylation · Src homology 2 domain · SH2 structure

Abbreviations

Abl	Abelson tyrosine-protein kinase 1;
APS	adaptor protein with PH and SH2 domains;
Cbl	Casitas B-lineage;
cSH2	C-terminal SH2 domain;
CSK	C-terminal Src kinase;
CTD	C-terminal domain;
ERK	extracellular signal-regulated kinase;
FERM	protein 4.1, ezrin, radixin, moesin;
GEF	guanine nucleotide exchange factor;
Grb	growth factor receptor bound protein;

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Hck	haemopoietic cell kinase;
ITAM	immunoreceptor tyrosine-based activation motif;
Lck	lymphocyte-specific protein tyrosine kinase;
MODA	membrane optimal docking area;
NEDD	neural precursor cell expressed developmentally down-regulated protein;
NMR	nuclear magnetic resonance;
nSH2	N-terminal SH2 domain;
PDGF	platelet-derived growth factor;
PH	pleckstrin homology;
PI3K	phosphoinositide 3-kinase;
PIP2	phosphatidylinositol-4,5-bsphosphate;
PIP3	phosphatidylinositol-3,4,5-trisphosphate;
PLC	phospholipase; pSer, phosphoserine;
PTK	protein tyrosine kinase;
PTP	protein tyrosine phosphatase;
pTyr	phosphotyrosine;
SAP	SLAM-associated protein;
SH2	Src homology 2;
SH3	Src homology 3;
Shp2	Src homology 2-containing phosphotyrosine phosphatase;
SLAP	Src-like adaptor protein;
SOCS	suppressor of cytokine signalling;
STAT	Signal Transducer and Activator of Transcription;
Syk	spleen tyrosine kinase;
Zap70	zeta chain-associated protein kinase of 70 kDa

1 Introduction to SH2 Domains

1.1 Biological Functions of SH2 Domains

Tyrosine phosphorylation contributes to only approximately 0.5 % of the total phosphoproteome, yet it plays critical roles in eukaryotic cell regulation (Cohen 2002). The enzymes which control tyrosine phosphorylation require careful spatiotemporal regulation in order to potentiate cellular responses to specific extracellular stimuli. The most crucial regulatory module is the *Src* Homology 2 (SH2) domain, which was originally identified in viral oncogenes. Tony Pawson's group first discovered the SH2 domain as a non-catalytic region of the *v-fps/fes* gene which is required for tumour transformation (Sadowski et al. 1986). Subsequent sequence alignments mapped SH2-like regions within a diverse array of intracellular signalling proteins found in eukaryotes. Since then, the importance of this module in signalling has been recognised as being central for regulating cell

growth, differentiation and migration, as well as mediating complex signalling pathways such as in the immune systems (Liu and Nash 2012). Tyrosine phosphorylation is cell-type specific as well as time dependent, and hence has provided a foundation for the development of diverse tissues, organs and organisms. These processes are fundamentally based on the differential expression and regulated activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) which often contain SH2 domains (Yaffe 2002).

In order to organize responses to diverse signals, elaborate networks of divergent SH2 domain-containing proteins have evolved that accurately detect and transform molecular events into appropriate cellular responses. SH2 proteins are widely considered to be the major readers of the tyrosine phosphoproteome and form the cell's primary means of phosphotyrosine (pTyr) recognition. The roles of SH2 domains within tyrosine phosphorylation-dependent pathways generally involve localizing the PTK and PTP enzymes to specific substrates and signalling effectors, as shown for the well-characterized Ras-MAPK, JAK-STAT and MEK/ERK pathways. The SH2 domain-containing proteins within these signalling cascades display modular architectures, with various other structural domains, such as pleckstrin homology (PH), PTB, PDZ, SH3 and WW domains, mediating interactions with proteins and membrane surfaces, as well as catalytic domains such as GTPases, inositol phosphatases and phospholipases. There are also SH2 proteins that do not possess enzymatic activity and instead act purely as adaptor proteins that link other proteins together. Prominent examples of such signalling adaptors include the Gab1, Grb2, SOS, SOCS and VAV1 proteins. Finally some SH2 proteins possess enzymatic functions but can also serve separately as adaptors, with distinct consequences in downstream signalling if either of these functions is disrupted. One such example is the PTP Shp2, where Noonan and LEOPARD syndromes arise from mutations that compromise its adaptor SH2 and catalytic domains (Gelb and Tartaglia 2006).

The primary biochemical function of the SH2 domain is to selectively recognize polypeptides containing a phosphotyrosine (pTyr) along with specific contiguous residues. As a small, stable structural unit (Fig. 1) that is generally flexibly tethered to a larger protein, the SH2 domain is able to diffuse through local intracellular spaces until a protein ligand is bound. The reversible interaction between SH2 domains and pTyr motifs thus provides regulated co-localization of multiple proteins within a signalling cascade, allowing further enzymatic modifications and propagation of signalling cascades. There are thousands of pTyr sites in the human proteome (Sharma et al. 2014) which mediate interactions with cognate binding partners in hundreds of interconnected pathways that each mount appropriate responses to changes in diverse cellular microenvironments (Hunter 2009). It is the SH2 domain that provides order to this signalling complexity, and its functional integrity is of paramount importance to the cell.

Extracellular signals are conveyed into the cell through growth factor receptors which generate intracellular tyrosine phosphorylation sites. These processes have been extensively studied in the cases of the cell surface receptors for insulin and various growth factors such as epidermal growth factor (EGF) and platelet-derived

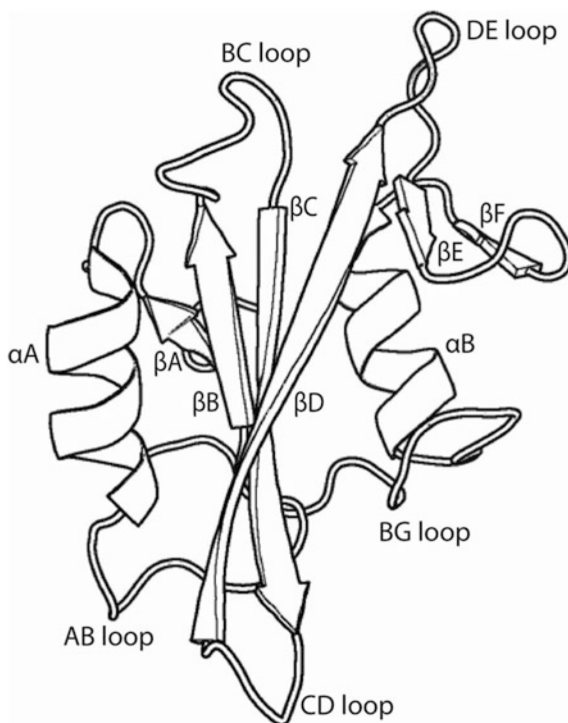

Fig. 1 Structure of a representative SH2 domain structure with the helices, strands and loops labelled

growth factor (PDGF). These transmembrane receptors multimerise and the activated cytoplasmic kinase domains phosphorylate tyrosine residues in the C-terminal tails of the other monomer. These pTyr sites serve as docking sites for various SH2 domains that act to potentiate the initial signal, with other post-translational modifications including serine and threonine phosphorylation, ubiquitination as well as GTP hydrolysis also coming into play (Deribe et al. 2010).

Some SH2 domains are known to directly regulate enzyme activity. In the PTKs Src, Fyn, Lyn and Yes the SH2 domain binds a phosphopeptide sequence near the C-terminus, thus occluding the kinase active site and autoinhibiting catalytic activity. In contrast, the Abl kinase can either be autoinhibited or activated by its SH2 domain, depending on its conformational state (Hantschel et al. 2003; Nagar et al. 2006; Filippakopoulos et al. 2009). The PTPs Shp1 and Shp2 contain two SH2 domains N-terminal to the catalytic domain, the first of these forms extensive contacts close to and occluding the PTP active site, thus stabilizing an inactive conformation (Hof et al. 1998). The PTP is activated through stable interaction of its tandem SH2 domains with pairs of pTyr motifs in cell surface receptors. Thus SH2 domains not only serve to connect the various components of signalling pathways in *trans* interactions but act in *cis* to modulate enzymatic function.

Depending on the signalling context of the SH2 domain-containing protein, a pTyr interaction may require specific residence times. Growth and differentiation

pathways require tight control over signal generation as even minor changes could lead to detrimental consequences. Residence times can be modulated by varying the affinity and specificity of a binary peptide:SH2 interaction, serving to fine-tune transient complexes in a cascade. Although SH2 domains are singularly important for the fidelity of intracellular signal transduction, other interaction domains within their host proteins and their homologs and complexes can compensate for disruption or loss. Such redundancy in a pathway is commonly observed in complex multicellular organisms and confers slack in the system (Kaneko et al. 2012b).

Although the fold of SH2 domains is highly structurally conserved, divergent features can endow them with specialized functions. For example, the adaptor protein Crk contains an atypical SH2 domain with a proline-rich loop (Fig. 2) that is recognized by the SH3 domain of Abl (Donaldson et al. 2002) in a phosphorylation-dependent manner (Anafi et al. 1996). The SH2 domain of the adaptor protein SOCS3 contains an unstructured 35 residue PEST motif sequence which is enriched in proline, glutamic acid, serine and threonine residues (Babon et al. 2006).

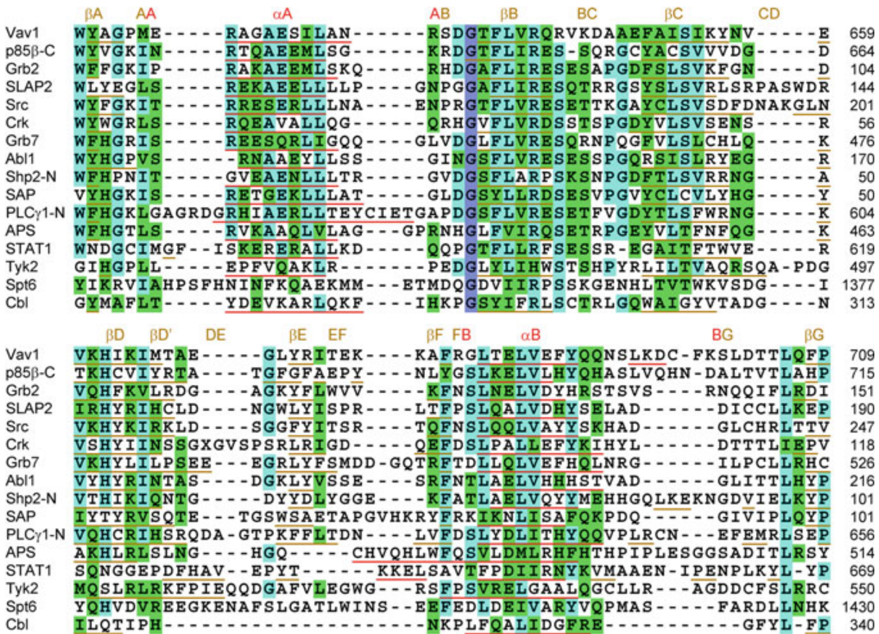

Fig. 2 Alignment of representative SH2 domain sequences showing secondary structure elements. Alignment of human SH2 domain sequences with secondary structures from Vav1 (2mc1), PI3K p85 β subunit (2y3a), Grb2 (3wa4), SLAP2 (4m4z), Src (1fmk), Crk (1ju5), Grb7 (2qms), Abl1 (1opk), Shp2 (3tkz), SAP (1d1z), PLCg1 (4fbn), APS (1rpy), STAT1 (1yvl), Tyk2 (4po6), Spt6, Cbl (3pfv), with "N" and "C" indicating the N or C terminal domain of a tandem SH2 proteins. Aligned with ClustalW using the BLOSUM weightings. Helices and strands are coloured *red* and *gold*, respectively, and labelled along with the intervening loops. Fully conserved, identical, and similar residues are highlighted in *blue*, *aqua* and *green*, respectively. A proline rich sequence in the Crk DE loop is indicated by an "X"

This PEST sequence undergoes phosphorylation as a means of modulating the half-life of the SOCS protein, thus providing a regulatory mechanism within cytokine/JAK-STAT signalling that utilises the ubiquitin-mediated degradation pathway (Yoshimura et al. 2007). Thus SH2 domains can both bind and be bound by partner proteins, and also expose unique surface features for regulating protein activity and degradation. As such, SH2 domains are multifaceted contributors to pTyr signalling systems and thus have been described as highly regulated readers of the pTyr signals that are produced and deleted by PTK and PTP enzymes respectively (Lim and Pawson 2010).

1.2 Evolution and Breadth of the SH2 Superfamily

Tyrosine phosphorylation first evolved as an intracellular signal in early metazoans (Liu and Nash 2012). The number and complexity of pTyr-based systems diversified with increasing organismal complexity (Liu et al. 2011). The expansion of the number of genes encoding SH2 domains during this period paralleled the evolution of divergent PTKs. The transcriptional regulator Spt6, which contains two SH2 domains—one canonical and the other uniquely folded and phosphoserine-binding, could be the progenitor of the superfamily, although another contender is the transcription activating STAT protein (Liu and Nash 2012).

Genome sequencing of the unicellular choanoflagellates has identified a large number of pTyr signalling proteins (Manning et al. 2008). Cross-species genome comparisons suggest that pTyr signalling networks emerged in these organisms, which are representative of the ancestral animal evolution. Gene duplication led to rapid growth of the number of SH2 domain-containing proteins, with domain gain and loss and mutational events leading to the development of novel functions and tissue-specific regulation whilst maintaining the basic conserved signalling network (Liu and Nash 2012).

To date 111 genes, encoding 121 SH2 domains, have been identified in humans (Liu et al. 2012). Those proteins containing pairs of SH2 domains include SYK, Zap70, Shp1, Shp2, the phospholipase C homologs PLCγ1 and PLCγ2, as well as the p85α and p85β regulatory subunits of phosphoinositide-3 kinase (PI3 K, Fig. 2). The presence of two SH2 domains in a protein confers additional affinity and specificity by imposing spatial constraints to a binary interaction (Eck et al. 1996), while single SH2 domains generally provide transient interactions with specific ligands.

1.3 Biomedical Significance of SH2 Proteins

At least 23 SH2 domain-containing proteins have been implicated in disease causation to date. This represents around 1 in every 5 members of the human SH2 superfamily, and effects chronic conditions such as diabetes, Noonan and

LEOPARD syndromes and immune system disorders. This has stimulated numerous efforts to design SH2-specific inhibitors, as reviewed recently (Kraskouskaya et al. 2013), although the most successful route to intervention to date has been from the design of competitive inhibitors of the kinase domains of these proteins.

Mutations within SH2 domains underpin the molecular pathogenesis of various diseases, thus understanding their mechanism of dysregulation is vital for designing clinical interventions. Functional effects of SH2 mutations range from enzyme hyperactivation to changes in pTyr:SH2 affinity and specificity. For the latter, a spectrum of deleterious mutations have been documented wherein pTyr:SH2 binding is completely abolished and in other cases altering either the residence times of phosphopeptide in the SH2 pTyr-pocket or their ligand specificity. Indeed it has been demonstrated that a single point mutation in the Src SH2 domain alters its peptide specificity to resemble the Grb2 SH2 domain (Kimber et al. 2000).

Phosphotyrosine-based signalling is widely utilised in growth factor-mediated cascades so it is unsurprising that mutations affecting these pathways are implicated in cancer initiation, progression and metastasis. A subset of these mutations lie within SH2 domains. The Shp2 protein was the first oncogenic PTP discovered, with germline mutations in its SH2 domains increasing the likelihood of hematological malignancies including juvenile myelomonocytic leukaemia. Germline and somatic mutations of Shp2 have also been implicated in cases of colon, skin, thyroid and brain cancer (Chan et al. 2008). Similarly, mutations in the C-terminal SH2 domain of the RASA1 protein which regulates the GTPase activity of Ras are found in Basal-cell carcinoma (Friedman 1995). A mutation in the first SH2 domain of PI3K's regulatory p85 subunit compromises the lipid kinase activity and may contribute to insulin resistance (Baynes et al. 2000), while a deletion near the second SH2 domain contributes to the development of solid tumours (Philp et al. 2001).

Several immune system disorders are caused by SH2 domain alterations. The X-linked Lymphoproliferative (XLP) syndrome is brought about by mutations in the *SH2D1A* gene encoding SLAM-associated Protein (SAP) and affects males only. Missense mutations localise to the SLAM-peptide binding site, suggesting abrogation of peptide binding to SAP drives pathogenesis of XLP by dysregulating pathways involved in adaptive immune system activation (Poy et al. 1999). Mutations in the Bruton's tyrosine kinase (BTK) gene including in its SH2 domain reduce the number of mature B-cells and thus cause a hereditary immunodeficiency known as X-linked agammaglobulinemia (Valiaho et al. 2006). Mutations in the Zap70 gene including in its SH2 domain can destabilise the protein, thus reducing T cell numbers and leading to a severe immunodeficiency (Cauwe et al. 2014).

Increased vulnerability to infection can also be caused by mutated SH2 domains. Dominant negative mutations in the SH2 domain of STAT1 alter the protein's phosphorylation and DNA-binding activity. This impairs the interferon and interleukin responsiveness of the cell and leads to susceptibility to mycobacterial infection (Tsumura et al. 2012). A mutation of the SH2 domain of STAT5B compromises cellular sensitivity to growth hormone, with the resulting immunodeficiency leading to growth failure, recurrent bacterial and viral infections (Kofoed et al. 2003).

2 SH2 Domain Structure and Function

2.1 SH2 Domain Fold

The fold of the SH2 domain is optimized for reading the pTyr signals which are dynamically created in higher eukaryotes and tuning the ensuing cellular responses. The three dimensional structures of ∼70 SH2 domains have been determined. They reveal a highly conserved topology comprising a β sheet consisting of seven anti-parallel strands (βA-βG) that are flanked at either end by a conserved pair of helices (αA and αB) (Waksman et al. 1992, 1993). The common nomenclature specifies the positions of residues relative to these secondary structure elements (Fig. 1), with βB5 being the fifth residue in the conserved second strand. Comparison of the SH2 domain structures in both free and phosphopeptide-bound states has collectively identified the architecturally conserved N-terminal region as the mediator of pTyr binding. This is in contrast to the C-terminal segment of the SH2 domain which exhibits much broader structural variability, owing to its specificity for hydrophobic residues that are found in the various peptide ligands and protein partners.

2.2 Phosphotyrosine Recognition by SH2 Domains

The elucidation of the key structural features governing SH2 recognition of pTyr containing sequences has not only provided key information on how pTyr residues are read, but also offers potential insights into the use of SH2 domains as diagnostic tools and as druggable targets for the design of therapeutic agents for an array of human diseases (Sawyer 1998).

Resolving the structural basis of pTyr recognition has been a critical step to informing our understanding of SH2 function. Accessing such information has relied on the utility of X-ray crystallography to illuminate the binding interfaces between signalling complexes at high resolution. Crystal structures for the majority of SH2-ligand complexes reveal that the pTyr-peptide adopts an extended conformation and binds perpendicular to the central β-strands of the SH2 domain. In almost all cases, the pTyr substrate is accommodated in a moderately deep binding pocket, with the phosphate oxygens participating in an extensive network of hydrogen bonds and electrostatic interactions that act to stabilise the complex. Conserved arginine residues Arg αA2 and Arg βB5 have been identified as key residues and mediators of phosphate group binding. The phenyl moiety of pTyr stabilises the interaction through the formation of an amino-aromatic bond to a conserved Arg within the FLVR motif (Fig. 2) in conjunction with additional interactions involving side chain and main chain groups from residues in the βD strand (Waksman et al. 1992; Waksman et al. 1993; Waksman and Kuriyan 2004). The second binding pocket within the SH2 domain provides hydrophobic

interactions for engaging residues positioned C-terminal to the pTyr and confers specificity for ligand interactions (Liu et al. 2012).

A central paradigm in SH2-dependent signalling asserts that the lifetime of global tyrosine phosphorylation is precisely controlled by the lifetime of the individual SH2-containing complexes that make up the signalling network. These signalling complexes function collectively as part of these networks to drive the propagation of phosphorylation cascades leading to the activation of a particular cellular response. The rate of decay of SH2-dependent interactions in any given network is therefore crucial to the maintenance of the signal and the downstream activation of diverse cellular processes. Several studies have reported the dissociation constant for the binding interaction of SH2 domains with pTyr ligands to have affinities in the range of 0.1–10 μM (Ladbury and Arold 2011; Jones et al. 2006). Despite the high specificity of these canonical interactions the moderate affinities reported are crucial to the transient association and dissociation rates necessary for complex formation, and for the subsequent control of cellular signalling cascades. SH2 domains artificially engineered to possess stronger binding (known as pTyr superbinders) of pTyr-containing sequences have detrimental consequences for signal transduction, illustrating the importance of maintaining the critical balance between binding affinities of signalling and adaptor proteins and the propagation of signals (Kaneko et al. 2012a).

The highly conserved pocket responsible for recognition of pTyr has been estimated to contribute approximately half of the energy associated with ligand binding (Waksman et al. 2004), while the comparatively variable hydrophobic pocket found in the C-terminal region of the SH2 domain provides essential specificity for hydrophobic residues on cognate ligands. The large energetic contribution of the pTyr residue to SH2-domain binding provides an efficient basis for discriminating between phosphorylated and non-phosphorylated sequences, and therefore provides a mechanism for the rapid termination of cell signalling events regulated by tyrosine phosphorylation. Additional structural features of SH2 domains also serve as crucial determinants for pTyr recognition, for example, distinct conformational states of the EF and BG loops are important for restricting ligand access and influence the specificity of SH2 domains for the residues occupying the second, third or fourth position that lies C-terminal to the pTyr residue (Liu et al. 2006, 2011).

2.3 Selectivity for Primary Sequence Motifs

The first SH2 structure solved in the presence of a pTyr ligand was that of the Src SH2 domain complexed with a high affinity ligand peptide derived from the middle T-antigen. The structure revealed the precise architecture of the pTyr binding pocket and illuminated both the hydrophobic pocket coordinating the +3 Ile (Waksman et al. 1993) and the canonical pY-E-E-I peptide recognition sequence (Fig. 3a). The SH2 domain structures that appeared subsequently exhibited highly conserved pTyr binding pockets, and showed notable diversity in their binding

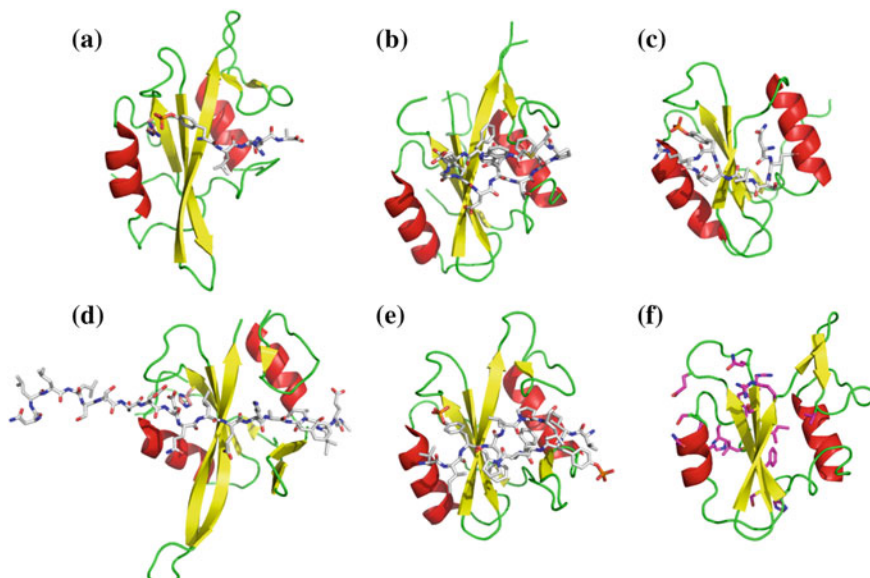

Fig. 3 Diversity of SH2 domain binding mechanisms. SH2 domains are shown as backbone ribbons and the bound peptide ligands are displayed using stick representations. **a** The classical orientation of the Src SH2 domain binding pY peptide is shown, with the key arginine residue which coordinates the phosphate of the pTyr shown in stick representation. **b** The Grb7 SH2 domain bound to a cyclical non-phosphorylated tyrosine peptide. **c** The Cbl SH2 domain bound to the phosphorylated Met receptor tail peptide in a reverse orientation. **d** The Tyk SH2 domain bound to a peptide with a glutamate in the usual pY position. **e** The Shp2 N-terminal SH2 domain complexed with two pTyr-containing peptides. **f** The Abl SH2 domain with residues that undergo chemical shift changes upon binding of PtdIns(4,5)P_2 shown in stick representation

modes for neighbouring ligand residues. For example, the Grb2 SH2 domain has been identified to have a strong propensity for a +2 Asn (pY-x-N-x motif) as a result of a Trp residue in the DE loop of the SH2 domain. Other SH2 structures have revealed key interactions extending beyond the +3 position. A combination of peptide array and structural data elucidated for the BRDG1 SH2 domain demonstrated a strong preference for a hydrophobic Leu residue at the +4 position. Additionally, SH2 domains belonging to PLC-γ1 have been shown to preferentially recognise longer more extended peptides with contacts forming up to the +5 position (Kaneko et al. 2010; Huang et al. 2008).

