Novel Src Homology 3 Domain-binding Motifs Identified from Proteomic Screen of a Pro-rich Region

Christina Y. H. Jia‡§, Jing Nie¶, Chenggang Wu‡, Chengjun Li‡, and Shawn S.-C. Li‡

The Src homology (SH) 3 domain has been shown recently to bind peptide sequences that lack the canonical PXXP motif. The diverse specificity in ligand recognition for a group of 15 SH3 domains has now been investigated using arrays of peptides derived from the proline-rich region of the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76). A screen of the peptide arrays using individual or mixed SH3 domains has allowed the identification of a number of candidate SH3-binding peptides. Although some peptides contain the conventional PXXP motif, most are devoid of such a motif and are instead enriched in basic residues. Fluorescent polarization measurements using soluble peptides and purified SH3 domains demonstrated that several SH3 domains, including those from growth factor receptor-bound protein 2 (Grb2), NCK, and phospholipase C (PLC)-γ1, bind with moderate affinities (10−100 μM) to a group of non-conventional peptides. Of particular interest, the PLC-γ1 SH3 domain was found to associate with SLP-76 through at least three distinct sites, two of which bore a novel KKPP motif and the other contained the classic PXXP sequence. Intriguingly mutation of critical residues for the three sites not only affected binding of SLP-76 to the PLC-γ1 SH3 domain but also to the Grb2 C-terminal SH3 domain, indicating that the binding sites in SLP-76 for the two SH3 domains are overlapped. Our studies suggest that the SH3 domain is an inherently promiscuous interaction module capable of binding to peptides that may or may not contain a PXXP motif. Furthermore the identification of numerous non-conventional SH3-binding peptides in SLP-76 implies that the global ligand pool for SH3 domains in a mammalian proteome may be significantly greater than previously acknowledged. Molecular & Cellular Proteomics 4: 1155–1166, 2005.

The SH3 domain is one of the most abundant protein modules found in eukaryotes (1−3). The human proteome is estimated to contain over 400 copies of SH3 domains spread among a diverse array of proteins (1). These non-catalytic domains of 50−70 amino acids have been shown to mediate protein-protein interactions by binding to short proline-rich sequences and thereby play important roles in the cell, ranging from signal transduction to regulation of cytoskeletal re-arrangements (4). The majority of SH3 domains characterized to date recognize the class I and/or II peptides that share a core PXXP motif (4–6). The two classes of SH3 ligands differ from each other principally in the location of a flanking basic residue (Arg or Lys) that dictates the orientation of the peptide with respect to the SH3 binding surface (7, 8). Thus, the class I ligands share the consensus motif (R/K)XXPX(XP, while the class II peptides conform to the degenerated sequence PXXPX(R/K) (where X represents any amino acid). When bound to SH3 domains, however, both classes of ligands adopt the polyproline type II helical structure (8, 9).

Extensive biochemical and biophysical analysis of numerous SH3 domains and their complexes in the past decade have provided tremendous insights into their specificity and structural basis of ligand recognition (4, 9). Nevertheless with the recent identification of a number of non-conventional SH3 domain-mediated interactions (10–13), it has become increasingly clear that our understanding of this important class of interaction module is far from being complete. A growing body of literature suggests that SH3 domains possess specificity beyond the recognition of the conventional class I and II ligands. For example, the SH3 domains from the tyrosine kinase substrate Eps8 and related proteins bind selectively to sequences containing a PXDDY motif (14). The SH3 domains of Fyn and Fyn-binding protein, Fyb/SLAP130, engage a site in the Src kinase associated protein of 55 kDa (SKAP55) with a consensus sequence RKXXXY that is devoid of proline (15). The SH3 domains of signal transducing adaptor molecule 1 (STAM1) and STAM2/EAST/Hbp, a family of proteins involved in cytokine signaling and receptor-mediated endocytosis and exocytosis, bind to a PX(V/I)(D/N)RXKP motif conserved in associated molecule with the SH3 domain of

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Novel SH3 Domain-binding Sequences

![Diagram of SLP-76 structure](image)

**Fig. 1. Structure of SLP-76 and amino acid composition of its proline-rich region.** The major functional domains of SLP-76 include three N-terminal Tyr phosphorylation sites, a central PRR, and a C-terminal SH2 domain. The amino acid sequence of the PRR (residues 162–412) is shown. Pro residues are identified in bold, whereas Arg and Lys residues are highlighted in bold and with underscores.

STAM (AMSH) and the deubiquitinating enzyme UBPY (16). Similar sequences harboring an (R/K)XX(K/R) core motif have also been identified in a group of proteins that play important roles in tyrosine kinase and/or immunoreceptor signaling, including Gab1/Gab2, BLNK (B cell linker), Fyb, hematopoietic progenitor kinase 1 (HPK1), and SLP-76 (10, 11, 17–19). In vitro and in vivo experimental data showed that the conserved (R/K)XX(K/R) motif in these proteins was responsible for their respective interactions with the C-terminal SH3 (SH3-C) domains of Grb2 and/or Gads (10, 11, 19). Collectively these observations suggest that SH3 domains possess a rather broad specificity. It should be noted that, although the affinity of an SH3 domain for its cognate ligand is usually weak with numerous positively charged residues (34 in total, 32%), it contains 10 μM range, the Gads C-terminal SH3 domain was shown to bind an RSTK-containing peptide derived from SLP-76 with submicromolar affinity (10, 11).

