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Distinct ligand preferences of Src homology 3 domains from Src, Yes, Abl, Cortactin, p53bp2, PLCγ, Crk, and Grb2

(signal transduction/protein–protein interactions/phage display)

ANDREW B. SPARKS*†, JAMES E. RIDER†, NOAH G. HOFFMAN†, DANA M. FOWLKES‡§, LAWRENCE A. QUILLIAM¶, AND BRIAN K. KAY*†‖**

*Curriculum in Genetics and Molecular Biology, Departments of †Biology, ¶Pharmacology, and §Pathology, and ‖Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599; and ‡Cytogen Corp., Princeton, NJ 08540-5237

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ABSTRACT **Src homology 3 (SH3) domains are conserved protein modules 50–70 amino acids long found in a variety of proteins with important roles in signal transduction. These domains have been shown to mediate protein–protein interactions by binding short proline-rich regions in ligand proteins. However, the ligand preferences of most SH3 domains and the role of these preferences in regulating SH3-mediated protein–protein interactions remain poorly defined. We have used a phage-displayed library of peptides of the form X_6PXXPX_6 to identify ligands for eight different SH3 domains. Using this approach, we have determined that each SH3 domain prefers peptide ligands with distinct sequence characteristics. Specifically, we have found that the Src SH3 domain selects peptides sharing the consensus motif LXXRPLPXΨP, whereas Yes SH3 selects ΨXXRPLPXLP, Abl SH3 selects PPXΘXPPPΨP, Cortactin SH3 selects +PPΨPXKPXWL, p53bp2 SH3 selects RPXΨPΨR+SXP, PLCγ SH3 selects PPVPPRPXXTL, Crk N-terminal SH3 selects ΨPΨLPΨK, and Grb2 N-terminal SH3 selects +ΘDXPLPXLP (where Ψ, Θ, and + represent aliphatic, aromatic, and basic residues, respectively). Furthermore, we have compared the binding of phage expressing peptides related to each consensus motif to a panel of 12 SH3 domains. Results from these experiments support the ligand preferences identified in the peptide library screen and evince the ability of SH3 domains to discern subtle differences in the primary structure of potential ligands. Finally, we have found that most known SH3-binding proteins contain proline-rich regions conforming to the ligand preferences of their respective SH3 targets.**

Recent efforts to elucidate intracellular components of the signal transduction apparatus have led to the discovery of a series of conserved noncatalytic domains within a number of signaling proteins. These domains, which include SH2, PTB, and SH3 domains, are thought to mediate protein–protein interactions by binding specific ligand sequences in target proteins (reviewed in ref. 1). Initial understanding of the nature of SH3 ligands derived from the identification of two cDNA clones, 3BP1 and 3BP2, whose protein products bind the Abl SH3 domain. The proteins both contain proline-rich regions; peptides from these regions only 10 amino acids long suffice as SH3 ligands (2). Subsequent studies have documented interactions between SH3 domains and proline-rich ligands in a variety of physiological contexts. Such interactions include that of Grb2 with SOS in growth factor-mediated activation of Ras (3–5); those of Src, Lyn, and Fyn with the p85 subunit of phosphatidylinositol 3′-kinase (PI3K) in stimulation of PI3K activity (6, 7); and those of $p47^{phox}$ and $p67^{phox}$ with $p22^{phox}$ and $p47^{phox}$, respectively, in assembly of the phagocyte NADPH oxidase (8–10). From these and other studies, it is clear that SH3 domains recognize proline-rich sequences; all known SH3-binding proteins contain proline-rich regions with at least one PXXP motif (11). However, the factors that determine the specificity of SH3-mediated protein–protein interactions have not been clearly defined.

Several recent reports suggest that one such factor may be the capacity of different SH3 domains to recognize ligands with distinct sequence characteristics. For example, phage-displayed peptide libraries have been used to identify Src SH3 peptide ligands with the consensus sequence RPLPPLP (12, 13). These ligands display reduced binding to many other SH3 domains (12). Similarly, Yu *et al.* (14) used a synthetic peptide library to identify Src and PI3K SH3 ligands with the consensus sequence RXLPPZP (Z = L for Src and R for PI3K) and demonstrated that each SH3 domain bound its respective ligand with higher affinity than did the other.