2.4 Specificity for Ligand Secondary Structures

Conformational Adaptability: Ligands for SH2 domains are typically found in unstructured sequences of cytoplasmic proteins. For example, the PLC-γ1 C-terminal SH2 domain binds disordered phosphopeptides derived from the PDGF

receptor. On contact, the mobility around the pTyr and +3 residues becomes restricted (Finerty et al. 2005). The bound peptide ligands typically assume an extended conformation that stretches between the pTyr and +3 pockets and lies across strand βD (Waksman et al. 1993). In the case of the Src SH2 complex (mentioned in Sect. 2.3), the four residues that are predominantly recognized are the pTyr and lle(+3) residues, which are buried, and the intervening Glu(+l) and Glu (+2) residues, which are largely solvent exposed and form complementary electrostatic interactions with positively charged residues on the SH2 surface. The binding conformations of such phosphopeptide ligands can be either extended or helical depending on the presence of an Ile or Leu at the +3 position, due to their respective intrinsic structural propensities (Nachman et al. 2010). In contrast, an alternative β-turn conformation is induced in Src ligands having an Asn at the +2 position, which can supplant the hydrophobic residue at the +3 position as a key determinant of SH2 binding specificity. Hence the Src SH2 domain can accommodate bound phosphopeptides having either extended, helical or β-turn conformations depending on their sequence context, indicating the plasticity of ligand recognition. Moreover, these various binding modes may be differentially regulated by phosphorylation, for example of Src residue Tyr213 within the EF loop of its SH2 domain by the PDGF receptor (Stover et al. 1996).

Ligands Forming Turns: In contrast to most SH2 ligands, a β-turn is the default binding conformation of peptides bound by the growth factor receptor bound protein Grb2, which serves as an adaptor that links receptors at the plasma membrane with cytoplasmic kinases. Its SH2 domain binds peptides containing the pY-V-N-V sequence in a type 1 β turn conformation, providing a basis for the design of selective inhibitors (Rahuel et al. 1996). The Asn residue at the pTyr +2 position forms hydrogen bonds with the protein backbone and the peptide itself, as well as contacting the tryptophan which is the first residue of the loop between βE and βF. One of Grb2's partners is the CD28 receptor on the T-cell surface, which offers a pY-M-N-M sequence as a ligand. The binding mode is that of a twisted U-shaped turn that deviates from a canonical type-I β-turn due to alternative packing of the +1 and +2 residues (Higo et al. 2013). In contrast, amyloid precursor protein-derived peptides bind to the Grb2 SH2 domain but have a proline residue at the pTyr+3 position that precludes formation of a β-turn and rather supports flipping of helix-turn-helix conformation (Das et al. 2011).

Thus, rather than each SH2 domain having a single preferred binding mode, each interaction must balance both the intrinsic structural propensity of the ligand motif as well as specific contacts with the SH2 domain at each position, particularly between the pTyr and +3 position. This indicates the adaptability of SH2 domains that allows them to recognize diverse ligands depending on their local sequence and conformational fit, both of which play significant roles in generating productive signalling complexes. It can be inferred that such complexes are not evolved for maximum affinity of a single ligand, but rather to allow reversible and specific binding profiles for arrays of potential biological partners.

2.5 Noncanonical Ligands of SH2 Domains

Phosphoserine Specificity: Although pTyr-containing peptide motifs are clearly the dominant ligands of most SH2 domains, there are well-documented exceptions. In fact, the earliest SH2 domain may have been a phosphoserine peptide binding module. The only SH2 domains in yeast are found in the transcription factor Spt6 (Lim and Pawson 2010). This protein is conserved in plants and slime moulds, and is known to bind to the C-terminal domain (CTD) of RNA polymerase II (Yoh et al. 2007). The CTD contains phosphorylated YSPTSPS repeats that are recognized by the intertwined pair of SH2 domains of Spt6 (Sun et al. 2010; Diebold et al. 2010). Only the N-terminal SH2 domain of the tandem has a functional phosphopeptide binding pocket, which includes the invariant arginine found in eukaryotic SH2 domains. In contrast, the C-terminal SH2 domain lacks this canonical site and instead contacts the CTD through a positively charged area on its surface. Thus Spt6 SH2 domains may represent progenitors of the pTyr-binding SH2 domains that emerged in parallel with protein tyrosine kinases during early eukaryotic evolution.

Backwards Binding: The divergent SH2 domain of the Cbl ubiquitin ligase binds pTyr peptides with inverted specificity. The Cbl protein structure possesses three closely interacting N-terminal domains: a four-helix bundle, a calcium-binding EF-hand and a divergent SH2 domain (Fig. 3c). Its SH2 domain is structurally unusual in that the neighbouring four-helix bundle completes the pTyr pocket, and strands D', E and F are missing as is the G loop (Meng et al. 1999). The ligand specificity of Cbl SH2 domain is atypical in that it prefers ligand sequences having an Asn, Aspartate or Arg residue immediately preceding the pTyr. Moreover, through interactions with the four-helix bundle this selectivity extends six residues N-terminal to the pTyr in the case of the adaptor protein APS (Hu and Hubbard 2005). As a further twist, the Cbl SH2 domain accommodates the Met receptor's ligand sequence in the reverse orientation, such that the residues N-terminal to the pTyr extend across the β sheet to occupy the positions where the C-terminal specificity determinant usually reside (Ng et al. 2008). This feature provides c-Cbl with a unique cohort of partners that are unavailable to other SH2 proteins, and allows ligands to bind in potentially two opposite orientations.

Phosphopeptide Independent Binding: Over time some divergent SH2 domains have lost the ability to recognize pTyr ligands. Those of the Rin2 and Tyk2 proteins contain a histidine rather than the conserved arginine in the βB5 position which is typically essential for pTyr binding, while SH2D5 contains a tryptophan here. A recent Tyk2 structure reveals that its tandem FERM and SH2 domains form an extensive interface with each other and contact adjacent box motifs in the interferon-α receptor tail (Wallweber et al. 2014). This unusual SH2 domain employs what is normally the pTyr binding pocket to instead recognize a glutamate residue in the receptor tail, while a hydrophobic groove formed by the βG1 strand and EF loop contacts extended aliphatic residues between five and seven residues C-terminal to this pTyr mimicking residue (Fig. 3d). Thus pTyr

ligands can over time be replaced by a glutamate to support constitutive, non-phosphorylation dependent interactions.

Another unique case involves the SH2 domain of the SLAM-associated protein (SAP), which binds a SLAM receptor sequence even when it is not phosphorylated on the target tyrosine residue. The affinity of the unphosphorylated ligand is four to five-fold lower than the pTyr-containing form, and involves recognition of residues at the +2 and -3 positions, leading to a three-pronged fit (Morra et al. 2001; Poy et al. 1999; Sayos et al. 1998). The preference for residues N-terminal to the tyrosine results from a parallel β sheet interaction with strand D as well as hydrogen bonding contacts with a glutamate at position αA6. The unphosphorylated tyrosine residue occupies the pTyr pocket along with ordered water molecules instead of the normal phosphate group. Thus although a phosphate group is usually required on a tyrosine ligand, some SH2 domain interactions with established partners are sufficiently robust that this dependency is no longer absolute. A further wrinkle is offered by SAP signalling. Its SH2 domain contains a loop that is recognized by the Fyn SH3 domain, thus allowing assembly of a ternary complex with the SLAM receptor. The structure of this signalling complex reveals that strand F and helix B of the SAP SH2 domain bind the Fyn SH3 domain through a non-canonical interaction. This interaction would preclude the Fyn's auto-inhibited conformation, thus promoting kinase activation (Chan et al. 2003). The SH2 domain remains free to bind a peptide from the SLAM receptor in its standard 3-pronged mode, thus illustrating SAP's ability to act as a multifaceted adaptor protein.

Grb7 belongs to a subfamily of SH2-containing adapters that includes Grb2, and interacts with both phosphorylated and unphosphorylated sequences in activated upstream partners (Pero et al. 2002). The structure of its SH2 domain bound to an unphosphorylated cyclic peptide reveals that the Tyr-Asp-Asn motif is positioned similarly to the canonical pTyr-containing ligands of its relative Grb2 (Ambaye et al. 2011). Despite the absence of a phosphate group, the tyrosine side chain of the ligand fits into the pTyr binding pocket, while the neighbouring residues make contacts that stabilize the bound pseudo-β-turn of the peptide and pack over the EF loop (Fig. 3b). The cyclic peptide binds specifically to Grb7 with an affinity of 35 μM despite the lack of a pTyr, providing a basis for the development of selective cell-permeable inhibitors for this putative cancer target.

Ligands that Double Up: While most peptide ligands bind as monomers to a SH2 domain, a unique class of peptides can bind as dimers. A pair of pY-F-V-P sequence containing peptides were co-crystallized with the N-terminal SH2 (nSH2) domain of Shp2 (Zhang et al. 2011b). They form an antiparallel β-sheet when bound such that one peptide's pTyr fits canonically while the other bound peptide extends across the central β sheet of the domain (Fig. 3e). Such dual peptide interactions with a single SH2 domain could conceivably serve as a scaffold for two ligand molecules by supporting their dimerization.

Lipid Interactions: Several studies have reported lipid binding activities within SH2 domains. The interactions of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) with the SH2 domains of PI3K, Src, Abl and Lck were discovered using radio-labelled vesicle binding experiments (Rameh et al. 1995). Lipid binding is

competed with phosphopeptide binding, indicating overlapping sites. The lipid-specific interaction was confirmed by assays including PIP_3-bead binding (Wang et al. 2000), radioligand binding, fluorescence titration, and gel filtration (Ching et al. 2001), and indicated an affinity in the lower μM range as well as a requirement for long chain lipid. This interaction was contested by an NMR study that showed lack of specificity for soluble phosphoinositides by the p85 cSH2 domain (Surdo et al. 1999), although a PIP_3 molecule was shown to bind weakly to the pTyr site. In a similar vein, the C-terminal SH2 domain of PLCγ binds PIP_3 with an affinity of 2.4 μM and in a manner that competes with the activated PDGF receptor (Rameh et al. 1998). PIP_3 binding to both the SH2 and PH domains of this protein suggests concerted stimulation of the catalytic activity of PLCγ, thus leading to inositol trisphosphate (IP_3) generation and intracellular calcium release (Bae et al. 1998). The Abl SH2 interaction with phosphatidylinositol-4,5-bisphosphate (PIP_2) was validated by NMR and vesicle binding experiments (Tokonzaba et al. 2006). The His-Tyr-Arg sequence on βD is crucial based on the NMR chemical shift and mutagenesis data (Fig. 3f). Hence various SH2 domains which are found in membrane-localized proteins appear to have acquired PI lipid binding capabilities that depend on electrostatic attraction within an area around the pTyr pocket. Although generally weak and of limited stereospecificity, such lipid interactions may orient signalling domains on membranes, particularly in the cases of myristoylated kinases and lipid-specific enzymes.

2.6 Accessory Interactions and Regulation Outside the PTyr Pocket

Several SH2 proteins act as scaffolds by relying on adjacent domains to form specific and tightly regulated complexes. This phenomenon can be illustrated by the Crk protein, which contains a single SH2 domain followed by a pair of SH3 domains (nSH3 and cSH3). Its SH2 domain associates with tyrosine-phosphorylated partner proteins but is negatively regulated by intramolecular binding of the nearby pTyr221. The SH2-ligand interaction promotes the accessibility of Crk's long DE loop such that its proline-rich motif can be recognized by the Abl SH3 domain (Donaldson et al. 2002). This stable complex is flexibly oriented due the dynamic loop, giving the Abl kinase access to a range of potential phosphorylation sites in Crk and its other partners. However, another negative regulatory mechanism is mediated by the SH3 domains. The SH2 residues Arg31, Tyr104, Leu114 can also interact with the nSH3 domain's docking site for proline rich motifs, and its DE loop can bind the cSH3 domain's C-terminus (Kobashigawa et al. 2007). The latter intramolecular interaction is thus in direct competition with Abl, and is modulated by phosphorylation of the linkers.

The Src-like adaptor protein 2 (SLAP2) contains adjacent SH3 and SH2 domains which form a continuous structural unit, which contrasts with most instances where

these domains are flexibly oriented. The crystal structure reveals that a short connector closely juxtaposes the domains, which are linked by pairing of strand βA of the SH2 domain with strand E of the SH3 domain. The interface is further stabilised by hydrophobic contacts mediated by SH2 residues Tyr96, Leu99, Lys103, Leu107 and Leu110, making it structurally dependent on the SH3 domain (Wybenga-Groot and McGlade 2013). The canonical binding pockets of the two domains remain accessible on one face of the protein, suggesting that they can act in a concerted manner.

A variety of secondary interaction sites within SH2 domains are used to assemble multi-protein complexes. For example, the growth-factor receptor-bound protein 10 (Grb10) recognizes the C2 domain of the E3 ubiquitin ligase NEDD4 through it SH2 domain. The structure of the complex reveals that Grb10's pTyr pocket is not used (Huang and Szebenyi 2010). Instead, the major interface involves antiparallel pairing of βF of its SH2 domain with the β strand C of the C2 domain. This allows the SH2 domain to simultaneously interact with NEDD4 in a pTyr-independent manner on one side, while also binding a kinase domain on its other side. Such multivalent interactions highlight the fact that SH2 domains are often bifunctional, playing a scaffolding role by bringing together multiple proteins into a productive signalling complex.

Another level of complexity is demonstrated by the SH2 domains that assemble PLC-γ1 and interleukin-2 tyrosine kinase (Itk) protein complexes. The latter nonreceptor PTK is recruited to the membrane by its PI-binding PH domain, whereupon its activation loop is phosphorylated by the Lck kinase. The activated Itk protein then initiates downstream lipid signalling events by phosphorylating PLC-γ1. A scaffolding role is played by PLCγ1's C-terminal SH2 domain, which docks to Itk to allow its kinase to selectively phosphorylate a critical tyrosine in the linker that precedes the SH2 domain (Min et al. 2009). PLCγ1 uses a basic patch formed by CD loop and C terminal residues within its SH2 domain to bind an acidic patch within the G helix of the Itk catalytic domain. Meanwhile the Itk SH2 domain can also dock to the Itk kinase, thus allowing it to phosphorylate a tyrosine within its own SH3 domain. However, the Itk SH2 domain uses a distinct basic patch on its surface for its engagement with the adjacent kinase. Thus the Itk kinase binds two different SH2 domains through distinct intramolecular and intermolecular docking events that leave the pTyr sites available for further signalling interactions.

In contrast, the fibroblast growth factor receptor (FGFR) kinase binds to the N-terminal SH2 domain of PLC-γ1 through yet another mechanism (JH Bae, ED Lew, 2009). The architecture of PLC-γ1 is unusual in that it contains a split PH domain containing a long loop that includes two tandem SH2 domains and an SH3 domain. The classical pTyr pocket of this nSH2 domain binds to an extended pY-L-D-L sequence in the C-terminal tail of the kinase. Moreover, it uses a secondary site involving hydrophobic and polar residues in β strand D and the BC and DE-loops to interact with the kinase helices αE and αI, β strand 8 and the loop between β7 and β8. The secondary site occupies an area similar in size to the pTyr site, and substantially strengthens the interaction, yielding a total binding affinity of 33 nM for this kinase and SH2 domain. The adjacent cSH2 domain binds a critical

phosphorylation site (pTyr-783) in the cSH2-SH3 linker of PLCγ1 (Bunney et al. 2012). The latter *cis* interaction is largely canonical, involving cSH2 Arg residues at positions 675, 694, and 696 contacting the pTyr and residues Phe706, Leu726, Leu746, and Tyr747 recognizing Val784 and Ala786 at positions +1 and +3 relative to the pTyr. Interestingly both the surfaces involved in this FGFR-SH2 complex differ completely from those observed for the complex between the Itk kinase and the C-terminal SH2 domain of PLC-γ1. Hence different interfaces are used to dock these kinase and SH2 domains, providing a basis for generating distinct complexes and signalling consequences.

The interactions between SH2 domains and kinases can play an auto-inhibitory role by limiting constitutive signalling. This is clearly seen in the Abl1 protein, for which structures of the complete protein have been resolved in the active and autoinhibited states (Hantschel et al. 2003; Nagar et al. 2006). Comparison of these two forms indicates that the SH2 domain autoinhibits the kinase by binding to the C lobe only when the N-terminal myristoyl group inserts into the base of the catalytic domain, inducing a bend in the kinase's C-terminal helix and generating a complementary surface for SH2 domain binding. In contrast, in the activated state, the SH2 domain appears to relocate 70 Å away to pack against the β sheet of the smaller N-lobe of the kinase principally via residues in the C-terminal half of helix αA including residues Ser143 and Ile145, strand βG, and the AB, CD, and BG loops. Thus the linkers between the structural domains are malleable, alternating between being sufficiently flexible to allow a range of interdomain motions, and packing within the interfaces between domains.

Phosphorylation appears to control the formation of Abl's states. A phosphorylated serine residue in Abl's N-terminal cap is bound by the His-Ser-Trp-Tyr128 sequence near the beginning of the SH2 domain and far from the pTyr site. Although this phosphorylation site is of unclear physiological significance, Tyr128 in βA of this SH2 domain is also often phosphorylated, suggesting a regulatory switch. Moreover, another residue involved in this pSer interaction site, Tyr167, is also frequently phosphorylated in cells (Hornbeck et al. 2004). Any of these three phosphorylation events would dramatically alter the interaction of the SH2 domain with the N-terminal cap of Abl. In addition, other phosphorylation switches appear to be operative in Abl signalling. The leukaemia-linked phosphorylation of Tyr139 in αA of the SH2 would block its interaction with kinase helix αE, thus compromising a key autoinhibitory interaction, as borne out by the effect of replacing this residue with an aspartate (Filippakopoulos et al. 2008). The cancer-linked phosphorylation of Tyr185 in the EF loop (Salomon et al. 2003) would interfere with SH2 ligand interactions at the +3 positions. Together this suggests that Abl is subject to control by phosphorylation at multiple SH2 interfaces. Interestingly, Abl1's phosphorylation pattern is generally also replicated in the related Abl2 protein, suggesting conserved regulatory mechanisms.

Like Abl, the Src kinase is controlled by intramolecular interaction of its SH2 and SH3 domain, and these are in turn controlled by phosphorylation sites at key interfaces. The structures of Src (Williams et al. 1997; Xu et al. 1997) and its

relative Hck (Sicheri et al. 1997) have been solved in both autoinhibited and phosphorylated states. These reveal that the SH2 and SH3 domains bind opposite the catalytic cleft and lock the kinase into an inactive conformation. The SH2 domain binds the phosphorylated C-terminal tail, although the presence of a glycine at the +3 position rather than the preferred isoleucine infers a purposefully weaker interaction. Nonetheless, residues at the −1 to +2 positions bind canonically, and complementary electrostatic interactions are found between the SH2 helix A and the kinase helix E. As a result of these autoinhibitory interactions the kinase helix C is relocated to displace the key catalytic residue Glu310 from the active site, the activation loop is rearranged to compromise substrate docking, and the N and C lobes are re-oriented. Together this prevents kinase activity through a cooperative network of individually weak regulatory interactions.

The activation of Src family kinases can be induced by dephosphorylation or loss of the C-terminal inhibitory pTyr, or by binding of ligands to the SH2 or SH3 domains. This leads to unravelling of the autoinhibited structure and decoupling of the SH3 and SH2 domains which are then available to recruit cellular partners. This can be seen in the structure of a partially activated state of dephosphorylated Src which reveals a distinct docking of the SH3 domain and linker to the N-lobe, leaving the SH2 unencumbered and free to interact with ligand proteins (Cowan-Jacob et al. 2005). Proteomics studies indicate that the Src and Hck SH2 domains are both frequently phosphorylated on a conserved tyrosine in strand E (Ballif et al. 2008; Guo et al. 2008), as is Src SH2 domain's BC loop (Hornbeck et al. 2004), these modifications would clearly affect their abilities to specifically recognize canonical SH2 ligands.

The Csk protein also consists of SH3, SH2, and kinase domains but differs from Src and Abl by the absence of conserved phosphorylation and acylation signals. The Csk structure reveals that the SH2 and SH3 domains pack on either side of the N-lobe of the activated kinase domain, assuming a variety of orientations in crystallized molecule but all involving conserved hydrophobic positions (Ogawa et al. 2002).

The Fes cytoplasmic tyrosine kinase is preceded by an SH2 and F-Bar domains. Its structure reveals the close interdomain packing of its active state, with the SH2 domain's N-terminal residues and Glu469 and Glu472 in helix A contacting the kinase helix C in the N-lobe (Filippakopoulos et al. 2008). These interactions are critical for placing the helix C correctly for catalysis in the active site, and allow the SH2 domain to feed substrates to the kinase domain by recognizing nearby pTyr-containing sequences. In this way the SH2 domain can help determine the substrate selectivity of the attached kinase.

The suppressor of cytokine signalling protein SOCS3 downregulates intracellular pathways by inhibiting the activity of Janus family kinases via its SH2 domain. Its ternary structure reveals that its SH2 domain simultaneously engages the kinase domain and a pTyr-containing peptide from the interleukin-6 receptor (Kershaw et al. 2013). The catalytic domain is bound by its conserved Gly-Gln-Met motif to SH2 residues including Tyr47 in strand A, and BC loop residues Asp72, Ser73 and Phe79 and Phe80. The linker before the SH2 domain also makes

important interactions with the kinase and precludes substrate access, emphasizing the ability of some SH2 domains to engage both receptors and kinases through canonical and accessory interactions as centrally positioned scaffolding modules.

Vav is a guanine nucleotide exchange factor (GEF) which is involved in B cell development and acts as an adaptor protein. Its SH2 domain associates with proteins including Syk, binding to the phosphorylated linker between its tandem SH2 domains. A doubly phosphorylated linker peptide binds to the Vav SH2 domain with pTyr342 and pTyr346 fitting in the canonical pTyr pocket and the specificity pocket, respectively, the latter involving a Lys at the βD3 position (Chen et al. 2013). This interaction leads to Syk phosphorylating the Vav protein on tyrosines in order to regulate its GEF activity.

