Searching for specificity in SH domains

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Elucidating protein–protein interactions has been a central feature to understanding intracellular signal transduction. Many of the binding sites of the interacting proteins in these pathways are within highly sequentially homologous and structurally conserved domains. We challenge the dogma that mutual exclusivity in signalling is derived from a high level of specificity in these domains.

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Chemistry & Biology 2000, **7**:R3–R8

1074-5521/00/$ – see front matter

The process whereby activation of a membrane-localised receptor leads to a signal being conveyed through the cytoplasm, giving rise to a cellular response, is dominated by a series of protein–protein interactions. For a given signalling pathway, such as that based on tyrosine kinase activity, the current dogma holds that activating the receptor gives rise to a linear pathway. The activated receptor therefore interacts with one protein that in turn interacts with a downstream effector or adaptor protein. Subsequently, a series of bimolecular interactions produce a relay of interactions that occur right down to the nucleus and ultimately result in a cellular response. This dogma has largely arisen because it provides a simple explanation and because of the types of experiments that have been used to address the issue of signal transduction. For example, immuno-precipitation experiments under most conditions will only show the protein(s) that interact(s) directly with the designated protein of interest.

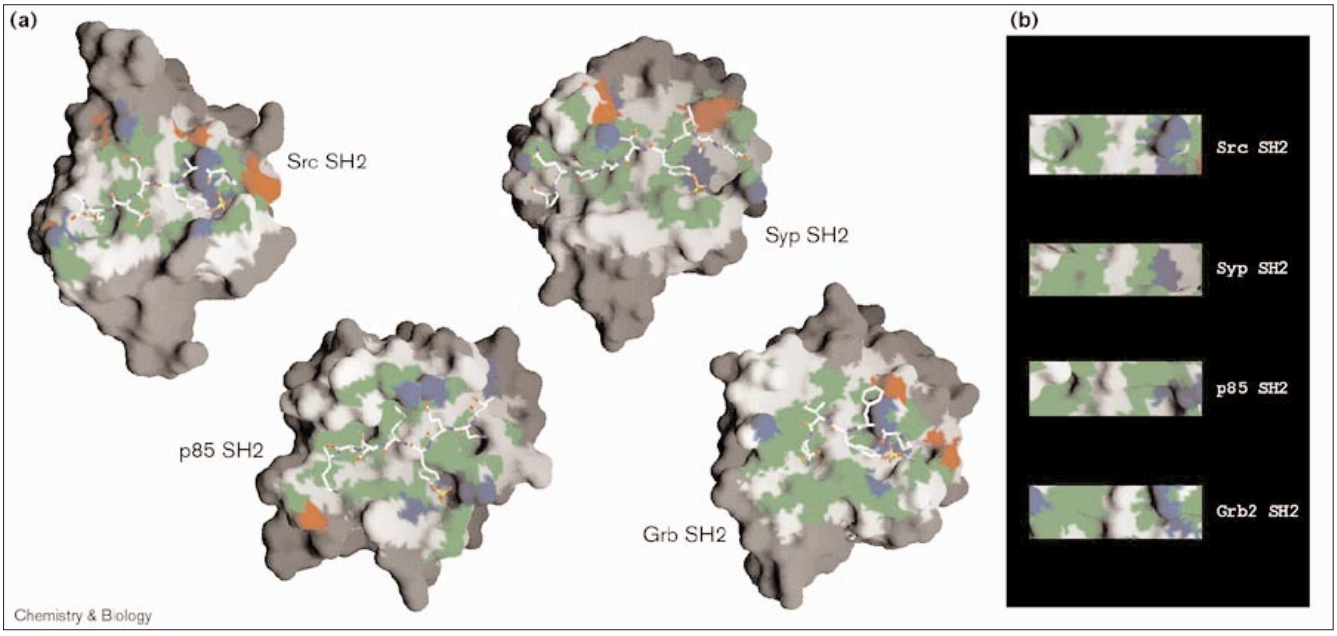
A linear signalling process requires that the interactions between the molecules in the transduction pathway are highly specific and do not allow the passage of the signal if interaction with the wrong upstream activator occurs. Perhaps the most extensively studied system exemplifying the necessity for specificity is that involving the tyrosine-kinase-mediated pathways. One prominent feature of the proteins involved in interactions in these pathways is that many of them contain similar structurally independent domains (for example SH2, SH3, PH and PTK; reviewed in [1]). Because similar domains are present in different pathways, which give rise to very different cellular responses, it suggests that a high level of specificity must prevail in the interactions of these domains. It is very difficult, however, to see how this exquisite specificity arises because the domains show such high sequence and structural homology. The interactions of SH2 and SH3 domains form the focus of this discussion of specificity.

