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The SH3 domain- a family of versatile peptide- and protein-recognition module

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1. ABSTRACT

Src homology 3 (SH3) domains were initially characterized as a prevalent protein module that recognizes proline-rich sequences, in particular those containing a PxxP motif. Recent studies have shown that the specificity and cellular function of SH3 domains are far more diverse than previously appreciated. Despite lacking distinguishing features, the ligand-binding surface of an SH3 domain can be molded to accommodate a variety of peptide ligands. Moreover, certain SH3 domains are capable of using surfaces distinct from the canonical ligand-binding site to engage a peptide or protein. The identification of novel motifs and domains recognized by the SH3 domain greatly expands the ligand pool and cellular function for this family. However, this also imposes the question as to how the specificity of the hundreds of human SH3 domains is regulated in a cell to ensure their proper functions. Here we review literature on the specificity of SH3 domains, with an emphasis on the structural basis of ligand recognition, and discuss mechanisms employed by SH3 domain-containing proteins to execute defined cellular functions through highly regulated SH3-ligand interactions.

2. INTRODUCTION

SH3 domains were first described as a polypeptide fragment conserved between the N-terminus of a Src family tyrosine kinase and blocks of sequences in the adaptor protein Crk (1). Subsequently this small modular domain was identified in many other signaling proteins (2, 3). Whereas the yeast harbors 28 SH3 domains, the human genome encodes approximately 300 such domains (2). With the decoding of numerous genome sequences, it is now clear that the SH3 domain is one of the most prevalent families of protein modules found in nature, a phenomenon that parallels with the wide range of cellular functions they are capable of performing in a cell. SH3 domains are involved in a plethora of important cellular processes including intracellular signaling and cell-environment communication, cytoskeletal rearrangements and cell movement, cell growth and differentiation, protein trafficking and degradation, and immune responses (3-5).

SH3 domains contain approximately 60 residues and share significant sequence identity and a common structure featuring a five-stranded anti-parallel beta-sheet

Table 1. Selectivity of an SH3 domain for the class I or II motif[1]

	Class I: [R/K]xxPxxP (%)	Class II: PxxPx[R/K] (%)
CRK_C SH3 domain	5	95
SRC SH3 domain	11	89
ABL1 SH3 domain	14	86
FYN SH3 domain	16	84
CRK_N SH3 domain	16	84
P85α SH3 domain	21	79
NCK_C SH3 domain	22	78
NCK_M SH3 domain	26	74
NCK_N SH3 domain	29	71
GRB2_N SH3 domain	29	71
GRB2_C SH3 domain	31	69
PLCγ1 SH3 domain	35	65

[1] Selectivity is represented by the percentage of class I or II peptides that showed significant binding to an SH3 domain in the peptide array experiment of Wu, *et al.* (16). The peptide array included 686 Class I and 686 Class II peptides. The SH3 domains are arranged in a descending order of Class II bias.

(6-10). The majority of SH3 domains characterized to date bind proline-rich sequences containing a core element, PxxP, where x denotes any amino acid, through a set of conserved surface residues (4, 6, 9, 11). Since approximately 25% human proteins harbor Pro-rich regions (5), it is mind-boggling how the hundreds of SH3 domains encoded by the human genome select for their respective physiological partners to execute or regulate specific cellular functions. This dilemma is compounded by recent findings that a number of SH3 domains are capable of binding to not only PxxP-containing sequences, but also non-PxxP sequences (reviewed in (5)). Furthermore, certain SH3 domains bind to proteins via tertiary contacts rather than through a defined sequence motif, thereby significantly expanding the pool of proteins these domains can interact with. The diverse modes of ligand-recognition displayed by the SH3 domain family are unexpected for such a small protein module, which serves as a reminder of how much there is still to learn about the SH3 domain nearly twenty years after its initial discovery. Here we review recent literature on the specificity and structures of SH3 domains in complex, respectively, with a variety of different ligands, with an emphasis on mammalian SH3 domains. We also discuss how an SH3 domain may overcome its mediocre affinity to perform highly defined functions in a cell.

3. CONVENTIONAL AND ATYPICAL SEQUENCE MOTIFS

3.1. Canonical peptide motifs recognized by the SH3 domains

Since the elucidation of the structures of the spectrin and Src SH3domains (7, 8), SH3 domains have been attractive targets for structural analysis by both crystallography and multi-dimensional nuclear magnetic resonance (NMR) spectroscopy due to their small size. Shortly after the initial structural characterization on isolated SH3 domains, it was discovered that proline-containing short peptides were specifically recognized by SH3 domains (7, 12). Chen *et al.* employed combinatorial peptide libraries to identify SH3-binding motifs and found that proline-rich peptides selected by SH3 domains could be classified into two related, yet distinct groups named classes I and II, respectively (13). The consensus sequences for these two classes of motifs have been written in various forms in the literature, which are represented here as the following: [R/K]xXPxXP (class I) and XPxXPx[R/K] (class II), where the capital 'X' signifies a non-glycine, hydrophobic residue while the small 'x' denotes any naturally occurring amino acid.

The structure of an SH3 domain in complex with either a class I (Figure 1, left) (14)) or II (Figure 1, right) (15)) peptide revealed that these two types of ligand bind to an SH3 domain in opposite orientations (6, 9). The peptide in either orientation forms a left-handed helix called the polyproline type II (PPII) that is characterized by three residues per turn. An interesting feature of a PPII helix is that it possesses a two-fold rotational pseudo-symmetry. Therefore, apart from the reversal of the N and C termini and the change of orientation for the XP dipeptide unit (see below), the peptide is essentially indistinguishable from its initial form after a 180° rotation along an axis perpendicular to the helical axis. Depending on the characteristics of the binding site, some SH3 domains prefer one orientation over the other while others make little distinction of the two. Wu *et al.* identified ligands selected by a dozen human SH3 domains by screening a peptide array containing 1536 peptides that represented 768 class I motifs and 768 class II motifs (16). The percentage of class I or II peptides favored by an SH3 domain was calculated and listed in Table 1. Although each SH3 domain is capable of binding to either motif, all exhibited a bias for the class II motif-containing peptides for this group of SH3 domains. It remains to be seen whether other SH3 domains have the same ligand preference.

The orientation of a peptide with respect to the binding site is determined by an Arg or a Lys residue that precedes or follows the PxxP core motif. In either case, the basic residue is recognized by a negatively charged pocket (or specificity pocket) on the SH3 domain (Figure 1) (5)). Apart from electrostatic interactions with acidic residues lining the specificity pocket, the sidechain of an Arg or a Lys makes favorable charge-aromatic interactions and/or Van der Waals contacts with a Trp residue that is conserved at the binding site for all SH3 domains (Figure 1) (5)).

