SH3 domains: complexity in moderation

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Summary
The SH3 domain is perhaps the best-characterized member of the growing family of protein-interaction modules. By binding with moderate affinity and selectivity to proline-rich ligands, these domains play critical roles in a wide variety of biological processes ranging from regulation of enzymes by intramolecular interactions, increasing the local concentration or altering the subcellular localization of components of signaling pathways, and mediating the assembly of large multiprotein complexes. SH3 domains and their binding sites have cropped up in many hundreds of proteins in species from yeast to man, which suggests that they provide the cell with an especially handy and adaptable means of bringing proteins together. The wealth of genetic, biochemical and structural information available provides an intimate and detailed portrait of the domain, serving as a framework for understanding other modular protein-interaction domains. Processes regulated by SH3 domains also raise important questions about the nature of specificity and the overall logic governing networks of protein interactions.

Key words: Protein-protein interaction, Modular binding domains, Signal transduction

Introduction
The idea that specific protein-protein interactions govern many biological processes is by now so deeply ingrained that it is easy to forget that it has only been a decade or so since this was widely appreciated. In that time the Src homology 3 (SH3) domain has become a standard-bearer for the protein interaction field, largely because it was one of the first modular protein interaction domains identified, is one of the most common and has served as a proving ground for new methods aimed at identifying partners or creating novel ligands through chemical synthesis. Indeed, given the unrelenting attention to such a modest domain, it seems hard to imagine that there could be much left to uncover. I will argue, that to the contrary, SH3 domains actually serve to remind us just how much remains to be learned. In fact their very omnipresence is one source of conceptual trouble - how can the cell reap meaningful information from networks based on such a common module, whose affinity and selectivity is modest at best? Because SH3 domains play a role in so many of the processes of interest to the cell biology and biochemical communities, it is without doubt important that we get a handle on what they can and cannot do, how we can use their properties to our advantage and how we can disrupt them in vivo. Here, I very briefly sketch out the general properties of SH3 domains, spend considerable time on recent advances in understanding the determinants of binding specificity (or lack of specificity) and its significance, and go on to discuss two specific recent examples in which these domains appear to play complex and varied roles in important biological processes.

A brief history
SH3 domains were first noted as regions of sequence similarity between divergent signaling proteins such as the Src family of tyrosine kinases, the Crk adaptor protein, and phospholipase C-γ (Mayer et al., 1988; Stahl et al., 1988). It was soon apparent that this ~60-residue region of similarity is present in many proteins (at this point data from genomic sequencing projects suggest that 63 proteins in Drosophila, 55 in Caenorhabditis elegans, and 25 in Saccharomyces cerevisiae contain at least one copy of the domain (Rubin et al., 2000); for a graphical representation of the domain structure of the ~1000 proteins in the database containing SH3 domains, browse the Pfam database [http://pfam.wustl.edu/] (Bateman et al., 1999)). Because the small size of the module seemed to preclude enzymatic activity, the search for function naturally focused on potential protein interactions, and screening of expression libraries using isolated SH3 domains soon identified seemingly specific binding partners (Cicchetti et al., 1992).

Early studies indicated that the region bound by SH3 domains is in all cases proline-rich and identified PxxP as a core conserved binding motif (Ren et al., 1993). A host of subsequent studies using alanine-scanning mutagenesis of known binding sites, phage display, combinatorial chemistry and high-resolution structure determination have revealed the specifics of binding in great detail (recently reviewed by Kay et al., 2000). What is clear is that the surface of the SH3 domain bears a relatively flat, hydrophobic ligand-bonding surface, which consists of three shallow pockets or grooves defined by conserved aromatic residues (Fig. 1). The ligand adopts an extended, left-handed helical conformation termed the polyproline-2 (or PPII) helix. The PPII helix has three residues per turn; this means it is roughly triangular in cross-section, and the base of this triangle sits on the surface of the SH3 domain. Two of the three ligand-binding pockets of the SH3 domain are occupied by two hydrophobic-proline (ΦΦ) dipeptides in register on two adjacent turns of the helix,
whereas the third ‘specificity’ pocket in most cases interacts with a basic residue in the ligand distal to the ΦPxΦP core. Remarkably, different binding modules such as WW domains and profilin have converged on very similar modes of interaction with proline-rich, PPII-helical ligands (Kay et al., 2000; Zarrinpar and Lim, 2000).