Proline-rich regions (PRRs), like SH3 domains, are highly prevalent in eukaryotic proteomes (2). The length of a PRR varies from a few to hundreds of amino acids. A PRR often nucleates interactions with proteins containing the SH3 domain or other proline recognition modules such as the WW, FYVE, and enabled Vasp homology (EVH) domains (9, 20). This is epitomized by SLP-76, a multidomain protein that plays an essential role in T cell development and activation (21). In addition to three N-terminal tyrosine phosphorylation sites and a C-terminal SH2 domain, SLP-76 contains a proline-rich region that spans more than 250 residues (Fig. 1). The SLP-76 PRR displays some features common to many known proline-rich sequences. For instance, it is very long and covers a nucleotide interaction domain, may overlook ligands that do not contain a preselected core motif (27). We present here a strategy by which to map potential SH3 domain-binding sites via unbiased screening of arrayed proline-rich sequences using a group of SH3 domains. In this study, we applied this strategy to the SLP-76 PRR. Through screening peptide arrays representing the PRR using a group of 15 SH3 domains, numerous potential SH3-binding sequences were identified. Remarkably although a small number of peptides identified from the array screen bear the conventional PXXP motif, the majority of peptides lack such a motif and are rich in positively charged residues. Synthetic peptides corresponding to the novel binding sequences displayed affinities in solution for the Grb2 and NCK C-terminal SH3 domains and/or the PLC-γ1 SH3 domain. Although the measured affinities were generally above 10 μM, they were comparable to that between an SH3 domain and a conventional, PXXP-containing ligand. Furthermore, the PLC-γ1 SH3 domain was found to engage at least three distinct sites in SLP-76 of which two contained a novel KKPP motif. Ala-scanning and permutation peptide arrays were used to define critical residues for binding in these sequences, and the physiological relevance of these binding sites was investigated using SLP-76 variants bearing mutations of one or more critical residues.

**EXPERIMENTAL PROCEDURES**

**Subcloning, Expression, and Purification of GST-SH3 Proteins—** cDNA fragments encoding the SH3 domains of v-Src, c-Src (chicken), Fgr, c-Abl, Fyn, Grb2 (C), Gads (C), spectrin, NCK (N, M, and C), Crk, PLC-γ1, and the p85 subunit of PI3K were amplified by PCR and subcloned into the pGEX4T2 vector. The letters N, M, and C in the parentheses denote the N-terminal, middle, and C-terminal SH3 domain, respectively. Proteins were expressed in *Escherichia coli* as GST fusions and affinity-purified on glutathione-Sepharose beads (Amersham Biosciences) according to the manufacturer’s protocols.Bound proteins were eluted in 20 mM glutathione, 50 mM Tris, pH 8.0, 100 mM NaCl and were concentrated in 20 mM phosphate buffer, pH 7.0, 100 mM NaCl prior to use in binding studies.

**Synthesis of Peptide Spot Arrays on Derivatized Cellulose Membranes and Probing of the Peptide Arrays by SH3 Domains—** The SLP-76 proline-rich region (residues 162–412) was represented by a series of unordered peptides. These peptides, 120 in total, were generated by “walking,” from the N to the C terminus, through the sequence of the PRR with a window of 11 amino acids and a frame-shift of two amino acids between consecutive peptides. These peptides were assembled in an array format on a functionalized cellulose membrane by multiple peptide synthesis (28) using the Auto-Spot Robot ASP 222 (Abimed).

The peptide array membrane was moistened sequentially with
ethanol and water before being probed by purified GST-SH3 proteins. Specifically the peptide array membrane was washed three times in TBS-T buffer containing 20 mM Tris-HCl, 140 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.6, and blocked in 5% BSA in TBS-T for 1 h at room temperature. Approximately 1.0 μM total GST fusion SH3 domains were added directly to the blocking solution and incubated with the membrane for 1 h at room temperature. The membrane was then washed three times in TBS-T and once in TBS before an anti-GST monoclonal antibody was added. After a 30-min incubation with the antibody, the array membrane was washed extensively in TBS and developed using either the enhanced chemifluorescence (ECF) or ECL Western blot kit (Amersham Biosciences). The binding signals on the peptide array were captured by and analyzed on a Fluor-S Multi-Imager (Bio-Rad).

**Peptide Synthesis and Fluorescence Polarization Measurements**—Individual peptides were synthesized at 0.1-mmol scale on a 431A Peptide Synthesizer (Applied Biosystems) using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. For fluorescein labeling, an appropriate amount of 5-(and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes) was added to a peptide resin, and the coupling reaction was allowed to proceed for 1 h at room temperature. Upon cleavage of a peptide from the resin using trifluoroacetic acid, the fluorescein-labeled peptide was separated from the unlabeled peptide by HPLC on a Luna C18 column (Phenomenex). Identities of the peptides were confirmed by mass spectrometry. Fluorescent polarization measurements were performed as described previously (28). A varied amount of a purified GST-SH3 protein was titrated to a fluorescent peptide solution in 20 mM PBS, pH 7.0, 100 mM NaCl. The SH3 domain–peptide mixtures were allowed to incubate in the dark for 30 min prior to fluorescent anisotropy measurements at 20 °C. Binding curves were generated by fitting the isothermal binding data to a hyperbola nonlinear regression model using Prism 3.0 (GraphPad Software, Inc., San Diego, CA), which also produced the corresponding dissociation constants (Kd).