Besides interactions mediated by the basic residue, which contributes significantly to affinity, the two XP dipeptide units (as in XPxXP) are engaged by two binding pockets on an SH3 domain through hydrophobic interactions. Although these pockets are shallow and lack distinguishing features such as deep grooves or ridges seen at the ligand-combining site of an antibody, they are remarkably specific for the XP dipeptide. Nguyen *et al.* (17) attributed this near-absolute specificity to N-substitution, rather than to the unique sidechain feature or the hydrophobicity of a Pro residue. Since proline is the only N-substituted amino acid, they investigated the basis of proline recognition by synthesizing a variety of N-

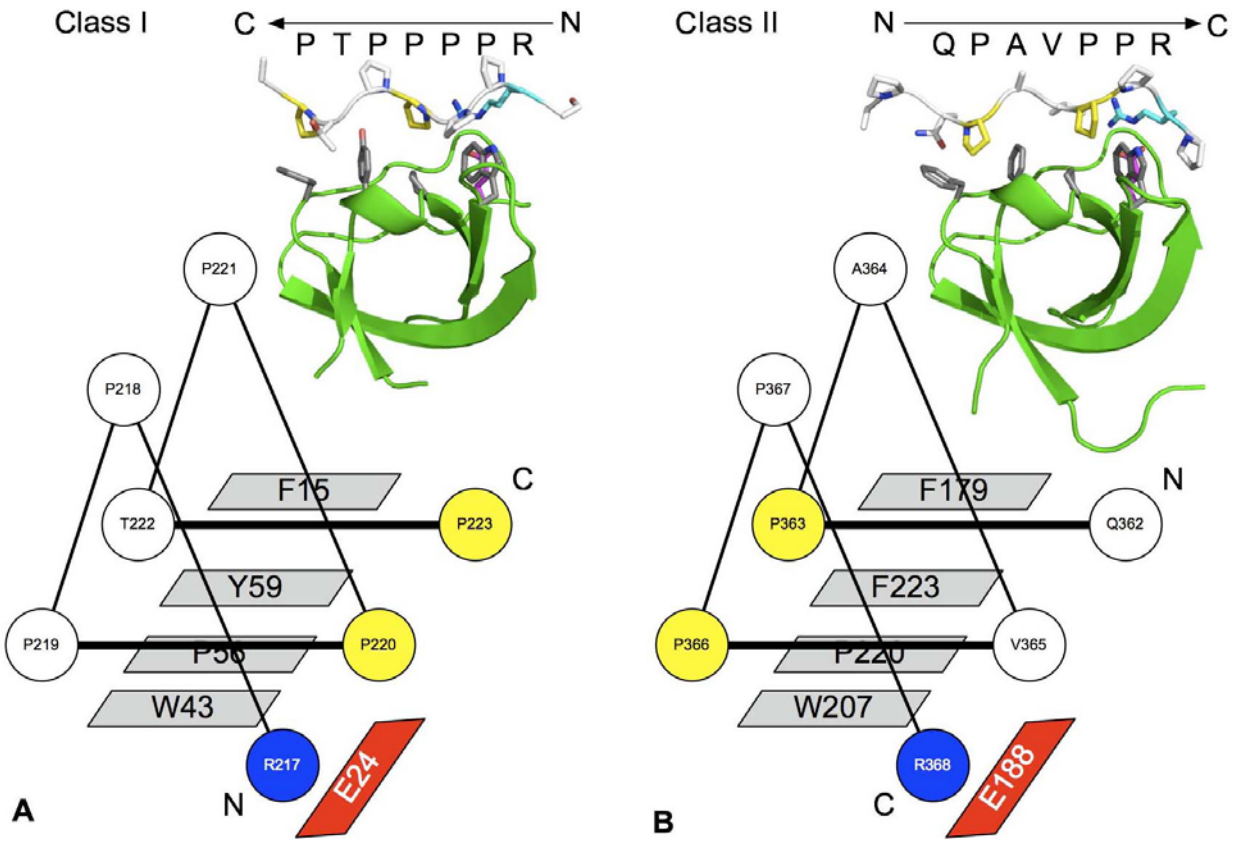

Figure 1. Structural basis of an SH3 domain binding to a Class I or II peptide. An schematic representation of SH3 domain recognition of a peptide in a 'plus' (C←N) (left) or 'minus' (N→C orientation (right) (9). The left-handed PPII helix of the peptide is shown as circles (residues) connected by sticks (amide bonds). Bold lines represent the XP dipeptide units. Conserved residues found at the ligand-binding site of the SH3 domain are shown in rectangles. (A) The beta-PIX SH3 domain in complex with a peptide derived from AIP-4 (PDB code: 2P4R) (14). The peptide contains the class I motif ([R/K]xXPxXP) and is bound in the plus orientation. (B) The $p40^{phox}$ SH3 domain in complex with a $p47^{phox}$-derived peptide (PDB code: 1W70) (15). The peptide contains the class II motif (XPxXPx[R/K]) and is bound to the SH3 domain in the minus orientation. The structures were drawn using PyMol (www.pymol.org).

substituted peptides (17). Each of the underlined prolines in the peptide PPPVPPR (class II) was replaced by either an alanine or a sarcosine (N-substituted glycine), and the affinities of the resulting peptides for the Sem-5 C-SH3 domain were then measured. Substitution of a Pro by an Ala residue completely abolished binding. However, replacement of the same Pro residue by a sarcosine did not significantly alter the affinity of the corresponding peptide for this SH3 domain (17). Moreover, replacing the first proline with an N-(S)-phenylethyl group produced a peptoid (peptide analogue) that bound to the Sem-5 C-SH3 domain with a 25-fold improved affinity over the natural peptide. Subsequent structural analysis demonstrated that the peptoid sidechain inserted more deeply into the XP-binding pocket, therefore making a better fit and more extensive contact with the SH3 domain (17). It is, however, unclear from this study whether the proline scaffold, which is essential for the formation of the PPII structure (5), is dispensable for binding since single Pro-substitution did not disrupt the overall structure of the peptide (17). Therefore, in the context of the PPII scaffold, substitution of a Pro residue by an N-substituted moiety could provide an effective means to generate specific, high-affinity inhibitors for SH3 domains (18).

3.2. Atypical SH3-binding motifs

Although most SH3 domains recognize the class I and/or II sequences, some have acquired ability to bind to the so-called "atypical motifs". For instance, the SH3 domains of the endocytic adaptor protein CIN85/CMS specifically bind to a proline-arginine motif, PxxxPR, that resembles a class II sequence (19). This motif is identified in a number of effector molecules for CIN85 including Cbl, ASAP1, SHIP1 and p115-RhoGEF (20). It has been shown that CIN85 associates with these effectors to control intracellular trafficking of epidermal growth factor receptors (20). A motif, RxxPxxxP, which is similar to the class I motif except for the extra residue inserted between the two conserved prolines, is found in the cytoplasmic regions of the calcium activated potassium (BK) channels and mediates binding to the cortactin SH3 domain. Importantly, this interaction links the BK channels to the actin cytoskeleton (21). Sequences that are not apparently related to the conventional motifs have also been identified

as ligands for some SH3 domains. For instance, PxxDY, a motif identified from a phage display library screen, is capable of binding to the SH3 domain of Eps8, a tyrosine kinase substrate (22, 23) (see below).

In the above atypical motifs, the conserved Pro residues were shown to be essential for binding. Can proline be spared for SH3-ligand interaction? The answer is yes, or at least in certain cases. Pex13p is a central component of the PTS1-receptor complex for protein import into the yeast peroxisome (24). The C-terminal SH3 domain of Pex13p engages at least two proteins, namely Pex5p and Pex14p, in this multimeric protein complex (24). Interestingly, the single SH3 domain of Pex13p is capable of binding simultaneously to a PxxP-motif in Pex14p in a conventional manner and to an alpha-helical peptide from Pex5p that is devoid of a proline (25-27). Since the binding surface for the Pex5p peptide is opposite to the one for the Pex14p peptide, the Pex13p SH3 domain therefore functions as a miniaturized adaptor to mediate the formation of the Pex14p-Pex13p-Pex5p ternary complex. That a modular domain plays the role of an adaptor is also seen for the SAP SH2 domain that binds simultaneously to an SH3 domain and a phosphotyrosine (pTyr)-containing peptide, and thereby promotes the recruitment of the kinase Fyn to the immunoreceptor SLAM (signaling lymphocyte activating molecule) in T cell activation (28, 29).