In an effort to develop a more detailed understanding of the specificity of SH3–ligand interactions, we have constructed a phage-displayed library (termed the PXXP library) encoding peptides of the form X_6PXXPX_6, where X represents any of the 20 naturally occurring amino acids and P represents invariant proline residues. Using this library, we have identified peptide ligands for SH3 domains of Src, Yes, Abl, Cortactin, p53bp2, PLCγ, Crk, and Grb2. Each SH3 domain selects a set of peptide ligands sharing a distinct consensus motif; these motifs reflect the unique ligand preferences of each SH3 domain.

MATERIALS AND METHODS

Preparation of Glutathione *S*-Transferase (GST)–SH3 Fusion Proteins. Constructs encoding GST fusions to the Grb2 N-terminal (Grb2 N, aa 1–58), Grb2 C-terminal (Grb2 C, aa 154–217), Nck N-terminal (Nck N, aa 1–68), Nck middle (Nck M, aa 101–166), Nck C-terminal (Nck C, aa 191–257), p53bp2 (aa 454–530), or Src (aa 87–143) SH3 domains were generated by PCR cloning of the appropriate cDNAs into pGEX-2T (Pharmacia). The integrity of the constructs was confirmed by DNA sequencing. pGEX-derived constructs expressing GST fusions to the SH3 domains of Yes, Cortactin, Crk, Abl, and PLCγ were kindly provided by M. Sudol (Rockefeller University), J. T. Parsons (University of Virginia), M. Matsuda (National Institutes of Health, Tokyo), A. M. Pendergast (Duke University), and S. Earp (University of North Carolina, Chapel Hill), respectively. GST–SH3 fusion proteins were prepared as described (15). The integrity and purity of the fusion proteins were confirmed by SDS/PAGE. Protein con-

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Abbreviations: SH3, Src homology 3; GST, glutathione *S*-transferase; PI3K, phosphatidylinositol 3′-kinase.

**To whom reprint requests should be sent at the † address.

centrations were determined using the BioRad protein assay (Bio-Rad).

Phage-Displayed Peptide Library Construction. The PXXP library was constructed as described (16). Peptides were expressed as N-terminal fusions to the mature protein III of bacteriophage M13. Two synthetic oligonucleotides (5′-ctgtgcctcgagk(nnk)$_6$cca(nnk)$_2$cca(nnk)$_6$tctagacgtgtcagt-3′ and 5′-actgacacgtctaga-3′, where k = g+t and n = g+a+t+c) were annealed and filled in with Sequenase (Amersham). The resulting DNA fragment was digested with *Xho* I and *Xba* I and was ligated into gene III of a phage display vector (mBAX) that allows for selection of recombinants by reversion of an amber codon between the *Xho* I and *Xba* I sites of the vector. The ligated DNA was electroporated into JS5 *Escherichia coli* and recombinant phage were propagated on 200 100-mm 2× YT (16 g of tryptone/10 g of yeast extract/5 g of NaCl/1 liter of H_2O, autoclaved)/0.8% agar plates (17). To minimize the recovery of sibling clones during affinity purification of binding phage, six distinct library fractions were prepared by dividing the plates into six equal groups. Each fraction was treated separately in all subsequent manipulations. Phage particles were harvested from each fraction by diffusion into 100 ml of PBS (137 mM NaCl/2.7 mM KCl/4.3 mM Na_2HPO_4/1.4 mM KH_2PO_4), concentrated by polyethylene glycol precipitation (17), and resuspended in 10 ml of PBS/10% glycerol. Each fraction contained $\approx 5 \times 10^7$ unique recombinants, for a total library complexity of $\approx 3 \times 10^8$.

Affinity Purification of SH3-Binding Phage. Library screens were performed as described (16). Briefly, wells of an ELISA microtiter plate were coated with 10 μg of GST–SH3 fusion protein in 100 mM $NaHCO_3$ (pH 8.5) for 3 h and blocked with Superblock (Pierce) for 1 h. Approximately 5×10^{11} infectious particles from each library fraction were diluted in 200 μl of PBS/0.1% Tween 20 and incubated in a GST–SH3-coated well for 3 h. The wells were washed five times with PBS/0.1% Tween 20, and bound phage were eluted with 50 mM glycine·HCl (pH 2.2). Recovered phage were propagated in 10 ml of 2× YT medium and 100 μl of a saturated DH5αF′ *E. coli* culture and affinity purified twice more as described above. Affinity-purified phage were plated onto 2× YT/0.8% agar plates to yield isolated plaques from which clonal phage stocks and DNA were produced. Phage binding was confirmed by incubating equal amounts of a clonal phage stock in wells coated with 1 μg of GST–SH3 or GST. The wells were washed five times with PBS/0.1% Tween 20, and bound phage were detected by anti-phage ELISA according to the manufacturer's instructions (Pharmacia). Clones with strong SH3-binding activity were selected for further analysis. The sequences of peptides displayed by these clones were determined by DNA sequencing of phage inserts.