Post-translational modifications other than phosphorylation can also regulate SH2 signalling assemblies. The interaction of Src with cortactin is unusual in that it can involve formation of an intermolecular disulphide bridge with the pTyr binding loop of the SH2 domain. Cortactin is a cytoskeletal protein that regulates actin-based cell motility, and is multiply phosphorylated by Src. The cross-link formed with Cys185 is transient and reversible, and may only occur during conditions of cytoplasmic oxidative stress, for example during the production of invadopodia (Evans et al. 2012). Although cysteines are present in the vicinity of the pTyr site in roughly a quarter of SH2 domains, the general utility of this alternative binding mode remain unclear.

2.7 *Multivalent Interactions by Tandem SH2 Domains*

Tandem pairs of SH2 domains are found in several proteins, and mediate particularly specific and tightly regulated interactions with signalling partners. The structures of zeta-chain-associated protein kinase of 70 kDa (Zap70), spleen tyrosine kinase (Syk), Shp1 and Shp2 phosphatases, PLC-γ1 and PI3 K have revealed how adjacent pairs of SH2 domains orient catalytic domains, providing insights into their intricate control mechanisms.

The Zap70 kinase mediates signalling downstream of the activated T-cell receptor, which becomes phosphorylated on a pair of tyrosine residues in its immunoreceptor tyrosine-based activation motif (ITAM). The tandem SH2 domains of Zap70 undergo a dramatic conformational change upon binding to the phosphorylated ITAM sequence. Their relative orientation changes from splayed apart to aligned for synergistic binding to an ITAM target. The structure of autoinhibited Zap70 reveals that a coiled coil forms in the linker between the SH2 domains. This docks onto the catalytic domain, and orients the two SH2 binding sites outward so as to receive pTyr ligands. Phosphorylation of a pair of tyrosine residues in the coiled linker uncouples this interface, allowing the kinase helix C to switch to the active conformation (Deindl et al. 2007).

A related mechanism is found to the Syk protein, which also binds ITAM sequences via its tandem SH2 domains. The phosphorylated ITAM peptide binds in

antiparallel orientation to the tandem SH2 domains, with the cSH2 motif interacting with the N-terminal pTyr and vice versa (Futterer et al. 1998). Their respective orientations are flexible, and a coiled coil forms in the intervening linker that mediates kinase regulatory interactions, as revealed by structures of full length Syk (Graedler et al. 2013). While the cSH2 domain remains accessible to canonical ligands in the autoinhibited state, the nSH2 would need to move significantly in order to bind a pTyr-containing sequence. Activation of the kinase involves phosphorylation of linker tyrosines, which dismantles their regulatory interface.

Structures of a PI3K complex reveal the autoinhibited states formed when the two SH2 domains of its p85β subunit bind the kinase domain in its p110β subunit (Zhang et al. 2011a; Mandelker et al. 2009). The nSH2 interferes with membrane binding by slotting its helix A between the membrane-binding C2 domain and a kinase loop that contains hotspot mutations and a Glu that projects into the pTyr pocket. A coiled coil linker fits against the kinase and connects to the cSH2 domain which locks the C-lobe of the catalytic domain into an inactive position. The pTyr site of the cSH2 domain remains exposed in the inhibited complex, which instead employs a loop between βF residue Ala674 and Tyr680 to bind the C-lobe. The cSH2 also binds to the A-Raf kinase via two separate sites that are pTyr-dependent and independent, respectively, thus increasing the potential specificity of the interaction (Fang et al. 2002). Interestingly this interface contains the two most frequently phosphorylated residues in the protein (Gu et al. 2010; Hornbeck et al. 2004). Indeed phosphorylation of serine in the PI3K SH2 domains directly prevents pTyr peptide binding, inferred a means of controlling recruitment and activation of this signalling enzyme (Lee et al. 2011).

The Shp2 protein transduces signals downstream of growth factors through the Ras-ERK1/2 pathway. The basal state of Shp2 is locked in an autoinhibited conformation by the nSH2 attaching to the catalytic site (Hof et al. 1998). In particular, the nSH2 domain inserts its DE loop into the active site of the phosphatase, engaging the catalytic cysteine and blocking substrate access. Residues in the nSH2 making contact with the catalytic domain include Asn58, Gly60, Asp61 and Ala72, and the register of αB packing changes. In contrast the cSH2 domain remains relatively free, and can orient flexibly relative to the nSH2 domain. Activation occurs upon interaction of the tandem SH2 domains with phosphorylated ligands including ITAM sequences, thus unencumbering the active site from allosteric inhibition. Germline mutations associated with Noonan and LEOPARD syndromes are clustered at this regulatory interface, and cause these dominant developmental disorders by altering the control of the phosphatase.

2.8 *Multimerization of SH2 Domains*

Although most SH2 domains are monomeric, the oligomeric assemblies formed by some SH2 domains can increase their ligand avidity and specificity. This phenomenon can be considered to be analogous to the interactions of tandem SH2 proteins.

For example, full-length Grb10 and its SH2 domain form dimers in solution under physiological conditions through interactions mediated by the αB helix (Stein et al. 2003). The dimer affinity is moderate at 2 μM, suggesting an equilibrium between the monomeric and dimeric states in cellular contexts, thus modulating membrane avidity of Grb10 through its adjacent phosphoinositide-binding PH domains. The similar Grb7 protein and SH2 domain also form dimers with similar affinity and interface, potentially contributing to cooperative binding to receptors, which are also dimeric when activated (Porter et al. 2007). High affinity binding of the insulin receptor by Grb14 also relies on its SH2 domain forming a homodimer mediated by αB, with Phe519 being centrally involved in the interface (Depetris et al. 2005). Thus all three relatives employ αB to mediate dimerization to facilitate receptor interactions.

In contrast, dimerization of the Grb2 SH2 domain found in crystals involves domain-swapping and compromises ligand binding affinity (Benfield et al. 2007), and hence may be considered an artefact of crystallization. An intermolecular disulphide is seen in crystals of the Src homologous and collagen-like (Shc) protein, where it stabilizes an extensive dimer interface (Rety et al. 1996). The dimer is further supported by contacts mediated by residues in the AB, CD and long BG loops, βC, and the C-terminal end of αB. Although the ligand binding pockets remain fully available, these dimers only form in solution at high protein concentrations and low pH, and hence their physiological relevance remains unclear.

The adaptor protein APS is a substrate of the insulin receptor kinase, which it binds via its SH2 domain. The APS SH2 domain was shown to form a dimer in cells. Its structure reveals a back to back dimer mediated by a long tilted αB helix that encompasses residues usually found in βE and βF (Hu et al. 2003). This dimer structure exhibits a noncanonical binding mode in which a pair of pTyr residues in the kinase's activation loop engage through the canonical pTyr site and a lysine in the βD3 position, respectively. Thus the dimer structure is responsible for the unique selectivity of this SH2 domain for doubly phosphorylated peptide ligands.

The STAT proteins derive their names from being signal transducers in the cytoplasm and activators of transcription in the nucleus. The structure of the unphosphorylated STAT protein indicates how the STAT SH2 domain binds a receptor through specific recognition of its pTyr motif (Neculai et al. 2005; Chen et al. 1998, 2003). The weak dimer interfaces which can be seen in crystals could be outcompeted by stronger SH2-mediated dimers formed upon receptor activation. The stable dimers translocate to the nucleus where the juxtaposed DNA binding domains can initiate transcription. Thus dimerization of SH2 domains directly influences the cellular destination of these signalling complexes.

3 Future Prospects

The wealth of structural information on SH2 domain-ligand complexes provides a depth of understanding into the molecular mechanisms of signal recognition. These signals are typically pTyr motifs in extended polypeptide conformations. However, other SH2 ligands are helical, turn or inverted pTyr sequences and can include unphosphorylated or serine phosphorylated peptides and even phospholipids. Hence identification of SH2 specificities within signaling proteins remains a challenge, due in part to the inherently transient and dynamic nature of these interactions, necessitating experimental validation. The multimerization surfaces and accessory protein-protein interaction sites outside the canonical SH2 pockets add further complexity, and reveal the importance of multivalency, interdomain contacts and adaptable linker elements in regulating and mediating biological interactions. These facets culminate in the subcellular localisation and activation of SH2 proteins, and must be anticipated if signalling events are to be accurately manipulated. Intelligent use of mutations and chemical probes can already be used to reprogram the biological behaviour of many SH2 proteins. As this yields quantitative insights into how pathological conditions develop and respond to intervention, SH2 domains will increasingly be seen as viable targets for the design of therapeutic agents that selectively block the progression of developmental diseases and cancers. The characterization of the various SH2-driven interactions have already revealed an astonishing diversity of ligand types and binding pockets. As this structural information is used in conjunction with phosphoproteomic and disease-linked mutation databases, more insights into SH2 signaling and deregulation will surely emerge, yielding further targets and biomarkers for mechanism-based intervention into a wider range of disease states.

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Cytoskeletal Signaling by Src Homology Domain-Containing Adaptor Proteins

Narcisa Martinez-Quiles

Abstract In a sense, the field of intracellular signaling began with the discovery of SH2 domains in 1989 by Tony Pawson's group (Mayer and Baltimore 1993). Dr. Pawson wrote in a farsighted review in 1991 that "the SH3 domain, together with the SH2, may modulate interactions with the cytoskeleton and membrane" (Koch et al. 1991). This prediction expresses the motivation for the present chapter, which aims to review how SH domain-containing proteins regulate the cytoskeleton, while focusing on how such proteins interact directly with factors that control actin remodeling.

Keywords Actin cytoskeleton · Wiskott-Aldrich syndrome · Nck · Crk · Cortactin

1 Actin Polymerization

Eukaryotic cells constantly remodel their actin cytoskeleton to accomplish a wide range of biological processes (Pollard and Cooper 2009). Consequently actin networks must simultaneously be structurally solid and dynamic. The actin cytoskeleton rearranges itself mainly through cycles of actin assembly and disassembly, collectively called "actin turnover". This remodeling sometimes affects nearly the entire cell, such as during cell migration, while on other occasions it is limited to specific areas, such as during dorsal ruffle formation. Actin cytoskeleton rearrangement even occurs in the nucleus, which is only beginning to be understood (Baarlink and Grosse 2014; Hendzel 2014).

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Actin polymerization occurs when the globular monomeric form (G-actin, 42 kDa) assembles into a double-stranded helical filament (F-actin). Nucleation factors help actin overcome the kinetic barriers to forming actin dimers or trimers, which are subsequently elongated into filaments. The two major groups of nucleation factors in eukaryotes are the Arp2/3 complex and formins. A third mechanism of nucleation, less well understood, involves proteins containing the Wiskott-Aldrich Syndrome protein (WASP) Homology 2 (WH2) domain (reviewed in (Carlier et al. 2013)).

The actin filament is a polar structure, with one terminus that incorporates new monomers (barded or plus end) and another terminus where depolymerization predominates (pointed or minus end). The two termini differ in their monomer concentration requirements, also known as the critical concentration. ATP-loaded actin monomers incorporate into the filament mainly on the barbed end; as the filament ages, the ATP hydrolyzes and the resulting ADP-bound monomer is more likely than the original ATP-loaded one to dissociate from the filament. According to the so-called "treadmilling" model, when the rate of polymerization at the plus end equals the rate of depolymerization at the minus end (steady-state equilibrium), the filament should maintain a constant length while nevertheless "pushing forward". This pushing is driven by a pool of available monomers originating in part from dissociation at the pointed end.

G-actin for filament elongation is provided primarily by proteins containing a **profilin**, **WH2 and β-thymosin domain** (WH2/βT; reviewed in (Xue and Robinson 2013)). Profilin is one of the major providers of monomeric actin in vivo because it not only binds G-actin but also favors exchange of ADP for ATP. The βT domain comprises a 43-residue repeat sequence, while the WH2 domain comprises only 17 residues, which is why it is often called a "motif" (Aguda et al. 2006). The WH2 domain is found in many proteins that promote actin nucleation (see Sect. 3.3). In addition to an extensive array of G-actin providers, several cellular processes, including gene regulation, help control the pool of available monomeric actin.

Similarly, several proteins and regulatory pathways contribute to the control of F-actin assembly and disassembly. Severing proteins, which include actin depolymerizing factor (ADF)/cofilins and are collectively referred to as cofilins, bind to and sever actin filaments, generating barbed ends that can be extended by polymerization (Bravo-Cordero et al. 2014). Severing proteins must first be activated by dephosphorylation or by dissociation from inhibitors. Capping proteins, for their part, bind to monomeric actin preferably on the barbed end, preventing its elongation. Still other proteins may exert multiple actions on actin. Gelsolin, for example, not only binds and sequesters G-actin but also severs and caps F-actin (Nag et al. 2013).

2 Some Major Cellular Actin Structures: The Leading Edge and Trailing Edge of Motile Cells …

Cells extend protrusions, such as filopodia and lamellipodia, when carrying out diverse cellular tasks. One generality that has emerged from research on cell morphology and movement is that proline-rich proteins "support protrusion" (Holt and Koffer 2001), and the same seems to be true for cytoskeletal remodeling. Proline-rich proteins are targets of SH3 domain-containing proteins, reflecting the latter's role in regulating the cytoskeleton. GTPases also contribute to the regulation of many actin structures formed during protrusion, as several seminal studies have shown (Nobes and Hall 1995; Ridley and Hall 1992; Ridley et al. 1992).

Cellular protrusions take on different shapes, such as thin and spiky filopodia, flat lamellipodia and wavy ruffles that detach from the substrate (Ridley 2011). Filopodia, as well as certain other cell structures such as microvilli in enterocytes, comprise parallel bundles of actin filaments that are cross-linked to one another via proteins such as α-actinin, fimbrin, and fascin. Filopodia are considered sensors of the environment (reviewed in (Heckman and Plummer 2013)), and macrophages can use them to catch bacteria (Moller et al. 2013).

An important milestone in the cell migration and motility field was the first observation of the **lamellipodium** (plural, lamellipodia) at the leading edge of motile fibroblasts (Abercrombie et al. 1970). The lamellipodium is defined as a thin sheet of cytoplasm enriched in intercrossed actin filaments but not microtubules. Actin turnover is fast inside the lamellipodium, which is consistent with the dendritic network model (see Sect. 3.1). This turnover contributes to the protrusion of the membrane. The actin concentration often forms a gradient, with the maximum occurring within the lamellipodium and the minimum towards the trailing edge of the cell. At the base of the lamellipodium is a so-called "transition zone", which gives way to a more stable area called the **lamellum** (plural, lamella), where actin turnover is slower and the filaments undergo slower retrograde movement (reviewed in (Bisi et al. 2013; Blanchoin et al. 2014; Ridley 2011)).

Cells can make **dorsal circular ruffles**, which are circular protrusions rich in F-actin that protrude from the apical cell surface and that close centripetally. They may be important for rapid internalization of receptors and membrane, for macropinocytosis and for preparation for cell migration (Hoon et al. 2012).

Stress fibers comprise bundles of actin filaments, some of which connect the actin cytoskeleton to the extracellular matrix via focal adhesion sites (Blanchoin et al. 2014). At these sites, **focal adhesions** (**FAs**) are multiprotein complexes containing plasma membrane-associated integrins that link the extracellular matrix and actin cytoskeleton. FAs are enriched in kinases, including focal adhesion kinase (FAK) and Src family kinases (SFKs), as well as SH2/3-containing proteins including p130Cas. A FAK-SFK complex phosphorylates p130Cas, converting it into a molecular platform that recruits many other adapters, including Nck and Crk (see below) (Ciobanasu et al. 2013).

Podosomes are dot-like adhesive structures only 0.5–1 micron long and 0.6 micron high with an F-actin core, which also contains F-actin-associated proteins such as cortactin. The core is surrounded by an adhesive ring (integrins, paxillin, vinculin, talin) (Linder and Wiesner 2015). Podosomes contribute to cell migration, matrix remodeling and antigen presentation in some myeloid-lineage immune cells (dendritic cells, macrophages). It also contributes to remodeling processes in other cell types, including endothelial cells (transendothelial migration) and osteoclasts (bone remodeling). Podosomes are sometimes observed to be grouped into rosettes.

Apparently similar to podosomes when visualized by low-resolution microscopy, **invadosomes** are usually bigger than those structures and they persist longer. They are frequently present in cancer cells and specialize in matrix degradation. Invadosomes can coalesce into giant rosettes, frequently observed in fibroblasts transformed with *v*-Src (Hoshino et al. 2013).

3 Cellular Actin Nucleators

3.1 The Arp2/3 Complex

The actin-related 2/3 (Arp2/3) complex was the first actin nucleator discovered (Machesky et al. 1994). It comprises seven subunits, two of which are called actin-related proteins 2 and 3 due to their structural similarity to G-actin. The complex nucleates actin by adding a ‘branch’ to the side of a preexisting filament, giving rise to characteristic filaments with branches that form 70° angles with the main fiber. This structure is consistent with the dendritic nucleation model (Blanchoin et al. 2000; Nicholson-Dykstra et al. 2005).

Normally present in the cell in a closed, inactive conformation, the Arp2/3 complex is activated by actin nucleation-promoting factors (NPFs), which have been divided into types I and II based on their mechanism of action. Type I NPFs belong to two protein families: the Wiskott-Aldrich syndrome proteins (WASPs) and the WASP family Verprolin-homologous proteins (WAVEs) (reviewed in (Rotty et al. 2013)). Type II NPFs include cortactin and its immune homolog hematopoietic specific protein 1 (HS1). The Arp2/3 complex is activated by the VCA domain in WASP-WAVE NPFs or by acidic motifs in cortactin and HS1. Several groups of proteins inhibit the Arp2/3 complex, including the cortactin antagonist coronin 1B (Cai et al. 2008).

3.2 Formins

Formins belong to a family of proteins discovered in the early 1990s and encoded by at least 15 genes in humans. They have been implicated in the formation of several cellular actin structures, including stress fibers, filopodia and lamellipodia,

so they are likely to participate in numerous actin rearrangement processes (Breitsprecher and Goode 2013). Some formins can regulate actin cytoskeleton in other ways, by promoting filament severing and bundling or even actin depolymerization. Formins also appear to help regulate the microtubule network, though this role is poorly understood. It is possible that formins help coordinate rearrangement of actin and microtubule networks (Ishizaki et al. 2001), since several cellular processes require remodeling of both rather than remodeling only of actin (reviewed in (Huber et al. 2014)).

Through the approximately 400-residue formin homology 2 (FH2) domain, formins bind directly to G- and F-actin and promote filament nucleation and/or elongation. Individual formins differ in how efficiently they promote each of these activities. FH2 domains form head-to-tail dimers that adopt an overall toroidal shape. This FH2 toroid binds the barbed end of actin filaments, giving rise to unbranched actin filaments. Formins may regulate actin through other domains as well, including WH2 domains (see Sect. 3.3) and DAD domains (see below). This makes unravelling the functions of these NPFs a complex challenge.

Formins contain a formin homology 1 (FH1) domain with proline-rich sequences (PRS), which are known to bind profilin and certain SH3 domains (Ren et al. 1993), including the SH3 domains in c-Src and c-Fyn SFKs. PRS has even been shown to bind with lower affinity to Abelson murine leukemia (Abl) kinase and PLCγ (Uetz et al. 1996). Studies are needed to determine whether the FH1 domain of formins interacts with the SH3 domains of type I NPFs such as cortactin, because it would support the possibility of functional interaction between the two known types of NPF.

Formins are recruited to particular subcellular locations through their interaction with GTPases, which thereby couples activation and localization. They may also be recruited by farnesyl and myristoyl modifications or even by direct interaction with phospholipids in the cell membrane.

A particular group of formins, named **Diaphanous-related formins** (**DRFs**) because they are encoded by the *Drosophila* gene *diaphanous* or its homologue in other organisms, have attracted interest because of their interaction with GTPases. They possess an N-terminal GTPase binding domain (GBD) that binds RhoA, Rac, and Cdc42 GTPases. Next in the sequence are an auto-inhibitory domain (DIF) and an autoregulatory domain (DAD). Binding of GTPases to the GBD domain partially unblocks the auto-inhibited conformation of DRFs. Other formins bind GTPases indirectly rather than through a GBD (reviewed in (Kuhn and Geyer 2014)). DRFs can also be regulated by post-translational modifications such as phosphorylation.

3.3 WH2 Domain

The WH2 domain is an approximately 30-residue actin-binding domain that is present in NPFs in one copy or as tandem repeats. Some examples of WH2-containing proteins are Spire and Cordon-Bleu, as well as the bacterial

effectors VopF and VopL from different *Vibrio spp*. Some of the best characterized WH2-containing proteins belong to the WASP family (see Sect. 3.4).

The WH2 domain plays a complex, poorly understood role in actin assembly. WH2 repeat proteins are able to nucleate actin in vitro, but their role in vivo seems to be more complex (reviewed in (Carlier et al. 2013; Qualmann and Kessels 2009)). Similarly to the case with formins, WH2 domains are often found in proteins that also contain PRS. It is thought that the binding of actin-loaded profilin to the PRS brings the WH2 domain into contact with G-actin (Khanduja and Kuhn 2014; Suetsugu et al. 1998).

3.4 Type I NPFs

WASPs were the first type I NPFs to be discovered. The prototypical member is WASP (Derry et al. 1994), a key immunological protein for which mutations in the gene give rise to three immune deficiencies: Wiskott-Aldrich syndrome (reviewed in (Massaad et al. 2013)), X-linked agammaglobulinemia and X-linked severe neutropenia (Al-Herz et al. 2011). The C-terminal domain of WASPs binds G-actin and activates the Arp2/3 complex, while the rest of the protein contains several domains that interact with various binding partners to coordinate activation and localization. The active site responsible for actin nucleation contains one WH2 domain in the case of WASP, or two WH2 domains in the case of N-WASP, which was initially identified in the nervous system and later found to be ubiquitous. These WH2 domains are also often called *verprolin homology domains* in the literature. In addition to these domains, the active site for actin nucleation contains a connecting sequence, previously called a cofilin homology region, and an acidic VCA domain (Fig. 1). WASPs also possess PRS that bind profilin, suggesting that the PRS interacts with the WH2 domain to provide G-actin. The PRS also contains several canonical binding sites for SH3 domains, which may have important functional consequences (see below).

Intramolecular interactions, involving primarily the basic region and GBD on one hand and the acidic VCA domain on the other, maintain N-WASP and WASP in a closed, auto-inhibited conformation. This auto-inhibition can be strengthened by the interaction between a unique domain in WASPs called Wiskott-Aldrich homology domain 1 (WH1) or Ena/VASP Homology domain 1 (EVH1) with Wiskott-Aldrich interacting protein (WIP) (Fried et al. 2014; Martinez-Quiles et al. 2001).

WASP and N-WASP are activated in a cooperative manner by multiple binding events (Fig. 2). The small GTPase Cdc42 binds to the GBD; phosphatidyl-inositol (4,5)-bisphosphate (PIP2), to the basic region; SH2 domain-containing proteins, to tyrosine 291 in the GBD; and/or SH3 domain-containing proteins, to the PRS (Padrick and Rosen 2010).