As discussed below, the affinity of SH3 domains for their peptide ligands is quite low, and selectivity is generally marginal at best; so it is reassuring that in a number of cases there is solid evidence for specific biologically significant SH3-ligand interactions. The first and most prominent example was the role of Grb2 (or Sem-5 in C. elegans and DRK in Drosophila) in activating Ras. Grb2 contains an SH2 domain that binds to tyrosine-phosphorylated proteins, which is flanked by two SH3 domains that bind to the proline-rich tail of Sos (as well as other proteins). Sos is a guanine-nucleotide-exchange factor (GEF) for the small GTPase Ras. Genetic and biochemical evidence demonstrated that Grb2 recruits Sos to

Fig. 1. Interaction of SH3 domains with their ligands. (A) Binding of class I (top) and class II (bottom) ligands to the surface of the SH3 domain is depicted diagrammatically. The specificity pocket that typically interacts with an arginine residue in the ligand is to the right, the two Φ-P pockets to the left. The orientation of the ligand is indicated beneath each diagram. Approximate positions of the variable RT and N-Src loops of the SH3 domain are indicated. (B) The Src SH3 domain bound to class I (top) and class II (bottom) ligands (adapted from Feng et al., 1994). The surface of the SH3 domain was rendered with the program GRASP; ligands are represented in stick format. The overall view corresponds to the diagram in A. (C) Schematic view of an X-P dipeptide of a class I (top) or class II (bottom) ligand on the surface of an SH3 domain (adapted from Nguyen et al., 1998). The view is down the axis of the PPII helix of the ligand from the perspective of the specificity pocket of the SH3 domain. The surface of the SH3 domain is represented in green, and the portions of the X-P dipeptide that contact the surface are represented in red. Note that the majority of the proline ring is not in contact with the surface of the SH3 domain and that it is the Cα atom of the proline ring (the atom directly bound to the main-chain nitrogen atom) that is in closest contact with the SH3 surface; N-substitution is thus the main feature recognized by SH3 domains. Note that in either orientation the Cα atom of the proline is directed away from the SH3 surface and does not contribute to the binding energy.
tyrosine-phosphorylated sites on the membrane, where Ras is localized by virtue of its covalent lipid modification, and that Grb2 thereby couples activation of Ras to changes in tyrosine phosphorylation (McCormick, 1993). While this remains the paradigm for SH3-mediated intermolecular interactions, there are now several other examples for which there is solid evidence, such as recruitment of the Pak kinase by the Dock SH2-SH3 adaptor during retinal axon migration in *Drosophila* (Hing et al., 1999).

The most prominent example of the importance of intramolecular SH3-mediated interactions comes from Src itself. Src and its relatives are normally held in an inactive conformation, in which a C-terminal phosphotyrosine engages in an intramolecular interaction with the SH2 domain of the kinase. When the X-ray crystal structures of Src and its close relative Hck were solved, the previously under-appreciated SH3 domain took center stage. The SH3 domain was found to bind to a linker region between the SH2 and catalytic domains of the kinase, which adopted the typical PPII helical conformation of an SH3 ligand despite the absence of sequence similarity to known Src SH3-binding sites (Sicheri et al., 1997; Williams et al., 1997; Xu et al., 1997). High-affinity SH3 domain ligands stimulate Hck activity in vitro (Moarefi et al., 1997; Nguyen et al., 2000), demonstrating that the intramolecular SH3-linker interaction seen in the crystal structures is critical for holding the protein in its repressed conformation. This example illustrates two points: first, that biologically important SH3-mediated interactions involving suboptimal, low-affinity ligands can occur if the local concentration is high enough (e.g. when the two interacting regions are found on the same molecule); and second, that SH3 domains can participate in complex regulatory interactions that depend on multiple contacts - in the case of the Src family kinases, a delicate conformational switch involving the SH2 domain, the SH3 domain and phosphorylation of at least two regulatory sites.

