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Proline-rich sequences that bind to Src homology 3 domains with individual specificities

(Abl/Src/protein-tyrosine kinase/protein–protein interaction)

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ABSTRACT **To study the binding specificity of Src homology 3 (SH3) domains, we have screened a mouse embryonic expression library for peptide fragments that interact with them. Several clones were identified that express fragments of proteins which, through proline-rich binding sites, exhibit differential binding specificity to various SH3 domains. Src-SH3-specific binding uses a sequence of 7 aa of the consensus RPLPXXP, in which the N-terminal arginine is very important. The SH3 domains of the Src-related kinases Fyn, Lyn, and Hck bind to this sequence with the same affinity as that of the Src SH3. In contrast, a quite different proline-rich sequence from the Btk protein kinase binds to the Fyn, Lyn, and Hck SH3 domains, but not to the Src SH3. Specific binding of the Abl SH3 requires a longer, more proline-rich sequence but no arginine. One clone that binds to both Src and Abl SH3 domains through a common site exhibits reversed binding orientation, in that an arginine indispensable for binding to all tested SH3 domains occurs at the C terminus. Another clone contains overlapping yet distinct Src and Abl SH3 binding sites. Binding to the SH3 domains is mediated by a common PXXP amino acid sequence motif present on all ligands, and specificity comes about from other interactions, often ones involving arginine. The rules governing *in vivo* usage of particular sites by particular SH3 domains are not clear, but one binding orientation may be more specific than another.**

Protein–protein interactions are central events in cellular signal transduction. These interactions are often mediated by noncatalytic conserved domains, three of which are the Src homology regions 2 and 3 (SH2 and SH3), first identified as part of the Rous sarcoma viral oncogene product (1, 2), and the plekstrin homology (PH) domain, more recently identified by protein sequence alignments (3, 4). To date, the best understood protein interactions are those mediated by the SH2 domains, which bind to phosphotyrosine residues on proteins that have been primed for signaling through phosphorylation.

The function of SH3 domains is less clear. In relation to the oncogenic potential of protein-tyrosine kinases, these domains play an inhibitory role because mutation or deletion of them activates the transforming potential of c-Src and c-Abl protooncoproteins (5–7). The first clue to the function of SH3 domains was obtained by the cloning of fragments of two protein ligands for the Abl SH3: 3BP1 and 3BP2 (8). In these proteins, proline-rich peptides only 10 aa long serve as targets of SH3 binding (9). Since then, a number of interactions between SH3 domains and proline-rich ligands have been described, showing that SH3 domains are involved in epidermal growth factor receptor signaling (10), cellular localization of cytoplasmic proteins (11), upregulation of the GTPase activity of dynamin (12), and activation of phosphatidylinositol 3-kinase in response to IgM crosslinking (13).

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From these and other reports it is now clear that interactions between SH3s and their ligands occur through proline-rich sites. It is less clear, however, how the specificity of interaction is achieved. In this study we report the isolation of peptide fragments from proteins that exhibit differential binding specificity to various SH3s. Specificity of interaction is mediated by unique sequences that interact with binding pockets found on the surface of SH3 domains. Our data correlate well with recent reports that identify specific artificial ligands for different SH3 domains from chemically synthesized and phage display libraries (14–16) and suggest that these ligands do occur *in vivo*.

MATERIALS AND METHODS

Library Screening. A 16-day mouse embryo cDNA expression library (Novagen) was screened for SH3-binding proteins according to the manufacturer's protocols and as previously described (8). Biotinylated glutathione *S*-transferase (GST)-SH3 fusion proteins of c-Abl, c-Src, c-Crk, and neuronal Src (n-Src) were used as probes to screen the library. Positive phage plaques were purified and recombinant phage were converted into plasmids through Cre-mediated excision from λEXlox by plating phage with the appropriate host. Recombinant plasmids (pEXlox) were then transformed into competent bacteria (Novagen) that permitted expression of the cloned cDNAs as fusion proteins of gene *10* of phage T7.

Filter Binding Assay. Bacteria cell lysates were prepared as described (9). Proteins were fractionated by electrophoresis through polyacrylamide gels and transferred to nitrocellulose filters that were processed as described (9). Immunoblotting with affinity-purified polyclonal antibody to GST was performed as described (9).