Enterohemorrhagic *Escherichia coli* (EHEC), a significant human pathogen, expresses an effector protein, EspFu, that contains several repeats that compete for binding to the GBD of N-WASP, thereby disrupting the auto-inhibited conformation

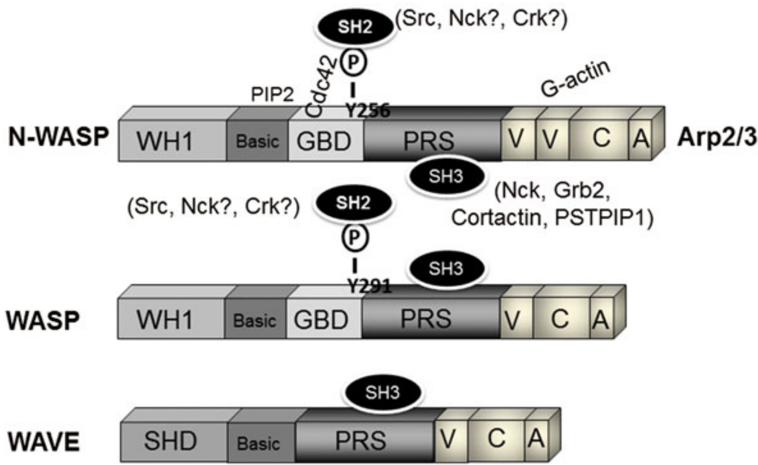

Fig. 1 Domain structure of the Wiskott-Aldrich protein (WASP) family and the main adapter partners. Despite their different N-termini, all WASPs possess proline-rich sequences (PRS) and a C-terminal VCA domain. WH1, WASP homology domain; B, basic domain; GBD, GTPase binding domain; PRS, proline-rich sequence; V, verprolin homology domain; C, connecting sequence; A, acidic sequence; WHD, WAVE homology domain. Question marks indicate no or inconclusive experimental demonstration

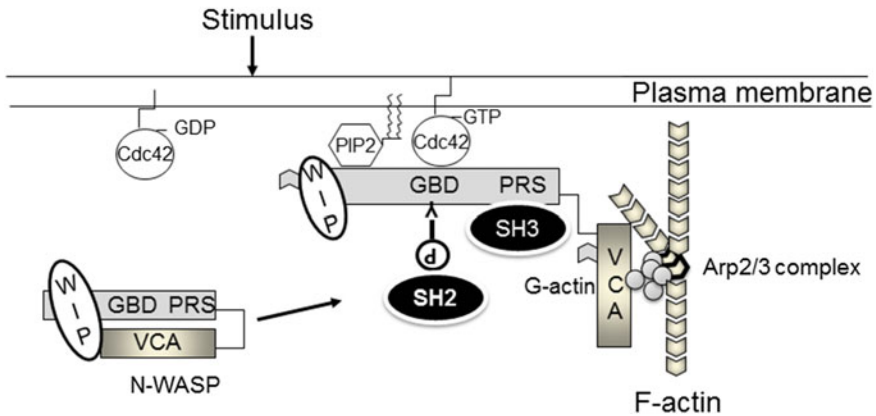

Fig. 2 Model of the activation of the N-WASP-WIP complex. The Wiskott-Aldrich proteins (WASPs), exemplified here by N-WASP, adopt an auto-inhibited conformation in complex with the Wiskott-Aldrich interacting protein (WIP). WASPs are activated cooperatively through the binding of phosphatidyl-inositol (4,5)-bisphosphate (PIP2), activated GTPases (GTP-loaded Cdc42) and SH domain-containing proteins. The released verprolin-connecting-acidic (VCA) domain binds G-actin and the Arp2/3 complex, promoting F-actin polymerization

and hijacking actin polymerization to support bacterial formation of pedestals, particularly actin-rich protrusions (Campellone et al. 2008). Similarly, mutations that give rise to X-linked severe neutropenia disrupt WASP auto-inhibition.

Post-translational modifications of WASPs, including tyrosine phosphorylation, increase the number of pathways involved in their regulation. Phosphorylation of tyrosine 291 in WASP creates an SH2 binding site, which is bound by Src kinase and potentially other kinases (Torres and Rosen 2003). The equivalent tyrosine 256 in N-WASP, located in the GBD, is phosphorylated by FAK, which causes sustained activation (Wu et al. 2004).

Phosphorylated WASP is in turn dephosphorylated by a protein tyrosine phosphatase named PTP-PEST because it recognizes sequences rich in proline, glutamate, serine and threonine (Badour et al. 2004). PTP-PEST interacts with WASP in a complex that contains proline, serine, and threonine phosphatase-interacting protein (PSTPIP) 1 (Cote et al. 2002). PSTPIP1 possesses an SH3 domain that interacts with the PRS of WASP. PSTPIP1 is mutated in autoinflammatory human diseases, and it would be interesting to see whether these mutations affect proper regulation of WASP activity (see Sect. 4).

More recently discovered type I NPFs include WASP and Scar homologue (WASH); WASP homologue associated with actin, membranes and microtubules (WHAMM); and junction-mediating and regulatory protein (JMY; reviewed in (Rottner et al. 2010)). Each of these NPFs has a different N-terminal domain that helps localize it to a specific part of the cell, namely the endosomes (WASH), Golgi membranes (WHAMM) or nucleus (JMY). Both WHAMM and WASH bind to microtubules. JMY has two nuclear localization signals (NLS) and was in fact discovered as a transcriptional cofactor of p53. JMY seems to act by linking the monomeric actin pool and the DNA damage response.

3.5 Type II NPFs

These NPFs comprise cortactin (reviewed in (Ammer and Weed 2008)) and its immune homologue HS1 (reviewed in (Martinez-Quiles 2011)). HS1 was so named because it is expressed in many cells of the immune system but not in other tissues. The protein is occasionally referred to as hematopoietic cell-specific Lyn substrate 1 (HCLS1) because it was identified as a substrate of Lyn kinase. Both cortactin and HS1 possess an N-terminal acidic (NTA) domain that directly binds and activates the Arp2/3 complex. In HS1, the NTA domain is followed by 3.5 tandem repeats of residues and then by a coiled-coil (CC) region. The repeats and CC region bind synergistically to F-actin (Hao et al. 2005) (Fig. 3). Cortactin contains 6.5 repeats of a sequence similar to that in HS1; the fourth repeat is required for binding to F-actin. Cortactin promotes not only Arp2/3 complex-mediated actin branching, but also the bundling of actin filaments. Interestingly, both cortactin and HS1 possess C-terminal SH3 domains. Detailed studies are needed to clarify whether expression

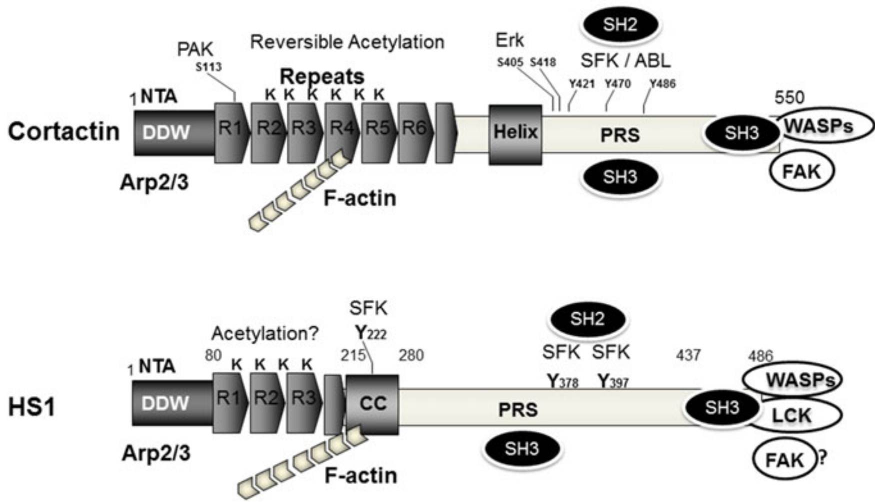

Fig. 3 Domain structure of cortactin and HS1 and the main adapter partners. Cortactin and its immune homologue hematopoetic specific protein 1 (HS1) are type I nucleation-promoting factors that activate the Arp2/3 complex through a conserved DDW amino acid motif located within the N-terminal acidic domain (NTA). One major structural difference is the number of amino acid repeats they possess. They are regulated by reversible phosphorylation and acetylation. Both proteins bind F-actin and possess a C-terminal SH3 domain that binds and activates the Wiskott-Aldrich proteins (WASPs). Other important binding partners include focal adhesion kinase (FAK) and lymphocyte-specific protein tyrosine kinase (Lck), a Src family kinase (SFK). Question marks indicate no or inconclusive experimental demonstration. CC, coiled-coil region; PRS, proline-rich sequence; ABL, Abl kinase; PAK, Serine/threonine-protein kinase

of the two NPFs overlaps in the immune system, and if so, to identify the specific functions performed by each one.

Cortactin participates in the remodeling of the actin cytoskeleton during diverse processes, including cell migration and invasion (Kirkbride et al. 2011; MacGrath and Koleske 2012). It participates in these processes mainly by helping to form lamellipodia, podosomes and invadosomes (Murphy and Courtneidge 2011); in fact, it is considered a reliable invadosome marker. Cortactin has also been implicated in other cell functions such as endocytosis and exocytosis, and many pathogens hijack the protein to gain entry into cells (Selbach and Backert 2005).

Cortactin is thought by many researchers to activate Arp2/3 complex only weakly, in part because it lacks a domain to bind G-actin directly (Helgeson et al. 2014). This may help explain why cortactin appears able to interact with N-WASP in multiple ways. Cortactin synergizes with the VCA domain of N-WASPs to activate the Arp2/3 complex (Weaver et al. 2001). In addition, in vitro studies using purified recombinant proteins indicate that the SH3 domain of cortactin can bind the PRS of N-WASP to activate it (Martinez-Quiles et al. 2004). This intriguing finding needs to be validated and extended in cell culture studies.

Cortactin regulation is multifaceted. The protein was identified both as the product of a gene (*Cttn,* formerly *EMS*) located in a chromosomal region overexpressed in carcinomas and as a cortical protein substrate of Src kinase. Several SFKs phosphorylate tyrosines in cortactin, while extracellular response kinase (Erk) 1/2 and p21-activated kinases (PAKs) phosphorylate serines in the protein. Cortactin can also undergo reversible acetylation, with the aceylated protein thought to be inactive (Zhang et al. 2007). How all these post-translational modifications relate to each other and how they influence cortactin's ability to regulate Arp2/3 complex-mediated actin polymerization remains unclear. Our group has addressed some of these questions, and we have found that tyrosine phosphorylation of cortactin promotes its deacetylation (Meiler et al. 2012), raising the possibility that the protein is regulated through sequential modifications. Work in this area is just beginning.

The complexity of cortactin regulation makes sense in light of the number of actin-remodeling processes it may participate in together with its numerous binding partners. In addition to activating the Arp2/3 complex and binding F-actin, cortactin via its SH3 domain binds to N-WASP and WASP homologue (Martinez-Quiles et al. 2004), as well as FAK, such as during *Helicobacter pylori* infection of cells (Tegtmeyer et al. 2011). Post-translational modifications of cortactin may alter the accessibility of the SH3 domain and thereby regulate the protein's activity; cortactin phosphorylated on tyrosines, for example, binds FAK more weakly than unmodified cortactin (Meiler et al. 2012). Future studies should clarify whether and how such modifications affect the cortactin-FAK interaction in particular. The cortactin-FAK complex has emerged as an important regulator of FA dynamics (Tomar et al. 2012; Wang et al. 2011), as well as a potential target in cancer therapy, since disruption of the cortactin-FAK complex sensitizes cancer cells to radiotherapy (Eke et al. 2012).

3.6 Adapters Implicated in Cytoskeletal Remodeling

While most SH2/3 domain-containing proteins were initially lumped together under the name "docking proteins" (Brummer et al. 2010), this term is no longer widely used because it implies that these proteins play a passive role in signaling. Nowadays, these proteins are more often described as "adapters" or "adaptor proteins", and the large multiprotein complexes in which they participate are often referred to collectively as "signalosomes" and "transducisomes" (Burack and Shaw 2000).

Some of the best characterized adapters involved in signaling to the cytoskeleton are the chicken tumor virus number 10 regulator of kinase (Crk), the non-catalytic region of tyrosine kinase (Nck), and the growth factor receptor-bound 2 (Grb2) (Buday 1999). All three proteins are widely recognized as activators of N-WASP/WASP proteins. PSTPIP proteins have also recently been reported to interact with WASPs and to regulate their activity.

3.7 Crk

The discovery of Crk adapter proteins was an important landmark in the signal transduction field (Pawson and Scott 1997). Crk was cloned as the product of the *v-Crk* oncogene (Mayer et al. 1988), and it was found to increase the tyrosine phosphorylation of many cellular proteins, even though it seemed to lack any enzymatic activity of its own. Further work revealed the existence of two alternatively spliced Crk isoforms, CrkI and CrkII (Fioretos et al. 1993; Matsuda et al. 1992). Both isoforms contain one SH2 domain, followed by one SH3 domain (CrkI) or two (CrkII). Crk-like (CrkL) is encoded by a separate gene (ten Hoeve et al. 1993) and shares a high degree of sequence homology with CrkII. CrkL was first identified as a protein highly phosphorylated in chronic myeloid leukemia (CML) cells, and it has been implicated with CML pathophysiology (ten Hoeve et al. 1994). To what extent CrkL and Crk are similar in their functions and regulation is unclear, especially since the SH2 domain of both CrkI and CrkII contains a PRS that is missing from the corresponding domain in CrkL.

The Crk family of proteins, ubiquitously expressed, has been implicated in transducing signals in a wide diversity of cellular processes, most of them related to cytoskeletal remodeling (Birge et al. 2009; Martinez-Quiles et al. 2014). Crk isoforms play a role in the formation of lamellipodia and filopodia, as well as in controlling FAs during cell spreading. One of the first Crk-mediated pathways regulating cell adhesion to be discovered involves paxillin, p130Cas and Abl.

Whether Crk isoforms have redundant or distinct functions is unclear, though studies suggest that the answer is likely to be complex and quite context-dependent. In knockout mouse models, the two isoforms show non-overlapping functions. In cell culture studies, however, at least some functional overlap (compensation) is observed. Our group has recently shown that CrkII and CrkL act redundantly in inhibiting actin polymerization at pedestals formed by enteropathogenic *Escherichia coli* (EPEC), and that they do so by competing with Nck adapters to bind the EPEC effector Tir. This finding is in agreement with a report that Crk adapters function in a heterocomplex during podocyte morphogenesis (George et al. 2014). Similarly, ablation of both isoforms together reduces cell spreading to a much greater extent than ablation of either isoform on its own, with cells adopting a rounded, detached morphology (Park and Curran 2014). This suggests that the two isoforms play complementary roles in focal adhesion formation in fibroblasts.

The C-terminal SH3 domains of CrkII and CrkL are thought to exert an autoregulatory allosteric effect, yet they are not considered typical SH3 domains because they are incapable of binding to classical polyproline motifs. This is due to the presence of several polar residues on the binding surface (Sriram and Birge 2012). CrkII and CrkL share a so-called “regulatory tyrosine” located in a linker region between the two SH3 domains. Phosphorylation of this tyrosine does not inhibit Crk activity, as one might predict based on the fact that its SH2 domain binds to it. Thus, phosphorylation of this tyrosine in CrkL still allows the SH3 domain to interact with its binding partners, including Abl kinase (Jankowski et al. 2012).

Abl kinase may play a role in integrating tyrosine phosphorylation and Crk activity. An elegant set of studies showed that integrin engagement activated Abl kinase, which phosphorylated CrkII, which in turn promoted interaction of Abl kinase with the SH3 domain of Nck (Antoku et al. 2008). The ultimate result was filopodium formation and delayed cell spreading. If CrkII was not tyrosine-phosphorylated, it promoted FA formation and Rac1 activation, thereby stimulating lamellipodium formation and cell spreading.

3.8 Nck

The Nck adapter family comprises the two isoforms Nck1 and Nck2, generally referred to simply as Nck. Each one contains three SH3 domains and one C-terminal SH2 domain. Little is known about Nck regulation except that ubiquitination down-regulates its expression (Joseph et al. 2014). Nck is one of the best studied adapters regulating actin cytoskeleton (Buday et al. 2002; Li et al. 2001). Like Crk adaptors, Nck was isolated and analyzed for its ability to bind growth factor receptors and cause oncogenic transformation. Thus Nck was soon implicated in growth factor signaling via the interaction of the SH2 domain with phosphorylated receptors. In contrast to Crk, however, Nck has never been associated with up-regulation of tyrosine phosphorylation (Chou et al. 1992; Margolis et al. 1992).

One important exception to the “rule” that the SH2 domain of Nck interacts with the phosphorylated form of growth factor receptors is signaling by the T-cell receptor (TCR). In this case, an activation-induced conformational change in the CD3ε subunits of the TCR exposes a PRS, to which the first SH3 domain of Nck binds. In addition, TCR activation of Nck can cause the SH2 domain of Nck to bind the leukocyte protein of 76 kDa (SLP76), which itself contains a phosphorylated SH2 domain (reviewed in (Lettau et al. 2009)). Perhaps reflecting this multimodal binding, TCR activation of Nck seems to trigger non-overlapping activities in Nck1 and Nck2 in Jurkat T cells. In this cell line, Nck1 seems to be the more important isoform (Ngoenkam et al. 2014).

The discovery that Nck activates N-WASP in synergy with phosphatidylinositol 4,5-bisphosphate (4,5-PIP_2) definitively implicated Nck in actin regulation (Rohatgi et al. 2001). Around the same time, Nck was shown to mediate the interaction between EPEC effector Tir and N-WASP, making it essential for pedestal formation (Gruenheid et al. 2001). After EPEC eliminate the intestinal microvilli, they induce the host cells to form actin-rich pedestals, which protrude under the plasma membrane and seem to contribute to bacterial adhesion. Other pathogens such as vaccinia virus recruit Nck to promote N-WASP-Arp2/3-dependent motility (Welch and Way 2013).

A major challenge to understanding how Nck participates in cytoskeleton remodeling has been determining whether the three SH3 domains bind to the same or different targets (Lettau et al. 2010; Wunderlich et al. 1999). All three domains are required for maximal N-WASP activation in vitro (Rohatgi et al. 2001), consistent with the idea that Nck adapters act synergistically with other activators (Rohatgi et al. 2001). Detailed experiments suggest a stoichiometry of 4:2:1 for the Nck:N-WASp:Arp2/3 complex (Ditlev et al. 2012).

Although initial studies suggested that Nck does not help activate the WAVE regulatory complex (Eden et al. 2002), subsequent work indicates a role in WAVE-dependent processes. In these processes, Nck appears to act as a bridge between a tyrosine-phosphorylated phagocytic receptor and the WAVE complex (Pils et al. 2012). In these ways, Nck controls a variety of complex functions by integrating many signals governing directional cell migration (Chaki and Rivera 2013).

More recent work has uncovered another link between Nck and actin dynamics. Nck-interacting kinase (NIK), a MAPK serine/threonine kinase, phosphorylates the Arp2 subunit, which contributes to activation of the Arp2/3 complex (LeClaire et al. 2015).

3.9 Grb2

The Grb2 adapter, which consists of an SH2 domain flanked by N- and C-terminal SH3 domains, has traditionally been thought to participate in receptor tyrosine kinase (RTK) signaling (Belov and Mohammadi 2012). In those studies, Grb2 was found to constitutively associate via its most N-terminal SH3 domain with Son-of-sevenless (Sos), a guanine nucleotide exchange factor for Ras GTPase. Phosphorylation of Grb2 then caused the Grb2-Sos complex to bind to RTKs and contribute to downstream signaling. More recent work complicates the picture by showing that dimeric Grb2 inhibits basal phosphorylation activity of RTKs; this inhibition occurs via the interaction of Grb2 SH3 domains with receptor PRS (reviewed in (Belov and Mohammadi 2012)).

Grb2 can activate actin polymerization through multiple mechanisms. In one pathway, the most C-terminal SH3 domain of Grb2 binds the PRS of N-WASP, activating it (Carlier et al. 2000). In another pathway, Grb2 activates N-WASP synergistically with Cdc42, suggesting that Grb2 can transduce RTK signaling into Arp2/3 complex-mediated actin polymerization (Carlier et al. 2000).

Grb2 localizes to podosomes, whereas Nck1 localizes to invadopodia but not to podosomes. This has led to the suggestion that Nck1 and Grb2 localization patterns can be used to distinguish podosomes from invadopodia (Oser et al. 2011).

3.10 PSTPIP Adapter and Other BAR Domain-Containing Proteins

The actin cortex is a thin meshwork of layers composed primarily of actin, myosin, and actin-binding proteins. The plasma membrane is anchored to the cortex to prevent the collapse of the cell. At the same time, the highly contractible cortex is constantly being remodeled to allow numerous cellular tasks to proceed, including cell migration and division. This remodeling is driven primarily by formins, the Arp2/3 complex, cortactin and the WAVE complex, with WAVE subunits SRA1 and NAP1 identified in the cortex (Bovellan et al. 2014). Cortex remodeling must occur hand-in-hand with plasma membrane deformation in order to prevent structural tension and allow cellular motility. The two processes are coupled by membrane-bending proteins called Bin/Amphiphysin/Rvs (BAR) domain-containing proteins (Mim and Unger 2012). BAR domain-containing proteins can possess a classical domain, inverse BAR domain (I-BAR) or extended Fer-CIP4 homology (FCH) domain (F-BAR) (Mim and Unger 2012).

Many F-BAR proteins have only one SH3 domain and they nucleate the assembly of a variety of protein complexes that contribute to membrane curvature. Several F-BAR proteins interact directly with WASPs and DRFs. The first such protein to be discovered was Toca-1, which binds both Cdc42 and the N-WASP-WIP complex, thereby activating the latter (Ho et al. 2004). Since the ability of many BAR domain-containing proteins to activate WASP/N-WASP has recently been reviewed (Aspenstrom 2014; Chen et al. 2013), we will focus here on proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1), a protein implicated in autoinflammatory human diseases.

PSTPIP1 (OMIM/606347) plays an important role in inflammation, in part due to its interaction with pyrin, the protein mutated in individuals with the familial form of Mediterranean fever (Shoham et al. 2003). Mutations in the *PSTPIP1* gene (also known as *CD2BP1*) cause several autoinflammatory syndromes, including pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome (Wise et al. 2002; Yu et al. 2007). These are complex diseases that usually involve fever, serositis, arthritis, skin symptoms and other manifestations. Most of these diseases are associated with deregulation of inflammasomes (Lamkanfi and Dixit 2014), which are supramolecular signaling complexes that activate a subset of inflammatory caspases, including caspase-1 and caspase-11. Activation of caspase-1 triggers an inflammatory form of cell death called pyroptosis.

PSTPIP1 is a ubiquitous protein containing FCH and SH3 domains; the latter binds to the PRS in WASPs (Wu et al. 1998b). PSTPIP2 is another isoform that lacks the SH3 domain (Wu et al. 1998a). PSTPIP1 was initially characterized as a binding partner of PTP-PEST. Through this interaction, PSTPIP1 helps regulate the phosphorylation of many proteins, including WASP. Some mutations in PSTPIP1 that have been identified in patients with PAPA syndrome block the interaction of PSTPIP1 with PTP-PEST. Another mutation lies within the SH3 domain, and it

decreases PSTPIP1 interaction with WASP, leading to increased matrix degradation by macrophages (Starnes et al. 2014).

Like WASPs, PSTPIP1 regulates the cytoskeleton and participates in filopodium and podosome formation (Starnes et al. 2014). In T cells, Fyn SFK phosphorylates tyrosine 291 in WASP (see Sect. 3.4), which is then dephosphorylated by PTP-PEST, which binds to WASP via PSTPIP1 (Badour et al. 2004). PTP-PEST also dephosphorylates p130CAS and paxillin, affecting the interaction of p130CAS with Crk and thereby regulating FA dynamics (Angers-Loustau et al. 1999). PSTPIP1 is itself regulated by tyrosine phosphorylation triggered by activation of epidermal growth factor (EGF) receptors and platelet-derived growth factor (PDGF) receptors (Cote et al. 2002), as well as other stimuli. How this phosphorylation regulates PSTPIP1 activity is poorly understood.

