Versatility of SH3 Domains in the Cellular Machinery

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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

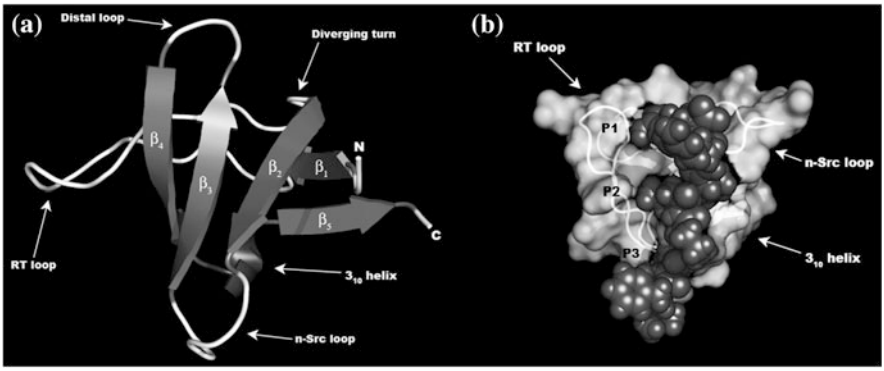

Fig. 1 Structure of an SH3 domain. **a** Tridimensional representation of the SH3 domain of α-spectrin (PDB entry: 1SHG) showing the main structural elements. **b** Representation of the 1:1 complex formed by the Src-SH3 domain and the PLR-1 nonapeptide, AFAPPLPRR, (PDB entry: 1PRM) showing the three 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 heterotrimeric 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

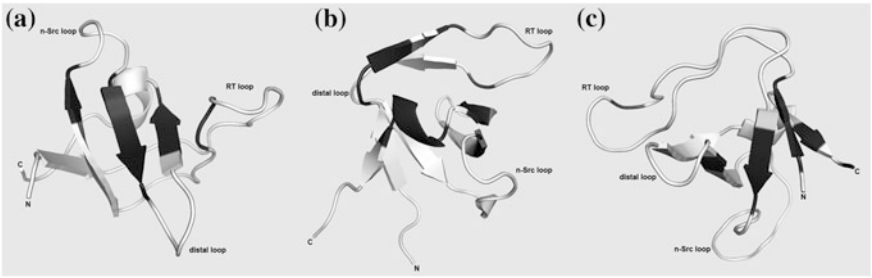

Fig. 2 Schematic representation of three anomalous binding surfaces for non-PxxP peptide recognition by SH3 domains. **a** $p67^{phox}$ SH3C domain (PDB entry:1K4U), **b** Grb2 SH3C domain (PDB entry: 1GFC) and **c** Pex13p SH3 domain (PDB entry: 1NM7). Residues involved in ligand recognition are colored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