**Cell Culture, GST Pull-down, Co-immunoprecipitation, and Western Blot**—Human embryonic kidney (HEK) 293 cells from ATCC were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin. Cells were transiently transfected with expression vectors. Bound SLP-76 was identified by Western blotting using an anti-FLAG antibody (upper panel). Equal application of GST-SH3 protein in each lane was verified in the lower panel by Coomassie Blue staining. WCL, whole cell lysate loaded at 20% of the amount used in a pull-down lane; WB, Western blot.

**RESULTS**

SLP-76 Is Capable of Binding to Multiple SH3 Domains—To explore the potential of the proline-rich sequence in SLP-76 to interact with multiple SH3 domains, we expressed a group of 15 SH3 domains in bacteria as GST fusions and assessed the ability of the purified proteins in pulling down SLP-76 from cell lysate. The SH3 domains used in the current study were taken from a variety of different proteins, including tyrosine kinases (i.e. v-Src, c-Src, Fgr, Abl, and Fyn), lipid kinase (i.e. PI3K), lipase (PLC-γ1), adaptor or docking proteins (i.e. Gads, Grb2, NCK, and Crk), and a structural protein (i.e. spectrin). Of the 15 SH3 domains examined, eight displayed significant affinities for SLP-76. The C-terminal SH3 domain of Gads and the PLC-γ1 SH3 domain exhibited strong binding to SLP-76 as expected. Interestingly the SH3 domains from several proteins that have not been shown previously to interact directly with SLP-76, such as Src, Fyn, NCK, and p85 (PI3K), also demonstrated robust binding to SLP-76. These data indicate that the SLP-76 PRR can accommodate multiple interactions mediated by different SH3 domains. Although the physiological relevance of novel interactions identified here awaits further investigation, it is worth noting that PI3K, Grb2, and NCK are components of a multiprotein complex in T cell signaling, referred to as the signalosome, that is nucleated by SLP-76 and the linker of activated T cells (LAT) (29).

**Conventional and Novel SH3 Domain-binding Sequences Identified through “Peptide Walking” through the Pro-rich Region of SLP-76**—To identify the potential SH3-binding sites in the SLP-76 PRR, we represented the PRR as an array of overlapping peptides using the SPOT technology of multiple peptide synthesis (28). Specifically a series of undecamer peptides was assembled in an array format on a nitrocellulose membrane and washed with 5% BSA in TBS-T for 1 h at room temperature. Approximately 1.0 μM total GST fusion SH3 domains were added directly to the blocking solution and incubated with the membrane for 1 h at room temperature. The membrane was then washed three times in TBS-T and once in TBS before an anti-GST monoclonal antibody was added. After a 30-min incubation with the antibody, the array membrane was washed extensively in TBS and developed using either the enhanced chemifluorescence (ECF) or ECL Western blot kit (Amersham Biosciences). The binding signals on the peptide array were captured by and analyzed on a Fluor-S Multi-Imager (Bio-Rad).

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likely caused by imperfection in the array synthesis.

To validate results obtained from the peptide array screening and to determine the binding affinities of individual SH3 domains for non-conventional peptides in solution, we selectively synthesized six peptides, namely p102, p127, p132, p142, p177, and p187, based on sequence novelty and peptides p142, p177, and p187, based on sequence novelty and strength of the corresponding binding signals on arrays probed using both individual and mixed SH3 domains (Table II). The peptides corresponded to those displayed on the array (Fig. 3B) but were extended by two residues on both the N and C termini to eliminate possible end effect. Consequently each peptide in Table 2 represents three consecutive spots on the PRR array. For example, peptide p102 encompassed spots 101–103, peptide p127 included spots 126–128, and so on. A fluorescein label was attached to the N terminus of each peptide to facilitate binding assays using fluorescent polarization. Because peptides p127, p132, and p142 exhibited binding to the SH3-C domains of Grb2, Gads, and/or NCK, we examined the interactions of the six peptides with these SH3 domains by fluorescence polarization. The spectrin SH3 domain, which showed no binding in the GST pull-down assay (Fig. 2), was used as a control (Fig. 4). Results from peptide-SH3 domain binding in solution are displayed in Fig. 4 and summarized in Table II. In agreement with previous reports, the Gads-binding peptide, p132, bound both the Gads and Grb2 SH3-C domains with high affinities (10, 11). These SH3 domains, however, differ in specificity for the motifs such as RXXK, KXXK, and/or HXXK. There was no apparent consensus for the remaining two-thirds of peptides in group II except for the enrichment of basic residues in their sequences. The lack of conventional SH3-binding sites in SLP-76 is intriguing as only one positive peptide (spot 108) has the hallmark of a class II SH3 ligand. Because overlapping peptides, such as those corresponding to spots 147–149, may represent a single SH3-binding site, the actual number of potential SH3-binding sites should be significantly smaller than the number of positive spots in the array.