4 Concluding Remarks

One guiding principle for making sense of the complex networks of adapters regulating actin dynamics and remodeling is “signaling by competition”: adapters can bind to the same motif in the same protein, and which adapter will prevail depends on many factors, including post-translational modifications, subcellular localization, affinity thresholds, and the presence of other proteins and receptor ligands (Sinzinger et al. 2013). For example, our group has demonstrated that the SH2 domain of CrkII/CrkL and the SH2 domain of Nck compete for binding to the EPEC effector Tir during actin polymerization at pedestals (Nieto-Pelegrin et al. 2014). This competition is important because when CrkII/CrkL binds, then actin polymerization is blocked; if Nck binds, however, the N-WASP-Arp2/3 complex pathway is activated and pedestals are formed. Another recent example of competition is that between the SH3 domain of Grb2 and the SH3 domain of PLCγ1 for binding fibroblast growth factor receptor 2 (FGFR2) (Timsah et al. 2014). At low Grb2 concentrations, PLCγ1 is preferentially recruited to the receptor, which upregulates its phospholipase activity and ultimately promotes cell invasion in the absence of extracellular FGFR2 activation. In this way, the involvement of one adapter or another is key for transducing different signaling processes into different types of actin remodeling, such as when Grb2 acts to promote podosome formation or when Nck acts to promote invadopodium formation.

A major challenge for future studies will be to elucidate the molecular details of the differential contributions of adapters to the numerous actin dynamics transduction pathways. Advances in experimental techniques have helped and will continue to help researchers assemble the many pieces of this puzzle (Johnson and Hummer 2013).

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Structure and Function of Jak3- SH2 Domain

Jayshree Mishra and Narendra Kumar

Abstract Janus kinase 3 (Jak3) is a non-receptor tyrosine kinase expressed in hematopoietic and non-hematopoietic cells. Though expression of Jak3 is necessary for adaptive immune functions, non-hematopoietic cell expression of Jak3 is also essential for the regulation of several physiological functions of gastrointestinal mucosa. Both in immune cells and intestinal epithelial cells (IEC), Jak3 regulates cell migration and mucosal restitution respectively by cytoskeletal remodeling through interactions with actin binding proteins (ABPs). Jak3 also regulates chronic inflammation of gastrointestinal tract through intestinal differentiation, mucosal homeostasis, regulation of proliferation and apoptosis, and maintenance of mucosa barrier functions. Molecularly, these functions are regulated through posttranslational and transcriptional regulation of Jak3, ABPs, and adapter proteins p52ShcA. For all these functions of Jak3, tyrosine phosphorylation of the SH2 domain plays a central role not only during common gamma chain induced auto-phosphorylation of Jak3 but also during phosphatases mediated deactivation of Jak3. In this chapter, we discuss the intramolecular and trans-molecular switches that regulate Jak3 interactions with other proteins and several aspects of its functions. Since SH2 domain plays a central role not only in Jak3 functions but the functions of all non-receptor tyrosine kinases, last section of the chapter discusses SH2 domain as drug target for the amelioration of different chronic inflammatory and other diseases.

Keywords FERM-domain · Jak3 kinase · Phosphatases · Apoptosis · SHP2 · SH2-domain

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1 Introduction

The Janus kinases (JAKs) belong to a family of four cytoplasmic tyrosine kinases that participate in the signaling of members of the cytokine receptor common gamma-chain family. They are widely expressed in birds, fishes, insects and mammals. The name "Janus" was taken from the two-faced Roman God of doorways, because the JAKs possess two almost identical phosphate-transferring domains. The expression of Jak3 is more restricted; it is predominantly expressed in hematopoietic cells and intestinal epithelial cells and highly regulated with cell development and activation. The human *jak3* gene is located on the short (p) arm of chromosome 19 at position 13.1 containing of 19 exons and 18 introns. Mutations in *jak3* gene are associated with autosomal SCID (severe combined immunodeficiency disease) (Riedy et al. 1996). Somatic mutations in Jak3 result in constitutively active kinases in myeloproliferative diseases and leukemia/lymphomas (Bellanger et al. 2014), though mutations that abrogate Jak3 functions cause various immunological disorders. The *Jak3* gene codes for a protein of 125 kDa that transduces a signal in response to its activation via tyrosine phosphorylation by interleukin receptors. Jak3 kinases are mainly involved in cell growth, survival, development, and differentiation of a variety of cells and also are critically important for immune cells and hematopoietic cells (Ghoreschi et al. 2009). Our group has extensively studied intestinal epithelial specific Jak3 and Jak3 mediated mucosal functions. Our study demonstrated that interleukin-2 (IL-2) enhances mucosal wound repair in an in vitro wound closure model in response to infection and inflammation. Specifically, IL-2 induces cell migration in the intestinal enterocytes through activation of Jak3. In these cells Jak3 interacted with the intestinal and renal epithelial cell-specific cytoskeletal protein villin in an IL-2-dependent manner. Inhibition of Jak3 activation resulted in loss of tyrosine phosphorylation of villin and a significant decrease in wound repair of the intestinal epithelial cells (Kumar et al. 2007). Since interactions of Jak3 with the actin binding proteins are important for epithelial wound closure process, we further demonstrated the molecular mechanism of these interactions as well as the structural determinants of Jak3 and tyrosine phosphorylation of the SH2 domain acted as an intramolecular switch for the interactions between Jak3 and cytoskeletal proteins. Tyrosine phosphorylation of the SH2 domain of Jak3 is essential for the interaction between Jak3 and villain because under nonphosphorylated conditions, intramolecular interactions between the FERM and SH2 domain of Jak3 prevent villin from interacting with Jak3. Tyrosine phosphorylation of the SH2 domain decreases its affinity for the FERM domain (F1–F3). As a result, disruption of the interactions between these two domains occurs that facilitates the interactions between the (now free) FERM domain of Jak3 and villin. Tyrosine phosphorylation of the FERM domain increased its affinity for the SH2 domain. However, there is a substantial decrease in affinity for the FERM domain after the SH2 domain is tyrosine-phosphorylated regardless of the phosphorylation status of the FERM domain. The intramolecular interaction between the FERM and SH2 domains of

nonphosphorylated Jak3 prevents Jak3 from binding to villin and tyrosine autophosphorylation of Jak3 at the SH2 domain decreases these intramolecular interactions and facilitates binding of the FERM domain to villin (Mishra et al. 2012a).

In a cell culture model, IL-2 regulates IEC homeostasis through mechanisms involving p52 ShcA and Jak3. At lower concentrations, IL-2 promotes tyrosine phosphorylation-dependent interactions between Jak3 and p52ShcA leading to increased proliferation. A higher concentration IL-2 recruits phosphatases (e.g., SHP1) at this complex through Shc leading to decrease in phosphorylation and disruption of this complex. Less phosphorylated form of Jak3 translocates to the nucleus inhibiting its own transcription, possibly with involvement of a transcription factor, and hence downregulation of *jak3*-mRNA leads to higher IL-2-induced apoptosis in IEC. Nuclear targeted Jak3 may also influence the transcription factors for Shc expression. Subsequent to our discovery that Jak3 localizes to nucleus and induces apoptosis in intestinal epithelial cells under higher IL-2 concentrations (Mishra et al. 2012b), the subcellular localization of Jak3 was also confirmed in human immunocytes. Jak3 was found to be located in the nuclei of primary CD4 lymphocytes. Comparative analysis showed that, far more phosphorylated JAK3 (pJAK3) was found in the nuclei of CD4 lymphocytes from HIV-infected patients than in those of healthy subjects. Since it is well known that HIV specifically infects CD4-T cells, it is possible that HIV mediated increase in nuclear localization may be responsible for T-cell apoptosis leading to depletion of these cells in HIV infected patients (Landires et al. 2013).

Jak3 plays a critical role in the pathogenesis of colitis induced by DSS (Dextran Sodium Sulphate); colonic expression of Jak3 is essential for a healthy mucosal barrier. Knock-out of Jak3 results in a low grade chronic inflammation in colon and Protein Loosing Enteropathy symptoms, which predisposes the mice toward increased severity of colitis. Under normal physiological condition, Jak3 is essential in the colon where it facilitates mucosal differentiation by promoting the expression of differentiation markers and enhanced colonic barrier functions through adherent junction (AJ) localization of β-catenin. Jak3 plays a key role in expression of differentiation markers and this role of Jak3 could be attributed to its activation by different cytokine receptors at the apical surfaces in nondifferentiated/differentiated cells. Specifically, in fully differentiated cells, Jak3 gets redistributed to basolateral surfaces where it facilitates cellular junction formation and mucin secretion (Mishra et al. 2013b).

2 Structural Aspects of Jak3

Jak3 protein contains seven Jak homology (JH) domains in common with other Jak proteins. The characteristic feature of the Jaks is the presence of a fully functional tyrosine kinase domain (JH1) and a catalytically inactive pseudokinase domain (JH2) (Cornejo et al. 2009). Apart from these two domains, Jaks also contain five other conserved regions. From the N- to C-terminus, Jak3 contains a FERM

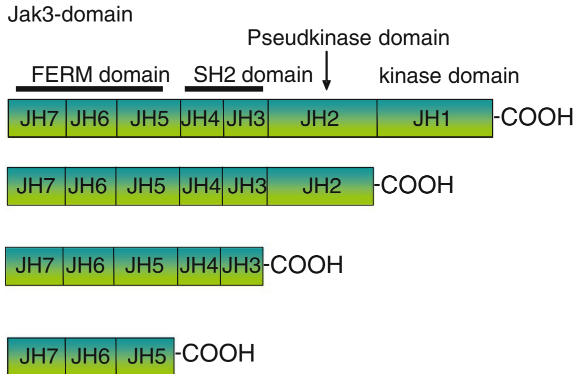

Fig. 1 Domain structure of Jak3. Jak 3 comprises seven Jak homology regions (JH) containing the catalytically active kinase domain (JH1), the enzymatically inactive pseudokinase domain (JH2), the SH2 domain (JH3, JH4), and a FERM domain (JH6, JH7) form the Jak protein. The FERM domain mediates Jak binding to the transmembrane cytokine receptor and regulates kinase activity. The SH2 domain mediates binding to specific signaling partner

domain, a SH2 domain, a pseudo kinase domain, and a kinase domain (Fig. 1). JH3-JH4 regions in Jak3 protein have homology with SH2 domains (Kampa and Burnside 2000) and presence of SH2 domain indicates interactions with other signaling molecules. The JH6-JH7 domains have homologies with the FERM domain found in molecules such as Band 4.1, ezrin, radixin, and myosin. Although, the FERM domains mediate intermolecular interactions with cytokine receptor (Huang et al. 2001), they are also involved in intramolecular binding to JH1 kinase domain, thereby enhancing the kinase activity (Zhou et al. 2001). The crystal structure of the JH1 kinase domain has been solved (PDB ID: 1YVJ) but there is no report on the functions and/or crystal structure of whole Jak3 (Zhou et al. 2001). The kinase domain is in the active conformation with both activation loop tyrosine residues being phosphorylated. The phosphate group on pTyr981 in the activation loop is in part coordinated by an arginine residue in the regulatory C-helix, suggesting a direct mechanism by which the active position of the C-helix is induced by phosphorylation of the activation loop. The structure of the kinase in an active, doubly phosphorylated state provides mechanistic insight into Jak activation and a detailed view of the kinase that facilitates development of Jak-specific inhibitors (Babon et al. 2014). The N-terminal half comprises a FERM domain and an SH2 domain that mediates JAK association with the cytoplasmic tail of a variety of cytokine receptors. The cell-based in vitro kinase assay results support a gain of function activity for the mutant JAK3 s. Interestingly, the JAK3 FERM domain's potentiation of kinase activity was suspected by O'Shea and colleagues (Zhou et al. 2001). In their studies, FERM domain mutations from SCID patients abolished γc binding and kinase activity in vitro. They also found that an intact FERM domain was required for Jak3 binding of an ATP analog. The crystal structure of the FERM

domain of focal adhesion kinase (FAK) revealed the structural basis for this kinase autoregulation (Ceccarelli et al. 2006). Amino acid side chains from the FAK FERM were shown to directly contact the intramolecular kinase domain causing occlusion of the substrate pocket. The pseudokinase domain has a protein kinase fold, but key catalytic residues are not conserved and the domain is thought to inhibit the adjacent tyrosine kinase domain, perhaps via an intramolecular interaction (Saharinen et al. 2000). Somatic gain-of-function mutations in Jaks underlie a number of hematologic malignancies (Ihle and Gilliland 2007), and the pseudokinase domain is the most frequent site of these activating mutations (Haan et al. 2010). In particular, the V617F mutation in the Jak2 pseudokinase is found in ~95 % of patients with polycythemia vera (PV) (James et al. 2005): myeloproliferative neoplasm (MPN) characterized by EPO-independent overproduction of red blood cells. This was due to constitutive signaling from Jak2 V617F that was in complex with the erythropoietin receptor (EPO-R) (Tefferi and Vainchenker 2011). In Jak3, pseudokinase domain is essential for JAK3 function due to its ability to regulate kinase catalytic activity and autophosphorylation. Pseudokinase domain regulates Jak3 function via intramolecular interaction with the kinase domain. Increased inhibition of kinase activity by the pseudokinase domain likely contributes to the disease pathogenesis in the patients with SCID (Chen et al. 2000). The region corresponding to domains JH3-JH4 represents a structural and functional SH2 domain. This domain binds to other proteins that specifically interact with JAKs and participate in the intracellular signaling pathway (Kampa and Burnside 2000). Tyrosine kinase 2 (TYK2), a member of the Janus kinase (JAK) family of non-receptor tyrosine kinases encompassing the FERM and SH2 domains, forms a complex with intracellular peptide motif from the IFNα receptor (IFNAR1). The TYK2–IFNAR1 interface reveals an unexpected receptor-binding mode that mimics a SH2 domain–phosphopeptide interaction (Wallweber et al. 2014). In our study we have also demonstrated the molecular mechanism of interactions between Jak3 and cytoskeletal proteins, where tyrosine phosphorylation of the SH2 domain acts as an intramolecular switch for the interactions between Jak3 and cytoskeletal proteins and this interaction is necessary for IL-2 dependent mucosal wound repair in IEC (Mishra et al. 2012a).

3 SH2 Domain and Its Role in Jak3 Activation

In mammalian tissues the phosphotyrosine (pTyr) mediated signaling depends upon mainly three classes of signaling molecules eluded as so called triad: (1) the protein-tyrosine kinases (PTKs) that add a phosphate onto substrate tyrosines (viewed as the ‘writers’); (2) the modular protein interaction domains which recognize these tyrosine phosphorylated ligands (viewed as the ‘readers’) and facilitate recruitment of the proteins containing these domains thereby specifying and facilitating downstream signaling events; and (3) the protein-tyrosine phosphatases (PTPs) which remove or dephosphorylate the phosphorylated tyrosine (and hence

viewed as the ‘erasers’) that terminate the signaling (Jin and Pawson 2012). Among the several modular interaction domains that have the capability to bind to the phosphorylated tyrosine residues as ligands, Src homology 2 (SH2) domain, phosphotyrosine-binding (PTB) domain, C2 domain and the Hakai pTyr-binding domain play major roles. Among these pTyr binding domains, the SH2 domain is the largest with 111 proteins containing at least one SH2 domain encoded in the human genome (Liu et al. 2011). In non-receptor tyrosine kinases, the SH2 domain is mostly located at the N-terminus of the catalytic kinase domain. It mainly mediates cellular localization, substrate recruitment, and regulation of the kinase activity (Li et al. 2008). Biochemical characterization reported through several studies showed that the presence of the SH2 domain is frequently required for catalytic activity, suggesting a crucial function of SH2 domain in stabilizing the active state of many cytoplasmic kinases. For example, the SH2 domain stabilizes the active kinase of Fes by direct interactions with the regulatory helix αC. These stabilizing interactions between the SH2 and the kinase domains are also observed in Csk and Abl (Filippakopoulos et al. 2009). The majority (71 %) of the disease-causing missense mutations in SH2 domains have been reported to affect functionally important amino acids either involved in direct ligand binding, or in mediation of interactions with the catalytic domain. The remaining missense mutations are located distantly from the SH2 domain and presumably affect its stability (Vihinen et al. 1994). Among these, one of the best studied examples of disease-causing mutations in SH2 domains is the Tec family member Btk. Mutations in the SH2 domain of this kinase cause X-linked agammaglobulinemia (XLA), a prototypical humoral immunodeficiency characterized by low levels of circulating B cells and a drastic reduction in serum concentrations of immunoglobulins (Lappalainen et al. 2008). So far as selectivity of pTyr is concerned, SH2 and PTB domains determine selectivity for pTyr ligands by recognizing pTyr and residues surrounding the pTyr. The preference for 1st to 5th residues downstream towards C-terminus of the pTyr in a sequence dependent manner is dictated by the surface residues on the SH2 domain that lie adjacent to the pTyr-binding pocket. In contrast, PTB domains recognize sequences located towards N-terminus of the pTyr with a core consensus of N-P-x-pY, yet only a small fraction of PTB domains are capable of binding pTyr peptides.

So far as cytoplasmic kinase Jak3 is concerned, it is involved in regulation of different signal transduction pathways mainly through its association and activation of common gamma chain of diverse cytokine receptors including IL-2 (Safford et al. 1997). Inactivating mutation of Jak3 leads to childhood immunodeficiency (Cornejo et al. 2009; Macchi et al. 1995), and its abnormal activation is associated with hematologic and epithelial malignancies (Cornejo et al. 2009; Lin et al. 2005), indicating that regulation of its activity is essential for normal hematopoietic and epithelial functions. Though non-hematopoietic expression of this protein was reported, the understandings of Jak3 functions in these cells is very limited (Takahashi and Shirasawa 1994; Safford et al. 1997). Our group has shown that Jak3 plays an essential role during mucosal wound repair and homeostasis (Kumar et al. 2007; Mishra et al. 2012b). We also demonstrated that Jak3 interactions with

cytoskeletal protein villin are essential for its wound repair function (Kumar et al. 2007) and determined the structural determinants responsible for Jak3 interactions with cytoskeletal proteins and the molecular mechanism that govern interactions of these actin binding proteins (ABPs) with Jak3. To achieve this, recombinant purified full length Jak3 and its substrate villin (and gelsolin) were expressed and purified. Characterization of the kinetic parameters for the auto-phosphorylation of Jak3 followed by determination of the kinetic parameters for trans-phosphorylation of these ABPs by Jak3 showed that not only the Jak3 kinase domain is sufficient to phosphorylate villin (Kumar et al. 2007), but the full length recombinant Jak3 is also able to phosphorylate villin and gelsolin. The $t_{1/2}$ (time taken to reach half of the maximum-phosphorylation) of villin/gelsolin trans-phosphorylation by the full length Jak3 is four fold lower than that of Jak3 auto-phosphorylation (Mishra et al. 2012a). These findings indicate that Jak3 auto-phosphorylation is rate-limiting during Jak3-cytoskeletal protein interactions. These observations were further confirmed by inhibition studies in which CP-690550, a Jak3 specific inhibitor, inhibited both Jak3 auto-phosphorylation and ABP trans-phosphorylation by Jak3. Though CP-690550 binds directly to the kinase domain of truncated Jak3 with IC_{50} of 35 nM (Chrencik et al. 2010), full length Jak3 binds to this inhibitor with a higher IC_{50} (128 nM). These indicates that presence of other domains including SH2 in Jak3-wt decreases the binding affinity of inhibitor to its kinase domain (Mishra et al. 2012a).

Subsequent to the discovery of direct interactions of Jak3 with villin/gelsolin, other ABPs are also found to interact with Jak3 (Ambriz-Pena et al. 2014). Jak3 is required for the actin cytoskeleton reorganization in T lymphocytes and homing to peripheral lymph nodes. In these cells Jak3 regulates actin polymerization by controlling cofilin inactivation in response to CCL21 and CXCL12. Jak3 also facilitates activation in these cells of small GTPases Rac1 and RhoA, which are indispensable for acquisition of the migratory cell phenotype. It is possible that Jak3 and heterotrimeric G proteins can use independent, but complementary, signaling pathways to regulate actin cytoskeleton dynamics during T-cell migration in response to chemokines (Ambriz-Pena et al. 2014). Since Jak3 is a non-receptor tyrosine kinase, understanding of Jak3-mediated intracellular signaling in different cells resulting in either hematological- (Landires et al. 2013) or epithelial- (Kumar et al. 2007; Kumar et al. 2012) cell migration would require adequate information on structure-function relationship between Jak3 and cytoskeletal proteins. Our lab reported that P(phosphorylated)-Jak3-wt interacted with P-villin-wt in a dose dependent manner with a K_d of 23 nM and a Hill's co-efficient of 3.7 indicating a high affinity and co-operative binding between Jak3 and villin. Recently, a new homologue of myosin heavy chain was also reported to bind Jak3 but in a tyrosine-phosphorylation-independent manner (Ji et al. 2000). Due to lack of crystallographic data on full length Jak3, the structure-function relationship between Jaks and their interacting partners still remains largely elusive. However most of the Jaks bind to their cytokine receptors through N-terminal FERM domain which encompasses from JH7 through a part of JH4 domain (Haan et al. 2006). In the case of Jak3, FERM domain also interacts with the JH1 kinase domain thereby

turning on its activity (Chrencik et al. 2010). We demonstrated that tyrosine phosphorylation of Jak3 is necessary for its interactions with villin and these interactions take place through direct contact between FERM domain of Jak3 and villin. Though tyrosine phosphorylation of FERM domain per se is not necessary for this interaction, however, the tyrosine phosphorylation of SH2 domain is essential because under non-phosphorylated conditions, intra-molecular interactions between FERM- and the SH2-domain of Jak3 prevent villin from interacting with Jak3. Tyrosine phosphorylation of SH2 domain decreases its affinity for the FERM (F1-F3)-domain leading to disruption of the interactions between these two domains and facilitated interactions between (now free) FERM domain of Jak3 and villin. Conversely and interestingly, our data also show that tyrosine phosphorylation of the FERM-domain increases its affinity for SH2-domain. However, there is a substantial decrease in affinity for FERM-domain when SH2-domain is tyrosine-phosphorylated regardless of the phosphorylation status of FERM-domain (Mishra et al. 2012a). Moreover, pair-wise binding shows that non-phosphorylated Jak3-FERM domain directly binds to villin. These results combined with data obtained using human intestinal epithelial cell model which showed that IL-2 activation resulted in tyrosine-phosphorylation dependent interactions between Jak3-V484 (that contained only FERM and SH2 domains of Jak3) and villin, indicated that tyrosine-phosphorylation of SH2-domain of Jak3 plays a major role during interactions between Jak3 and villin. Clover-shaped FERM domain comprises three sub-domains: F1 with a ubiquitin-like β-grasp fold, F2 with an acyl-CoA-binding-protein like fold, and F3 that shares the fold of phosphotyrosine binding (PTB) or pleckstrin homology (PH) domain (Cornejo et al. 2009). We predict that the F3 sub-domain may be responsible for the inter-molecular interactions between tyrosine phosphorylated villin and the FERM domain (Fig. 2). Since Jak3 regulates IL-2-induced wound closure and proliferation in IEC

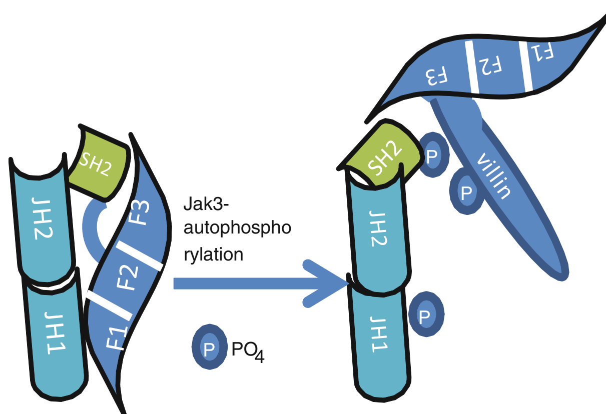

Fig. 2 SH2 domain regulates Jak3 interactions with ABP. Tyrosine phosphorylation of SH2 domain of Jak3 disrupts the interactions between Jak3-FERM domain and Jak3-SH2 domain making the F3 sub-domain of FERM-domain available to interact with phosphorylated villin

(Mishra et al. 2012b; Kumar et al. 2007), these studies show that though Jak3-V484* interacts with villain, lack of kinase and pseudo-kinase domains compromises Jak3-mediated physiological functions. This could be due to defective F-actin turn-over and failed signal-integration leading to surface defects induced by Jak3-mutants and loss of coble-stone morphology as seen in HA-Jak3-wt expressing epithelial cells (Mishra et al. 2012a). Based on distinctive domain architectures that place SH-2 domain in the context of other modular protein domains, such as SH3, PH, kinase, phosphatase, PTB, FERM and others, there are 38 families of SH2 domains in humans that encode a heterogeneous range of proteins that connects pTyr signaling to a diverse array of cellular systems. Since 111 SH2-domain containing proteins contain a diverse array of additional protein interaction domains (e.g. SH3, PTB etc.) including catalytic domain (kinase, phosphatase etc.), either gain or loss of these domains generate diversity in signaling (Xie et al. 2011; Jin et al. 2009). SH-2 domains may also exert allosteric regulation over other aspects of their host proteins. For example, intramolecular contacts between the SH-2 domain and tyrosine kinase domain directly regulate the enzymatic substrate activity for SH2-domain-containing kinases such as Fes and Abl (Filippakopoulos et al. 2008). In Jak3, presence of FERM domain adjacent to SH-2 domain exemplifies this point: SH-2 domain not only regulates ABP interactions with the adjacent FERM domain but it also regulates the auto-activation of Jak3 through rate-limiting auto-phosphorylation and change in conformation (Fig. 2).