Specificity in SH2 domains

SH2 domains consist of ~100 amino acids that fold into a structure dominated by a β sheet sandwiched by a pair of α helices (see, for example, [2]). The role of SH2 domains in intracellular signal transduction was shown to be centred around the interaction with proteins that have phosphorylated tyrosine residues (pY). These interactions can, potentially, be mimicked by tyrosyl phosphopeptides. High-resolution structural studies reveal that the basis for this binding involves the insertion of pY into a deep pocket on the SH2 domain. The pockets are, for the most part, very similar in all SH2 domains, forming an anchoring point for the ligand but offering nothing in the way of specificity. A highly conserved arginine residue interacts with one of the negative charges on the phosphate group, pinning it into the pocket. The tyrosyl phosphopeptide is generally held in an extended conformation, but there are a few exceptions, such as in the binding of peptides to the SH2 domain of Grb2. Structural studies reveal that, in this case, the peptide adopts a β-turn conformation (see Figure 1a) [2].

The identification of many proteins containing SH2 domains revealed their role in mediating different linear signalling pathways that produce diverse cellular responses. The involvement of SH2-containing proteins in different pathways suggested that there was an inherent specificity associated with their interactions. Studies using tyrosyl phosphopeptides with random sequences proximal to the pY appeared to reveal where this specificity emanated from. The interactions of individual SH2 domains were shown to have preferences for a given sequence of amino acids, either amino- or carboxy-terminal of the pY. In many cases the interaction of the third residue carboxy-terminal of the pY (pY + 3) was deemed important [1]. For example, the SH2 domain of Src was shown to require the sequence pYEEI (using single-letter amino-acid code), whereas the amino-terminal SH2 domain from the p85 subunit of PI3-kinase recognised the sequence pYMXM (where X is any amino acid) [3]. The experiments that determined these preferences by their nature (i.e. using successive rounds of binding, washing and hence enrichment of the library towards tighter binding epitopes) would inevitably select tighter binding ligands, but the level of difference in affinity that this corresponds to does not have to be high.

Figure 1



(a) Space-filling models of SH2 domains from four proteins. The binding surfaces are shown with the nonpolar (green), polar (grey) and charged (positive – blue; negative – red) residues highlighted. Despite the SH2 domains being from proteins from varied signalling pathways the similarity of the binding surfaces is dramatic. **(b)** Focusing in on the binding sites of the SH2 domains shown in (a) to highlight the similarities of the surfaces as are experienced by potential ligands.

The expected high level of specificity should be reflected in a large difference in the binding affinity of a specific compared with a nonspecific ligand for a given SH2 domain. A series of interactions have been studied using a variety of SH2 domains and tyrosyl phosphopeptides. These studies showed that the difference between a so-called specific and a nonspecific interaction amounts to less than two orders of magnitude in affinity. For example, Table 1 shows the binding data for interactions of a range of tyrosyl phosphopeptides with the SH2 domain from the protein Src. These data raise the question of whether the specificity demonstrated in these peptide-binding studies is sufficient to guarantee mutual exclusivity in signalling pathways in cells that might have numerous SH2-domain-containing proteins. For example, the peptide based on the platelet-derived growth factor (PDGF) receptor (with the motif pYVPM) binds to the SH2 domain only about an order of magnitude less tightly than the peptides containing the specific pYEEI motif. Thus, potentially in the presence of high, localised concentrations in a given cell line the PDGF receptor would compete for the Src SH2 domain at the expense of the physiological ligand. Activation of Src through stimulation of the PDGF receptor could therefore result, leading to an erroneous signal. Table 1 also reveals that the expected specificity from the interaction in the pY + 3 pocket is very limited and makes little apparent difference to the overall binding. Furthermore, the binding of a peptide that has a sequence very different from that of the specific peptide, such as that of the carboxyl terminus of Src (pYQPG), binds with an affinity only of approximately two orders of magnitude weaker.