The C-terminal SH3 Domains of Grb2 and NCK Are Capable of Binding to Multiple Sequences Lacking the PXXP Motif—The binding sites for individual SH3 domains were subsequently mapped using freshly synthesized arrays to eliminate background signals resulting from incomplete stripping of a used array (data not shown). The binding profiles of the C-terminal SH3 domains of Grb2, Gads, and NCK are shown in Supplemental Fig. S1. Two methods of signal detection, ECF and ECL, were used to identify positive binding peptides. It should be pointed out that, although the two methods produced comparable results, ECF detection seemed more sensitive. Taken together, the Gads SH3-C domain appeared to bind specifically to a site represented by spot 132, consistent with results from a previous report (10). In addition to the Gads-binding site, the Grb2 SH3-C domain bound to several other sites, including the one represented by spots 123–127. Similarly the NCK SH3-C domain appeared to engage multiple sites in the SLP-76 PRR (Supplemental Fig. S1).

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Novel SH3 Domain-binding Sequences

List of SLP-76 peptides that showed significant binding to SH3 domains in the peptide-walking array screen

Peptides containing a PXXP core motif are listed in the left column, while those that lack such a motif are listed in the right column. Proline residues are underlined and basic (Arg, Lys, and His) residues are identified in bold.

| Spot no. | Conventional sites (group I) | Motif | Sequence | Spot no. | Non-conventional sites (group II) | Motif | Sequence |
|----------|-------------------------------|-------|----------|----------|----------------------------------|-------|----------|
| 108      | PQQPVPPQRP                   | PXXPXR|          | 102      | SMYIDRPSPSKG                     | Novel |          |
| 111      | PQROPMAALPP                  | PXXP  |          | 103      | YIDRPPSGKTP                      | Novel |          |
| 112      | QREMAMALPPPP                  | PXXP  |          | 123      | QTNHEEPRSR                      | Novel |          |
| 114      | AALPPPAPGRN                   | PXXP  |          | 125      | EEPRSRNHK                        | RXXK |          |
| 118      | GRNHSPFLPQ                   | PXXP  |          | 126      | PSHRNRHHTAKL                    | RXXK | KXXK    |
| 147      | PPFSDKPSIAR                   | PXXP  |          | 127      | RSRRNHHTAKL                      | RXXK | KXXK    |
| 148      | FSDKPSIPAGR                   | PXXP  |          | 128      | RNRNHHTAKLP                      | RXXK | KXXK    |
| 149      | DKPSIPAGRSL                   | PXXP  |          | 129      | HKTAKLPSI                       | KXXK |          |
| 156      | LPKIQKPPPLP                  | KXXK | PXXP     | 130      | TAKLPSSIPD                       | Novel |          |
| 157      | KIOKKPLPPTT                   | KXXK | PXXP     | 131      | KLPSSIPD                        | Novel |          |
| 166      | PLPGKPPVPK                   | PXXP  |          | 132      | PAPSIDRSTKP                     | RXXK |          |
| 189      | RSTKPSMNPL                   | RXXK | PXXP     | 133      | PSIDRSTKPL                      | RXXK |          |
| 215      | NPPLPNKPR                    | PXXP  |          | 134      | IDRSTKPLDR                      | RXXK |          |
| 216      | PLIPLPKPRP                   | PXXP  |          | 135      | RSLAPFDREP                       | Novel |          |

| Dissociation constants \( (K_d, \mu \text{M}) \) of selected SH3 domain–peptide complexes

Dissociation constants reported were determined by fluorescence polarization using purified SH3 domains and fluorescein-labeled peptides. ND, not determined.

| Peptide | Sequence | SH3 domains |
|---------|----------|-------------|
| p102    | SNSMYIDRPPSGKTP                   | NCK-C: 34 ± 3 | Grb2-C: 40 ± 8 | Gads-C: ND | Spectrin: 282 ± 62 |
| p127    | PSRRSRNHHTAKLPAP                  | NCK-C: 111 ± 65 | Grb2-C: 55 ± 9 | Gads-C: >300 | Spectrin: 218 ± 28 |
| p132    | KLPSSIPDSTKPL                   | NCK-C: 185 ± 74 | Grb2-C: 5.8 ± 1.2 | Gads-C: 0.30 ± 0.04 | Spectrin: >300 |
| p142    | DREPFTLGGKPPFD                   | NCK-C: 11 ± 3 | Grb2-C: 16 ± 5 | Gads-C: ND | Spectrin: 195 ± 55 |
| p177    | NDEDDVHORPLPQA                   | NCK-C: 98 ± 39 | Grb2-C: 77 ± 11 | Gads-C: ND | Spectrin: 251 ± 55 |
| p187    | SNTFPSRSTKPSMN                   | NCK-C: 159 ± 30 | Grb2-C: 232 ± 154 | Gads-C: >300 | Spectrin: 301 ± 102 |

*Peptides are named according to their corresponding spot numbers in Fig. 3B. Basic residues are identified in bold, while prolines are underlined.