RKxxY, another motif that lacks a proline, is identified in the adaptor protein SKAP-55 to mediate its interaction with the C-SH3 domain of ADAP/SLAP (or adaptor adhesion and degranulation promoting adaptor protein; also known as SLP-76-associated protein) (30, 31). A unique feature of this interaction is that the Tyr residue, corresponding to Y294 in SKAP-55, can be phophorylated by Fyn, which in turn abolishes the interaction between the SKAP-55 motif and the ADAP/SLAP SH3 domain (31).It was also shown that a Y294F mutation blocked LFA-1-mediated adhesion and cytokine production in T cells, suggesting that Y294 plays an important role in regulating the SKAP-55-SLAP interaction (31). Moreover, this observation demonstrates that binding of a peptide to an SH3 domain may be regulated by phosphorylation, which provides a means to couple an SH3 protein-ligand interaction to phoshotyrosine signaling. In a similar vein, Kesti *et al.* (23) found that the Eps8 or the Nck1 N-terminal SH3 domain bound to a PxxDY motif present in CD3epsilon that shared its tyrosine residue (Y166) with a nearby immunoreceptor tyrosine-based activation motif (ITAM). Phosphorylation of Y166 abolished binding of Eps/Nck1 SH3 domain to CD3epsilon both *in vitro* and *in vivo*. Therefore, tyrosine phosphorylation serves as a molecular switch during T cell activation that controls whether CD3epsilon is coupled to an SH3 or SH2 domain-containing protein (23).

The first proteomic scale analysis of SH3-binding motifs was carried out in yeast. Tong *et al* (32) identified an SH3-mediated interaction network by combining phage display with yeast two-hybrid screening. While most SH3-binding motifs deduced from the phage display screening conformed to the class I or II category, three motifs (each of which binds to a different SH3 domain of the 20 tested) were considered "unusual" (i.e., PPxVxPY, RxxRxxS, and RxxxxY). This study lent support to the notion that, although the 'PxxP' core motif is commonly recognized by an SH3 domain, other unconventional sequences may target a distinct set of SH3 domains. These non-canonical motifs therefore increase the ligand space of the SH3 domain family.

More recently, Jia *et al* screened peptides derived from SLP-76 (or SH2 domain-containing leukocyte protein of 76 kDa) proline-rich region for their ability to bind a panel of 15 mammalian SH3 domains (33). A total of 120 peptides arrayed on a nitrocellulose membrane were examined, and 59 peptides were found to display significant affinities for the SH3 domains. Interestingly, 39 peptides contained no PxxP motif. Even though these peptides had overlapping sequences, a number of unique, atypical sequences were identified from this study, suggesting SH3 domains are capable of recognizing a variety of sequences that do not conform to the conventional motifs. It remains to be seen, however, how many of these non-canonical sequences mediate physiologically relevant interactions. Because the peptide array method employed by Jia at al. was inherently sensitive, it is likely that many weak interactions were detected. Nonetheless, a proteome-scale peptide array screen of Arg/Pro-rich regions should help establish the full spectrum of sequence motifs recognized by the SH3 domain family.

3.3. The RxxK motif

The RxxK motif was first described as a novel sequence, Px(V/I)(D/N)RxxKP, in the deubiquitinating enzyme UBPY recognized by the STAM2 SH3 domain (34). This motif was subsequently found to mediate a physiological interaction between SLP-76 and Gads (35-37). Structural analysis performed on the Gads C-SH3 and the STAM2 SH3 domains in complex, respectively, to RxxK motif-containing peptides explained why the two basic residues are essential for binding (35, 38, 39). As shown in Figure 2A, an RxxK-containing SLP-76 peptide APSIDRSTKPA binds to the Gads C-SH3 domain in a unique fashion but sharing some features of a class II peptide. The first XP dipeptide from either the class II or RxxK motif occupies the same binding pocket on the SH3 domain surface and an arginine residue in either motif mediates electrostatic interaction with an acidic patch on the corresponding SH3 domain (35). Despite these similarities, the RSTK fragment adopts a 3_{10} helix such that the sidechains of both the Arg and Lys residues are oriented towards the acidic residues in the RT loop of the Gads C-SH3 domain, thereby drastically increasing electrostatic interactions between the SH3 domain and the ligand (35, 38). The structure of STAM2 SH3 in complex with an RxxK-containing 11-mer peptide derived from UBPY showed a similar mode of binding (39).

What sets the Gads C-SH3-SLP-76 peptide interaction apart from other SH3-ligand complexes lies not only in the novel mode of ligand recognition, but also in its unusually high affinity. The affinity (Kd) of the Gads C-SH3 for SLP-76 falls between nano- and submicromolar, depending on