The literature reveals many additional examples in which different peptide sequences do not seem to bind to SH2

Table 1

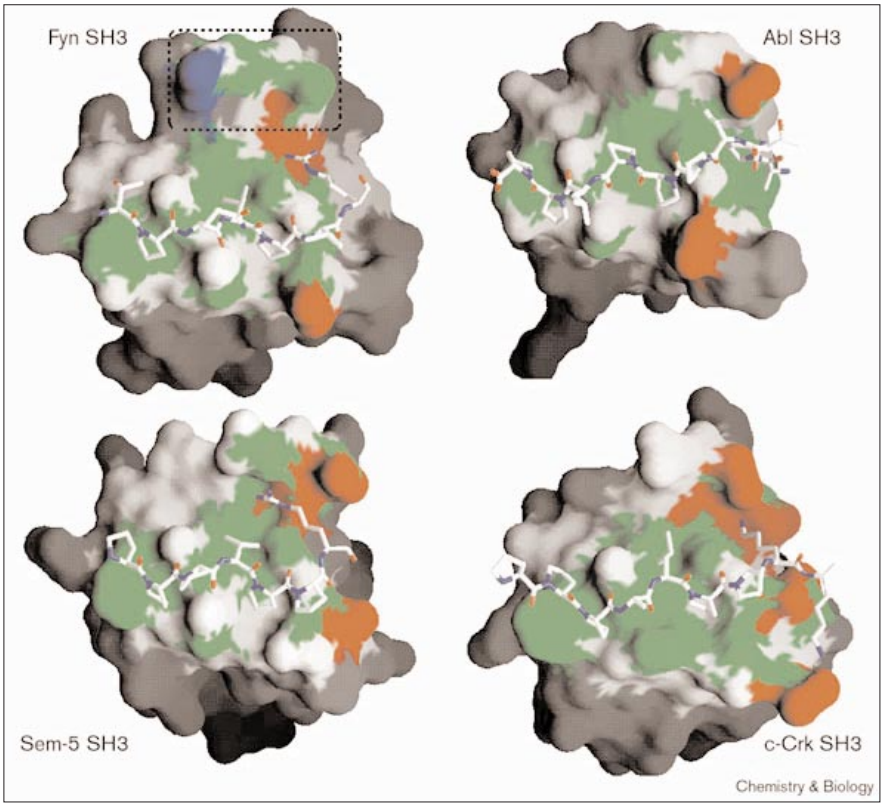
Binding of Src SH2 domains to tyrosyl phosphopeptides.

Tyrosyl phosphopeptide	K_D (μM)	Reference
KGGQpY**EEI**PIP*	0.55	[20]
EPQpY**EEI**PIYL†	0.09	[21]
EPQpY**EEI**	0.18	[22]
PQpY**EEI**PI	0.20	[23]
PEGDpY**EEV**L	0.16	[22]
EPQpY**EEV**PIYL	0.16	[21]
EPQpY**EEE**PIYL	0.21	[21]
TQpY**VPM**LE‡	5.88	[23]
EPQpY**QPG**EN§	14.3	[21]
PQpY**QPG**EN	29.4	[23]

All binding studies were performed under the following conditions unless indicated: 25°C and 20 mM MES buffer, 50 mM NaCl, pH 6.0. 10 mM HEPES, 90 mM NaCl, pH 7.4. †Derived from the hamster middle T antigen; ‡derived from the platelet-derived growth factor receptor; §derived from the carboxy-terminal sequence of Src. Bold indicates key residues involved in recognition by the SH2 domain.