*Peptide sequences corresponded to those on the spot array (Fig. 3B). However, each peptide was extended by two residues at both the N and C termini to eliminate possible end effects. A fluorescein moiety was coupled to the N-terminal amine of each peptide to facilitate \( K_d \) measurements by fluorescence polarization.
remaining peptides. Thus, although the Grb2 SH3-C domain displayed weak to moderate affinities for peptides p102, p127, and p142, the Gads SH3 domain did not exhibit significant binding to the same peptides (Table II), suggesting that the interaction between Grb2 and SLP-76 involved more than one site. Binding of peptide p102 to the Grb2 SH3-C domain was surprising because it did not produce a positive signal in the peptide array screen (Supplemental Fig. S1). This discrepancy suggests that correlation between peptide binding on the membrane and in solution was imperfect.

Similar to the Grb2 SH3-C domain, the NCK SH3-C domain was capable of binding to multiple peptides. In particular, peptides p102 and p142 displayed moderate affinities (34 and 11 μM, respectively) for the latter SH3 domain. That these two peptides were capable of binding both the Grb2 and NCK SH3-C domains was interesting. It suggests that not only can an SH3 domain bind to multiple sites in the SLP-76 PRR but that a given site may be recognized by distinct SH3 domains. Despite this dual promiscuity in SH3 domain-ligand recognition, the pairwise SH3-ligand interactions observed here are likely specific. This was supported by the observation that the spectrin SH3 domain did not show significant binding to any of the peptides in Table II. Furthermore the affinities displayed by some peptides were within the realm of conventional SH3 domain-ligand interactions.

The PLC-γ1 SH3 Domain Recognizes Multiple Sites in the SLP-76 PRR—Upon the activation of the T cell receptor, SLP-76 associates with PLC-γ1. This interaction involves the PRR of the former and the SH3 domain of the latter. Two regions of the SLP-76 PRR are thought to directly mediate its interaction with PLC-γ1: the P-1 region that encompasses residues 157–223 and the P-2 region between residues 245 and 314 (23). Deletion of either region significantly compromised the ability of T cell receptor in signaling through PLC-γ1 (23). To map the precise binding sites for the PLC-γ1 SH3 domain, we synthesized a subarray targeting specifically the P-1 and P-2 regions of SLP-76. Peptides in the subarray were made two residues longer than those in the whole PRR array such that each peptide now contained 13 amino acids. Probing the subarray by purified PLC-γ1 SH3 domain led to the identification of three major binding sites: one was located in the P-1 region (designated p1a), and the other two resided in the P-2 region (named p2a and p2b, respectively) (Fig. 5A). The PLC-γ1 SH3 domain is known to bind to class II ligand with the preferred sequence PPVPPRP (5). Although peptide p1a has the characteristics of a class II ligand, neither peptide p2a nor p2b contains a similar sequence. Interestingly peptide p2a overlaps with peptide p142 that was shown earlier to bind the Grb2 and the NCK SH3-C domains (Table II).

To identify critical residues in peptide p1a or p2a that mediate its binding to the PLC-γ1 SH3 domain, an alanine-scanning array was synthesized for peptide p1a, and a permutation array was assembled for peptide p2a. These arrays were then probed with purified PLC-γ1 SH3 domains. As shown in Fig. 5B, substituting the C-terminal Arg residue or those in the PVPP motif led to either complete or partial loss of binding for peptide p1a. In contrast, replacement of Pro residues outside the PVPP core motif by Ala had no significant effect. Interestingly substituting an Ala for the first Lys or the last Gln residue augmented binding, suggesting that peptide hydrophobicity played a role in binding to PLC-γ1 SH3 domain (Fig. 5B).

Notwithstanding the above observations, permutation analysis on peptide p2a revealed that the positively charged Lys residues were the most important determinants in binding to the PLC-γ1 SH3 domain. In particular, the pair of lysines located near the N terminus of the peptide could only be replaced by either another basic or an aromatic residue (Fig. 5C). In comparison, a proline in the peptide could be charged to any amino acid, except Asp and Glu, without incurring significant loss of affinity. Acidic residues appeared to be disfavored at all positions of the peptide except being immediately before the C-terminal lysine as in the native sequence. Even at this position, a negative charge was not necessary for...
binding because replacement of the Asp residue in the native sequence by any other amino acid either maintained or enhanced the affinity of the peptide (Fig. 5C). Collectively these data indicate that positively charged residues play a pivotal role in mediating the interaction between peptide p2a and the PLC-γ1 SH3 domain. The dual Lys residues in peptide p2b were found to play a similar role in binding the PLC-γ1 SH3 domain (data not shown).

Peptide p2b resembles a class II ligand in that it contains a PPVP core motif. However, it lacks an Arg residue that is normally C-terminal to the core. To determine the importance of the PPVP motif and the minimal sequence required for binding the PLC-γ1 SH3 domain, we synthesized a series of truncated versions of peptide p2b by progressive deletion of residues from either the N or the C terminus (Fig. 6). Truncation of the C-terminal two residues of the PPVP motif did not affect binding. However, a decrease in affinity was evident when the dual Pro residues were omitted (Fig. 6). A complete loss of binding was observed when the dual Lys residues were taken out of the peptide, signifying the importance of the KK pair, instead of the PPVP motif, in binding the PLC-γ1 SH3 domain. In contrast, deleting residues N-terminal to the central proline had a negligible effect on binding. Taken together, these data suggest that the minimal sequence for p2b binding to the PLC-γ1 SH3 domain is PGKKPP (Fig. 6). A similar study conducted on peptide p2a resulted in a minimal sequence of KKPP for binding (Fig. 6). Thus, despite the apparent discrepancies in sequence for the two peptides, they share a common motif, KKPP, that is important for binding to the PLC-γ1 SH3 domain.