**Specificity**

The target specificity of particular SH3 domains is an important issue, because if we understand the rules governing specificity we will be able to predict ligands for particular proteins of interest and perhaps develop ways to inhibit specific interactions in vivo. Because the PPII helix of an SH3 ligand is quite similar in overall structure when viewed from its N- or C-terminus, ligands can bind in either of two orientations: so-called class I ligands have the general consensus +xFPhxFp and class II ligands have the general consensus FpPhxFp+ (where x is any amino acid, and + is in most cases a basic residue, usually arginine) (Feng et al., 1994; Lim et al., 1994; Mayer and Eck, 1995). This means that the invariant proline residues of the Fp scaffold (more commonly denoted PxxP) restricts the variability that can be used to generate specificity, recent efforts have addressed how selectivity can be achieved both in nature and in the test tube. One obvious potential source of specificity is the third specificity pocket of the domain. Although most SH3 domains prefer an arginine residue at this site, the Crk SH3 has high selectivity for class II sites that instead contain a lysine residue (Wu et al., 1995). The Abi SH3, which ironically was the first for which specific targets were identified, is atypical in that it prefers class I ligands in which hydrophobic residues contact the third specificity pocket; this is because it lacks conserved acidic residues found in other SH3 domains, which make specific contacts with the arginine residues of typical ligands (Ren et al., 1993; Weng et al., 1995).

For some time, we have known that ligand residues outside the core binding motif can also contribute specificity by interacting with surfaces on the SH3 domain outside the PPII binding groove, such as the highly variable RT and N-Src loops (see Fig. 1) (Feng et al., 1995). For example, peptide-binding sites for the second SH3 domain of the adaptor Nck have been carefully mapped, showing a strong requirement for a serine residue downstream of the core class II binding motif (PxxPRxS); furthermore, phosphoserine, acidic and proline residues are not tolerated in positions immediately adjacent to the core motif (Zhao et al., 2000).

The binding of the SH3 domain of the Src family kinase Hck to the HIV Nef protein illustrates how regions of a ligand protein distant from the PPII helix can also modulate specificity. In this case, a single residue in the RT loop of the Hck SH3 confers high-affinity binding to native Nef, but not to the isolated PxxP-containing peptide (Lee et al., 1995). After screening a library of mutant Hck SH3 domains (in which six RT loop residues were randomized) by phage display for binding to native Nef, Saksela and colleagues...
were able to isolate mutant domains that have up to 40-fold higher affinity than the wild-type Hck SH3 (Hiipakka et al., 1999). They also generated mutant SH3 domains that have very high affinity and selectivity for a Nef variant bearing a point mutation in the region predicted to contact the RT loop. These results demonstrate that it is possible to generate SH3 domains that bind very selectively to a specific target protein. This might in some cases allow the experimental blocking of particular SH3-binding sites, while leaving other, closely related sites untouched. This also raises the question of why naturally occurring SH3-mediated interactions tend to be of relatively low affinity - why has evolution not taken full advantage of the potential affinity and selectivity available in the system? There must be some evolutionary advantage to maintaining relatively low affinity and selectivity for SH3-mediated interactions.

A smattering of recent data indicate that SH3 domains can in some circumstances bind to ligands lacking the canonical PxP core binding motif - for example, the Pix SH3-binding site in Pak (PPVIAAPPETK5) (Manser et al., 1998), the Eps8 SH3-binding consensus (PxXDY) (Mongiovì et al., 1999) and the Hbp SH3-binding sites on UBPy (Px(V/I)(D/N)xxKP) (Kato et al., 2000). It can be argued that, for most of the phage display and combinatorial library experiments published to date, non-PxxP ligands could never have been detected, because the original libraries were biased by fixing proline in the two ‘invariant’ positions. However, enough SH3 ligands have been detected by direct filter binding or yeast two-hybrid approaches to suggest that, if non-PxxP ligands were common, they would have been detected much more often by now. It is probably safe to assume that for most SH3 domains the PxxP core is required, with the caveat that exceptions always exist.

One fascinating recent development is the chemical synthesis of novel SH3 domain ligands that have much greater selectivity than do authentic peptide-binding sites found in naturally occurring proteins. Lim and colleagues proposed that the requirement for proline in the two Pφ dipeptides of the PφxPφP core might be due at least in part to the unique structure of proline – it is the only N-substituted amino acid. To test this idea they constructed synthetic peptoid ligands in which one or both of the proline residues were replaced with various non-natural N-substituted residues. Remarkably, not only could some of these peptoid ligands bind to SH3 domains with higher affinity than known natural (proline-containing) ligands, but they also found ligands that had greater selectivity than natural peptides (Nguyen et al., 2000; Nguyen et al., 1998). For example, one compound, in which both prolines were replaced with synthetic N-substituted residues, bound to the Crk SH3 domain with high affinity in the absence of detectable binding to either the Src or Grb2 SH3 domain (Nguyen et al., 2000). This implies that SH3 domains do not generally recognize the entire ring structure of proline, but instead recognize only a small portion of the ring near the backbone nitrogen (Fig. 1c). As a consequence there is a great deal of structural variability among SH3 domains in the vicinity of the Pφ pockets, which can be exploited if different functional groups are appended to this nitrogen (by contrast, the cell is limited in its ability to optimize binding by the fact that proline is the only N-branched amino acid at its disposal). The potential to inhibit specifically a particular SH3 domain while sparing others has obvious potential both as a research tool and as a source of lead compounds for pharmaceutical development.