Subcloning. Construct GST-10a (see Fig. 2*D*) contains a 92-codon fragment of the library clone derived by oligonucleotide-directed polymerase chain reaction (PCR) and subcloned in frame into the pGEX2T prokaryotic expression vector. Primers for the PCR contained 24 bp of coding sequence, a *Bam*HI site at the 5′ end of the sense strand oligonucleotide, an *Eco*RI site at the 5′ end of the antisense strand oligonucleotide, and six additional nucleotides at the 5′ end of each oligonucleotide for stability and recutting efficiency. The amplified fragment was subcloned into pGEX2T in frame at both ends. The remaining GST fusion constructs represent potential binding sites and were constructed by using complementary oligonucleotides containing coding sequences with *Bam*HI and *Eco*RI sites at the 5′ and 3′ ends, respectively, with five additional nucleotides at each end for cutting efficiency. Complementary oligonucleotides were annealed by boiling for 2 min and then cooling slowly to room temperature. The DNA fragments were digested with *Bam*HI and *Eco*RI restriction enzymes and cloned into pGEX2T. The Btk binding site was cloned as described (17).

Abbreviations: SH3, Src homology 3; GST, glutathione *S*-transferase.

Sequencing of the library clones and the GST fusion constructs was carried out with the Sequenase kit (United States Biochemical).

Biotinylated Proteins. The c-Abl, c-Src, n-Src, and v-Crk GST-SH3 fusion proteins were prepared as described (8). Cloning of the GST-SH3 fusion proteins for Fyn, Lyn, and Hck has been described (17). Biotinylation of the SH3 domains was performed as described (18).

RESULTS

Phage plaques expressing members of an embryonic mouse cDNA library were screened with a mixed, biotinylated SH3 probe and partial cDNA clones expressing peptides that bound to SH3 domains were isolated. All of the clones derived from proteins with no counterpart in the GenBank data base (November 1994). Total bacterial lysates expressing the isolated clones as phage T7 gene *10* fusion proteins were fractionated by electrophoresis, transferred to filter paper, and probed with individual biotinylated SH3 domains (Fig. 1). Two clones, 2b (and the identical 9c) and 9h, bound to all the SH3 domains tested, whereas clone 10a interacted with only c-Src and n-Src SH3 domains. Clones 10b, 10g, and 10l exhibited binding affinity for only the Abl SH3. Clone 10c and GST alone did not bind to any of the SH3 domains tested. Certain of the clones were examined further.

To determine how specificity of interaction is achieved, we first examined the interaction of clone 10a with the Src SH3. GST fusions of 10a subfragments were generated (Fig. 2*A*) and tested by filter binding. An 18-aa peptide (peptide 2) bound the SH3 (Fig. 2*B*). An N-terminal arginine was indispensable for binding, since deletion of this arginine (peptide 1) or substitution of a lysine for this arginine (peptide 3), abolished or greatly reduced the binding affinity of this peptide (Fig. 2*B*). The first 7 aa of peptide 2 (peptide 4) had the full binding affinity of the longer peptide. This binding was quite strong—a previously reported binding site for the Src SH3 on the p85α subunit (peptide 5) showed very little binding when compared with the RPLPALP sequence of 10a (Fig. 2*B*). An extended construct that contained the C-terminal half of this peptide and 2 aa immediately downstream (peptide 6) showed increased binding when compared with peptide 5, but the binding was still lower than with peptide 4. We then searched the database for other proteins that contained the RPLPALP motif. Two other proteins, a K^+ channel and the calcitonin receptor, contained sequences (peptides 7 and 8, respectively) that closely matched the Src SH3 binding site on 10a. When these were tested in filter binding assays they exhibited high binding affinity for the Src SH3 that matched that of 10a. The binding activity of the 10a peptide was compared with that of peptides from a biased combinatorial peptide library recently shown to bind to the Src SH3 with high affinity (14). Fragment 10a was tested against peptides 9 and 10 and exhibited binding