4 SH2 Domain in Jak3 Deactivation

Jak3 mediates signals initiated by cytokine through interactions with the common γ (gamma) chain of cytokine receptors (Safford et al. 1997). Abnormal activation of Jak3 leads to both hematologic and epithelial malignancies in human (Cornejo et al. 2009; Lin et al. 2005) and its functions are essential for epithelial development (Mishra et al. 2013a). Since constitutive activation of Jak3 leads to various cancers, it is essential not only to understand the physiological regulation of Jak3 deactivation but also to determine the mechanism that leads to its constitutive activation in cancerous cells. Jak3 contains seven Jak homology (JH) domains. JH3-JH4 regions have homology with SH2 domains and JH6-JH7 domains have homologies with FERM domain found in such molecules as Band 4.1, ezrin, radixin, moesin (Cornejo et al. 2009). While the FERM domain mediates inter-molecular interactions with cytokine receptor (Huang et al. 2001), it is also involved in intra-molecular binding to SH2 domain thereby maintaining the closed conformation in Jak3 (Mishra et al. 2012a). Though Jak3 regulated mucosal wound repair in human epithelial cells through interactions with villain, however, Jak3-mediated mucosal homeostasis was dependent on its interactions with p52ShcA (Mishra et al. 2012b; Kumar et al. 2007). Jak3 mediated homeostasis is also dependent on IL-2 concentration: lower concentrations promote mucosal proliferation whereas higher

concentrations promote epithelial apoptosis. Inside the cell, this changes in IL-2 concentration regulated Jak3 interactions with an adapter protein p52ShcA (Shc). Shc is identified as proto-oncogenic protein with three members viz. *shcA*, *shcB*, and *shcC*, that are involved in regulation of growth factors signaling (Wills and Jones 2012). Among members, ShcA is ubiquitously expressed, while ShcB and ShcC are restricted to neuronal cells (Sakai et al. 2000). ShcA is involved in mitogenic signaling through activation of Ras/MAPK pathways (Bonfini et al. 1996) and KO of *shcA* gene results in embryonic lethality (Lai and Pawson 2000). The CH1 domain of p52ShcA contains three critical tyrosine residues, Y_{239}, Y_{240}, and Y_{317} that become phosphorylated upon engagement of a number of cell surface receptors. The SH2 domain of Grb2 binds to both Y_{239} and Y_{317} which leads to MAP-Kinase activation (Wills and Jones 2012). It was reported that Jak3-wt trans-phosphorylates Shc-wt. The $t_{1/2}$ of Shc trans-phosphorylation is lower than that of Jak3 auto-phosphorylation (Mishra et al. 2012a; Mishra and Kumar 2014), indicating that Jak3 auto-phosphorylation is rate-limiting during Jak3-Shc interactions. These results were further confirmed by inhibition studies in which CP-690550 (Chrencik et al. 2010), inhibited Shc-wt trans-phosphorylation by Jak3. Activation of Shc signaling is associated with poor prognosis in cancer patients (Ursini-Siegel and Muller 2008; Wills and Jones 2012). Therefore, understanding of Shc-mediated signaling inside cell is essential. In colonic epithelial cells, P-Jak3-wt interacts with P-Shc-wt in a dose dependent manner with a K_d of 0.22 μM and a Hill's co-efficient of 1.08 indicating non-cooperative binding. In contrast, Jak3 interactions with cytoskeletal proteins are cooperative and have higher affinity. Thus Jak3-mediated cell migration may be an early step during mucosal restitution which might be followed by cell proliferation and apoptosis to maintain cellular homeostasis through interactions with Shc.

Understanding structure-function relationship between Jaks and their interacting partners is essential but availability of literature on this topic is limited. Studies suggest that tyrosine phosphorylation of Jak3 is necessary for its interactions with Shc and these interactions take place through direct contacts between FERM domain of Jak3 and PID and CH1 domains of Shc (Mishra and Kumar 2014). Isolated SH2-domain of Shc is reported to bind its ligand in solution; however, it loses binding ability in full-length Shc (George et al. 2008). The binding between isolated FERM-domain of Jak3 and P-Shc is significantly higher than between P-Shc and full-length Jak3 indicating that presence of other domains of Jak3 lowers the binding affinity. Additionally, tyrosine phosphorylation of Shc by cytosolic kinases shows that while v-Src phosphorylates Shc at both $Y_{239/240}$ and Y_{317}, c-Src phosphorylates only at $Y_{239/240}$ (Sato et al. 1998). On the other hand, Jak3 phosphorylates a total of four tyrosine residues in Shc. Jak3 phosphorylates Y_{420} and Y_{458} in SH2 domain of Shc. The remaining two tyrosine residues are one each in CH1 and PID domains of Shc (Mishra and Kumar 2014).

Adapter functions of Shc during intracellular signaling depend on various factors including phosphorylation/dephosphorylation of its residues (Wills and Jones

2012). In IEC, Shc is involved in the regulation of Jak3 deactivation through regulation of Jak3 interactions with phosphatases. Constitutive activation of Jak3 is associated with various cancers. For example, mutation in *jak3* gene has been indicated as a cause of constitutive activation (Koo et al. 2012). However, it is not known if the impairment of Jak3 interaction with other proteins could also lead to constitutive activation of Jak3. Recent studies suggest that in epithelial cells, even when there was no mutation in *jak3* gene, Jak3 is still constitutively active because the interactions between Jak3 and Shc are impaired. Phosphatases SHP2 and PTP1B dephosphorylate activated Jak3 and Shc facilitating Jak3 interactions with these phosphatases. Protein phosphatase PP2A associates with Shc through PID domain. Phosphorylation of CH1 domain facilitates their dissociation (Ugi et al. 2002). However, CH1-domain of Shc is essential for its direct association with both the phosphatases PTP1B and SHP2 and presence of both SH-2 and CH1 domains of Shc is essential for Jak3 interactions with SHP2. Also, presence of only CH1 domain of Shc is essential for Jak3 interactions with PTP1B in a tetra-molecular complex of Jak3-PTP1B-SHP2-Shc inside an IL-2-activated human IEC because Jak3 may partially interact with PTP1B through direct interactions in a tetra-molecular complex (Fig. 2).

While mutation in *jak3* gene is reported in various immunological disorders in human, reports on relevance of *Shc* mutations in these diseases are limited. Knock-out of the *shcA* gene results in embryonic lethality (Lai and Pawson 2000) in mice and *jak3* KO results in predisposition to colitis (Mishra et al. 2013a). Shc also regulates cell survival through expression of bcl-2/bcl-xl (Lord et al. 1998). Moreover, disruption in Jak3 interactions with Shc results in increased sensitivity towards staurosporin-induced apoptosis and compromises wound repair in human IEC in Shc domain dependent manner because in these cells, increased tyrosine phosphorylation of Jak3 leads to an increased content of severed actin filaments which could be due to activated Jak3-mediated sustained tyrosine phosphorylation-driven increased severing activities by cytoskeletal proteins (2004, Kumar et al. 2007; Mishra et al. 2012a). Overall review of this literature not only shows the characterization of Jak3 interaction with Shc, but also demonstrates the molecular mechanism of intracellular regulation of Jak3 deactivation in which Jak3 interactions with Shc acts as a regulator of Jak3 dephosphorylation through direct interactions of Shc with both Jak3 and tyrosine phosphatases. These experiments show that Jak3 auto-phosphorylation is the rate limiting step during Jak3 trans-phosphorylation of Shc (and/or villin/gelsolin). Jak3 directly phosphorylates two tyrosine residues in SH2-domain and one tyrosine residue each in CH-1, and PID domains of Shc. Direct interactions between mutants of Jak3 and Shc shows that while FERM domain of Jak3 is sufficient for binding to Shc, CH-1 and PID domains of Shc are responsible for binding to Jak3. Functionally, Jak3 is auto-phosphorylated under IL-2 stimulation in epithelial cells. However, Shc recruits tyrosine phosphatase SHP-2 and PTP-1B to Jak3 and thereby dephosphorylates Jak3 (Fig. 3).

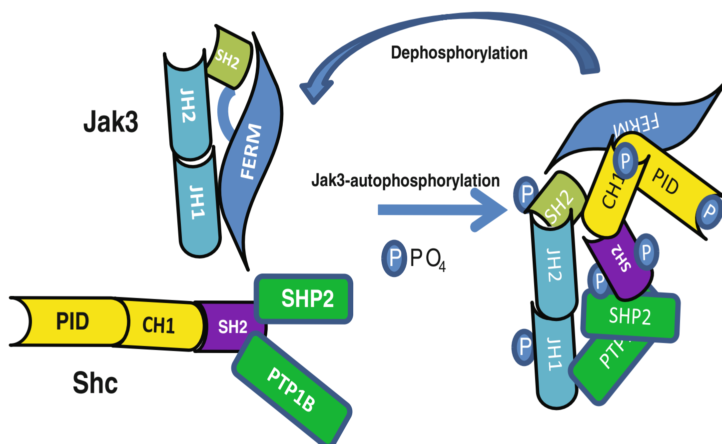

Fig. 3 SH2 domain regulates of Jak3 deactivation. While Jak3-autophosphorylation at the SH2 domain is necessary for its interactions with Shc, the SH2 domain of Shc in turn facilitates Jak3 interactions with tyrosine phosphatase SHP2 and PTP1B thereby de-phosphorylating Jak3

5 SH2 Domain as Drug Target

SH2 domains are small protein modules that mediate protein-protein interactions in signal transduction pathways that are activated by protein tyrosine kinases. They bind to short phosphotyrosine (pY)-containing sequences in growth factor receptors and other phosphoproteins (Schlessinger 1994). For example Stat3 (Signal transducer and activator of transcription 3) is constitutively activated in a number of human cancers and in many cancer cell lines. As mentioned above, in cancer cells, Stat3 participates in aberrant growth, survival, angiogenesis, and invasion signals and becomes a validated target for anticancer drug design. It transmits signals from growth factors and interleukin-6 family of cytokines by binding to their receptors via its SH2 domain. Through its SH2 domain, Stat3 is recruited to phosphotyrosine residues on intracellular domains of cytokine and growth factor receptors, whereupon it is phosphorylated on Tyr705, dimerizes, translocates to the nucleus and participates in the expression of various genes related to angiogenesis, metastasis, growth and survival (Mandal et al. 2013). SH2 domains present the paradigm of phosphotyrosine (pY) protein recognition modules and mediate numerous cancer-promoting protein-protein complexes. Effective SH2 domain mimicry with pY-binding coordination complexes offers a promising route to develop new and selective disruptors for pY-mediated protein-protein interactions (Drewry et al. 2012). SH2-containing inositol 5'-phosphatase proteins (SHIP2) are potential targets for type 2 diabetes. Their ability to dephosphorylate the lipid messenger phosphatidylinositol 3, 4, 5-trisphosphate [PtdIns (3, 4, 5) P3] is important for insulin signaling, thereby making these proteins important targets against type 2 diabetes (Saqib and Siddiqi 2011). Depending on the nature of neighboring protein

module(s), such as catalytic domains and other protein binding domains, SH2-containing proteins also play many roles in cellular protein tyrosine kinase (PTK) signaling (Machida and Mayer 2005). SH2 domain-containing protein Grb7 and the erbB2 receptor tyrosine kinases are over-expressed in certain subsets of human breast cancers and associate with each other in vitro (Janes et al. 1997). Ras GTPase-activating protein (GAP) contains two SH2 domains which are implicated in binding to tyrosine-phosphorylated sites on activated growth factor receptors and to a cytoplasmic tyrosine-phosphorylated protein, p62 (Marengere and Pawson 1992). Investigations of the peptide binding specificity of the SH2 domain of the Src kinase (Src SH2 domain) have defined the EEI motif C-terminal to the phosphotyrosine as the preferential binding sequence (Lubman and Waksman 2002). The structural and functional characterization of SH2 domains and their relationship to catalytic proteins (e.g., kinases, phosphatases, and lipases) or non-catalytic proteins (e.g., upstream adapters, and downstream transcription factors) has significantly impacted our understanding of signal transduction pathways and the identification of promising therapeutic targets for drug development. The ShcA adapter protein transmits activating signals downstream of receptor and cytoplasmic tyrosine kinases through the establishment of phosphotyrosine-dependent complexes. In this regard, ShcA possesses both a phosphotyrosine-binding domain (PTB) and SH2 domain, which bind phosphotyrosine residues in a sequence-specific manner (Ursini-Siegel et al. 2012). Modeling of the SH2 domain-peptidomimetic complexes is essential to better understand and design drugs for cancer treatment. Synthesis of a potential Src family SH2 domain inhibitor incorporating a 1,4-cis-enediol scaffold is reported (Dhanik et al. 2011). The synthetic route offers straightforward and highly selective access to the enediol and its associated chiral centers (Marian et al. 2011). SH2 domains and protein-tyrosine phosphatases expand alongside PTKs to coordinate cellular and organismal complexity in the evolution of the eukaryotes (Liu and Nash 2012). SH2-containing inositol 5-phosphatases 1 and 2 (SHIP1 and SHIP2) are capable of dephosphorylating the second messenger PtdIns (3, 4, 5) P3 (phosphatidylinositol 3,4,5-trisphosphate) and interacting with several signaling proteins (Giuriato et al. 2003). Some of the challenges are in formulation of the structural principles that govern peptide-binding specificity in SH2 domains (Gay et al. 1997). There are also reports on novel series of non-peptide ligands designed using a combined computational and NMR-driven approach that inhibit the growth factor receptor-bound protein 2 (Grb2) binding to SH2 domain. The lead compound has 1n (IC_{50}) = 56 μM), which is cytotoxic in HER2-positive breast cancer cells and disrupts the interaction between HER2 and Grb2. Thus, growth factor receptor-bound protein SH2 (Grb2-SH2) plays an important role in the oncogenic Ras signaling pathway, which involves cell proliferation and differentiation. Thus, the antagonist of Grb2-SH2 has become a potential target for developing anticancer agents. Recently, a peptide (Fmoc-Glu-Tyr-Aib-Asn-NH) was discovered with high affinity for the Grb2-SH2 domain by using surface plasmon resonance (SPR)-biosensor technology (Chen et al. 2010). Though SH2 domains provide fundamental recognition sites in tyrosine kinase-mediated signaling pathways which, when aberrant,

give rise to disease states such as cancer, diabetes, and immune deficiency, designing specific inhibitors that target the SH2 domain-binding site, however, has presented major challenges (Taylor et al. 2008). Thermodynamic and kinetic studies of bimolecular interactions give insight into specificity of molecular recognition processes and advance rational drug design. Binding of phosphotyrosine (pY)-containing peptides to Src- and Grb2-SH2 domains was investigated using a surface plasmon resonance (SPR)-based method (de Mol et al. 2005). In an effort to understand the mechanism of ligand binding and more specifically the role of water, a general computational model was developed based on the potential of mean force to compute the thermodynamics of water molecules at the protein-ligand interface for two SH2 domain complexes of the Src kinase, those bound to the two peptides Ac-PQpYEpYI-NH2 and Ac-PQpYIpYV-NH2 where pY indicates a phosphotyrosine (De Fabritiis et al. 2008). The structural basis of the SH2 domain diseases was elucidated based on the bioinformatic analysis (Lappalainen et al. 2008). The issue of specificity in tyrosine kinase intracellular signaling mediated by SH2 domains has great importance in the understanding how individual signals maintain their mutual exclusivity and affect downstream responses. Several proteins contain tandem SH2 domains that, on interacting with their ligands, provide a higher level of specificity than cannot be afforded by the interaction of a single SH2 domain (O'Brien et al. 2000). Since the interactions in the specificity determining region of SH2 domains are weak, single SH2 domains show only a modest level of specificity for tyrosine phosphorylated targets (Bradshaw and Waksman 2002). Various SH2 domains are selective for distinct phosphopeptides, and the function of a given SH2 domain is often dictated by the specific motifs that it recognizes. Therefore, deciphering the phosphotyrosyl peptide motif recognized by an SH2 domain is the key to understanding its cellular function (Huang et al. 2008).

6 Concluding Remarks

During the last couple of decades remarkable advances have been made in the field of cytokine signaling and functions of non-receptor tyrosine kinases. Starting with the elucidation of the major pathways of Jak3 signaling that culminated in the transfer of knowledge from basic science to introduction of new therapeutic, we have now come very close to a full circle in our thinking about the role of Jak3 in mediating various adaptive immune as well as innate immune functions. Currently, Jak3 inhibitors are widely used in the treatment of various immunological disorders including IBD, allergic asthma, transplant, and cancers. Previous study focused almost exclusively on the role of kinase domain in activation of Jak3 while role of other domains of Jak3, its activation and other physiological functions were not known. Limited understanding of the structure-function relationship of Jak3 has also led to several complications associated with the use of Jak3 inhibitor as drug. Studies from several groups including ours have demonstrated clearly the importance of other domains of Jak3 not only in its auto-activation but also in its

interactions with other proteins. Together, these interactions regulate various aspects of adaptive immune functions and gastrointestinal physiology. Phosphorylation of SH2 domain is an important regulator of the dynamic and functional properties of Jak3. Specifically, SH2 domain phosphorylation seems to be crucial for Jak3 activation and its effect on various epithelial functions such as epithelial restitution, cell-migration and cell-homeostasis. Besides, Jak3 interactions are also important for T-cell migration and homing. Generation and investigation of Jak3-KO mice as well as intensive observations and diagnosis in several human diseases have helped to delineate the signaling pathways that show the importance of SH2 domain. Recent studies have improved our understanding of the domains that play essential role in regulatory mechanisms that control the functions of these non-receptor tyrosine kinases, including the SH2 domains, tyrosine phosphorylation sites, and interactions with associated regulatory proteins. Though we have made substantial progress, there is long way to go before the realization of these domains as drug targets and development of rational drugs make these molecules available for the treatment of various chronic inflammatory diseases including IBD, diabetes, and cancers.

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Helical Assemblies and SH Domains

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Abstract Multiprotein assemblies are essential components of many biological processes. Response to bacterial and viral infection, protein synthesis, sorting and targeting to various organelles, degradation, shuttling between cellular compartments, signaling and communication are governed by numerous complexes composed of tens or even hundreds of proteins. In this chapter, molecular assemblies involved in these processes are described. The emphasis is made on proteins for which function depends on protein-protein interactions with involvement of SH domains and helical assemblies.

Keywords SH domain · Helical assembly · Protein conformation · Src-family · Protein repeats

1 Introduction

Multiprotein assemblies are essential components of many biological processes. Response to bacterial and viral infection, protein synthesis, sorting and targeting to various organelles, recycling, degradation, shuttling between cellular compartments, signaling and communication are governed by numerous complexes composed of tens or even hundreds of proteins. Assembly of multiprotein complexes depends on proteins that recognize specific motifs. Proteins motifs range from small peptides to domains of various folds, such as β-barrel of SH3, 8α/8β barrel of triose

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phosphate isomerase, helical toroids of ankyrin, Armadillo/Heat, leucine rich, and pumilio homology repeats, 6α/6α barrel of fucosyltransferase, and coiled coil. Helical assemblies and SH domains in spite of differences in structure recognize similar sequence motif; however, each type exhibits specificity toward bound atomic groups. In this chapter, molecular assemblies involved in cellular processes are described. The emphasis is made on proteins for which function depends on protein-protein interactions with involvement of SH domains and helical assemblies.

2 Structure of SH Domains and Helical Assemblies

SH3 domains consist of five to eight β-strands that form two antiparallel β-sheets or a barrel (Mayer 2001; Kurochkina and Guha 2014). SH2 domains comprise a β-sheet flanked by one α-helix on each side (Filippakopoulos et al. 2009).

Protein assemblies of ankyrins, ARM/HEAT, and tetratricopeptide (TPR) contain a repeating unit for which major part represents a pair of antiparallel α-helices. These repeating units are packed parallel to each other in a spiral or solenoid (Andrade et al. 2001a; Michaely et al. 2002). Ankyrin repeats form a left-handed whereas ARM and HEAT repeats a right-handed spiral. Ankyrin and HEAT repeat unit consists of two antiparallel α-helices, A and B (Andrade et al. 2001b). ARM repeat contains three α-helices H1, H2, and H3 in which H2 and H3 form an antiparallel interface and shorter H1 is almost perpendicular to H2-H3. Tetratricopeptide is a right-handed super-helix (Blatch et al. 1999; Allan and Ratajczak 2011; Kajander et al. 2007). Leucine-rich repeats (LRR) (Kobe and Deisenhofer 1996; Kajava 1998) and TIM-barrel (Wierenga 2001) contain one external row of alpha-helices and one internal row of beta-strands. Circular 6α/6α barrels have two rows of α-helices, one wrapped around another (Dong et al. 2012). Most of the repeats function as modules for binding other proteins (Smith 2004; De Wit et al. 2011). However, repeating character of structural motifs is property of many proteins including leucine zipper (O'Shea et al. 1991), myohemerithrin (Hendrickson and Ward 1997) and TIM-barrel (Wierenga 2001).

Structure of proteins is an assembly of secondary structure elements as compact globules or elongated rods. Specific interactions of secondary structure elements and domains driven by amino acid composition of secondary structure elements and domain interfaces to a large extent determines structure of proteins (Kurochkina 2008; Kurochkina and Choekyi 2011; Teramoto et al. 2014).