The larger specificity question - are we missing something?

A central question that remains unresolved is this: do meaningful biological outputs necessarily depend on specific, pairwise SH3-ligand interactions, or is it more realistic to think of these interactions in a probabilistic way - i.e. does a particular SH3 domain or ligand site simultaneously engage in multiple interactions in dynamic equilibrium? The conceptual problem arises from the fact that there are many different SH3 domains and potential ligand sites present simultaneously in the cell – certainly many tens and probably hundreds of each. From what we know about peptide-SH3 interactions, affinities for natural ligands and selectivity for a particular SH3-ligand pair versus potential competing interactions are relatively low. Ladbury and Arold have argued that there is insufficient inherent specificity in most SH3-mediated interactions to account for specific biological outputs without one having to invoke additional mechanisms (Ladbury and Arold, 2000). Two extreme resolutions of this apparent paradox exist: effective selectivity could be increased in vivo by a variety of means, such as compartmentalization of potential interaction partners, additive effects of multiple separate interactions between two partners, and the cooperative assembly of multiprotein complexes; alternatively, selectivity might not be strictly important in vivo - in this case, the central organizing principle would not be the linear pathway but a vast and ever-shifting web of interactions, from which output is gauged by global changes in complex binding equilibria.

Certainly, in at least some situations, selectivity is enhanced by multiple interactions - for example, the combination of the intramolecular interaction of the Src SH3 domain with its own linker peptide and multiple additional interactions among the SH2, SH3 and catalytic domains. Grb2 provides a different type of example: its two SH3 domains are likely to engage multiple PxxP sites in the C-terminus of Sos simultaneously (Simon and Schreiber, 1995). Other proteins that have multiple SH3 domains, such as the Nck adaptor, presumably use a similar strategy to build selectivity through multiple, relatively low-affinity interactions (Adler et al., 2000; Wunderlich et al., 1999). A change in the subcellular localization of a domain or ligand will certainly alter dramatically the repertoire of proteins with which it can interact owing to changes in their local concentration – this is the case for SH2-containing proteins that relocalize to sites of tyrosine phosphorylation during signaling. In this regard, the modest intrinsic affinity of SH3 domains for their ligands means that off-rates are fast, and binding partners can exchange relatively rapidly upon relocalization. Such plasticity is almost certainly advantageous in some contexts, allowing the rapid remodeling of interactions in response to changes in the environment.

Despite such mechanisms, in many situations a particular SH3 or ligand will inevitably interact simultaneously with many different partners. Although we may not be comfortable with pathways that cannot be described in a linear (A→B→C) fashion, nonlinearity seems to be the logic of many biological networks. A combinatorial, probabilistic set of interactions (A interacts with partners U, V, W, X, Y and Z; X, in turn,
interacts with B, C, D, E and F in addition to A; and so forth) could potentially generate far more specific information than unique interactions, because the number of possible combinations of interactions is vastly greater than the actual number of interacting proteins (Fig. 2). One can also easily envision how the gradual process of evolution could weave and shape such a web of overlapping interactions: if a protein gains an SH3 domain (or proline-rich binding site) by exon shuffling, it will then engage in many new interactions that subtly alter the overall equilibrium of such interactions in the cell. There is no need to invoke the simultaneous evolution of both a unique binding domain and a unique ligand site for every biologically important interaction. Perhaps an apt analogy can be made to the webs of interactions among species: although mice eat corn, and owls eat mice, the linear pathway of owl→mouse→corn does not do justice to the facts that the owl will eat many other prey besides mice given the opportunity, that mice have good reason to fear many predators beside owls, and that mice will happily eat many things in addition to corn. An owl’s nest in the barn might create a specific output (less corn eaten by mice), but it would certainly be misleading to attempt to define the system solely in terms of the ‘specificity’ of owls for mice or mice for corn.