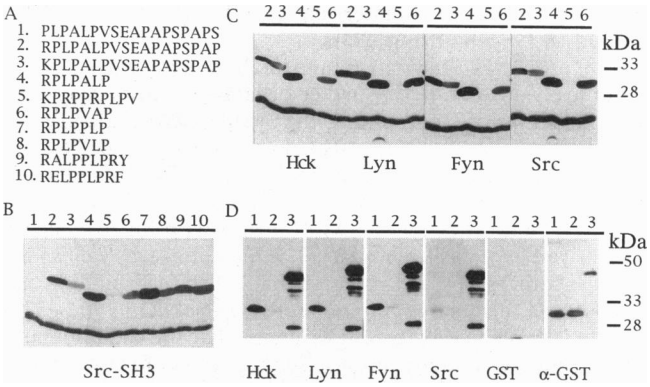

FIG. 2. A peptide fragment with the consensus sequence RPLPXXP binds to SH3 domains of Src and Src-related kinases. (*A*) Sequences of peptides that were screened. Peptides 1–4 were derived from the 10a protein fragment. Peptides 5 and 6 were from the p85α subunit of phosphatidylinositol 3-kinase. Peptides 7 and 8 were from proteins identified in the GenBank database. Peptides 9 and 10 were high-affinity Src SH3-binding peptides defined in a peptide library screen by Yu *et al.* (14). (*B*) Complementary oligonucleotides encoding the 10 peptides were cloned into pGEX2T and were expressed as GST fusion proteins. Blot transfers of electrophoretically fractionated bacterial lysates were probed with biotinylated Src SH3. (*C*) Blot transfers of bacteria lysates expressing GST fusion peptides 2–6 were probed with various SH3 domains as shown. (*D*) Blot transfers of GST fusions of KKPLPPTPEE (lane 1) and KKPLPPEPTA (lane 2)—high- and low-affinity sites, respectively, present on Btk—and a 92-aa fragment of 10a (lane 3) were probed with SH3 domains from Src and Src-related kinases. Biotinylated GST and antibodies to GST (α-GST) were included as controls.

constants of 7.8 and 9.6 μM, respectively. The binding of 10a to the Src SH3 was at least as good, if not slightly better, that of peptides 9 and 10, suggesting that 10a does indeed bind to the Src SH3 with high affinity as compared with published data.

Peptides 2–6 bound to the SH3 domains of the Src-related kinases Fyn, Lyn, and Hck with the same affinity as to the Src SH3 (Fig. 2*C*). This is not surprising, since the three-dimensional structures of these SH3 domains overlap closely. However, differences in binding to proline-rich sequences do exist between the members of the Src kinase family. It has been shown recently that the Fyn, Lyn, and Hck SH3 domains, but not the Src SH3, bind to Btk (Bruton tyrosine kinase) through a KKPLPPTPEE motif (17). This fulfills the requirements of the RPLPXXP motif except for the R $\rightarrow$ K substitution. We therefore compared the affinities of the Src SH3 and Src-related SH3 domains for the Btk and 10a binding sites. As predicted, all the SH3 domains bound to GST-10a, whereas only Fyn, Lyn, and Hck SH3 domains bound to the Btk site (Fig. 2*D*). The affinity of the SH3 domains for GST-10a was higher than that for Btk, since stronger binding was observed

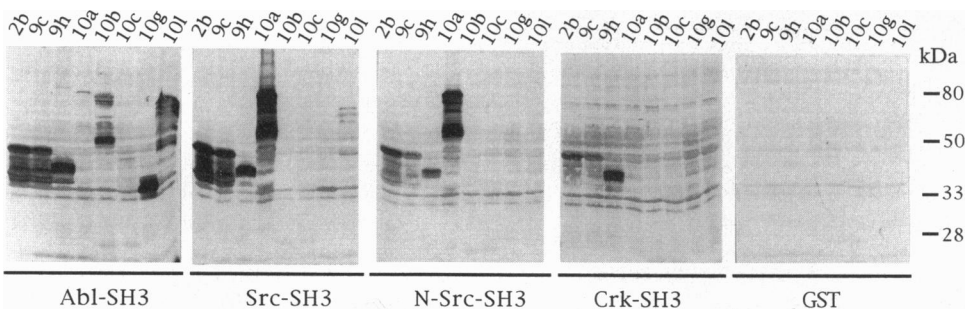

FIG. 1. Determination of peptide binding specificities. Bacteria were transformed with pEXlox expressing fusion proteins made by linkage of gene *10* of phage T7 (*Materials and Methods*) and cDNAs cloned into an *Eco*RI–*Hin*dIII site of pEXlox. Expression of fusion proteins was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 2 hr. Identical blotted transfers of bacterial lysates were probed with biotinylated GST-SH3 fusion proteins or with GST as indicated. The blots were developed with streptavidin-conjugated alkaline phosphatase.

with 10a when approximately equal amounts of protein were loaded (α-GST blot, Fig. 2*D*).