RESULTS AND DISCUSSION

PXXP Library Design and Screening. All known SH3 ligands contain at least one PXXP motif. We therefore designed the PXXP library to encode peptides of the form X_6PXXPX_6. This bias results in a population of peptides highly enriched in SH3 ligands. In test screens of various phage-displayed peptide libraries with the Src SH3 domain, the PXXP library produced titers of SH3-binding phage ≈100-fold greater than random peptide libraries of equivalent complexity (data not shown). With a complexity of 3×10^8 unique clones, the PXXP library has the capacity to encode all PXXP-containing 7-mers with 99% confidence, assuming a Poisson distribution of sequences within the library.

Immobilized GST–SH3 fusion proteins containing the Src, Yes, N-Src, Abl, Cortactin, p53bp2, PLCγ, Crk N, Grb2 N, and Grb2 C SH3 domains were used to affinity purify SH3-binding phage from each of the six fractions of the PXXP library. After three rounds of affinity purification, phage titers were 10^2- to 10^5-fold higher than background for all SH3 constructs except Grb2 C. Repeated attempts to enrich binding phage with Grb2 C SH3 met with failure. For each of the other SH3 domains, four isolated clones from each of the six library fractions were tested for binding to their respective SH3 targets; ≈90% of tested clones exhibited strong SH3-binding activity and were subsequently characterized. Although most clones were isolated only once, several clones were recovered up to four times. Significantly, sibling clones were never recovered from different library fractions or from screens with two different SH3 domains.

Src SH3

LASRPLPLLPNSAPGQ*
LTGRPLPALPPPFSDF
PAYRPLPRLPDLSVIY
RALRVRPLPPVPGTSL
DAPGSLPFRPLPPVPT
LKWRALPPLPETDTPY
ISQRALPPLPLMSDPA*
LTSRPLPDIPVRPSKS
NTNRPLPPTPDGLDVR
MKDRVLPPIPTVESAV
LQSRPLPLPPQSSYPI
FINRRLPALPPDNSLL
FRALPLPPTPDNPFAG
LYSAIAPDPPPRNSSS†
LXXRPLPXΨP

Abl SH3

PPWWAPPPIPNSPQVL*
PPKFSPPPPPYWQLHA
PPHWAPPAPPAMSPPI
PPTWTPPKPPGWGVVF
PPSFAPPAAPPRHSFG
PTYPPPPPPDTAKGA*‡
GPRWSPPPVPLPTSLD
APTWSPPALPNVAKYK
PPDYAAPAIPSSLWVD
IKGPRFPVPPVPLNGV
PPAWSPPHRPVAFGST
APKKPAPPVPMMAHVM
PPXΘXPPPΨP

p53bp2 SH3

YDASSAPQRPPLPVRKSRP*
EYVNASPERPPIPGRKSRP
WNGIAIPGRPEIPPRASRP
SMIFIYPERPSPPPRFSRP
GVEEWNPERPQIPLRLSRP
WVVDSRPDIPLRRSLP
VVPLGRPEIPLRKSLP*
GGTVGRPPIPERKSVD
YSHAGRPEVPPRQSKP
FSAAARPDIPSRASTP
LYIPKRPEVPPRRHEA
NNISARPPLPSRQNPP
MAGTPRPAVPQRMNPP
RPXΨPΨR+SXP

Crk N SH3

GQPAGDPDPPPLPAKF*
FEQTGVPLLPPKSFKY
IFGDPPPPIPMKGRSL
SNQGSIPVLPIKRVQY
NYVNALPPGPPLPAKN
SSDPERPVLPPKLWSV
HFGPSKPPLPIKTRIT
DWKVPEPPVPKLPLKQ
ATSEGLPILPSKVGSY*
NANVSAPRAPAFPVKT
EMVLGPPVPPKRGTVV
AGSRHPPTLPPKESGG
SVAADPPRLPAKSRPQ
ΨPΨLPΨK