Binding of the PLC-γ1 SH3 Domain to Both Conventional and Novel Peptide Sequences—To gauge the relative affinities of conventional versus non-canonical sequences for the PLC-γ1 SH3 domain, peptides p-1a, p-2a, and p-2b were individually synthesized and purified for binding studies in solution. These peptides were made two residues longer than they were on the array to facilitate fluorescein labeling (Table III). A series of p-2a analogues were also generated to investigate the contribution of the KKPP motif to SH3 binding. Moreover to assess the contribution of the KKPP motif to SH3 binding, a GKKPP minipeptide was synthesized. Finally a peptide identified as a PLC-γ1 SH3 domain ligand in a phage display library screen (5) was synthesized and used as a positive control in subsequent binding studies by fluorescent polarization.

As shown in Fig. 7 and Table III, binding of peptides p-1a...
and p-2a to the PLC-γ1 SH3 domain assumed saturable patterns with a similar $K_d$ value of $\sim 11 \mu M$. It is remarkable that both peptides were capable of binding to the PLC-γ1 SH3 domain with a similar affinity despite drastic differences in their primary structures. Peptide p-2b, on the other hand, displayed a significantly lower affinity than did either peptide p-1a or p-2a despite both peptides p-2b and p-2a containing a KKPP motif. Although peptide array studies indicated that this motif was dispensible for binding, the minipeptide GKKPP failed to bind the PLC-γ1 SH3 domain in solution. This is analogous to a class I or II ligand in which residues flanking the core sequence play an important part in governing the affinity and specificity of the ligand (30). Using phage-displayed libraries, Sparks et al. (5) found that the PLC-γ1 SH3 domain selected for the motif PPVPQPRXXT. A 16-residue peptide, p-ctrl, containing this extended motif bound with high affinity (3 $\mu M$) to the PLC-γ1 SH3 domain (Fig. 7 and Table III). The relatively low affinities displayed by the SLP-76 peptides suggest that they are not optimized for binding to the PLC-γ1 SH3 domain.

The contributions of selected residues in peptide p-2a to binding were examined using peptide analogues. Substitution of the pair of Lys residues in the peptide by a pair of Arg, as in p2a-(KK/RR), or a pair of Gln, as in p2a-(KK/QQ), led to an $\sim 50$ and $70\%$ reduction in affinity (Table III). These results reinforced our earlier observation that the pair of Lys residues was important for binding. However, replacing the Asp residue in peptide p2a by an Ala did not have a significant effect. In comparison, changing the Gly residue to a Tyr slightly increased the affinity of peptide p-2a(G/Y) (Table III). Except for peptide p-2a(D/A), these in-solution binding data agreed in principle with those obtained from the peptide array studies (Fig. 5C).

Role of Distinct Sites in SLP-76 Binding to the PLC-γ1 SH3 Domain—To address the question of whether the PLC-γ1 SH3-binding sites identified from the peptide studies function in the context of full-length proteins, we generated a series of SLP-76 variants containing mutations, at each site, of residues identified in Table III. The contributions of selected residues in peptide p-2a to binding were examined using peptide analogues. Substitution of the pair of Lys residues in the peptide by a pair of Arg, as in p2a-(KK/RR), or a pair of Gln, as in p2a-(KK/QQ), led to an $\sim 50$ and $70\%$ reduction in affinity (Table III). These results reinforced our earlier observation that the pair of Lys residues was important for binding. However, replacing the Asp residue in peptide p2a by an Ala did not have a significant effect. In comparison, changing the Gly residue to a Tyr slightly increased the affinity of peptide p-2a(G/Y) (Table III). Except for peptide p-2a(D/A), these in-solution binding data agreed in principle with those obtained from the peptide array studies (Fig. 5C).
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Dues found to be important for binding (Fig. 8A). For instance, the C-terminal Arg residue was shown to be indispensable for binding of peptide p-1a to the PLC-γ1 SH3 domain. The corresponding residue, Arg-192 in SLP-76, was mutated to a Gln to create mutant R192Q or M-1a. It was expected that this mutation would maintain the hydrophilic character of the Arg side chain but eliminate its positive charges. Similarly mutant M-2a contained mutations of Lys-259 and Lys-260 in SLP-76 that corresponded to the double Lys in the KKPP motif of peptide p-2a. The equivalent pair of Lys residues of peptide p-2b was mutated to two Gln residues in mutant M-2b. In addition to these single site mutants, double and triple site mutants were also created to ascertain whether different sites act in an independent or cooperative manner in binding. To assess their relative affinities for the PLC-γ1 SH3 domain, these mutants were expressed in HEK 293 cells and subjected to pull-down assays using purified GST-PLC-γ1 SH3 domain. As shown in Fig. 8B, although the GST-PLC-γ1 SH3 domain was able to pull down the wild-type SLP-76, its ability to precipitate mutants M-1a and M-2a was either abolished or drastically reduced. In contrast, most of mutant M-2b in the lysate was brought down by the GST-SH3 beads, indicating that the p2b site played a relatively minor role in SLP-76-PLC-γ1 SH3 domain interaction. Intriguingly although the triple mutant M-1a/2a/2b was completely incapable of binding to the SH3 domain as expected, the double mutants M-1a/2a, M-2a/2b, and M-1a/2b exhibited weak to moderate affinities for the PLC-γ1 SH3 domain. This latter result suggests that the three binding sites in SLP-76 may not function independently from one another in binding to the PLC-γ1 SH3 domain. Alternatively the conformation of the PRR may be affected differently by the various combinations of mutations.