Clone 9h was one that bound to both Src and Abl SH3 domains; we examined it further because it had no apparent RPLPXXP motif for binding to the Src SH3. We could localize the binding sites for both SH3 domains to peptide 9h-3, a proline-rich region of sequence PPPPPPPLPPR (Table 1). The C-terminal arginine was essential for binding to SH3 domains, as were at least some of the five N-terminal prolines (compare 9h-derived peptides in Table 1). The data above, as well as the work of others (14, 16), suggest that a Src SH3-binding site needs an N-terminal arginine, but that is lacking in 9h-3. This puzzle is explained by recent evidence that the SH3 domains of Src and phosphatidylinositol 3-kinase select for two classes of ligands from a biased random peptide library (14) (15) that bind in opposite orientations to the SH3: class I ligands, containing RPLPPXP motif, and class II ligands, containing the consensus XXXPPLPXR (15). From these data we can conclude that clone 10a is a class I ligand whereas 9h is a class II ligand and that the two bind to the Src SH3 in opposite orientations. It is perhaps significant that 9h binds to the Abl SH3 as well as to the Src SH3, whereas 10a is specific for Src-family SH3 domains. This suggests that peptides binding in the class I orientation may be more specific than those binding in the class II orientation.

A third clone, 2b, that also exhibited broad binding specificity was mapped for sites that interacted with different SH3 domains. Two proline-rich sites were evident by inspection (2b-1 and 2b-5, Table 1). Peptide 2b-1 bound to both the Abl and the Src SH3 with high affinity, whereas 2b-5 interacted only weakly. A Src SH3 site, RPLPPPP, was immediately apparent in 2b-1. Peptide 2b-5, which did not bind to the Src SH3, contained a closely related sequence, RAPPPPP, showing that leucine at position 3 is important for ligand binding to the Src SH3. Binding to the Abl SH3 seemed more complex. Because 2b-3 did not bind, but 2b-2 and -4 bound well (Table 1), it appears that binding to the Abl SH3 is mediated by either of two overlapping sites. These sites (as well as 9h-3 and segments of 10g and 10l in Fig. 1) fulfilled the previously suggested requirements for Abl SH3 binding—namely, prolines at positions 2, 7, and 10 (see Table 2) (9, 19). Thus proteins can contain sequences that bind to more than one SH3 domain by using different subsites.

Our data and those of others (14–16) imply that SH3 domains from Abl and Src (and Src-related kinases) recognize ligands with different consensus sequences. A recent report, however, suggests that short peptides from proteins 3BP1 and 3BP2, previously identified as prototypical Abl SH3 ligands, bind the to the Abl and Fyn SH3 domains with the same affinity (K_d of 34 μM) (19). These peptides lack basic amino acids (see top lines in Table 2) and should be unable to bind to the Src-like Fyn SH3 domain. Therefore, the different sites were tested in parallel for binding to SH3 domains from Abl, Src, and Fyn (Fig. 3). Under the conditions used, we could not detect binding of the Src, and Fyn SH3 domains to 3BP1, 3BP2, and 10g, all of which specifically recognize the Abl SH3 (Fig. 3, lanes 1–3). In the same experiments 9h and 2b exhibited binding to all SH3 domains tested, as expected (Fig. 3, lanes 5 and 6), whereas 10a was recognized only by SH3 domains from Src and related kinases (Fig. 3, lanes 4). The differences between our data and those of Musacchio *et al.* (19) may be due to the different experimental conditions used for these experiments, although our filter binding assay data correlate well with the structural studies published by Yu *et al.* (14).