Figure 2

Space-filling models of SH2 domains from four proteins. The binding surfaces are shown with the nonpolar (green), polar (grey) and charged (positive – blue; negative – red) residues highlighted. Again, as with the SH2 domains, although the SH3 domains being from proteins from varied signalling pathways the similarity of the binding surfaces is clear. The surface within the black dots represents an area on the surface of the Fyn SH3 domain where additional interactions have been observed in the structure of the complex with Nef (see text for details).



domains with high levels of specificity. The tyrosyl phosphopeptides GHDGpYQGLSTATK and ATKDTpYDALHMQA bind to the SH2 domain from Shc with K_D values of 50 μM and 650 μM, respectively [4]. This difference of little more than an order of magnitude for two apparently quite different peptides suggests a low level of discrimination in binding.

Some level of promiscuity in SH2 domain binding has been demonstrated in *in vivo* studies. Mutations in the SH2 domain of Sem-5 produce a vulva-less phenotype in *Caenorhabditis elegans*. A chimeric form of Sem-5 in which the SH2 domain was replaced by that of Src was able to rescue vulval development at nearly 30% of the wild-type efficiency on micro-injection into a cell line containing a strong *sem-5* allele displaying the completely vulva-less phenotype. The ability of the SH2 domain from a very different protein to rescue activity by binding to the site on the receptor defined as specific for Sem-5 emphasises that the level of specificity is not very high [5].

The lack of specificity in these interactions might be expected when one considers the binding sites presented to ligands by SH2 domains from a variety of proteins (Figure 1). It is clear from the alignment of these proteins showing the positively (blue) and negatively (red) charged, the nonpolar (green) and polar (light grey) residues in the binding sites that a potential ligand does not have much to distinguish one site from another. The general topology of the sites is very similar; the flexible peptide traverses a hydrophobic patch with a stripe of polar residues running vertically across it. In all the structures there is a positively charged patch that includes a highly conserved arginine residue, which accommodates the pY phosphate moiety (see above). Figure 1b shows the region occupied by the peptide on the SH2 domain surface. The alignment of all the peptide sites shows that they all have very similar features. A possible analogy to this would be to consider the peptide as an aircraft searching for its required landing field (the cognate SH2 domain binding site). On looking at Figure 1b there is little identify the airport to the pilot.

Specificity in SH3 domains

Like SH2 domains, SH3 domains play a role in the localisation of proteins to defined sites, anchoring them through interactions with sites containing proline-rich amino acid sequences. The first reports of ligands for SH3 domains demonstrated that large polypeptides containing these sequences could bind more tightly than short peptides containing the proline-rich sequence in isolation [6,7]. It was generally accepted, however, that the specificity in binding was dominated by the proline-rich regions alone

(other interactions were largely forgotten). Differences in binding motifs containing the sequence PXXP were shown to offer different levels of specificity.

The SH3 domain structure consists largely of two β sheets that form a partly open β barrel. Structural studies have revealed that the binding site for the proline-rich motif is a largely featureless hydrophobic patch (Figure 2). The issue of specificity of ligands for SH3 domains was again addressed using an approach based on screening with limited random peptide libraries (e.g., [8,9]). Peptide binding studies suggest that some additional specificity could emanate from some residues carboxy-terminal of the proline-rich motif. This was emphasised in studies showing that for some SH3 domains the proline-rich peptide could be orientated in two opposed directions depending on the flanking sequence of the residues in the peptide [10,11]. There are a large number of reports of the binding of peptides to SH3 domains from a variety of proteins and these generally confirm that differences in K_D values only encompass about two orders of magnitude (Table 2). As with the SH2 domain interactions, there does not seem to be a high level of specificity invoked. For example, the SH3 domain from Fyn binds equally well to peptides bearing the proline-rich motifs from Sos and the p85 subunit of PI3-kinase. This SH3 domain (based on binding studies with peptides) cannot, therefore, discriminate between binding sites on two very different proteins that occur in the same cell lines. Structural details on SH3 domains in complex with intact proteins suggest that other interactions outside those mimicked by peptides might be important [12], but binding studies suggest that affinities do not increase by more than an order of magnitude when additional interactions are incurred [13]. Furthermore, in one of these studies the SH3 domain interacts with a viral protein (Nef) that has potentially evolved a mechanism to exploit additional interactions to allow it to better compete with pre-existing cellular proteins [14] (Figure 2).