Yes SH3

ITMRPLPALPGHGQIH*
LPRRPLPDLPMAAGKG
LGSRPLPPTPRQWPEV
STIRPLPAIPRDTLLT
RSGRPLPPIPEVGHNV*
IGSRPLPWTPDDLGSA
LAQRELPGLPAGAGVS
IPGRALPELPPQRALP
FVGRELPPTPRTVIPW
DPRSALPALPLTPLQT
SPHDVLPALPDSHSKS
ΨXXRPLPXLP

Cortactin SH3

LTPQSKPPLPPKPSAV*
SSHNSRPPLPEKPSWL
PVKPPLPAKPWWLPPL
TERPPLPQRPDWLSYS
LGEFSKPPIPQKPTWM*
YPQFRPPVPPKPSLMQ
VTRPPLPPKPGHMADF
VSLGLKPPVPPKPMQL
LLGPPVPPKPQTLFSF
YKPEVPARPIWLSEL
GAGAARPLVPKKPLFL
+PPΨPXXPXWL

PLCγ SH3

MPPPVPPRPPGTLQVA*
LSYSPPPVPPRPDSTL
VLAPPVPPRPGNTFFT
YRPPVAPRPPSSLSVD
LQCPDCPRVPPRPIPI
VPPLVAPRPPSTLNSL*
LTPPPFPKRPRWTLPE
YWPHRPPLAPPQTTLG
PPVPPRPXXTL

Grb2 N SH3

KWDSLLPALPPAFTVE*
RWDQVLPELPTSKGQI
RFDFPLPTHPNLQKAH
RLDSPLPALPPTVMQN
RWGAPLPPLPEYSWST
YWDMPLPRLPGEEPSL*
RFDYNLPDVPLSLGTA
TKKPNAPLPPLPAYMG
KWDLDLPPEPMSLGNY
+ΘDXPLPXLP

YYQRPLPPLPLSHFES*
YYRKPLPNLPRGQTDD
YFDKPLPESPGALMSL
YFSRALPGLPERQEAH*
YΘX+PLPXLP

SLWDPLPPIPQSKTSV*
SYYDPLPKLPDPGDLG
KLYYPLPPVPFKDTKH
DPYDALPETPSMKASQ*
ΘDPLPXLP

+ΘDXPLPXLP

FIG. 1. Amino acid sequence of peptides expressed by SH3-binding phage clones. Similar sequences within the peptides have been aligned to yield the SH3 ligand consensus motifs, which are listed below each group of peptides in boldface. Aliphatic, aromatic, and basic residues are represented by the symbols Ψ, Θ, and +, respectively. Invariant proline or flanking residues are underlined. *Clones used in subsequent assays. †Putative class II peptide (see text). ‡Clone contained a 3-nucleotide deletion in the random peptide coding sequence.

Table 1. Alignment and classification of SH3 ligand consensus motifs

Class I	**+ p Ψ P p Ψ P**
Src	L X X **R** P **L** P X Ψ **P**
Yes	Ψ X X **R** P **L** P X L **P**
Abl	P **P** X Θ X P **P** P Ψ **P**
Grb2 N	+ Θ D X P **L** P X L **P**
	Y Θ X R P **L** P X L **P**
	Θ D P **L** P X L **P**
Class II	**Ψ P p Ψ P p +**
Cortactin	+ **P** P Ψ **P** X **K** P X W L
p53bp2	**R** P X Ψ **P** Ψ **R** + S X P
PLCγ	X **P** P **V** P P **R** P X X T L
Crk N	Ψ **P** Ψ **L** P Ψ **K**

Each SH3 ligand consensus motif was assigned to class I or II based on its agreement with the class I or II consensus motif. Highly (>90%) conserved positions in each SH3 ligand consensus motif are listed in boldface and were interpreted as SH3 contact residues.

SH3 Ligand Consensus Motifs. Fig. 1 lists the amino acid sequences of peptides expressed by SH3-binding clones. Similar sequences within the peptides have been aligned to yield consensus motifs; these motifs reflect the ligand preferences of each SH3 domain.