**MAGUKs - controlling assembly of large complexes**

The MAGUK (membrane-associated guanylate kinase) family of scaffold proteins illustrates the potential role of both intramolecular and intermolecular SH3-mediated interactions in assembly of macromolecular complexes. MAGUKs are a large family of related proteins consisting of 1-3 PDZ domains, an SH3 domain and a guanylate-kinase-like (GK) domain (Fig. 3a); they are thought to play a role in assembly of large protein complexes at specialized regions of the membrane such as synapses, neuromuscular junctions and other cell-cell junctions (Dimitratos et al., 1999). The PDZ domain, another protein-interaction module, typically binds to sites at the extreme C-terminus of target proteins that have the consensus Exx(V/I) (Songyang et al., 1997). The GK region, although bearing all the sequence hallmarks of a catalytically active domain, appears not to have enzymatic activity but instead probably mediates protein-protein interactions. In addition to the three highly conserved domains, some members of the MAGUK family also contain additional motifs, including a region with similarity to the catalytic domain and calmodulin-binding site of calcium/calmodulin-dependent protein kinase (CaM kinase), and a so-called HOOK domain that interacts with the band 4.1 protein and its relatives (Dimitratos et al., 1999).

Given their generous complement of protein-interaction domains, MAGUK proteins are well-equipped to mediate the assembly of large, multimolecular protein complexes through interactions with other proteins and self-assembly into multimers. The PDZ domains interact with transmembrane or membrane-associated proteins such as receptors and ion channels; clustering of these proteins presumably facilitates signaling by generating high local concentrations (Garner et al., 2000). The role of the SH3 domains in scaffolding by MAGUK proteins has until recently been elusive. It has long been known that the SH3 is somehow important, because in *Drosophila*, SH3 domain mutants of the MAGUK protein Discs Large (Dlg) are oncogenic, leading to epithelial overgrowth and malignant transformation (Woods et al., 1996). This observation, and the well-known role of SH3 domains as protein interaction modules in other systems, led to a largely unsuccessful hunt for ligands for the SH3 domains of various MAGUK family members.

As it turns out, researchers did not have far to go for those ligands - they are located in the same protein. Several groups independently found in yeast two-hybrid experiments that the SH3 domains of various MAGUKs, including PSD-95, chapsyn-110, SAP97, DLG, CASK and p55, all interact with their own GK regions (McGee and Bredt, 1999; Nix et al. 2000; Shin et al., 2000). Unsurprisingly, given the conservation of the SH3 and GK domains among MAGUK proteins, heterotypic interactions also occur: in some (but not all) cases, the SH3 domain of one MAGUK can bind to the GK domain of a second (as assayed by yeast two-hybrid, coimmunoprecipitation and GST-pulldown assays). In all cases examined, however, a MAGUK molecule that contains *both* an SH3 and a GK domain cannot interact in trans with either the SH3 or GK domains of a second MAGUK molecule in vitro; this suggests that the intramolecular cis interaction is favored under normal circumstances, which might be expected given the high local concentration of the two domains on the same molecule. Nix et al., have provided evidence for heterotypic interaction in vivo, however, by co-immunoprecipitating endogenous hCASK and hDLG from human colon cancer cell lysates and colocalizing the two proteins by immunofluorescence at cell junctions in these and other epithelial cells (Nix et al., 2000). Of course, whether this apparent complex depends on SH3-GK interactions in trans remains to be proved, but, given that trans interaction of isolated domains can be demonstrated in vitro, the full-length proteins are likely to do so under some circumstances in vivo.

Interestingly, not all MAGUK SH3-GK interactions are allowed - there is some specificity in which heterotypic interactions can occur. For example, the hDLG GK binds to its own SH3 domain, but not to those of hCASK or p55, whereas the GK domains of hCASK and p55 bind equally well to SH3 domains of all three proteins (Nix et al., 2000); similarly, the SH3 domain of SAP 97 binds to the GK domains of all the closely related MAGUKs in the PSD-95 family but not to that of the more distantly related ZO-1 (Shin et al., 2000). Therefore, although all MAGUKs appear to be capable of intramolecular (monomer) as well as homotypic (dimer or higher-order) interactions, only some heterotypic interactions will be favored. In some cases, heterotypic interactions are allowed only in an asymmetric fashion: for example, hDLG-hCASK heterodimers can form through interactions between the hDlg SH3 and the hCASK GK, but since the hCASK SH3 and the hDlg GK cannot bind to each other they are presumably free to interact with other proteins (Nix et al., 2000).