Binding Sites for the PLC-γ1 and Grb2 SH3-C Domains

Overlap in SLP-76—Like the PLC-γ1 SH3 domain, the Grb2 SH3-C domain appeared to engage multiple sites in SLP-76. In addition, peptide p142, which was shown to bind weakly to the Grb2 SH3-C domain, overlaps in sequence with peptide p2a. This suggests that binding sites for the two SH3 domains in SLP-76 may also partially overlap. To explore the role of the various binding sites of the Grb2 SH3-C domain, we used a group of seven SLP-76 mutants in a GST-pull down study (Figs. 8 and 9). Besides the mutants described for the PLC-γ1 SH3 domain, two additional mutants, namely M-gb and M-1b, were generated (Fig. 8A). Mutant M-gb or R237A was created in a previous study to investigate the role of the RSTK motif in peptide p132 to Gads SH3-C binding (11). Mutant M-1b or E171A, E174A) Glu residues at the ligand binding site as described in the text. Equal expression of SLP-76 or a mutant was verified by Western blotting of the corresponding lysate. Pull-down of SLP-76 from cell lysate by the Grb2 SH3-C domain or mutant. The Grb2 SH3-C mutants contained mutations of one (e.g. E171A and E174A) or more (e.g. E171A, E174A) Glu residues at the ligand binding site as described in the text. Equal expression of GST or GST-SH3 was confirmed by Coomassie Blue staining.

bodies. As shown in Fig. 9A, Grb2 was found in SLP-76 immunoprecipitates, implying that the two proteins interacted with each other in the cell. In subsequent studies, we used purified GST-Grb2 SH3-C domain immobilized on agarose beads to precipitate SLP-76 or a mutant from cell lysate. Although the wild-type SLP-76 was readily pulled down by the SH3 domain, mutant M-gb was not, suggesting that the Grb2 SH3-C domain mediates binding to Grb2. In comparison, mutant M-1b still bound to the Grb2 SH3-C domain albeit with significantly reduced affinity than did the wild-type SLP-76, suggesting that the p127 site plays an important but non-dominant role in binding. Curiously mutant M-1a also had a drastically reduced affinity for the Grb2...
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SH3-C domain. Moreover the triplet mutant M-1b/2ab and the quadruplet mutant M-1ab/2ab failed completely in binding the Grb2 SH3-C domain. These results suggest that the Grb2 SH3-C domain may recognize multiple sites in the SLP-76 PRR, a high affinity site represented by peptide p132 and several low affinity sites represented by p127, p1a, p2a, and/or p2b. It is not clear whether these low affinity sites were directly involved in binding or whether their disruptions affected binding of SLP-76 to the Grb2 SH3-C domain due to conformational changes. Nonetheless it is remarkable that mutations that affected SLP-76 binding to the PLC-γ1 SH3 domain also impaired its interaction with the Grb2 SH3-C domain.

Sequence alignment suggests that the C-terminal SH3 domains of Grb2, Gads, and NCK are closely related to the PLC-γ1 SH3 domain (Supplemental Fig. S2). One characteristic of this group of SH3 domains is the presence of multiple (at least three) acidic residues in the RT loop (Supplemental Fig. S2). Because a pair of Glu residues present in the RT loop of the Gads SH3-C domain was previously shown to play a pivotal role in binding SLP-76 (11), we predicted that equivalent residues in Grb2 SH3-C would have a similar function. To test this hypothesis, we generated three variants of the Grb2 SH3-C domain that contained mutation(s) of either one or both acidic residues (Glu-171 and Glu-174) in the RT loop. Binding studies using these mutants confirmed the critical importance of the two acidic residues in binding to SLP-76 because mutants with either or both Glu residues changed to an Ala (Fig. 9C) failed to bind SLP-76.

DISCUSSION

Deciphering the specificity of protein interaction modules such as the SH3 domain is an important step toward the understanding of their cellular functions and the reliable prediction of protein-protein interactions mediated by these domains (31, 32). The large number of SH3 domains contained in a mammalian proteome makes such a task daunting. Despite tremendous progress made in the past decade toward understanding the specificity and function of SH3 domain-mediated interactions, this age old family of domains continues to generate surprises. The identification of an RXK motif recognized by the C-terminal SH3 domain of the hematopoietic protein Gads (11, 33) challenges the dogma that PHX is the signature motif recognized by all SH3 domains. Novel modes of peptide recognition by the SH3 domain have also been identified under a variety of other biological settings. For instance, a family of Phox proteins, including p47phox and p67phox, regulates the activation of the NADPH oxidase through non-conventional SH3-mediated interactions (for reviews, see Refs. 20 and 34). The Fyn SH3 domain is capable of mediating distinct binding events either by engaging a conventional class II motif (35) or through surface-surface interactions (36). These examples illustrate the extreme flexibility and promiscuity of SH3 domains and call for new strategies in uncovering unique features of these interaction modules.