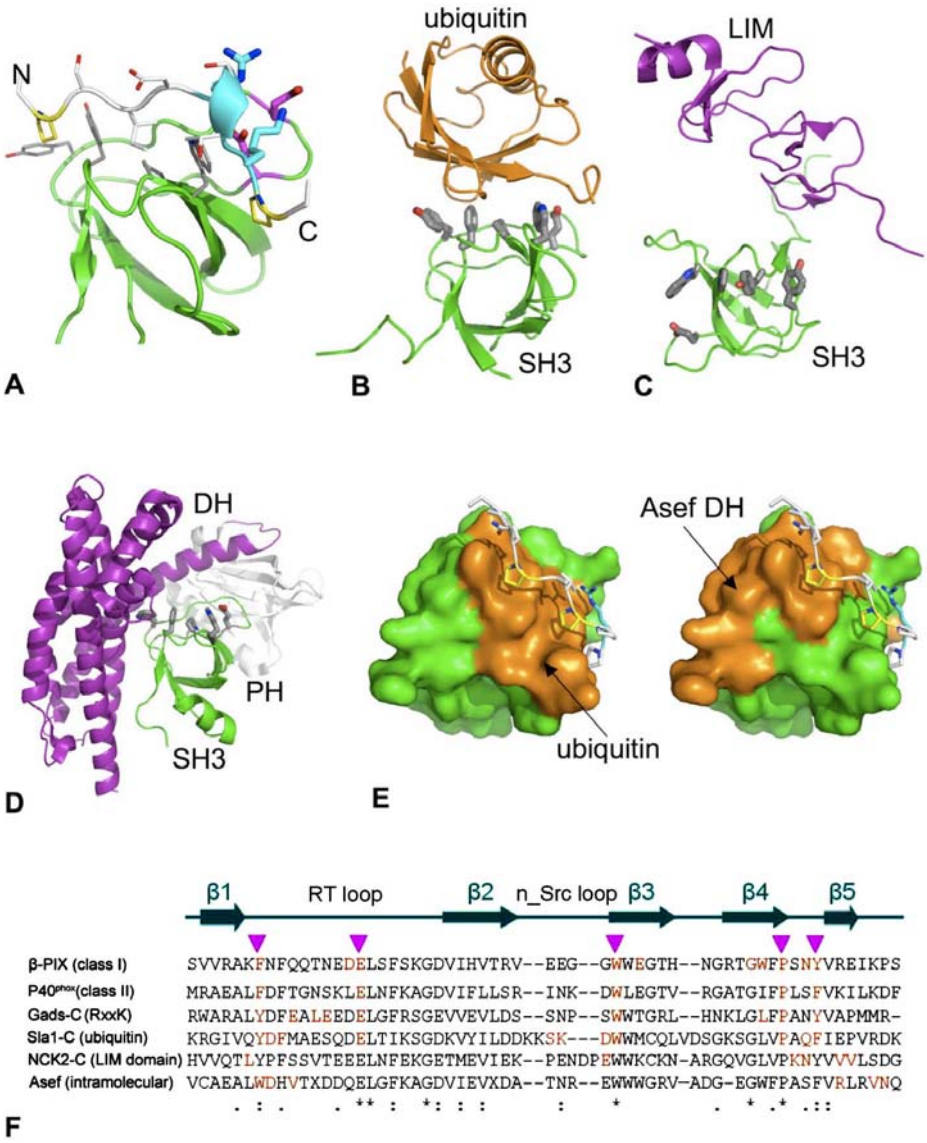

Figure 2. Diverse modes of recognition of peptide or protein ligands by the SH3 domain. In each complex structure, the SH3 domain is colored in green. The five residues corresponding to those identified at the canonical ligand-binding site in Figure 1 are depicted in gray sticks. (A) Gads C-terminal SH3 domain in complex with an RxxK-containing peptide derived from SLP-76 (PDB: 1H3H) (35). Here, the unique RSTK 3_{10} helix is colored in cyan. Two acidic residues at the ligand-binding site, namely Glu278 and Glu281, are shown in magenta. (B) The third SH3 domain of Sla1 bound to ubiquitin (PDB: 2JT4) (43). Sla1 is the yeast ortholog for CIN85. The canonical binding surface of the SH3 domain is used for binding ubiquitin. (C) The ultraweak interaction between the third NCK2 SH3 domain and the fourth LIM domain of PINCH-1 (PDB: 1U5S) (44). The SH3 domain uses a small area distinct from the canonical ligand-binding site to engage the LIM domain. (D) Structure of the Rac-specific guanine nucleotide exchange factor (GEF) Asef in its autoinhibited conformation (PDB: 2DX1) (45). The structure shown depicts the SH3 domain in green, the DH domain in purple, and the PH domain in gray. The SH3 domain is bound to the DH domain in a non-conventional manner, ie., the SH3 domain occupies the Rac-binding surface on the DH domain such that the GEF activity of Asef is inhibited. (E) The ubiquitin or Asef DH domain-binding surface (identified in orange) mapped onto the $p40^{phox}$ SH3 domain (as in Figure 1B, PDB: 1W70). The bound $p47^{phox}$ peptide is also shown to indicate the canonical binding site for a PxxP ligand. While the binding sites for a PxxP ligand and ubiquitin largely overlap (left), the Asef DH domain occupies an area on the SH3 domain that is significantly different from the PxxP-binding area (right). (F) Structure-based sequence alignment of the six SH3 domains shown in Figs. 1 & 2. The secondary structure boundaries are based on the beta-PIX SH3 domain. The five canonical ligand-binding residues are identified in triangles. Ligand-binding residues are colored in red. Conserved residues are marked with asterisks (*) whereas highly and fairly conserved ones are indicated with colons (:) and dots (.), respectively.

the length of the peptide used (35-38, 40), which is significantly greater than the 1-100 microM affinity for a typical SH3-ligand interaction. Seet *et al.* measured the affinity of the Gads C-SH3 for a 14-mer SLP-76 peptide, APSIDRSTKPPLDR, and obtained a Kd of 8 nM (41). This is perhaps the strongest interaction reported for an SH3 domain with a short peptide. It is intriguing that, by extending the peptide by a few residues C-terminal to the RSTK sequence, the affinity of the resulting peptide is significantly increased (41). While the molecular basis underlying this super-strong interaction awaits further investigation, it is likely that the extra C-terminal residues make additional contact with the Gads C-SH3 domain and thus contribute favorably to binding.

The distinction between the PxxP and RxxK motifs is blurred in certain instances. For example, the Gads C-SH3 domain was found to also bind to HPK1 (hematopoietic progenitor kinase 1) (40). The structure of a 16-mer HPK1 peptide in complex with the Gads C-SH3 domain was recently determined by X-ray crystallography (40). Interestingly, the peptide ligand, GQPPLVPPRKEKMRGK, is a chimera of the class II and the RxxK motifs where an Arg residue is shared by the two motifs. In the complex structure, the PPLVPPR fragment of the peptide forms a PPII helix while the RKEK motif adopts a 3_{10} helical structure. However, the Kd value for the complex was approximately 2.4. microM, which is two orders of magnitude greater than the interaction between Gads C-SH3 and SLP-76. These data suggest that the mere presence of an RxxK motif in a peptide does not necessarily increase the affinity of that peptide. But rather, the role of the RxxK motif in enhancing SH3-ligand binding may be further determined by the sequence surrounding this motif and by the specific SH3 domain under concern.

4. LIGAND-RECOGNITION *VIA* TERTIARY CONTACTS

In the majority of the cases, SH3 domain-mediated protein-protein interaction can be recapitulated by the corresponding SH3-peptide motif binding *in vitro*. This is, however, not true for some SH3-mediated interactions. Mounting evidence suggests that, in addition to peptide recognition, SH3 domains can also associate with another protein *via* tertiary contacts that involve no defined motif. Because a binary interaction is often rationalized by shape and chemical complementarity, tertiary contacts may be regarded as a general scenario of molecular recognition while motif-recognition a special case.

Binding of an SH3 domain to another modular domain has emerged as a recurring theme in protein-protein interaction, and in most cases (Table 2), this involves the association of the two domains *via* complementary surfaces. A subset of SH3 domains has been shown recently to bind ubiquitin, suggesting that they may function in the ubiquitination pathway (42). In a screen for novel ubiquitin-binding proteins using monoubiquitin immobilized on Sepharose beads, Stamenova *et al.* found that the yeast protein Sla1 and its mammalian homologue CIN85 were capable of binding to ubiquitin. Subsequent biochemical and NMR analysis identified the Sla1 SH3 domain as a novel ubiquitin-binding module (42). The CIN85 SH3 domain, but not the Grb2 N-SH3 domain, also interacted specifically with ubiquitin, suggesting that ubiquitin-binding is a conserved function for the Sla1/CIN85 protein family. A critical Phe, corresponding to Phe409 in the Sla1 SH3 domain, appears to play an important role in mediating ubiquitin-binding since its mutation to a Tyr residue abolished the above SH3-ubiquitin interaction. Furthermore, SH3 domains that contain the conserved Phe residue, such as those from amphiphysin I and II, were shown to be capable of binding ubiquitin. Subsequent structural analysis of the Sla1 SH3-ubiquitin complex identified that this Phe, together with a group of hydrophobic amino acids, form the binding site for ubiquitin (Figure 2B) (43). Intriguingly, the ubiquitin-binding site on Sla1 SH3 domain overlaps with its predicted binding site for a PxxP-containing ligand (see Figure 2E), suggesting flexibility in ligand-recognition by an SH3 domain. The Kd value for the SH3-ubiquitin interaction is measured at ~40 microM, which falls within the range for a typical SH3-PxxP peptide interaction (1-100 microM). Although the physiological relevance of an SH3-ubiquitin interaction remains to be further defined, it is likely that ubiquitin and PxxP ligands compete for interaction with an SH3 domain, thereby adding another layer of control over the dynamic nature of SH3-mediated protein-protein interactions in endocytosis and/or actin polarization (42).

The interaction between the Sla1 SH3 domain and ubiquitin does not involve a specific sequence motif, it is instead mediated by hydrophobic, tertiary contacts between the two interacting partners. The same mode of tertiary contact-mediated binding has also been observed in other SH3-domain interactions. For instance, the Fyn SH3 domain binds to the SAP SH2 domain through a surface-to-surface interaction that involves neither a conventional SH3- nor SH2-binding sequence (28). Surface as well as charge complementarity appear to underpin the ultra-weak interaction between NCK-2 third SH3 domain and PINCH-1 fourth LIM domain (Figure 2C). This extremely weak, and likely transient interaction, is explainable by an unusually small and polar interface between the two domains (44).