One potential role for SH3-GK interactions might be to orient individual MAGUKs with respect to other proteins in large, polymeric complexes. Individual MAGUKs, which can dock onto larger, assembling complexes through other protein-interaction domains, such as the PDZ domains, would effectively snap into place as the intramolecular SH3-GK interaction is out-competed by trans interactions once it has assumed the correct spatial orientation for proper assembly (Fig. 3b). The assembled complex would be stable despite the
relatively low affinity of the individual SH3-GK interactions owing to the additive effect of many simultaneous interactions. The fact that Sheng and colleagues have identified a PSD-95 SH3 domain mutant that cannot cluster the Kv1.4 potassium channel, and yet is targeted to the membrane and binds to the channel normally, is consistent with this idea (Shin et al., 2000). There are some interesting twists to the SH3-GK interaction that highlight the dangers of overgeneralizing SH3 domain properties. One outstanding issue concerns the actual binding
sites for the SH3s, which have yet to be mapped precisely. In the case of PSD-95, no canonical PxxP site is present in the C-terminal GK region. Although three proline residues do lie in potential degenerate class II sites (PxR), changing those residues to alanine individually or in combination did not eliminate binding (Shin et al., 2000). Furthermore, the GK region required for SH3 binding extends beyond the borders of the actual GK domain (defined by sequence similarity) both N- and C-terminally, which suggests that a larger folded structure is recognized by the SH3 domain (McGee and Bredt, 1999; Shin et al., 2000). MAGUK family SH3 domains differ from the typical SH3 consensus at several highly conserved sites in the ligand-binding regions (see: http://pfam.wustl.edu/cgi-bin/getdesc?name=SH3); their ligands might therefore also differ from the canonical SH3-binding consensus. Mutation in the PSD-95 SH3 of the invariant tryptophan (which defines the ridge between the second P pocket and the specificity pocket; Mayer and Eck, 1995) apparently does not eliminate the intramolecular SH3-GK interaction, although it does eliminate binding to GK domains in trans. This tryptophan is generally assumed to be essential for binding to canonical PxxP ligands, and so the milder effect in the case of PSD-95 is again consistent with the idea that its ligand is atypical.

Finally, the effect of the intra- or inter-molecular SH3-GK interaction on other interactions must be considered. The kainate receptor is the only MAGUK SH3 ligand (other than GK domains) identified to date (Garcia et al., 1998), but it is possible that others exist. Their binding would depend on the disruption of intramolecular SH3-GK interactions to expose the SH3 domain and would necessarily expose the GK domain for interaction with other SH3 domains in trans. Proteins that lack SH3 domains also bind to the GK domain, although in the case of PSD-95 and its relatives binding of one of these, GKAP, to the GK domain is not affected by the intramolecular SH3-GK interaction (Shin et al., 2000). Although this demonstrates that two independent binding sites are present in the GK domain, the effects of SH3 binding on other GK-domain-binding partners have yet to be examined. The possibility that a novel protein-binding interface is created by interaction of the SH3 and GK domains also needs to be explored. Clearly, the permutations of inter- and intra-molecular interactions that can and do occur, and their effects on macromolecular assembly and interactions with other partners, are complex, and additional experiments will be required to sort these out.