Amino acid composition of each secondary structure element contributes to the shape of the assembly since arrangement of edges of α-helices and β-sheets is critical for the direction of the assembly and depends on interactions of amino acids atomic groups (Kurochkina 2010). Intrinsic properties of α-helices are determinants of the curvature and chirality of the helical assemblies (Kurochkina and Iadarola 2015).

SH3/SH2 domains and helical assemblies as modules of protein-protein interactions and signaling are present in many proteins and protein complexes. Their specific interactions and shape complementarity allow to bring together multiple participants of signaling pathways.

3 Adaptor Protein Complexes

Assembly of multiprotein complexes occurs with participation of various adaptor proteins involved in recognition of specific motifs.

Development- and differentiation enhancing factor 1 (DDEF1) is GTPase-activating protein of the ADP ribosylation factors, regulators of endocytosis, vesicle trafficking, and cytoskeletal remodeling. DDEF1 contains ankyrin repeat, PH, and SH3 domains. Its SH3 domain binds to the adenomatous polyposis coli (APC) using SAMP (Serine, Alanine, Methionine, and Proline) motif. These two proteins are involved in regulation of cytoskeleton. APC is known for its tumor suppressor function, ability to associate with microtubules, and downregulate Wnt pathway through the formation of a large multiprotein complex involving Axin1, glycogen synthase kinase 3b and other proteins. APC contains Armadillo repeats domain followed by four 15-mer repeats and seven 20-mer repeats, some of which contain SAMP motif. Repeats show homology in their N-terminal region. SAMP sequence adapts polyproline II conformation and is one of sequences that deviate from the canonical PxxP SH3 binding motif. DDEF1 SH3 domain also interacts with the PxxP motif of focal adhesion kinase on the same surface. If we compare two complexes, DDEF1-SH3 with APC-SAMP1 and Axin1-RGS with APC-SAMP3 (Fig. 1a, b), we can see that amino acid residues of SAMP motif involved in complex formation differ in the two complexes although SAMP sequence is involved in both complexes (Kaieda et al. 2010/1emu 2rqt/). Another protein, with which APC interacts using these repeat sequences 15 or 20 residues long, is β-catenin. Targeting of β-catenin that is not in adherence junctions for degradation/recycling by ubiquitin-proteasome system requires assembly of a multiprotein complex comprising APC, glycogen synthase kinase–3b that phosphorylates β-catenin on serine, and adaptor protein Axin1 (Spink et al. 2001/1jpp/). Crystallographic structures of β-catenin ARM repeat with APC 15-mer repeat (Fig. 1c) and APC 20-mer repeat in phosphorylated/unphosphorylated state were determined (/1jpp 1v18 1t08/). Armadillo domain of APC also interacts with SH3 domain of guanine exchange factor Asef (Fig. 1d), which otherwise is autoinhibited by binding to Dbl homology domain intramolecularly. Asef forms an interface with H3 helices of ARM1–ARM4 and H1 helix of ARM3. Amino acid sequence of APC binding groove is highly conserved (Zhang et al. 2012/3nmx/).

β-catenin interaction via ARM repeats with a RHO/CDC42/RAC GTPase-activating protein RICS links cadherins to actin cytoskeleton and regulates synaptic plasticity. Large multiprotein complex includes RICS, β-catenin, N-cadherin, PSD-95, NMDA receptors and other proteins (Okabe et al. 2003).

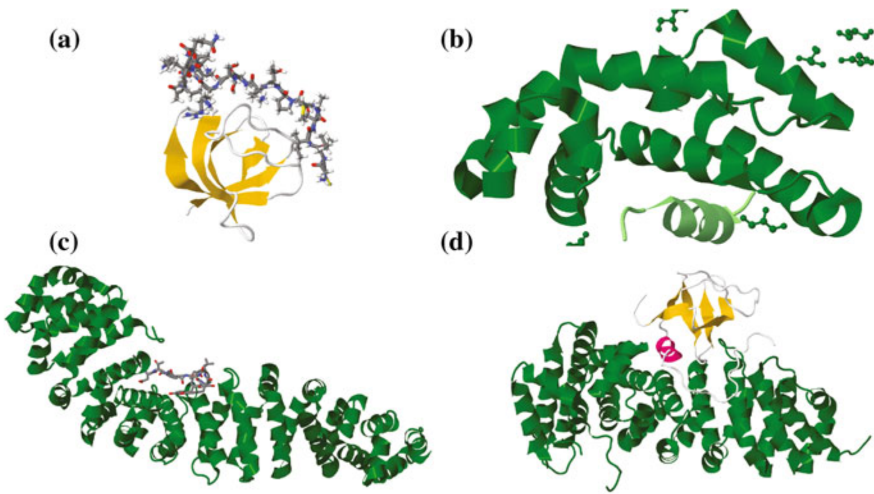

Fig. 1 Adaptor protein complex APC. **a** Complex between the DDEF1 SH3 domain and the APC SAMP1 motif/2rqu/. **b** Complex between the AXIN RGS-homologous domain and the APC SAMP3 motif/1emu/. **c** Complex between the β-catenin Armadillo domain and the APC 15-mer repeat sequence/1jpp/. **d** Complex between the SH3 domain and the APC Armadillo domain/3nm2/

Interaction of β-catenin with members of MAGUK (membrane associated guanylate kinases) family of proteins occurs through SH3 or PDZ domains and is important for assembly of signaling complexes (Dobrosotskaya and James 2000). Association of β-catenin with transcription factors, such as members of SOX family, plays important role in early embryonic development (Mou et al. 2009; Rudrabhatla et al. 2014). Sox proteins also associate with adaptor proteins and components of nuclear import machinery. Large multiprotein complexes that SOX proteins assemble at gene promoters and enhancers suggest their involvement in determining cell specificity (Wilson and Koopman 2002; Wegner 2010).

MAGUK proteins cluster and anchor glutamate receptors and other proteins at synapses. The MAGUK family of proteins includes PSD95, PSD93, SAP102, SAP97, essential components of postsynaptic density (PSD). They contain PDZ domains, a guanylate kinase (GK) domain, and an SH3 domain. Stabilization of SAP102 at the PSD depends on SH3/GK domain. Actin, a core skeletal component in spines, interacts with multiple proteins of PSD (Zheng et al. 2010). MAGUKs indirectly bind to actin via complexes SAP97—MyosinVI—actin, PSD-95—SPAR—actin, PSD-95—GKAP—Shank—cortactin—actin, and PSD-95—NMDAR—actinin—actin. Proteins of this family function at the sites of cell-cell contacts and include Discs-large protein (Dlg), PSD95, and SAP90. Dlg is important for regulation of cell polarity. Drosophila Dlg contains several protein interactions domains: SH3, PDZ, and a guanylate kinase homology domain. Deletion of SH3 domain does not affect expression pattern of Dlg but mutations or deletion of its carboxyl

terminus are important for its localization in the midbody during cytokinesis (Massimi et al. 2003).

Sorting of proteins to be targeted to lysosomes or endosomes occurs through coated areas of membranes and involves recognition of sorting signals on the cytoplasmic side of cargo integral membrane proteins and protein coats (Bonifacino and Traub 2003). Clathrin adaptor protein (AP) complexes play important role in the budding process of vesicular transport in which they regulate cargo sorting and vesicle formation (Nakatsu et al. 2014). Together with clathrin and other accessory molecules they assemble cargo at the donor membrane for the delivery and fusion to the acceptor membrane. Cargo sorting relies on specific recognition sequences, for example, tyrosine, dileucine, or short proline rich motifs (Ren and Hurley 2011). These motifs on the cargo protein selectively bind to the AP complexes. Some complexes (AP1A, AP2, AP3A, AP4, and AP5) are expressed ubiquitously, whereas other (AP1B and AP3B) in a tissue specific manner. Adaptor proteins 1 and 2 form a tetrameric core of two large subunits (α, β, γ, δ, ε, or ζ), one medium subunit (μ1-μ5) and one small subunit (σ). Both AP-2 (subunits α, β2, μ2, σ2) and AP-1 (γ, β1, μ1, σ1) are heterotetramers (Ren et al. 2014; Heldwein et al. 2004/1w63/).

AP2 σ2 subunit recognizes dileucine motifs of Nef, CD4, and other proteins (Fig. 2a–c). PI(4,5)P2-containing membranes induce AP-2 conformational change from locked to open conformation and increased affinity toward binding protein (Nef or CD4) (Jackson et al. 2012/2xa7/). PI(4,5)P2 binds to two sites on AP2, one on α and one on μ2 subunits; dileucine [ED]xxxL[L/I] cargo motif binds to σ2 subunit; tyrosine motif binds to the C-terminus of μ2 subunit. The membrane-bound AP-2:Nef complex reconstructed from all available data and its interaction with CD4 result in ternary complex that provides insight into mechanism of the regulation of CD4 by Nef (Ren et al. 2014/4nee/). Similarly, molecular detailed view of AP-1:Nef regulation of MHC class I emerges (Jung et al. 2011; Ren et al. 2013/4hmy 4en2/) (Fig. 2d).

Nef is HIV-1 accessory protein. Structure of Nef consists of the N-terminal polyproline type II (PPII) helix, two antiparallel α-helices and four antiparallel β-strands. It interacts with SH3 domain of Fyn and Hck by its conserved N-terminal polyproline sequence PxxP important for virus replication. R96I mutation in the RT loop of the Fyn SH3 domain increases affinity of binding. The mode of interaction of Nef PPII with Fyn SH3 domain is very similar to the mode of guanine exchange factor Sos peptide interaction with c-Crk SH3 or other peptides with SH3 domains of Sem5, Grb2, and Src (Lee et al. 1996/1efn/). N-terminal domain of Nef does not exhibit folded structure in non-myristoylated state (Geyer et al. 1999/1qa4 1qa5/).

Crystallographic structure of Nef complex with SH3 domain of Hck provides very good illustration of assembly of β-barrel of SH3 interacting with polyproline sequence in PPII conformation that is located N-terminal to α-helical domain of Nef (Fig. 6b). Nef also contains endocytic dileucine sorting motif at the C-terminus that interacts with cellular adaptor protein complex, mediator of internalization of cell surface receptors (/3rbb/ Horrenkamp et al. 2011).

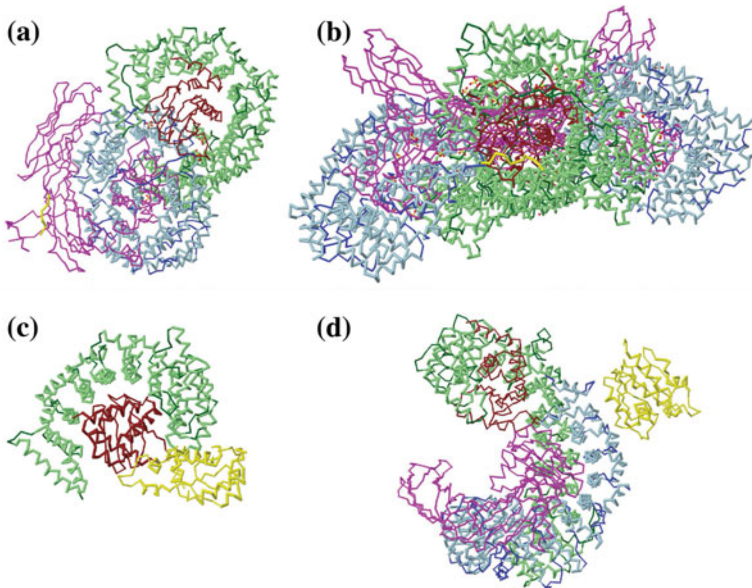

Fig. 2 Adaptor complexes (AP1 and AP2). Subunits α/γ (*green*), β (*blue*), μ (*magenta*) and σ (*brown*), and cargo (*yellow*). **a** AP2 subunit with bound TGN38 cargo peptide (EQKLI)/2xa7/. **b** AP -2 with bound CD4 cargo peptide/2jkr/. **c** AP -2 with bound Nef. **d** AP -1 with bound Arf1/4hmy/

VHS domains of the Golgi-localized γ-adaptin ear-containing ARF-binding proteins (GAG), monomeric clathrin adaptors, recognize cargo motifs and mediate the sorting of cargo at the trans-Golgi network and endosomes. Hinge domain binds clathrin whereas γ-adaptin ear (GAE) regions bind accessory proteins (Mattera et al. 2004). GGA1-VHS domain binds dileucime sorting cargo peptide in extended conformation (Fig. 2b) (Zhu et al. 2012; Misra et al. 2002; Jackson et al. 2012; Cramer et al. 2010/1yp1 2jkr 2xa7 3g2s 4hmy/).

Adaptor proteins are implicated in the process of the establishment and maintenance of cell polarity, asymmetric arrangement of organelles and cytoskeleton and differentiation of the plasma membrane into domains of distinct protein/lipid composition (Bonifacino 2014).

Clathin mediated endocytosis is characterized by the formation of tubules and membrane vesicles. Cdc42 influences clathin mediated endocytosis by regulation of the formation and activity of Toca1/N-Wasp complex. Coexpression of Toca1/N-Wasp leads to induction of motile membrane vesicles. Cdc42, Toca1 and N-Wasp associate as a complex on tubules and membrane vesicles. Tubulation involves deformation of lipid vesicles. F-BAR domain mediates membrane deformation activity. FBP17, CIP4, and their F-BAR domains tubulate membranes. Toca1 or its F-BAR domain alone does not tubulate membranes. Toca1

coexpressed with Toca1 N-Wasp tubulate membranes and leads to induction of motile membrane vehicles (Bu et al. 2010).

Various types of helical assemblies participate in cargo recognition process. Kinesins, motor proteins that transport cargo molecules along microtubules, are able to recognize over forty different molecules. The kinesin heavy chain contain three domains: the N-terminal motor domain that binds ATP and microtubules, central coiled coil involved in dimerization, and C-terminal regulatory domain. The kinesin light chain also carries three domains. The N-terminal coiled coil domain binds to the heavy chain whereas two C-terminal domains, tetratricopeptide helical assembly and C-terminal tail, bind cargo molecules (Zhu et al. 2012/3nf1/).

Helical assemblies and SH domains in spite of differences in structure recognize very similar sequence motifs. Ankyrin repeat proteins and SH3 domains bind sequence motif that contains prolines. Ankyrins, with a repeating unit of two anti-parallel α-helices stacked parallel to each other to form a solenoid, bind peptides using several consecutive repeats. Binding of a peptide by inner surface of ankyrin repeats was studied for several proteins. Protein complexes of ankyrin repeats protein 2 (ANKRA2) with HDAC4, HDAC5, HDAC9, megalin, and regulatory factor 5 (RXF5) (Xu et al. 2012/3v2o/), show that ANKRA2 and RFXANK interaction with proteins occurs via common sequence these target proteins share, PSLPxI, in spite of their different structure and function. However, precise arrangement of interacting atomic groups is unique for each type (Xu et al. 2012).

ArfGAP with SH3 domain, ankyrin repeat and PH domain protein, ASAP, regulate vesicular transport and sorting. ASAP1 in the Golgi, is important component of a multiprotein complex compising polycystin 1, ciliary-targeting signal, several GTPases and GTPase-activating protein. ASAP1 stimulates vesicle budding and Golgi exocytosis (Ward et al. 2011; Li et al. 2012). ASAP2 is involved in regulation of cellular migration and autophagy. Surrounded by conserved binding sites of the transcription factor CTCF, it participates in chromatin remodeling. The region around the ASAP2 gene contains binding sites of vitamin D receptor (Seuter et al. 2014). ASAP3 regulates cancer cell migration and invasion. Its modulation of expression of γ-actin-1 affects cytoskeletal reorganization (Luo et al. 2014).

Movement of proteins between cellular compartments involves specific recognition motifs, SH2/SH3 domains and helical structures. Btk shuttles between cytoplasm and nucleus. Two important phosphotyrosines, one in kinase domain and another in SH3 domain, regulate the kinase activity. Ankyrin repeat domain 54 protein (ANKRD54), or Lyn-interacting ankyrin repeats protein, interacts with Btk in SH3-dependent manner and facilitates Btk shuttling to cytoplasm. Similarly, ANKRD54 modulates localization of another tyrosine kinase of Tec family, Txk/Rlk, but not other nucleocytoplasmic proteins, such as Abl. Its ankyrin repeats were found to interact with SH3 domains of Lyn, HS1, ESE2L, Vav1, Hip55, and LASP1. One possible explanation of the mechanism is that similarly to ankyrin repeats protein IκBα, masking the NF-κB NLS sequence and leading the NF-κB to the cytoplasm, ANKRD54 NES is automasked in the unbound state but exposed in complex with Btk leading both protein out of the nucleus (Gustafsson et al. 2012).

4 Apoptosis

Highly specific interactions of proteins regulate process of apoptosis.

Apoptosis-stimulating protein of p53, ASPP, contains 4 ankyrin repeats and SH3 domain that mediate its interactions with apoptosis-related proteins p53, Bcl2, and NFκB. Three members were identified: ASPP1, ASPP2, and iASPP. ASPP1 and ASPP2 promote apoptosis whereas iASPP inhibits it (Benyamini and Friedler 2010; Liu et al. 2014). For iASPP, proposed mechanism suggests that it may induce apoptosis by blocking NFκB or inhibit apoptosis by blocking p53 (Pinto et al. 2010).

Crystallographic structure of the complex between ankyrin and SH3 domains of ASPP2, or 53BP2, and core domain of p53, p53CD, shows that both domains of ASPP2 interact with P53 (Gorina and Pavletich 1996/1ycs/). Proline rich domain of ASPP2 compete for the same binding site on ASPP2 Ank-SH3 (Rotem-Bamberger et al. 2013). Binding sites of ASPP2 to p53CD, Bcl-2, and NFκB are located on the same side of the molecule but differ in the involvement of specific groups (Benyamini and Friedler 2010). ASPP2 binds p53 and p73 DNA binding domain (Fig. 3) and cytotoxin associated gene. P53 proteins are recognized as atypical SH3-binding molecules: they interact with SH3 domain not as extended chain but via two separate loops, L3 loop segments (Canning et al. 2012/2ocj 2xwc/; Nešić et al. 2014/4irv/) (Fig. 3). ASPP2 regulates early embryonic growth and by modulating cell growth and apoptosis through interaction with IRS-1 and IRS-2 influences organ size. SH3 and ankyrin domains are conserved among ASPP2 family indicating that interactions with p53 play important role in its function (Liu et al. 2014). Stat1/ASPP2 pathway shows involvement of ASPP2 in the cellular response to inflammation and may link tumor suppression and cell polarity to neuroinflammation (Tordella et al. 2013; Turnquist et al. 2014). Crystallographic structure of the ankyrin repeats and SH3 domains of iASPP was determined (Robinson et al. 2008/2vge/). A module that consists of ankyrin repeats domain followed by SH3 domain is also predicted to be part of CASKIN1 and CASKIN2 proteins (Tabuchi et al. 2002).

5 Ubiquitination

The suppressor of cytokine signaling (SOCS) mediates protein polyubiquitination and degradation by proteosome 26S. Its 40-amino acid residue motif called SOCS box is an essential module of many intracellular signaling molecules. SOCS proteins recognize substrates and assemble a functional complex that comprises adaptor proteins (elongins B and C, Rbx2) and scaffold proteins (Cullin5). SOCS1-7 act on E3 ubiquitin ligase pathway (Linossi and Nicholson 2012). SOCS1-3 and cytokine-inducible SH2 protein regulate cytokines receptor signaling via JAK-STAT pathway; SOCS4-7 regulate receptor tyrosine kinase signaling with

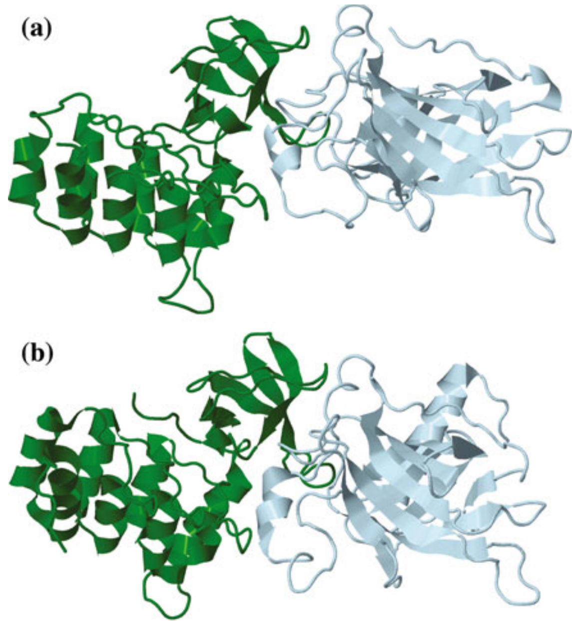

Fig. 3 Ankyrin and SH3 domains of ASPP2. **a** Complex with p53/1ycs/; **b** Complex with p73/4a63/. ASPP2 (*left*); p53 and p73 (*right*)

SH2 domain interacting phosphotyrosine of their target (Trengove and Ward 2013). N-terminal domain of SOCS–SH2, SPRY, WD40, Ankyrin, GTPase, Leucine repeats, or β-domain—serves as specific recognition module (Fig. 4). SOCS box domain is 40–60 residues long and is disordered in unbound form; its structure is determined in complexes including one with cullin and elongin (Babon et al. 2008/2jz3/). N-terminal α-helix of SH2 domain, extended SH2 domain (ESS), stabilizes phosphotyrosine binding loop (Bullock et al. 2006; Babon et al. 2006). SH2 domain containing SOCS proteins regulate important cytokines, such as leukemia inhibitory factor (LIF) and interleukin 6 (SOCS3), IRS and PI3 K (SOCS6-7), and T-cell specific Src family kinase Lck (SOCS6), leading to inhibition of T-cell receptor signaling. Inactivation of SOCS3 may result in human hepatocellular carcinoma progression (Calvisi et al. 2006).

6 Carbohydrate Processing

Extracellular matrix contains glycosaminoglycan (GAGs), storage of sugars. Carbohydrate components of GAGs are repeating disaccharide units. Many microorganisms degrade GAG using GAG lyases: chondroitinases, hyaloluronidases, and heparinases. Polysaccharide lyases are subdivided into 22 families PL1-PL22. Differences in structure are observed for many members; for example, parallel β-helix fold dominates in members of PL-6 family whereas 6α/6α and

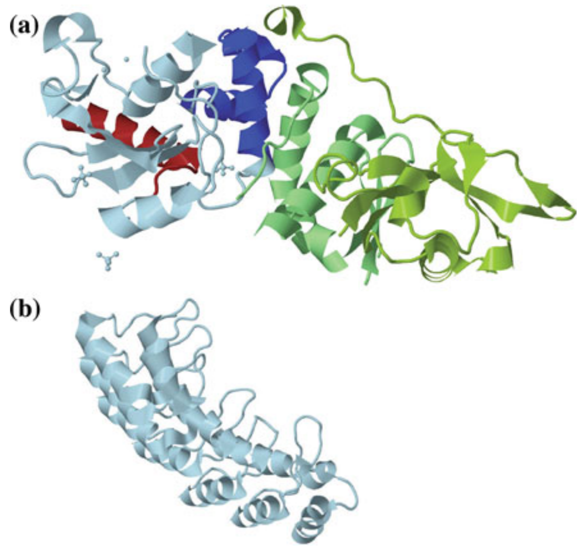

Fig. 4 SOCS box proteins. **a** SOCS2 in complex with elonginB and elonginC/2c9w/; SOCS box (*blue*), extended SH2 (*brown*), elonginB and elonginC (*green*). **b** Ankyrin domain of SOCS box protein ASB11/4uuc/

antiparallel β-sheet domains in members of PL-8 family. Chonroitinase ABC are enzymes that are used for therapies of spinal cord injuries due to their ability to dissolve glial scars that prevent regrowth and repairs (Huang et al. 2003/1hn0/; Shaya et al. 2008/2q1f/). Chondroitinase attached to SH3 domain is a novel system for controlled protein release and delivery (Pakulska et al. 2013).