Table 2

Binding of peptides containing proline-rich motifs to SH3 domains from a range of proteins.

SH3 domain	Peptide	K_D (μM)	Reference
Abl	RAPTMPPPLPP (3BP-1)	34	[24]
Abl	PPAYPPPPVP (3BP-2)	5	[24]
Fyn	PVRPQVPLRPPMT (Nef)	202	[12]
Fyn	PPRPTPVAPGSSKT (p85)	50	[25]
Fyn	HSIAGPPVPPR (Sos1-4)	20	[24]
Fyn	RAPTMPPPLPP (3BP-1)	34	[24]
Fyn	PPAYPPPPVP (3BP-2)	34	[24]
Fyn	PPRPLPVPPGSSKT (p85)	16	[16]
Grb2	VPPPVPPRRR (Sos)	5	[26]
Grb2	GTDEVPVPPPVPPRRRPESA (hSos)	21	[27]
Hck	PVRPQVPLRPPMT (Nef)	91	[15]
p85	RKLPPRPSK (libraries)	9	[28]
Src	RALPPLPRY (libraries)	8	[29]
Src	HSIAGPPVPPR (Sos1–4)	26	[24]

As in the SH2 domain, the SH3 binding site has few distinguishing features among SH3 domains from a diverse sample of proteins (Figure 2). The bound peptide appears to follow a hydrophobic furrow running along the surface of the domain. All the domains seem to have a pair of negatively charged patches, one of which forms a recognition site for a positively charged residue if it appears in the PXXP motif (e.g. arginine in the sequence from Nef PLRP). Again, the pilot trying to identify the correct landing strip would struggle to identify whether a particular binding site was London Heathrow or London Gatwick.

Is the specificity in SH domain interactions sufficient?

For both SH2 and SH3 domains the difference between a specific and a nonspecific interaction is less than two orders of magnitude in affinity. A difference in K_D of approximately two orders of magnitude in some interactions is considered to be specific. For example, the difference between a specific interaction and a nonspecific interaction for gene repressor proteins is often not more than two orders of magnitude. In these interactions, however, the control of the downstream event is based on having the operator site on the DNA occupied and hence repressing gene expression. Different concentrations of protein will only affect the outcome if they drop below the point where the operator is not occupied. Elevated concentrations above this have no effect on the biological outcome. The transduction of a defined signal from one protein to another in a linear manner requires a high level of integrity, however. With many similar domains present in the cell, over-expression of one can lead to competition for the available binding sites. Two orders of magnitude difference in affinity can even be considered promiscuous because, for example, it represents the range exhibited by the protein OppA for the binding of tripeptides. OppA will bind peptides with any of the naturally occurring amino acids in any of the three positions. As this protein is involved in mopping up peptides in the bacterial periplasm, it ideally should not show any specificity for its ligands [15]. In the case of SH domains, is a difference in affinity of two orders of magnitude enough to ensure that only the specific interaction prevails?

In a cellular context, specificity is not an intrinsic characteristic of a protein interaction (such as affinity) but is a relative characteristic defining the ability of a protein to discriminate in favour of its cognate ligand. Given the fact that more than 100 different SH2 and SH3 domains are encoded by genes in eukaryotic cells, an SH domain has to potentially compete for its 'specific' ligand against an

ensemble of potentially 100 times more numerous non-specific interactions, that is to say, the bulk of structurally similar SH domains. If the nonspecific SH domains are in concentrations equal to the specific SH domain, they will therefore compete for the ligand and predominate in binding, despite a difference of two orders of magnitude in affinity.

How might specificity be induced in SH domain signalling?

The fact that activation of a given signal transduction pathway will produce a defined response suggests that erroneous pathways could frequently be activated. As this does not appear to be the case (or at least the outcomes of these are not frequently apparent) the cell must have mechanisms to combat such potentially deleterious effects.