In another interesting example, the SH3 domain of Asef, a Rac-specific GEF, binds to the DH domain through an intramolecular interaction mediated by tertiary contacts that buries an area of 350 A^2 (45) (Figure 2D). Intriguingly, the DH domain interacts with the SH3 domain in an orientation that is perpendicular to that for a PxxP-containing peptide. Although only part of the canonical ligand-binding groove of the SH3 domain is utilized for interacting with the DH domain (Figure 2E), binding of the latter blocks the end of the groove and therefore prevents the binding of additional proteins. Moreover, this intramolecular interaction blocks the Rac-binding site on the DH domain and is essential for Asef autoinhibition (45).

Table 2. A list of SH3 domain-ligand complex structures[1]

SH3 domain	PDB ID	ligand secondary structure	ligand protein or peptide in structure with comments	Ref
PPII helix ligands				
ABL1 human	1JU5	PPII helix	Crk SH2 domain	64
ABL1 mouse	1ABO	PPII helix	3BP-1 peptide	65
beta-PIX rat	2DF6	PPII + 3_{10} helices	PAK2 peptide containing PxxxPR motif, P**P**VIA**PR**PEHTKSIYTRS	66
beta-PIX rat (Figure 1A)	2P4R	PPII + class I PPII helices	AIP4 peptide, GGFKPSRPPRPS**R**PP**P**PT**P**RRPASV	14
BIN1 human	1MV3	PPII helix	intramolecular contact, class I motif	67
BIN1 human	1MV0	PPII helix	c-Myc peptide, class II motif (binding orientation opposite from 1MV3)	67
CMS human	2J7I	PPII helix	CD2 peptide, PxxxPR motif	19
CRK mouse	1CKA	PPII helix	C3G peptide	68
CSK mouse	1JEG	PPII helix	PEP peptide, SRRTDDEI**PPPLPER**TPESF**IV**VEE, PxxP as well as hydrophobic contacts at peptide C-terminal region	69
FYN human	1AVZ, 1EFN	PPII helix	HIV-1 Nef protein	70, 71
GRB2 human (N-terminal)	1AZE, 4GBQ	PPII helix	Sos peptide	72, 73
ITK mouse	1AWJ	PPII helix	intramolecular contact	74
LYN human	1WA7	PPII helix	herpesvirus peptide	75
PLC-gamma1 rat	1YWO	PPII helix	SLP-76 peptide, PxxPxRP motif	76
SRC chicken	1PRL, 1RLP	PPII helix	designed peptides, 1PRL with class II peptide, 1RLP with class I peptide	77
3_{10} helix ligands containing RxxK motif				
GADS mouse (C-terminal) (Figure 2A)	1H3H, 1OEB, 2D0N	3_{10} helix	SLP-76 peptide	35, 38, 78
GADS mouse (C-terminal)	1UTI	PPII & 3_{10} helices	HPK1 peptide, PxxP/RxxK combined sequence forming both PPII & 3_{10} helices, GQP**P**LV**PP**R**R**KE**K**MRGK	40
STAM2 mouse	1UJ0	3_{10} helix	UBPY peptide	39
tertiary contacts / unusual sequences				
ASEF human (Figure 2D)	2DX1, 2PZ1	tertiary	intramolecular	45, 79
FYN human	1M27	tertiary	SAP SH2 domain	28
NCK2 human (C-terminal) (Figure 2C)	1U5S	tertiary	PINCH-1 4th LIM domain	44
SLA1 yeast (C-terminal) (Figure 2B)	2JT4	tertiary	ubiquitin	43
GRB2 human (C-terminal)	1GCQ	tertiary	Vav N-SH3 domain	80
CRKL human (C-terminal)	2BZY	tertiary	SH3 homodimer, exchange of the first beta-strand	81
IB1 rat	2FPD, 2FPE, 2FPF	tertiary	SH3 homodimer, dimerizing by facing the canonical ligand binding sites of both molecules	82
53BP2 human	1YCS	loop	p53 protein, non-PxxP sequence	83
beta-PIX human	1ZSG	turns	PAK peptide	84
SH3-peptide-SH3 (SH3 homodimer bound to single PPII helix)				
CORTACTIN human (Figure 3A)	2D1X	PPII helix	AMAP1 peptide	52
beta-PIX rat	2AK5	PPII helix	Cbl-b peptide, PxxxPR motif	51
CIN85 human (N-terminal) (Figure 3A)	2BZ8	PPII helix	Cbl-b peptide, PxxxPR motif	51
CMS (N-terminal) (Figure 3A)	2J6F	extended	Cbl-b peptide , PxxxPR motif	19
CMS (N-terminal)	2J6O	extended	CD2 peptide, PxxxPR motif	19
NADPH oxidase subunits				
$p47^{phox}$	1NG2, 1UEC	PPII helix	intramolecular, SuperSH3	50, 85
$p47^{phox}$ (Figure 3A)	1OV3, 1WLP	PPII helix	$p22^{phox}$ peptide, SuperSH3	50, 86
$p67^{phox}$ (C-terminal)	1K4U	class II PPII + helix-turn-helix	$p47^{phox}$ peptide derived from the tail of $p47^{phox}$, SKPQPAVPPRPSADLILNRCSESTKRKLASAV	27
$p40^{phox}$ (Figure 1B)	1W70	PPII helix	$p47^{phox}$ peptide derived from the tail of $p47^{phox}$, KPQPAVPPRPSAD	15
Src family kinases				
ABL1 human, ABL1 mouse	1OPL, 2FO0, 1OPK	PPII helix	intramolecular, linker sequence K**P**TV**Y**, second P of PxxP is replaced by Y which is phosphorylated upon activation	87, 88
HCK human	1QCF, 2HCK, 1AD5	PPII helix	intramolecular	89, 90
SRC human	1FMK, 1Y57, 2SRC, 1KSW	PPII helix	intramolecular, linker sequence K**P**QT**Q**, lacking second P of PxxP motif; 1Y57 is in open conformation but SH3 retains its binding to the linker	61, 91-93

[1]Note that all structures of SH3-ligand complexes are not listed in the table. Moreover, this list concerns primarily with mammalian SH3 domains. In cases where multiple structures exist for a single SH3 domain, only one is listed unless the ligands involved are significantly different in structure and/or binding mode.

The six SH3 domains depicted in Figure 1 and Figure 2 are compared in a structure-based sequence alignment (Figure 2F) in order to extract sequence patterns that underpin their diverse specifity. Despite unique binding modes exhibited by individual SH3-ligand complexes, a group of conserved residues important for class I or II ligand binding are involved in mediating most of these unconventional interactions. A ligand-binding

surface can overlap fully (ie., Sla1-ubiquitin), partially (ie.,Asef), or not at all (ie., NCK2-LIM) with the canonical peptide-binding site, suggesting that the ligand-binding surface of an SH3 domain is highly adaptable and that tertiary contacts occurring outside of the canonical site may contribute to the diverse ligand specificity observed for the SH3 domain family.