SH3 domains from the closely related Src family members Src and Yes select peptides sharing similar consensus motifs: LXXRPLPXΨP and ΨXXRPLPXLP, respectively. (In this and subsequent motifs, Ψ, Θ, and + represent aliphatic, aromatic, and basic residues, respectively.) Previously, we (12) and others (13, 14) have shown that Src SH3 selects peptides with the consensus sequence RPLPPLP; peptides containing this sequence exhibit significant binding to the SH3 domains of several other Src family members (A.B.S. and B.K.K., unpublished data). Similarly, Rickles *et al.* (18) have recently demonstrated that SH3 domains from the Src family members Src, Lyn, and Fyn select peptides with the consensus sequence RPLPPLPXP, RXXRPLPPLPxP, and RPLPP(I/L)P, respectively. Taken together, these data suggest that the closely related Src, Yes, Lyn, and Fyn SH3 domains possess very similar ligand preferences.

Whereas the Src family SH3 domains select similar peptides, more distantly related SH3 domains exhibit distinct ligand preferences (Fig. 1). For example, Abl SH3 selects peptides sharing the consensus motif PPXΘXPPPΨP. This motif is consistent with sequences found in Abl SH3-binding proteins (see below) as well as a recently identified Abl ligand consensus motif (PPPYPPPPLP) (18). The Cortactin and p53bp2 SH3 domains are unique in that they both select peptide ligands with conserved positively charged residues flanking the PXXP core; Cortactin SH3 prefers +PPΨPXKPXWL and p53bp2 prefers RPXΨPΨR+SXP. In contrast, PLCγ SH3 and Crk N SH3 both select peptides with a conserved basic residue C terminal of the PXXP core; PLCγ SH3 prefers PPVPPRPXXTL and Crk N SH3 prefers ΨPΨLPΨK. Finally, Grb2 N SH3 selects ligands with the consensus +ΘDXPLPXLP. Interestingly, Grb2 N SH3 ligands may be grouped into three subpopulations sharing related consensus motifs +ΘDXPLPXLP, YΘX+PLPXLP, and ΘDPLPXLP. Thus, each of the SH3 domains we have investigated selects peptide ligands with distinct sequence characteristics.

SH3 Ligand Binding Orientation. Peptide ligands bound to SH3 domains have been shown to assume a left-handed polyproline type II (PPII) helix conformation (14). SH3 ligands are pseudosymmetrical and may therefore bind in one of