Heparin lyases, Hep I, II, and III, hydrolyze 1,4-glycoside bond between uronate and amino sugar. Structure of Hep II and Hep III comprises 6α/6α barrel and antiparallel β-sheet. Cleft between these two domains hosts an active site (Fig. 5).

Heparinase II, a heparin degrading lyase, hydrolyzes oligosaccharide chain. Its structure in complex with disaccharide product shows that disaccharide binds to three consecutive repeats of the N-terminal α-helical domain of the enzyme, mainly inner row helices of the toroid (Shaya et al. 2006/2fut 2fuq/).

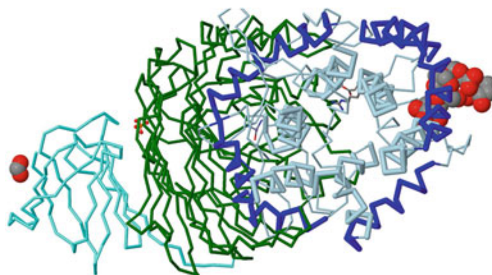

Fig. 5 Binding of saccharides by helical assemblies. **a** Heparinase II in compex with disaccharide product. Three domains of Heparinase II are N-terminal helical toroid domain, inner (*light blue*) and outer (*blue*) rows, central domain (*green*), and C-terminal domain (*cyan*); disaccharide (cpk)

Heparinase III is specific for heparan sulfate (Hashimoto et al. 2014/4mmi 4mmh/). Heparinases II and III are similar in the three-dimensional structure and the active site (Dong et al. 2012/4fnv/).

Phosphorylation and glycosylation are posttranslational modifications that regulate activity and signal transduction. Similarly to attachment and removal of a phosphogroup by kinase and phophatase, *O*-GlcNAc is attached or removed by *O*-GlcNAc transferase or *O*-GlcNAcase. Sugar donor, UDP- GlcNAc, is one of the major products of the glucose metabolism. Reversible *O*-GlcNAc modifications along with tyrosine and serine/threonine phosphorylation were proposed to carry important role in IRS-1 and IRS-2 receptors signaling (Ball et al. 2006).

FUT glycosyltransferases catalyse synthesis of fucosylated glycoconjugates (De Vries et al. 2001). The α1,6-fucosytransferase, FUT8, catalyses the reaction of the transfer of GDP-fucose to oligosaccharides attached to proteins, lipids, and sugars. FUT8b transfers fucose on α1-6 on the GlcNAc-containing acceptor (Javaud et al. 2000). FUT8 overall structure does not show high similarity to known proteins; however, each of its domains that include coiled coil, proline rich region with SH3 binding motif, Rossmann fold, and SH3 domain is similar to one of known proteins. Catalytic domain resembles glycosyltransferases GT-A and GT-B. SH3 domain also carries many features of other SH3 domains and might be responsible for binding to substrate glycoproteins and regulation (Ihara et al. 2007; Brzezinski et al. 2012/2de0 3six 3siw/). Activity of FUT8 is essential for growth and regulates binding of EGF to its receptor (Wang et al. 2006; Liu et al. 2011).

Sonic Hedgehog (Shh), well known morphogen, plays an important role in embryonic development and production of stem cells in adults. Hh ligand, Shh binds several receptors: Ptch1, Ptch2, CDO, BOC, Gas1, HSPGs. The glycosaminoglycans attached to core proteins (perlecan, glypican 3, glypican 5 in vertebrates and Dally, Dally-like proteins in invertebrates) are important for Hh signaling. Heparan sulfates consist of repeating disaccharide units of *N*-acetyl-d-glucosamine (GlcNAc) and d-glucuronic acid (GlcUA). Heparan sulfate proteoglycans promote Shh signaling and neural precursor cell proliferation (Witt 2013). The complex of ankyrin repeats protein ANKMY2 and tetratricopeptide protein FKBP38 regulate Shh activity (Saita et al. 2014). Involvement of PI3 K in Shh signaling presents many therapeutic opportunities (Gruber-Filbin et al. 2013). Ptch1 contains many SH2 and SH3 binding sequences, potential sites of interaction with c-Src, and tyrosine phosphorylation sites; this interaction is inhibited by Hh binding and plays an important role in Shh signaling (Harvey et al. 2014).

LysBP13, a putative endolysin found in the genome of bacteriophage BPS13 and classified as N-acetylmuramyl-L-alanine amidase, contains a catalytic domain and a cell wall binding SH3 domain. Ability to cleave bond between N-acetylmuramic acid and L-alanine makes endolysin valuable antimicrobial agent carrying potential to act against food-borne pathogens including emerging antibiotic-resistant strains (Park et al. 2012).

Interesting example of regulation by glycosylation is Notch. The Notch protein contains an extracellular ligand binding domain of 36 EGF-like repeats subject to *O*-glucosylation and *O*-fucosylation, negative regulatory region, transmembrane

domain, and intracellular region of RAM, two nuclear localization signals, seven ankyrin repeats, transcription activation domain, and proline, glutamic acid, serine, threoine rich sequence PEST (Rana and Haltiwanger 2011; Okajima and Irvine 2002).

7 Bacterial and Viral Assemblies

Bacterial pathogens, such as *Salmonella enterica*, major cause of food poisoning and typhoid fever, or *Borrelia Burgdorferi*, cause of Lyme disease, act through a multiprotein assembly of more than 20 proteins (type III secretion system, TTSS) to inject bacterial proteins into host eukaryotic cell. Major part of this assembly is a needle complex that comprises envelope-associated base and needle projected from bacterial surface and linked to the base by an inner rod. Timing of the assembly-dependent processes is important in the establishment of the hierarchical organization of the secretion process.

Effector proteins that target Rho GTPases are key molecules in this process: effectors SopB, SopE, SptP and Rho GTPases Cdc42 and Rac1. SopB activation of SH3-containing guanine nucleotide exchange factor for RhoG, for example, regulates cellular responses and contributes to actin cytoskeleton remodeling. SptP reverses cytoskeleton reorganization. The activation of Rho family GTPases turns on MAPK signaling pathway and results in activation of transcription machinery and altered gene expression in the host cell (Buchwald et al. 2002; Burkinshaw et al. 2012; Patel and Galán 2006; Lefebre and Galán 2014; Zhang et al. 2011; Zhang et al. 2013; Zhao et al. 2013/1gzs 2jow 4did/).

Binding of GTPase Rac to $p67^{phox}$, a component of a mutiprotein enzyme complex NADPH oxidase that generates superoxide ions in response to pathogen infection, results in the assembly of active complex comprising four cytosolic (Rac, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$) and two membrane bound ($gp91^{phox}$, $p22^{phox}$) proteins. SH3 domains of $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ bind to proline rich sequences of $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$. The $p67^{phox}$ consists of four TPR repeats, activation domain, proline rich domain, and two SH3 domains. RAC/GTP binds to the N-terminal domain of $p67^{phox}$ between two TPR motifs. The right-handed super-helical assembly of nine α-helices, or four complete TPR repeating units and one C-terminal helix C, forms a groove on the inner surface of the assembly. The 18-residue fragment C-terminal to C helix, last helix of the TPR toroid, binds to this mostly hydrophobic groove in extended conformation. The C-terminal peptide bound to the inner groove contributes to the binding site of GTPase. Together with an insertion between TPR3 and TPR4, which comprises two antiparallel β-strands and a 3_{10} helix, it forms an extensive network of polar and nonpolar interactions with GTPase. Mutations at this site as well as in TPR repeats distant from this site influence binding. Cdc42 and Rac show amino acid sequence similarities at the site of binding to $p67^{phox}$. However, Cdc42 does not bind to $p67^{phox}$ unless few key residues are mutated (Lapouge et al. 2000/1e96/).

Flaviviruses, such as mosquito-borne Dengue, West Nile, and Yellow Fever viruses, are pathogens that contain single-stranded RNA genome which is translated to a single polyprotein and then processed to virion and nonstructural proteins (Padmanabhan and Strongin 2010). The hepatitis C virus (HCV), RNA virus responsible for liver damage, is an assembly of three structural and seven non-structural proteins. Nonstructural proteins NS3, NS4A, NS4B, NS5A, and NS5B are important components of the replication process. The largest and most conserved protein, NS5, consists of three domains: MTase, importin-binding and NS3-interacting site, and C-terminal domain of RNA-dependent RNA polymerase (RdRp) (David et al. 2014; Yap et al. 2007/2j7u/). NS5A interacts with SH3 domains of the Src family of tyrosine kinases, such as Hck, Lyn, Lck, and Fyn, and adaptor proteins, such as Grb2, amphiphysin II, PI3 K p85 subunit, and Bin1. Three proline rich motifs in NS5A were identified as potential sites of interactions with SH3 domains. Crystal structures of the c-Src-SH3 domain alone and in complex with one of these proline rich sequences show major conformational changes in RT loop upon peptide binding (pdb codes/4jz4 4qt7/) (Fig. 6a). This interaction may be important for the NS5B/NS5A/c-Src tyrosine kinase binding that is necessary for virus replication (Bacarizo et al. 2015). NS5A comprises N-terminal helix that attaches protein to the endoplasmic reticular membrane, conserved zinc- and RNA-binding domain and less conserved C-terminal domains with two PPII sequences interacting with SH3 domains (Feuerstein et al. 2012).

Another example demonstrates that mutations of the SH3-binding domain of HBv genome affects virus replication (Li et al. 2010).

Replication complexes of viruses are characterized by extensive network of interactions between viral and host proteins. Particularly important are interactions between NS4B and NS5A in which NS5A exhibits several phosphorylation steps. Mutations leading to reduction of the amount of hyperphosphorylated NS5A or inhibition of NS5A hyperphosphorylation by kinase inhibitors result in increased viral replication (David et al. 2014). Interactions of adaptor protein Grb2 SH2 and SH3 domains were shown to be important for HIV-1 and marine leukemia virus entry (Chen et al. 2011).

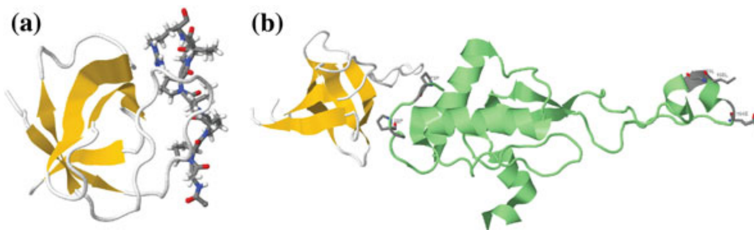

Fig. 6 Assembly of components. **a** c-Src SH3 domain in complex with PPII of Hepatitis C virus. **b** Hck SH3 domain in complex with PPII of HIV-1 Nef domain/3rbb/. SH3 domain (*ribbons*); HCV peptide in PPII, HIV-1 Nef polyproline sequence in PPII, and dileucine motif (cpk)

Protein tyrosine phosphorylation is not unique to animals and can be found in simple life forms (Zhao and Zhao 2014). Genome of unicellular protist *Monosiga brevicollis*, for example, contains tyrosine phosphatase (PTP) with two SH2 domains just like in animals.

8 Channels

Transient receptor potential (TRP) channels are cation channels that form a large family of receptors activated in response to noxious stimuli. They contain six transmembrane segments hosting a cation pore flanked by cytoplasmic N-terminal ankyrin repeats domain and C-terminal tail involved in the regulation of a channel function. TRPs are subdivided into six subfamilies: TRPC, TRPV, TRPM, TRPP, TRPML, and TRPA. Ankyrin repeats domain length varies: 4 repeats in TRPC, 6 in TRPV, 14 to 18 in TRPA. N-terminal short sequence stretches with polyproline sites are subject to regulation by SH3 domains. In TRPV4, the region preceding ankyrin repeats is SH3 domain binding site. TRPV2 region N-terminal to ankyrin repeats domain may also contain SH3 specific sequence. The N-terminus of TRPV6, 40 amino acids with no similarity to any known sequence, contains nine prolines and is proposed to interact with SH3 domains. TRPV4 proline rich region binds SH3 domain of PACSIN family of proteins which carries BAR domain and is involved in endocytosis, plasma membrane remodeling, cell morphology, motility, and neurotransmission. PACSIN3 regulates TRPV4. N-terminal region of TRPM2 20 amino acids long also contains two PxxP SH3 binding motifs and deletion of this stretch of amino acids results in a dysfunctional channel. N-terminal domain of TRPA including ankyrin repeats is predominantly the site of response to stimuli and modulation by calcium. Besides, tyrosine phosphorylation of ankyrin repeats by Src kinase is observed (Xu et al. 2003; Cuajungco et al. 2006; D'Hoedt et al. 2008; Nillius and Flockerzi 2014; Cordero-Morales et al. 2011; Jin et al. 2006/2eta 2etb/). Phosphorylation and tetramerization are important regulatory mechanisms of TRPV channels (Pareek et al. 2006; Kaszas et al. 2012). Crystal structures of TRPV1 conformations in two activated states were determined. In TRPV1, as in voltage gated ion channels, transmembrane segments S5 and S6 align ion permeation route, the central pore, and are surrounded by S1–S4, voltage sensors. However, segments S1-S4 of TRPV1 in apo and activated state remain static in contrast to these segments of VGICs (Liao et al. 2013; Cao et al. 2013). Structure of TRPV1 ankyrin repeats provides details of channel ATP and calmodulin binding (Fig. 7) (Lishko et al. 2007/2nyj, 2pnn/). Crystal structures of ankyrin repeats domain of TRPV2, TRPV4, and TRPV6 were determined (Jin et al. 2006; McCleverty et al. 2006; Landouré et al. 2010; Phelps et al. 2011/2eta, 2etb 4tzi 2f37 3jxj 2rfa/).

TRPV1 region N-terminal to ankyrin repeats domain contains binding site for synaptotagmin and snapin of SNARE, soluble NSF attachment protein receptor (Morenilla-Palao et al. 2004). SNAP (soluble NSF attachment protein) is an adaptor protein between SNARE and NSF (N-ethylmaleimide-sensitive factor). The yeast

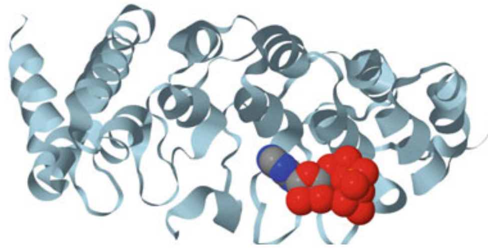

Fig. 7 Ankyrin repeats domain of TRPV1 in complex with ATP. TRPV1 (*ribbon*); ATP (cpk)

homolog of α-snap, Sec17, has an N-terminal α-helical hairpin, similar to tetratricopeptide but exhibiting different twist, and C-terminal four-helix bundle (Rice and Brunger 1999/1qqe/). Crystal structures of SNARE complexes reveal coiled coil domains that bring together multiple interaction domains (Hu et al. 2007, 2011 2pjx 3puj). SNARE domains of Syntaxin-1, SNAP-25 and VAMP-2 form a neuronal SNARE complex that is important for the fusion of vesicles, carrying neurotransmitter and exiting cell via exocytosis, with presynaptic membrane. Syntaxin-1 also interacts with actin, Munc18, Tomosyn, ion channels, CDCRel, and Abi1 and regulates many cellular functions. Abi1 is Abl tyrosine kinase interactor protein in the tips of lamellipodia and filopodia that contains SH3 and SNARE domains, shuttles between cytoplasm and nucleus, and regulates actin dynamics (Echarri et al. 2004; Messa et al. 2010).

Endocytosis requires interaction in timely manner of 20 or more proteins including coat protein clathrin, clathrin assembly protein AP2, phosphoinositide phosphatase synaptojanins, SH3 domain proteins amphiphysins, accessory proteins AP180 and auxilin, EPS15, EPS15r, and dynamin. Polyproline sequence of dynamin interacts with multiple SH3 domains and contributes to the assembly of the components. Calcium triggered exocytic fusion of synaptic vehicles with the presynaptic plasma membrane and consequent endocytosis and recycling are coupled. EH domain/SH3 domain containing protein, EHSH1, has two N-terminal EH domains and five C-terminal SH3 domains joined by a central charged coiled coil domain. The third SH3 domain is alternatively spliced. EHSH1 binds to SNAP23 and SNAP25 and several molecules of dynamin at the same time. Dynamin binds to three of the five domains of EHSH1. Similarly to amphiphysin, EHSH1 stimulates clustering of dynamin modules since each SH3 domain binds one dynamin molecule but amphiphysin with its only one SH3 domain creates multimers. High concentration of dynamin at plasma membrane is necessary for ring formation around caveolar necks. EHSH1 and its isoform EHSH2, also called intersectins, are proteins that provide the link between endocytosis and exocytosis. Another protein, synaptotagmin, acts as calcium sensor in exocytosis and facilitator of AP2 regulated clathrin assembly in endocytosis. (Okamoto et al. 1999; Predescu et al. 2003). Assembly of proteins at Golgi membranes involves association via specific interactions domains. Proline rich domain of dynamin II interacts with SH3 domain of

syndapin II isoform that colocalizes with syntaxin 6. Syndapin-dynamin complex mediates vehicle formation in the Golgi network (Kessels et al. 2006).

TrpC4α regulation by PLCβ1b and SH3/ankyrin repeats protein 3 (SHANK3) at the sarcolemma is associated with cardiomyocyte hypertrophy (Cooley et al. 2014).

Nerve injury leads to increased expression of ion channels, receptors and neuropeptides, activation of Src-family kinases in hyperactive microglia and subsequent phosphorylation. Not only primary sensory neurons but also glial cells play a role in hyperalgesia and allodynia associated with injury. Src expressed in spinal cord contributes to the development of inflammatory pain. Activation of Src-family kinase, such as Src, Lyn, and Lck, results in increased ERK phosphorylation in spinal microglia and development of hypersensitivity. TRPV1 and TRPA1 are activated in response to noxious stimuli. TRPA1 and TRPV1 were shown to be upregulated in response to damage. Src-family kinases were not shown to influence expression levels of TRPV1/TRPA1 but possibly activate receptors by phosphorylation. Therefore, inhibition of this activation in Src/ERK pathway can provide new candidates for treatments of neuropathic pain (Katsura et al. 2006).

Huntington's disease is associated with impairment of the gene Huntingtin (Htt) caused by expansion of coding for glutamine repeats. Huntingtin interacting protein 14 (HIP14) contains ankyrin repeats domain for which substrate recognition function is proposed. Adaptor protein Grb2 that contains SH2 domain and two SH3 domains also interacts with Htt. C-terminal SH3 domain of Grb2 interacts with mutant form of Htt and affects its interaction with Grb2-Sos1-Gab1 complex that involves Ras-MAPK signaling pathway (Gao et al. 2009/3eu9/ Baksi et al. 2014).

9 Postsynaptic Density

Shank proteins, Shank1, Shank2, and Shank3, contain protein-protein interactions domains SH3, PDZ, ankyrin repeats, SAM, and proline rich sequences. These scaffold proteins bind cytoplasmic and membrane proteins such as G-protein coupled receptors, postsynaptic density proteins, spectrins, and fodrins, and mediate cellular signals. Alternative splicing of Shank is subject to regulation and its isoforms show different domain composition: some of Shank2 splice variants do not contain SH3 domain; many interdomain sites contain short sequence inserts and alternative start or stop codons. Splice variant of Shank2 that lacks SH3 and ankyrin domains interacts via polyproline region with SH3 domain of cortactin, a substrate of Src tyrosine kinase and an F-actin-binding protein. Shank3 N-terminal ankyrin repeats domain binds α-fodrin, SH3 domain interacts with PLCβ1b, PDZ domain binds Ret, and proline rich sequence binds Homer and cortactin. Splice variant specific sequences of PLCβ, one in PLCβ1a and another in PLCβ1b, were shown to mediate selectivity of binding. Each of Shank proteins shows unique pattern of tissue distribution. Extensive network of SHANK interactions links together NMDAR, mGluR, and AMPAR, major postsynaptic receptors. Although function of alternative splicing of Shank proteins is not yet well studied, its important role in

brain development is proposed (Sheng and Kim 2000; Grubb et al. 2011). Molecular morphogens, such as Shank, Homer, and SPAR, regulate organization of dendritic spines, which exhibit large variety of shapes. Many environmental, developmental, pathological, and hormonal factors contribute to shaping of spines and shape change responses (Ehlers 2002; Lim et al. 1999). Members of the SSTRIP/Shank/ProSAP1/CortBP1 family possess common domain architecture (Kreienkamp et al. 2000; Rubini et al. 2009; Haeckel et al. 2008). Disc-shaped post synaptic density (PSD) is an assembly of more than a hundred of different proteins on the postsynaptic side of the synapses that adapt composition and structure as a result of neuronal activity. PSD Shank3 was proposed to assemble this huge complex of proteins at the PSD in the presence of zinc. Structure of the assembly reveals dimensions of the disk that is 40 to 50 nm thick and 500 nm wide. Crystal structure of PSD Shank that forms helical fibers packed in a sheet was determined. Mutations at the interfiber interface can solubilize the protein (Baron et al. 2006).

Shank3 is one of proteins involved in abnormalities associated with autism spectrum disordered (Roberts et al. 2014). ASD is associated with mutations in many genes including PSD95, neurexin, neurologin, synaptin, cadherin, cortactin, gamma-aminobutyric acid receptor and glutamate receptor (Chen et al. 2014).

Src, Fyn and N-methyl-D-aspartate receptor (NMDAR) are localized in PSD. NMDAR subunits NR2A and NR2B are tyrosine phosphorylated. Ability of phosphotyrosines to bind SH2 domains contribute to the assembly of PSD signaling complexes. Src and Fyn regulate NMDAR ion channel and induce long term potentiation (LTP) (Takagi et al. 1999).

PSD95 consists of three PDZ, SH3 and guanylate kinase (GK) domains. Crystallographic structures of SH3 and GK domains were determined (Fig. 8). Binding of phosphatidylinositol-3-kinase C2α (PI(3)KC2α) to the SH3 domain of PSD95 is associated with inflammatory response. Mutation at the PxxP binding site to SH3 domain that disrupts PSD95/PI(3)KC2α interaction or use of PI(3)KC2α inhibitor abrogates inflammatory pain sensitization but does not affect other forms of neuronal plasticity: peripheral nerve injury and LTP (Arbuckle et al. 2010). Another group of proteins in PSD is family of end-binding (EB) proteins which regulate interactions between the ends of protein complexes, microtubules and organelle and are important for microtubules stability. Three members are identified. Mutations in the EB3 polyproline sequence impair EB3 binding to PSD95. EB3 interaction with SH3 domain of PSD95 affects microtubule organization and

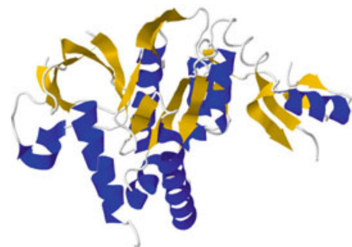

Fig. 8 PSD95. SH3 and guanilate kinase domains

leads to decreased dendrite branching in developing hyppocampal neurons (Sweet et al. 2011).

10 Summary

SH domains and helical assemblies are important modules of protein protein interactions, each exhibiting unique geometry and recognizing specific ligands. They bring together multiple modules of signal transduction complexes that regulate cellular pathways. Their organization shapes the way proteins interact with each other, nucleus acids, carbohydrates, and lipids and provide basis for highly precise nature of structure and function of living organisms.

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