5. LIGAND-MEDIATED SH3 DIMERS

Although dimerization has been documented for a number of modular interaction domains such as PDZ (46), PB1 (47), DD (48) and BRCT (49), the SH3 domain has not been known to dimerize until recently. SH3 dimers differ from other domain dimers in that the former is often mediated or stabilized by a peptide ligand that simultaneously engages the two SH3 domains while the latter can form directly between two homo- or hetero-domains. For instance, the two SH3 domains in the phagocyte oxidase protein $p47^{phox}$, namely SH3A and SH3B, form a superSH3 domain, and in its inactive state, bind to an extended C-terminal sequence in the same protein through an intramolecular interaction (50). The crystal structure of $p47^{phox}$ that includes the tandem SH3 domains and this C-terminal region demonstrated a unique mode of peptide recognition by the tandem SH3 domains (Figure 3A). These two SH3 domains form a heterodimer with a two-fold rotational symmetry such that two ligand-binding surfaces are facing each other. This creates a deep and extended hydrophobic groove for ligand-binding. Interestingly, the peptide fragment occupying this groove, RGAPPRRSS, possesses characteristics of both the class I and II motifs and binds simultaneously to the two SH3 domains. Although this peptide has a mediocre affinity (Kd ~ 29 microM) for the tandem SH3 domain, it failed to bind to either the SH3A or SH3B domain alone, suggesting the importance of SH3 dimerization to this interaction. The relatively low affinity of the core fragment is augmented by interactions mediated by a stretch of basic residues to the C-terminus of the peptide. Indeed, a 36-mer peptide containing the core sequence was found to bind the tandem SH3 domains with 1.5. □M affinity, but exhibited no binding to either SH3 domain alone. This superSH3 domain-mediated intramolecular interaction presumably locks the $p47^{phox}$ in an inactive conformation until the phosphorylation of a group of Ser residues in the polybasic region to relieve this autoinhibition (50). In the activated state, the tandem SH3 domains also function together to engage its physiological ligand $p22^{phox}$, suggesting that these two SH3 domains have been evolved to recognize its physiological partner in a dimeric form with drastically enhanced affinity and specificity (Figure 3A).

Variations to the SuperSH3 domain found in $p47^{phox}$ have been subsequently documented for other SH3 domains. Notably, a Pro/Arg-rich peptide, PARPPKPRPRR, in the ubiquitin ligase Cbl-b has been shown to bind to two copies of either the CIN85 SH3 domain, the CMS SH3 domain or the beta-PIX SH3 domain, thereby promoting the formation of a heterotrimeric $(CIN85)_2$-Cbl-b, $(CMS)_2$-Cbl-b or $(beta\text{-}PIX)_2$-Cbl-b complex in solution as confirmed by isothermal titration calorimetry and co-precipitation assays (19, 51). Structures of the corresponding SH3 domain-peptide complexes support a model of Cbl-b peptide-mediated dimerization of two SH3 domains (19, 51). This is made possible by the pseudo-symmetrical characteristic of the peptide such that it can simultaneously engage the two SH3 domains, one in the class I orientation and the other in the class II orientation (Figure 3A). This mode of peptide-induced SH3 dimerization is also observed for AMAP1 and cortactin both in solution and in the corresponding crystal structure (52) (Figure 3A). In this case a proline-rich peptide from AMAP1 was shown to mediate the binding of two cortactin SH3 domains. It is worth noting that peptide-mediated SH3 dimerization observed above is different from the $p47^{phox}$ superSH3 domain. In the former cases, the two identical SH3 domains are not covalently linked, and the change in affinity between single SH3 to double SH3 binding is not as drastic as for the $p47^{phox}$ SH3 domains. However, since Arg/Lys and Pro often co-exist in the proline-rich region of a protein, it is likely that other peptides that possess a palindrome-like pseudosymmetry, i.e., a pair of positively charged residues flanking a central proline-rich motif, could mediate the dimerization of additional SH3 domains. Peptide-assisted SH3 dimerization therefore provides an effective means for the formation of multimeric protein complexes.

Do these different SH3 domains dimerize in a similar manner or differently? To address this question, we overlaid the structures of CIN85-Cbl-b, CMS-Cbl-b, and cortactin-AMAP1 complexes onto the structure of the $p47^{phox}$-$p22^{phox}$ complex so that one SH3 domain (i.e., SH3B in $p47^{phox}$) in each dimer is aligned. As shown in Figure 3B, although the location of the peptide ligand is similar in the these dimers, the second SH3 domain (corresponding to SH3A in $p47^{phox}$) is scattered around the peptide and showed little overlap between each other. This observation suggests that the inter-domain angle between the two SH3 domains can be different from complex to complex and the dimer is not limited to one particular orientation.

6. *IN VITRO* VERSUS *IN VIVO* SPECIFICITY

The abundance of SH3 domains and their binding motifs in a proteome raises the question as to how the specificity of an SH3 domain is governed in a cell? Besides the structural fit between the ligand and the binding site on an SH3 domain, Lim and coworkers proposed an evolutionary principle referred to as “negative selection” which, in addition to positive selection, may account for the specificity of the SH3-ligand interaction *in vivo* (53). The yeast SH3 proteome, which contains 27 SH3 domains, was used to explore this hypothesis. The Sho1 SH3 domain interacts with its physiological ligand Pbs2 to regulate response to changes in osmotic pressure in yeast (53-55). It binds to a canonical proline-rich motif in Pbs2 with a moderate Kd of 1.3. □M. Zarrinpar *et al* first assayed the binding of a synthetic Pbs2 peptide to a group of 12 non-yeast SH3 domains and found that 6 of them could bind to Pbs2, implying that the Pbs2 peptide is not a specific target