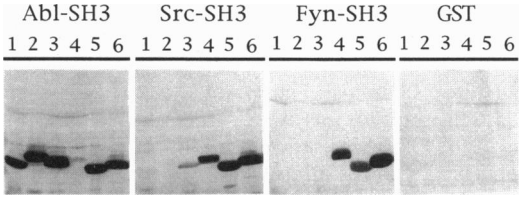

FIG. 3. Differential binding of peptide ligands to the Abl, Src, and Fyn SH3 domains. Western blots of lysates are shown of GST fusion peptides of the Abl SH3 ligands 3BP1-10 (9), 3BP2-12 (9), and 10g (lanes 1–3), the Src SH3 ligand 10a (lanes 4) (peptide 2, Fig. 2*A*), and 9h-3 and 2b-1 (lanes 5 and 6). Sequences are given in Table 2.

DISCUSSION

We have identified and characterized peptide ligands on unidentified cellular proteins that are binding sites for the Abl, Src, and Src-related SH3 domains. The rules for binding specificity and affinity that emerge from investigating these ligands are similar to those implied from studies of artificial peptide ligands. Binding is mediated through proline-rich sites that share common elements but also have residues that contribute to specificity.

Combining the data from this study with data from other studies, we can distinguish four types of SH3 binding sites (Table 2). All require a PXXP motif, but then they differ. The first difference is the class I/class II distinction, which determines the N-terminal to C-terminal ligand binding orientation. Among the class I sites are those specific for Src and those specific for Abl. The sites for Abl SH3 binding are about 10 aa long and conserve three prolines, those at positions 7 and 10, which are in the PXXP sequence, and another at position 2. Abl SH3 binding sites do not necessarily have any basic amino acids. The Src SH3 ligands are 7 aa long and have an arginine at position 1 and the PXXP from 4 to 7. Src-related kinases have SH3 domains that bind to the Src peptide but also, although less well, to peptides with a lysine in the place of the arginine.

Table 1. Identification of Src and Abl SH3 binding sites on clones 9h and 2b

Peptide	Sequence	Src SH3	Abl SH3
9h-0	AARAGQLEPQLRAPAHSSPLPPPPPPPPPPLPPRGAARSSPRSRSR	++++	+++
9h-1	AARAGQLEPQLRAPAHSSPLPPPPPPPPPPLPP	−	−/+
9h-2	PPLPPRGAARSSPRSPSR	++	+
9h-3	PPPPPPPLPPR	++++	+++
2b-0	RLGAPDSRPPARPLPPPPPAPRPPRCRRRLRFLRGGLRPSRRRLRAPPPPPPSTM	++++	++++
2b-1	PPARPLPPPPPAPRPPR	++++	++++
2b-2	PPARPLPPPPPA	++++	+++
2b-3	ARPLPPPPPA	++++	−
2b-4	ARPLPPPPPAPRPPR	++++	+++
2b-5	RPSRRRLRAPPPPPPST	−	+

Peptide 9h-0 is a segment of clone 9h that retains full binding to Src and Abl SH3 domains. Peptides 9h-1, -2, and -3 are deletion peptides of 9h-0. Peptide 2b-0 is a segment of clone 2b that retains full binding affinity for Src and Abl SH3 domains. Peptides 2b-1 to -5 are deletion peptides of 2b that define overlapping yet distinct Src and Abl SH3 binding sites. Blot transfers of bacterial lysates expressing the peptides as GST fusion proteins were probed with Src and Abl SH3 proteins. The relative binding of the peptides was graded from very strong (++++) to not detectable (−).

Table 2. Alignments of binding sites for various SH3 domains

SH3 domains	Peptide	Sequence
	Class I	
Abl	3BP-1	APTMPPPLPP
	3BP-2	PPAYPPPVP
	2B-1a	PPARPLPPPP
	2B-1b	RPLPPPPPAP
	2B-1c	PPPPPAPRPP
	10g	PPQLAPPPPR
Src, Fyn, Lyn, Hck	10a	RPLPALP
	p85a	RPLPVAP
	2b	RPLPPPP
Fyn, Lyn, Hck	BTK1	KPLPPTP
	Class II	
Abl, Src, Fyn, Crk, Src+*	9h	PPPPPPPLPPR
Grb-2	Sos	PVPPPVPPRRR
Crk, Grb-2	AB3-1	APELPTKTR
Crk	AB3-2	EPAVSPLLPRKER
Grb-2, Nck	AB3-3	MAPTPPKR

Amino acids that are important for binding within each group of ligands are boxed.
*n-Src indicates neuronal Src.