We used peptide spot array in conjunction with peptide and protein binding assays to identify a group of novel SH3 ligands from the Pro-rich region of SLP-76. Several findings from this small scale proteomic study are worth noting. First, the SLP-76 PRR is capable of supporting a large number of SH3 domain-mediated interactions. Although it is not clear how many of these interactions are physiologically relevant and, if they are, how they are regulated in the cell, our data suggest that it may be necessary to characterize these interactions simultaneously and systematically to achieve a comprehensive understanding of the function of SLP-76. The same argument is likely applicable to other proline-rich regions that harbor multiple binding sites for Pro recognition domains. Second, the majority of potential SH3-binding sites identified in the SLP-76 PRR do not conform to either the class I or II consensus. Rather these sites are rich in basic residues. Therefore, apart from recognizing conventional PXK motif-containing sequences, SH3 domains are capable of binding to positively charged peptides. Numerous peptides identified in SLP-76 PRR as potential SH3 ligands bear one or more basis motifs such as RXK, KXXK, and KKPP. Although several SH3 domains, including those from Grb2, Gads, and PLC-γ1, bound with moderate affinities to these motifs, it is likely that they are recognized by many more SH3 domains, especially by SH3 domains that contain multiple acidic residues in their RT loops. It is interesting to note, in this regard, that a basic peptide devoid of prolines can confer sufficient affinity to an SH3 domain under physiological conditions. For example, the Bin-1 SH3 domain binds to a basic motif, (K/R)XXXXKX(K/R)(K/R), present in the same molecule through an intramolecular interaction and thereby blocks the binding of the SH3 domain to its physiological target dynamin (37). Third, a single SH3 domain can engage multiple, distinct sites, and a given site may be recognized by more than one SH3 domain. For example, both the Grb2 SH3-C and the PLC-γ1 SH3 domains bind to multiple sites in SLP-76, and the two SH3 domains commonly recognize at least one site represented by p142/p2a. Fourth, our studies not only identified the binding sites for known ligands of SLP-76 such as PLC-γ1, they also uncovered many potentially interesting interactions. Although the biological significance of these interactions awaits further investigation, direct binding of SLP-76 PRR to the SH3 domains of Grb2, PI3K-p85, and NCK may be of functional relevance as these proteins coexist in a signalosome nucleated by LAT following T cell receptor activation. Moreover both Grb2 and PI3K can associate with LAT through their respective SH2 domains (29). The NCK SH2 domain can also bind to phosphorylated Tyr residues at the N-terminal region of SLP-76 upon its recruitment to LAT by Gads. The array of SH3 domain-mediated interactions observed in the present study may thus cooperate with the SH2 domain the formation of the T cell signalosome.
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Compared with the high affinity displayed by the RXKK-containing peptide p132 for the Gads SH3-C domain, most novel peptides identified from the current study exhibited weak or moderate affinities for their receptor SH3 domains. Although it is reasonable to assume that high affinity often translates to high specificity (38), most SH3 domain-mediated, physiological interactions are of moderate affinities. Cellular signal transduction is a dynamic process that requires the formation of a particular signaling complex when the stimulus is on and the dissociation of the complex when the signal is attenuated or switched off. This dictates that most protein-protein interactions occurring in cellular signal transduction be transient. Furthermore the presence of multiple weak to moderate binding sites in a single polypeptide chain for an SH3 domain may be a mechanism used by the cell to regulate SH3-mediated interactions (20). Although the SH3 domain may dissociate quickly from one site, it can be recapitulated by a neighboring site. This way, the presence of multiple binding sites effectively increases the local concentration of the SH3 domain and thereby promotes binding. This mechanism of regulation is exploited by the CD2 binding protein 2 (CD2BP2) GYF domain that binds only weakly to a single copy of a Pro-rich motif in CD2. However, when two copies of the same motif are present in a single polypeptide, its affinity for the GYF domain increases by 10-fold (39). It is likely that a similar mechanism underpins the interaction between SLP-76 and PLC-γ1 SH3 domains.

In conclusion, we have presented here an effective method by which to map the specificity and interaction partners of SH3 domains. The peptide-walking array approach can be readily expanded to include a larger set of Pro-rich regions and SH3 domains. It may also be adapted to investigate other proline recognition domains. Similar approaches have been developed for the identification of binding partners for the WW domains on a proteome scale (40). Peptide arrays, in conjunction with bioinformatics and other proteomic and genomic strategies, would prove to be a powerful tool for the identification of the global ligand pool for peptide interaction domains.

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∥ A scientist of the National Cancer Institute of Canada with funds made available by the Canadian Cancer Society. To whom correspondence should be addressed: Dept. of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1, Canada. Tel.: 519-850-2910; Fax: 519-661-3175; E-mail: sli@uwo.ca.

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