**WASP and friends – controlling actin**
A second emerging story in which SH3 domains are implicated

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**Fig. 4.** Protein interactions implicated in nucleation of actin filaments by WASP family members. Wasp, Nck, WIP/verprolin, class 1 myosin and Pak are depicted approximately to scale; important domains and regions are shown as labeled boxes. Interactions implicated in regulation are indicated by arrows; SH3-mediated interactions are depicted in red. Sites of interaction indicated by arrows are intended to be approximate only.
is the regulation of actin dynamics by members of the WASP family of proteins. WASP was originally isolated as the gene mutated in Wiskott-Aldrich Syndrome, a severe X-linked immunodeficiency (Derry et al., 1994). WASP and its relatives play a pivotal role in organizing the actin cytoskeleton, a process that must be exquisitely controlled in time and space to allow directed movement (Higgs and Pollard, 1999; Mullins, 2000; Ramesh et al., 1999; Takanawa and Miki, 2001; Zigmond, 2000). Both WASP and its more ubiquitously expressed relative N-WASP contain several modular domains, including an EVH1 domain, a PtdIns(4,5)_2-binding site, a region that binds the Rho-family GTPase Cdc42, an extensive proline-rich region, and a C-terminal VCA (for verprolin-cofilin-acidic) region involved in regulating actin polymerization. The EVH1 domain engages proline-rich ligands, but the mode of recognition differs from that of SH3 or WW domains (Prehoda et al., 1999). The Cdc42-binding region binds to and inhibits the VCA region when Cdc42 is not bound; thus this region is a conditional switch that responds to changes in GTP-Cdc42 levels (Abdul-Manan et al., 1999; Kim et al., 2000; Miki et al., 1998). The VCA region interacts with the Arp2/3 complex, which nucleates de novo actin filament formation and induces filament branching (Blanchoin et al., 2000; Mullins et al., 1998; Pantaloni et al., 2000), and the filament-nucleating activity of the Arp2/3 complex is greatly increased when bound to the VCA region (Machesky et al., 1999; Rohatgi et al., 1999; Winter et al., 1999; Yarar et al., 1999). Thus Cdc42 can activate Arp2/3-mediated actin polymerization by disrupting the intramolecular interaction between the PBD and VCA regions (reviewed by Fawcett and Pawson, 2000).

Where do SH3 domains fit into this picture? The long proline-rich region found in all WASP family members is an obvious potential SH3-binding site, and there is no shortage of suspects for ligands. The Nck SH2/SH3 adaptor, for example, is localized on the surface of vaccinia virus particles and plays a role in recruiting N-WASP and the Arp2/3 complex, thereby serving to nucleate the formation of actin ‘comet tails’ that propel the virus in the cytosol (Frischknecht et al., 1999; Moreau et al., 2000). Because Nck has an SH2 domain, it could recruit and cluster WASP family members at sites of tyrosine phosphorylation. Indeed, a phosphotyrosine-containing site whose phosphorylation is necessary for actin tail formation is present on the vaccinia surface and can bind to the Nck SH2 domain (Frischknecht et al., 1999). In T cells Nck helps to mediate the phosphotyrosine-dependent assembly of a complex containing WASP and Vav, a GEF for Rho family GTPases, and dominant-negative experiments suggest that disruption of this complex inhibits localized actin polymerization following T cell receptor stimulation (Bubeck Wardenburg et al., 1998). Furthermore, a close relative of Nck, Nckβ, is implicated in actin rearrangements downstream of PDGF receptor stimulation (Chen et al., 2000).

Other potential partners for WASP family members are the unconventional SH3-containing myosins. In yeast the WASP relative Bee1p/Las17p is essential for proper formation and localization of cortical actin patches and for endocytosis (Li, 1997; Naqvi et al., 1998). Loss of both type I myosins Myo3p and Myo5p, or mutation of their SH3 domains, leads to severe defects in the actin cytoskeleton (Anderson et al., 1998; Evangelista et al., 2000; Geli et al., 2000; Goodson et al., 1996). As it turns out, the SH3 domains of Myo3p and Myo5p bind the proline-rich region of Bee1p (Evangelista et al., 2000; Lechler et al., 2000). Surprisingly, Myo3p and Myo5p can also bind directly through their acidic tails to the Arp2/3 complex, which suggests that, in a Bee1p-Myo3p or Bee1p-Myo5p complex, there is redundancy in Arp2/3 binding (Evangelista et al., 2000; Lechler et al., 2000). Myosin motor activity is important for cortical actin structures (Lechler et al., 2000), perhaps to transport the complex to the barbed ends of actin filaments or to drive movement of actin filaments relative to the membrane (Machesky, 2000).

Phosphorylation of type 1 myosin at a specific site, presumably by the Pak family kinases Ste20p and Cla4p, is essential for Cdc42-stimulated actin assembly in yeast (Lechler et al., 2000), and phosphorylation of amoeboid type 1 myosin by Pak-like kinases is essential for its activity (Brzeska and Korn, 1996; Novak and Titus, 1998). Remarkably, in mammalian cells Nck binds via its SH3 domains to Pak kinases (Bokoch et al., 1996; Galisteo et al., 1996; Lu et al., 1997), which suggests a mechanism whereby Nck might modulate the activity of unconventional myosins associated with WASP-Arp2/3 complexes. In Drosophila the Nck homolog Dock is important for the directed migration of retinal axons, probably regulating localized actin polymerization in the growth cone in response to environmental cues (Garrity et al., 1996), and Pak has been shown to function downstream of Dock in this system (Hing et al., 1999).