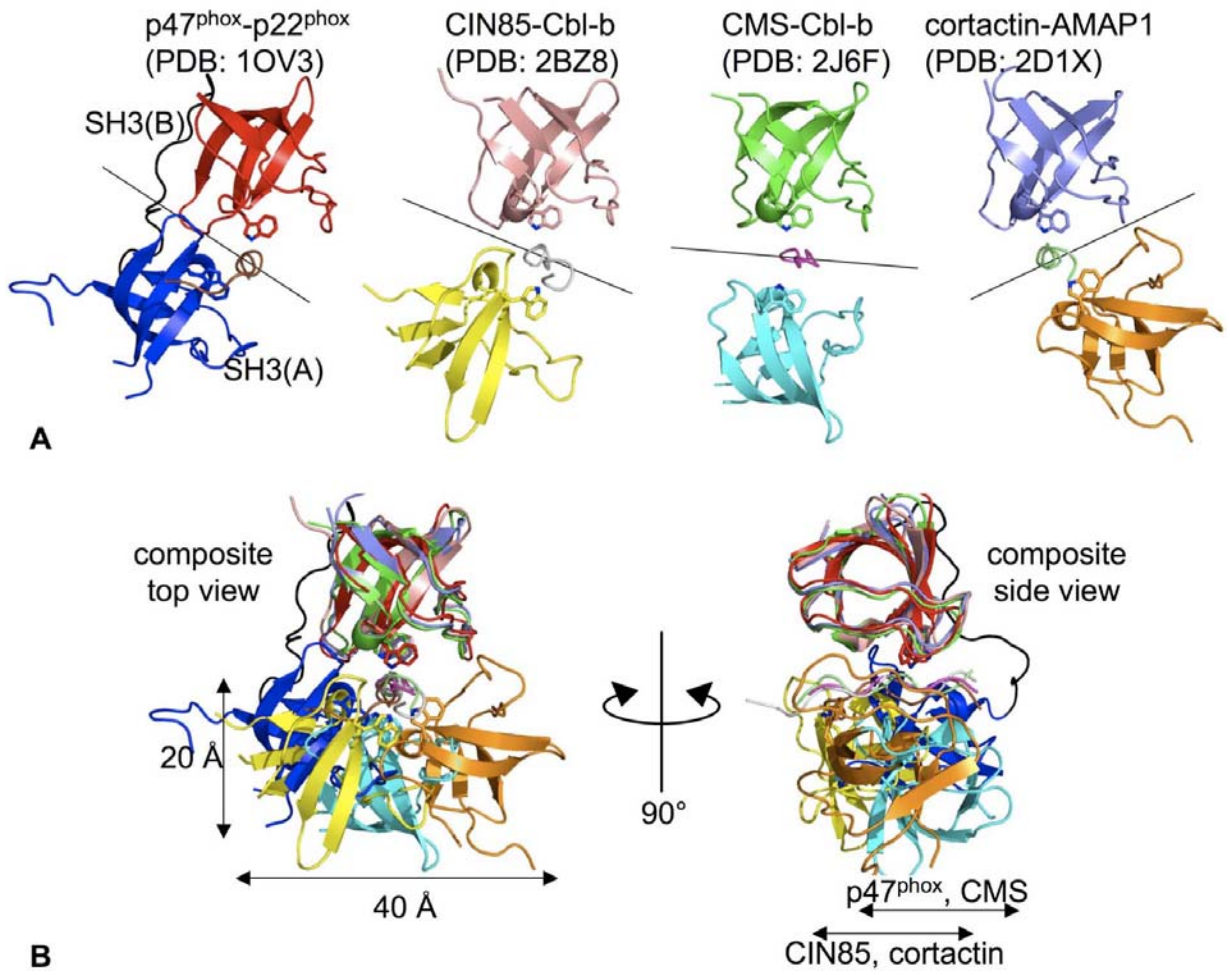

Figure 3. Ligand-assisted formation of SH3 dimers. (A) The structures of four $(SH3)_2$-peptide complexes. The two SH3 domains in a complex are related to each other in an approximately 2-fold rotational symmetry (the black line between the domains denotes the rotational axis). The conserved tryptophan residue at the ligand binding site is shown in stick representation. (B) Superimposition of four SH3 dimers onto the $p47^{phox}$ superSH3 domain. Herein we designate each upper SH3 domain as SH3B and lower one as SH3A. The SH3B domains and the corresponding peptide ligands superimpose with an RMSD of 1.2.4 Å for the 54 SH3B and 6 peptide $C_{\square}$ atoms (calculation not shown). In contrast, the position of the SH3A domain varies from one complex to the other. The side view suggests that the position of the SH3A domain in each complex is restricted by the bound peptide such that it aligns with the SH3B domain roughly on the same plane. The MultiProt server was used to generate the multiple structural alignment shown (94).

for these SH3 domains. In contrast, when the same binding assay was carried out on 27 yeast SH3 domains, the peptide showed absolute selectivity for the Sho1 SH3 domain and exhibited no detectable binding to the remaining 26 SH3 domains (53). This result clearly demonstrates that the proline-rich motif in Pbs2 is evolved for recognition only by the Sho1 SH3 domain in the yeast proteome, a phenomenon that was confirmed in a subsequent proteomic screen (56). Even though Pbs2 can potentially target non-yeast SH3 domains in a rather non-selective fashion, these SH3 domains are alien to the yeast system. Thus the Pbs2-Sho1 interaction exploits a narrow region in the yeast SH3-peptide interaction space that is exclusive to other SH3 domains to achieve high specificity. The concept of negative selection is illustrated in Figure 4 (53). The Pbs2 peptide motif occupies a niche in the specificity space of the Sho1 SH3 that is not shared by any other yeast SH3 domains (Figure 4A). However, non-yeast SH3 domains are independent of the evolutionary context of yeast interactome, and explore specificity spaces that encompass this niche (Figure 4B). This explains why extrinsic SH3 domains are capable of binding to the Pbs2 peptide sequence.

The importance of negative selection was further elaborated by mutagenesis. The concept of mutational drift implies that if a mutation is introduced into the Pbs2 motif, the position of the mutated motif in the specificity space will drift away from its original locus and becomes accessible to other SH3 domains in addition to the Sho1 SH3 domain (Figure 4A, arrows). The introduced

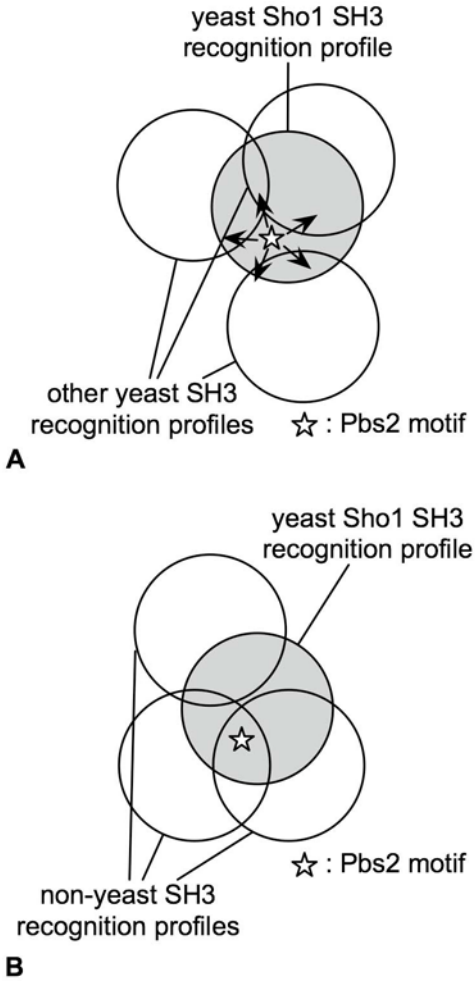

Figure 4. Negative selection contributes to the *in vivo* specificity of a Pbs2 peptide for the Sho1 SH3 domain in yeast osmotic response (53). The specificity space of the yeast Sho1 SH3 domain is represented by a shaded circle. (A) Although ligand spaces of other yeast SH3 domains (open circles) may overlap with that of the Sho1 SH3 domain due to their intrinsically low specificity, the former SH3 domains do not recognize the Pbs2 peptide (denoted with a star) which explores a niche within the Sho1 SH3 domain ligand space unoccupied by any other yeast SH3 domains, thereby ensuing a specific interaction between Pbs2 and Sho1. This type of selection was termed 'negative selection' by Lim and associates (53), since the 'absolute' specificity of the Pbs2 is conferred by selection against other SH3 domains rather than for the Sho1 SH3 domain. However, once a mutation is introduced to the Pbs2 peptide, its position in the sequence space drifts away from the niche (arrows) and the peptide interacts with non-Sho1 SH3 domains,resulting in promiscuity. (B) The same Pbs2 peptide occupies ligand spaces of non-yeast SH3 domains that were not under evolutionary pressure as the yeast SH3 domains to stay away from the 'specific' niche of the Sho1 SH3 domains. The latter SH3 domains were shown to indeed cross-interact with the Pbs2 peptide. This figure was modified from Zarrinpar *et al.* (53).

mutations indeed increased cross-reactivity with other yeast SH3 domains, indicating that the wild type Pbs2 is optimized for binding Sho1.