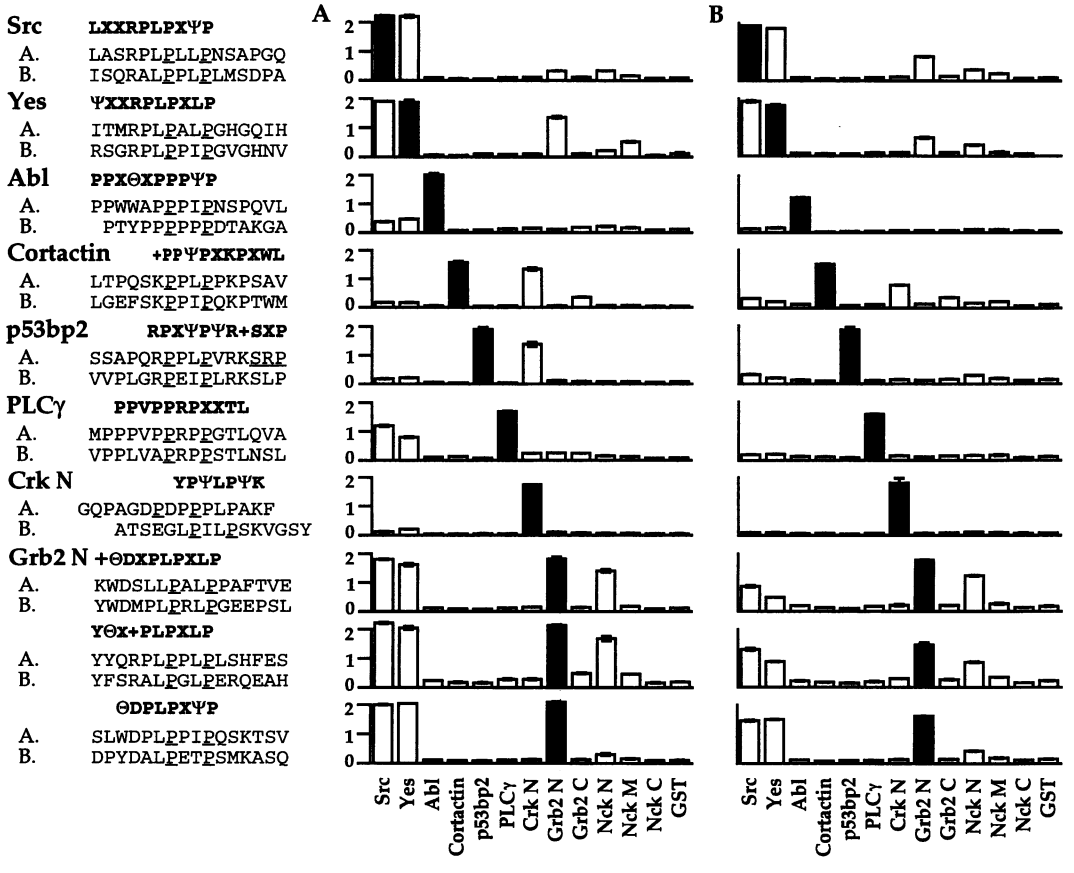

FIG. 2. Relative binding of SH3-selected phage clones to various SH3 domains. Two clones (*A* and *B*) representing each consensus motif were assayed for binding to 1 μg of each immobilized GST–SH3 fusion protein. Bound phage were detected by anti-phage ELISA. Sequences of peptides displayed by each clone are aligned with their respective consensus motifs. Invariant proline residues are underlined. Solid bars, specific binding; open bars, cross-reactive binding. Values are average OD_{405} ± SD (N = 3).

two opposite orientations (19). Feng *et al.* (19) have demonstrated that peptides that bind in one or the other orientation share different consensus motifs. Specifically, ligands that bind in the class I or class II orientation conform to the consensus +pΨPpΨP or ΨPpΨPp+, respectively, where uppercase positions represent conserved residues that contact the SH3 domain and confer specificity, and lowercase positions represent scaffolding residues that tend to be proline.

According to this model, we predict that the peptides selected by the Src, Yes, Abl, and Grb2 N SH3 domains bind in the class I orientation, whereas the peptides selected by the Cortactin, p53bp2, PLCγ, and Crk N SH3 domains bind in the class II orientation (Table 1). Interestingly, most of the SH3 ligand consensus motifs identified in this work contain additional conserved residues flanking the SH3-binding core defined by Feng *et al.* (19). Furthermore, these conserved residues are situated N and C terminal of the SH3-binding core in class I and class II motifs, respectively, and are therefore predicted to interact with equivalent regions of their target SH3 domains (Table 1).

The Src SH3 domain is capable of binding both class I and class II peptides (19). Although class I peptides predominate the population of Src SH3 ligands selected from the PXXP library, one clone conforms well to the class II consensus (Fig. 1). Previously, we (12) and others (14) have isolated class II Src SH3 ligands sharing the consensus PPΨPPR. Similarly, whereas the Grb2 N SH3 domain has been shown to bind peptides from SOS with the class II consensus sequence PPΨPPR (5), we have isolated Grb2 N SH3 ligands that conform to the class I consensus (Table 1). Thus, both the Src and the Grb2 N SH3 domains apparently have the capacity to bind both class I and class II peptide ligands.

Despite the availability of both class I and II ligands in the PXXP library and the ability of at least some SH3 domains to bind both class I and II ligands, we have found that each of the SH3 domains investigated in this work tends to select ligands that bind in one particular orientation. Whether this bias reflects the preferences of different SH3 domains for ligands that bind in a particular orientation or biases inherent in the phage selection process is not known.

SH3 Ligand Binding Characteristics. To explore further the capacity of SH3 domains to discriminate between different SH3 ligands, we investigated the binding of phage expressing various peptide ligands to a panel of SH3 domains. Equal titers of clonal phage stocks were incubated in microtiter wells coated with different GST–SH3 fusion proteins. The wells were washed several times, and bound phage were detected with an anti-phage antibody (Fig. 2). Positive ELISA signals were equivalent to those obtained with previously characterized Src SH3-binding clones (12) and are indicative of SH3:peptide affinities in the 5–75 μM range (14, 18). Whereas the Src, Yes, Crk, and Grb2 N SH3 domains cross-reacted with a few phage clones selected with other SH3 domains, the Abl, Cortactin, p53bp2, and PLCγ SH3 domains displayed considerable specificity. Significantly, only 33 of 220 potential instances of cross-reactivity were observed, suggesting that SH3 selectivity is the rule rather than the exception.