The SH3 domains of Myo3p and Myo5p have also been shown to bind another proline-rich protein, verprolin, which is also implicated in organization of the actin cytoskeleton (Anderson et al., 1998; Evangelista et al., 2000; Geli et al., 2000). The mammalian homolog of verprolin, WIP, binds directly and with high affinity to the N-terminal EVH1 domain of WASP (Ramesh et al., 1997), and this interaction is conserved in yeast (Naqvi et al., 1998); such a verprolin-Bee1p complex would thus contain multiple binding sites for type 1 myosin SH3 domains. Furthermore, WIP is required for Nck to recruit N-WASP to vaccinia virus particles, which suggests that binding of the Nck SH3 domains to N-WASP is too weak to be stable without the additional contacts provided by Nck-WIP and WIP-N-WASP interactions (Anton et al., 1998; Moreau et al., 2000). Finally, WIP might also function to bundle actin filaments together (N. Ramesh and R. Geha, personal communication), thereby modulating the higher-order structure of filaments initiated by the Arp2/3 complex.

SH3 domains might also directly activate complexes containing Arp 2/3 and WASP family members. Carlier and colleagues have shown that SH3 domains from the Grb2 adaptor enhance the activity of the N-WASP–Arp2/3 complex in vitro (Carlier et al., 2000). We find that Nck SH3 domains are more effective than those of Grb2, and multiple Nck SH3 domains are required for optimal activation (R. Rohatgi, B. J. Mayer, and M. Kirschner, unpublished). This requirement for multiple SH3 domains raises the possibility that simultaneous engagement of two or three SH3-binding sites on N-WASP can fix it in an open, activated conformation in which its VCA region is exposed. In these in vitro assays GTP-Cdc42 and Nck seem to play redundant roles in the activation of N-WASP, in both cases synergizing with PtdIns(4,5)_2 and Pak-like kinases (R. Rohatgi, B. J. Mayer, and M. Kirschner, unpublished). A Cdc42-independent mode to nucleate actin polymerization in the cell through high...
local concentrations of Nck (or other SH3-containing proteins) might therefore exist. This indeed seems to be the case for vaccinia actin tail formation, which appears to be independent of GTP-Cdc42 (Moreau et al., 2000).

What emerges from these studies is a picture in which a vital function, nucleation of new actin filaments at the appropriate time and place in the cell, is controlled by a multicomponent complex of proteins assembled through SH3-mediated and other protein-protein interactions (Fig. 4). The individual components of the complex provide multiple functional outputs (filament nucleation, filament bundling and transport along filaments), multiple levels of control (by guanine nucleotide exchange, phosphorylation and local concentration), and functional redundancy. Of course many other functions can be envisioned. The central role played by SH3 domains promotes flexibility in possible interactions, because their relatively low affinity and modest specificity allows for the assembly of large but ‘informal’ complexes that can also disassemble rapidly in response to changes in the environment.

Perspectives

The WASP-mediated actin nucleation system is likely to be an excellent paradigm for the role of the ubiquitous SH3 domain in many other complex cellular processes. They will play varied and important roles wherever it is advantageous to bring together proteins with moderate specificity and affinity. Proteomics and genomics will soon provide us with the entire compendium of interactions that can occur among all SH3-containing proteins and their ligands. The overriding challenge will then be to tease out specific functional effects from this ‘probability cloud’ of potential interactions. Recent progress in constructing mutant SH3 domains that have very high selectivities for specific targets, or synthetic ligands that have high selectivities for specific SH3 domains, might allow us to inhibit discrete subsets of interactions without disturbing the entire equilibrium (although one suspects it will be difficult to perturb any aspect of the system significantly without some global effects). New approaches designed to direct the specific, pairwise association of proteins in the absence of other competing interactions might allow the consequences of any particular protein-protein interaction to be assessed. Advances in imaging techniques such as fluorescence resonance energy transfer (FRET) should allow us to visualize SH3-mediated interactions as they occur in the living cell. Furthermore, synthetic peptoid compounds that have high affinities and selectivities might eventually fulfill the longstanding dream of intervening in SH3-mediated interactions for the treatment of human disease. We have come a long way in the past ten years, but our progress has brought a more mature appreciation of just how much there is left to do.

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References


SH3 domains: complexity in moderation