Cellular co-localisation of proteins

Specificity only becomes an important issue if elevated concentrations of nonspecific ligands can be found to compete for the specific sites. Little is known about the localised concentrations of proteins in cells. Some proteins can potentially be compartmentalised by, for example, pH gradients in the cell or by other proteins (e.g. chaperones). The cell membrane itself can potentially give rise to high local concentrations of given proteins. Proteins that bind preferentially to a given lipid can be localised by the partition of lipids in the membrane into rafts. For example, there is some evidence that activation of protein kinase C is enhanced by lateral heterogeneities of lipids in the membrane bilayer [16].

A way of maintaining mutual exclusivity in the absence of high levels of specificity in the interactions of individual domains, and one that is gaining wider acceptance in the field, is to invoke the assembly of large signalling complexes involving a multitude of interacting proteins. Rather than a system of individual interactions of proteins building up to produce a linear pathway, stimulation of a membrane-bound receptor results in the recruitment of a large number of proteins that assemble at the membrane. The involvement of a number of proteins means that, although the affinity of any given interaction might be low, the presence of this protein is necessary for the complete assembly and only once the correct assemblage of proteins is in place will downstream signalling be affected. The order of assembly, the time for which the complex prevails and conformation of particular proteins in the complex are likely to be of importance in the integrity of the signal. The multi-protein aggregate therefore forms a gate to signal transduction like a capacitor in an electric circuit.

Parallel processing of signalling pathways

Another way of circumventing the requirement for a high level of specificity in any given SH domain interaction is to invoke the parallel processing of signals. In this case the activation of a receptor might recruit a protein containing a modular domain. This protein can interact with more than one downstream effector, which can result in the initiation of more than one signalling pathway. For a signal to be transduced, however, the pathways emanating from the activated receptor ultimately converge on a protein that forms a 'junction' in the pathways. One way in which any aberrant pathways can be removed is if the downstream signal from the junction protein can only be activated if two or more incoming signals arrive at the junction protein simultaneously. As the downstream effect of receptor activation will occur when both pathways are activated and reach the junction protein, then this parallel processing of signals can preclude activation of a pathway by the wrong ligand. These junction proteins can therefore interact with proteins from different signalling pathways.

The effect of multiple domains

In many proteins, SH2 and SH3 domains are juxtaposed with other ligand-binding domains (reviewed in [17]). In some cases it is clear that the combined effect of the interaction of two or more domains with a given ligand adds a higher level of specificity [18]. This occurs not only from the combined effect of the favourable free energies of interaction of the individual domains, but also from the steric restrictions imposed on interactions by the relative positions of the domains. It is clear, however, that not all interactions involve the effects of multiple domains; for example, Grb2 binds to receptors with only its SH2 domain. The adaptor protein links the β-adrenergic receptor with c-Src via the SH3 domain of the latter protein [19]. The SH2 domain of Src appears to play no role in this complex.

Implications for pharmaceutical research

The lack of specificity shown by both SH2 and SH3 domains might provide an explanation for the general failure of the pharmaceutical industry to develop suitable inhibitors of these interactions. Much effort has been focused on the screening of SH domains for compounds to interfere with defined signalling pathways. Originally, these appeared to be highly suitable targets because an appropriate compound could selectively knockout a specific aberrant pathway, whilst leaving other cell functions unaffected. The lack of specificity might lead to any potential drug compound interacting with numerous SH domains in the cell with undesired effects.

On the basis of the low levels of specificity observed in the interactions and the large number of proteins containing SH domains that often play a crucial role in distinct signalling pathways, it seems that the integrity of signals has to be guaranteed by mechanisms other than simple linear transduction. Here we have tried to highlight the potential inadequacies in invoking linear pathways to explain intracellular signalling and attempted to highlight other more complex mechanisms. To fully understand how higher order structures or protein localisation processes might

provide appropriate signalling requires a new approach to experiments and, potentially, a different interpretation of existing data.

Acknowledgements
We thank M.A. Lemmon for his thought provoking ideas and for stimulating the writing of this article. Gratitude to Molly's Academy, N.Y.C. Thanks also to M. Eck for the co-ordinates of the p85 SH2 domain and additional comment. J.E.L. is a Wellcome Trust Senior Research Fellow.

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