Each instance of cross-reactivity may be explained by similarities between the sequences of the peptides and the ligand preferences of the cross-reactive SH3 domains. For example, Crk SH3 cross-reacted with three phage clones selected with other SH3 domains; each of these clones coincidentally expressed peptides conforming to the Crk SH3 preferred ligand consensus motif. Similarly, the cross-reactivity observed between the Src, Yes, and Grb2 SH3 domains and clones selected by other SH3 domains within this group may be a consequence of the fact that these SH3 domains prefer the same proline-rich core. Finally, the Src and Yes SH3 domains cross-reacted with the PLCγ SH3 ligand MPPPVPPRPPGTL, which contains the class II Src SH3-binding sequence PPVPPR. Taken together, these data demonstrate the capacity of SH3 domains to discern subtle differences in the primary structure of potential ligands.

Natural SH3 Ligands: SH3-Binding Proteins. To evaluate the contribution of SH3 selectivity to natural SH3–ligand interactions, we have compared the SH3 ligand consensus motifs with sequences of known SH3-binding proteins (Fig. 3). The Src SH3-binding proteins Dynamin, hnRNP K, p62, PI3K p85, and Shb all possess proline-rich regions conforming to the Src SH3 class I or II consensus motifs. However, the Src

		Ref
Src SH3 Class I	**LXXRPLPXΨP**	
Hs AFAP-110 (62-73)	PPQMP**L**P**E**I**P**QQ	20
(76-87)	PPDNGP**P**P**L**P**T**S	
Hs CDC42 GAP(250-261)	TAPKP**M**P**P**R**P**PL	21
Hs hnRNP K (302-313)*	SRAR**N**L**P**LP**P**PP	22
Mm p62 (328-339)	TVT**R**GV**P**PP**P**TV	22
Hs PI3K p85 (90-101)*	R**P**PR**P**L**P**VA**P**GS	6
Hs Shc p52 (296-307)	VRKQM**L**P**P**PP**P**CP	22
Src SH3 Class II	**PPΨPPR**	
Hs Dynamin (810-820)	GGA**P**PV**P**S**R**PG	23
(827-837)	GPP**P**QV**P**S**R**PN	
(838-848)	RAP**P**GV**P**S**R**SG	
Hs hnRNP K (308-318)*	PLP**P**P**P**P**P**RGG	22
Mm p62 (294-304)	APP**P**P**P**VP**R**GR	22
Hs Paxillin (42-52)	AVP**P**PV**P**P**P**PS	24
Hs PI3K p85 (302-312)*	QPA**P**AL**P**P**K**PP	6
Hs Shb (50-60)	GGP**P**PG**P**G**R**RG	25
(103-113)	TKS**P**PQ**P**P**R**PD	
Yes SH3	**ΨXXRPLPXLP**	
Hs Yap65 (240-251)	PVKQP**P**P**L**AP**Q**S	26
Abl SH3	**PPXθXPPPΨP**	
Mm 3BP-1 (265-276)*	RA**P**TMP**P**P**L**P**P**V	2
Mm 3BP-2 (200-211)*	YP**P**A**Y**P**P**P**P**VP**V**	2
Dm Ena (350-361)	PG**P**G**Y**GP**P**PVP**P**	27
PLCγ SH3	**PPVPPRPXXTL**	
Hs Dynamin (812-823)	A**P**PV**P**S**R**PGASP	23
(829-840)	P**P**QV**P**S**R**PNRNR	
Hs c-Cbl (493-504)	L**P**PV**P**P**R**LDLLP	28
Crk N SH3	**PΨLPΨK**	
Hs Abl (524-533)*	QA**P**EL**P**T**K**TR	29
(568-577)*	VS**P**LL**P**R**K**ER	
(758-767)	EK**P**AL**P**R**K**RA	
Hs C3G (282-291)*	PP**P**AL**P**P**K**KR	30
(452-461)*	TP**P**AL**P**E**K**KR	
(539-548)*	KP**P**PL**P**E**K**KN	
(607-616)*	PP**P**AL**P**P**K**QR	
Grb2 N SH3 Class I	**+θDXPLPXLP**	
	YθX+PLPXLP	
	θDPLPXLP	
Hs c-Cbl (560-571)	PQRR**P**L**P**CT**P**GD	31
(589-600)	WLPR**P**I**P**KV**P**VS	
Grb2 N SH3 Class II	**PPPΨPPR**	
Hs Abl (523-533)*	LQA**P**EL**P**TKTR	29
(567-577)*	AVS**P**LL**P**RKER	
(609-619)*	KTA**P**TP**P**KRSS	
Hs c-Cbl (491-501)	ASL**P**PV**P**P**R**LD	31
Hs Dynamin (810-820)	GGA**P**PV**P**S**R**PG	23
(827-837)	GPP**P**QV**P**S**R**PN	
(838-848)	RAP**P**GV**P**S**R**SG	
Hs SOS1 (1148-1158)*	PVP**P**PV**P**P**R**RR	5
(1177-1187)	DSP**P**AI**P**P**R**QP	
(1209-1219)*	ESP**P**LL**P**P**R**EP	
(1287-1297)*	IAG**P**PV**P**P**R**QS	
Rn Synapsin I (592-602)	NLPEPA**P**P**R**PS	32
(670-680)	PPGPAG**P**I**R**QA	