The concept of negative selection in SH3-mediated interaction was illustrated elegantly in another example involving the recognition of RxxK motif by the Gads SH3 domain. The affinity of the Gads C-SH3 for the SLP-76 RxxK peptide is unusually high (Kd = 8 nM). Seet *et al.* used SH3 domain array to investigate this remarkable interaction in terms of specificity and affinity (41). The SLP-76 RxxK peptide was found to bind to as few as four SH3 domains (namely Gads C-, STAM1, STAM2 and Grb2 C-SH3) out of the 147 unique human SH3 domains tested. The authors argued that Gads C-SH3 is the only possible physiological binding partner for SLP-76 in T-cell receptor (TCR) signaling because the STAM proteins have different cellular localization from TCR while the Grb2 C-SH3 binds to SLP-76 with a much reduced affinity (i.e., ~3 microM). In comparison, an RxxK-containing peptide derived from either Gab1 or HPK1 interacted with more than ten SH3 domains, suggesting that these peptides are less selective than the SLP-76 RxxK peptide. Remarkably, an HPK1-derived PxxP peptide displayed binding to 53 SH3 domains in the array, suggesting that the PxxP motif is intrinsically more promiscuous. Therefore, the concept of negative selection seems to account for the near absolute selectivity of the SLP-76 RxxK peptide for the Gads C-SH3 domain. It should be noted that, although negative selection may play an important role in certain SH3-ligand interactions, spatiotemporal factors and scaffolding as described below may impart specificity to SH3-mediated interactions at large.

7. CONTROL OF SPECIFICITY – MANY ROADS LEADING TO ROME

The affinity of an SH3 domain for a peptide or a protein ligand typically ranges from 1 to 100 microM (4) (5). This mediocre afffinity may be desirable under certain circumstances where signaling complexes need to form and dissolve in accordance to environmental cues. On the other hand, some complexes of unusually high affinity have been described. However, we have seen that an SH3-ligand binding affinity may not translate directly into specificity. For instance, the highly specific interaction between the Gads C-SH3 domain and the SLP-76 RxxK peptide was attributed to the principle of negative selection rather than to its unusual high affinity, since mutated forms of the SLP-76 peptide impaired its intrinsic selectivity for the Gads C-SH3 while retaining extremely high affinities (41). Another recent study examined binding of PAK2, ADAM15 and Nef, respectively, to the human SH3 proteome (2). Screening of the phage-displayed SH3 domains and subsequent binding studies identified a number of SH3-protein interactions with Kd values ranging from nano- to submicromolar. Despite the comparable affinity for all identified SH3-protein interactions, Nef was found to exhibit an exclusive selectivity for the Hck SH3 domain while PAK2 displayed strong, yet promiscuous binding to nine proteins (2).

In vitro binding studies using peptides and isolated SH3 domains demonstrated that an SH3-peptide interaction is usually of low affinity and specificity. On the other hand, however, SH3 domain-mediated interactions seem to be remarkably specific *in vivo*. For example, although the NCK SH3 domains have a tendency to bind to multiple peptides in an *in vitro* ligand mapping study (33), NCK interacts specifically with the Wasp and WIP proteins to regulate the actin cytoskeleton (57, 58). How does the Wasp or WIP proline-rich regions (PRR) select for the NCK SH3 domains *in vivo*? Apparently peptide motifs may not play a vital role here since the above PRR do not contain particular sequence motifs that distinguish them from PRRs in other proteins that co-exist in the cell. Although the molecular basis for this 'positive' selectivity is currently unknown, it is likely that these SH3-mediated interactions are facilitated by the formation of a multimeric protein complex (4). In addition, the SH2 domain in NCK may help target the protein to a specific location of the cell, such as where receptor activation occurs, to promote its interaction with its physiological partners. An interesting example of this scenario is found in the NCK1-nepherin receptor interaction that serves to promote localized actin polarization in the kidney podocytes. The cytoplasmic tail of nephrin contains multiple YDxV sequences that are preferred binding sites for the NCK SH2 domain once phosphorylated by Src-family kinases (59). Presumabl, recruitment of NCK to the activated nepherin receptors helps nucleate the WASP/Arp2/3 complex to initiate localized actin polarization.

Accumulating structural evidence (Table 2) suggests that unique modes of ligand recognition such as through tertiary contacts or SH3 domain dimerization as shown above (Figs. 2 & 3) may be a way for certain SH3 domains to escape from the promiscuous PxxP recognition problem (Table 1). However, this type of interaction occurs only for certain SH3 domains and should therefore not be extrapolated to all SH3 domains. A more general solution for the specificity issue utilized by many SH3 domains is spatial confinement or compartmentalization of the binding partners. Whereas most signaling proteins are expressed in micromolar or less amounts in a cell, their concentrations can be increased significantly when segregated into a local compartment of the cell. For instance, an SH3 domain that is anchored to the plasma membrane is expected to have a higher tendency to interact with a transmembrane or membrane-associated protein than with a cytoplasmic molecule. In the same vein, the Src family kinases are anchored to the plasma membrane by N-terminal myristoylation (60), and this membrane localization should facilitate the phosphorylation of receptors or membrane-associated proteins. By associating with the plasma membrane, the motion of the interacting proteins is restricted to the two-dimensional space of the lipid bilayer and the local concentrations of the interacting partners are greatly enhanced. Many signaling complexes form at or near the inner leaflet of the plasma membrane, apparently exploring this 'local concentration' effect. Alternatively, an SH3 protein may be localized to the membrane through an interaction mediated by a different part of the protein. Indeed, membrane-proximal signaling events initiated from a receptor tyrosine kinase serves as a primary example on how membrane localization of signaling molecules can greatly enhance the fidelity and efficiency of signal transduction.

Intramolecular SH3 domain-mediated interactions are frequently explored to regulate the activity of an enzyme. It is conceivable that an intramolecular interaction would take place in preference to an intermolecular one due to local concentration as well as entropic effects. And often, a weak interaction that would not take place between two isolated binding partners occurs favorably when they are parts of the same protein. Besides examples cited in earlier sections, such as the intramolecular SH3 interactions found in Asef that inhibits its GEF activity (Figure 2D) and in $p47^{phox}$ that keeps it in an autoinhibitted conformation (Figure 3A) (45, 50), the regulation of the Src family kinase serve another prime example of how intramolecular interaction involving an SH3 domain may be used to control biological activity. Src is normally kept in an inactive state in the cell by two intramolecular interactions, one involving the binding of its SH2 domain to a C-terminal phosphotyrosine and the other involving binding of its SH3 domain to a peptide sequence that links the SH2 and kinase domains (61). Breaking one or both intramolecular interactions by extramolecular interactions allow the activation of the kinase. Such intramolecular domain-motif interactions that underpin the autoinhibited conformation of a Src family kinase appears to be a prevailing mechanism used by other kinases outside the Src family (eg., Abl and Tec (60)) .

8. PERSPECTIVES

Our knowledge on how modular domains mediate specific protein-protein interactions has undergone a drastic expansion in the last decade. Numerous modular domain families, including SH3, SH2, PDZ, WW, to name just a few, have been shown to recognize linear peptide sequences (62). Deciphering the specific motifs recognized by a modular domain would be tremendously helpful for understanding its cellular function. Huang *et al.* recently determined the phosphotyrosyl peptide motifs selected by 76 human SH2 domains (63). Intriguingly, only a handful of truly distinctive motifs were uncovered from this comprehensive study, suggesting that, similar to SH3 domains, SH2 domains exhibit overlapping specificity. One important aspect of future research is to correlate *in vitro* specificity of a modular domain to the corresponding *in vivo* protein-protein interactions. While the specific motifs recognized by a modular domain can help predict its potential targets, it should be recognized that elaborate regulatory mechanisms, including those alluded above, exist in a cell to ensure which interactions would actually occur *in vivo* in response to a specific signal or environmental cue. Detailed knowledge on the *in vitro* and *in vivo* specificity of modular domains is necessary for engineering proteins with tailor-made signaling properties and for developing therapeutic strategies based on the principles governing modular domain-motif recognition.

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Abbreviations: SH: Src homology, PPII helix: polyproline type II helix

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