The class II ligands are often sites for SH3 domains of adapter proteins such as Crk (20) and Grb-2 (21–24) and have the general form XPXXPXR (15). Sequence alignments of Crk SH3 binding sites on c-Abl (20) and the Sos site that binds to Grb-2 indicate that lysine can substitute for arginine at the C terminus of Crk-SH3 ligands (Table 2). The Grb-2 site on Sos contains three arginine residues at the C terminus, all of which are required for high-affinity binding to Grb-2 SH3 domains (15). Extended peptides for the Crk SH3 contain additional arginine residues that may be important for binding (unpublished results). Therefore, more flexibility in the distance between the PXXP and the arginine may exist in class II ligands. That flexibility, as well as the presence of arginine or lysine at the C terminus, may be a source of specificity, since many of the class II sites are specific for one or another of the adapter proteins (20).

The structures of many SH3 domains with bound peptides are known at atomic resolution (25–31). From these structures, the binding rules can be rationalized. SH3 domains have an extended hydrophobic patch that binds the proline-containing ligands into two pockets, sites 1 and 2. In either orientation, the prolines bond with aromatic and proline residues. The arginine residue, which is a key feature of most ligands, fits into site 3, where it is anchored by interaction with acidic resides at the base of the site. The Src SH3 has only one acidic residue (position 99), whereas the Fyn, Lyn, and Hck SH3 domains have two (positions 98 and 99), perhaps explaining the ability of the latter to bind ligands where lysine has replaced arginine. Only the Abl SH3 lacks any acidic residue in this region, and the Abl SH3 binds to a ligand that lacks an arginine. The Abl SH3 ligand is longer and has a key proline near its N-terminal end that binds in the hydrophobic pocket adjacent to site 3. Being longer than the Src SH3 sites, it makes a 2-aa turn before binding back to the SH3. The class II ligands all contain a C-terminal arginine that can bind at site 3. Because they bind in opposite orientation from the class I ligands they are formally equivalent to the Src sites, although the presence of multiple basic residues on the C terminus suggests that more flexibility in the distance between the PXXP and the arginine may exist. This distance may correlate with specificity of binding, although no mutagenic studies of the role of that distance in binding specificity have been reported.

One apparent contradiction to this picture of SH3 specificity is that Musacchio *et al.* (19) found that an Abl SH3 ligand (the 3BP2 peptide) bound as well to the Fyn SH3 in spite of not having an arginine. From their crystal structure, it is evident that the Fyn SH3 binding can take place and that it is like the binding of the ligand to the Abl SH3 in that the site adjacent to site 3 is used to bind a proline residue. However, we found that the binding to the Fyn SH3 was so weak that it was not detectable by our assay, and therefore this is unlikely to be a physiological mode of binding.

Class II peptides seem more promiscuous in their binding than class I ligands. For instance, fragment 9h exhibited binding to Abl, Src, n-Src, Crk, and Fyn SH3 domains (Table 2). This may be a consequence of the flexibility in the distance from the PXXP to the arginine. It is important to note that 9h ends with the sequence PPPPLPPR, in which PXXP can be in either of two positions, and it could bind in one way to some SH3 domains and in another to others. This we found was the interpretation of the binding of fragment 2b to both the Src and the Abl SH3 domains.

In this work we have focused only on the binding of short peptides, but we have occasionally noted that longer peptides bind more strongly than short ones (unpublished results). This suggests that in real protein ligands, there may be SH3 contacts from more than one region of the protein. In one case—the binding of Hck SH3 to the human immunodeficiency virus Nef protein—we have developed direct evidence for interactions from two widely spaced sites on Nef (32). Because the extent of specificity evident in our studies is still quite crude, we believe that another level of specificity, perhaps deriving from multiple interactions with SH3, explains finer distinctions. In fact, the interaction with Nef occurs well with the Hck SH3 but poorly with the Fyn SH3, indicating that a complex ligand can make distinctions not evident when peptides are studied.

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