FIG. 3. Alignment of sequences from SH3-binding proteins. Sequences of proteins known to interact with each SH3 domain were compared to its ligand consensus motif(s). Cortactin SH3-binding proteins have not been reported. Each entry lists species and protein from which the sequence was derived. Amino acid positions of each sequence are listed in parentheses. Residues within protein sequences agreeing with highly (>90%) conserved residues in the consensus motifs are listed in boldface. *Protein sequences previously demonstrated to bind their respective SH3 domain.

SH3-binding proteins AFAP-110, CDC42 GAP, and Shc contain sequences lacking at least one critical consensus residue. Each of the Abl, Crk, and PLCγ SH3-binding proteins possess at least one proline-rich region conforming to the consensus motifs of their respective SH3 targets. Most of the Grb2-binding proteins do not possess sequences conforming to the class I Grb2 N SH3 ligand motif identified in the random peptide screen. However, each Grb2-binding protein contains at least one region in agreement with the class II Grb2 N SH3 motif derived from Grb2-binding sequences in SOS. Why Grb2 selects class I peptide ligands yet seems to prefer class II protein ligands is unknown.

Thus, most of the SH3-binding proteins contain proline-rich regions conforming to the ligand consensus motifs of their respective SH3 targets. Many of these regions have been implicated in SH3 binding. However, the sequences responsible for SH3 recognition by several of these proteins have not been clearly defined. The sequences listed in Fig. 3 represent a reasonable starting point for future investigations.

Several of the SH3-binding proteins possess proline-rich regions that do not conform to the consensus motifs of their respective SH3 targets, suggesting that factors other than the local structure of the ligand may contribute to the regulation of SH3-mediated protein–protein interactions *in vivo*. Such factors might include subcellular localization of SH3 or ligand proteins, cooperativity between different binding domains within a protein or complex of proteins, steric limitations upon the access of an SH3 domain to its ligands, and the existence of discontinuous SH3-binding regions in folded proteins.

Similarities between the SH3 ligand preferences and sequences in naturally occurring SH3-binding proteins suggest that these preferences may reflect functionally significant biases of different SH3 domains for specific sequences within target proteins. Thus, we have searched several protein data bases for sequences related to each of the SH3 ligand consensus motifs. By identifying putative SH3-binding sites in candidate proteins, this approach can yield testable predictions regarding specific SH3-mediated protein–protein interactions *in vivo*. These searches identified most of the sequences listed in Fig. 3 as well as sequences in a large number of proteins not previously implicated in SH3 interactions. The results are available via the Internet: http://www.unc.edu/depts/biology/kayref.html.

Note Added in Proof. After acceptance of this manuscript for publication, a paper was published describing the use of biased phage libraries to analyze the ligand preferences of the Src, Lyn, Fyn, Yes, and PI3K SH3 domains (33).

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