Identification of Itk/Tsk Src Homology 3 Domain Ligands*

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The tyrosine kinase Itk/Tsk is a T cell specific analog of Btk, the tyrosine kinase defective in the human immunodeficiency X-linked agammaglobulinemia and in xid mice. T lymphocytes from Itk-deficient mice are refractory to mitogenic stimuli delivered through the T cell receptor (TCR). To gain insights into the biochemical role of Itk, the binding properties of the Itk SH3 domain were examined. An optimal Itk SH3 binding motif was derived by screening biased phage display libraries; peptides based on this motif bound with high affinity and selectivity to the Itk SH3 domain. Initial studies with T cell lysates indicated that the Itk SH3 domain bound Bcl, Fyn, and other tyrosine phosphoproteins from TCR-stimulated Jurkat cells. Under conditions of increased detergent stringency Sam 68, Wiskott-Aldrich Syndrome protein, and hnRNP-K, but not Cbl and Fyn, were bound to the Itk SH3 domain. By examining the ability of different SH3 domains to interact with deletion variants of Sam 68 and WASP, we demonstrated that the Itk-SH3 domain and the SH3 domains of Src family kinases bind to overlapping but distinct sets of proline-rich regions in Sam 68 and WASP.

The T cell specific tyrosine kinase Tsk/Itk/Emt (1–4) (hereafter referred to as Itk) is a member of the Tec family of nonreceptor tyrosine kinases. This family of kinases consists of Tec, Btk, Itk, and Bmx (5–7), and is defined by the presence of SH3 and SH2 domains (8) adjacent to the catalytic domain and an amino-terminal region containing a pleckstrin homology (PH) domain (9), a Tec homology (TH) domain (10), and a proline-rich region (11). Btk, the best characterized member of this family, is an important component of antigen receptor signaling pathways in B cells and mast cells (12–15). The expression pattern of Itk complements that of Btk; Itk is expressed in T cells whereas Btk is expressed in most hematopoietic cells other than T cells (16). This reciprocal pattern of expression suggests that these two kinases play analogous roles in antigen receptor signaling pathways, as is the case with ZAP-70 and Syk, which are capable of functionally sub-

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The abbreviations used are: Btk, Bruton’s tyrosine kinase; SH3, Src homology 3; SH2, Src homology 2; PH, pleckstrin homology; TH, Tec homology; GST, glutathione S-transferase; WASP, Wiskott-Aldrich Syndrome protein; PAGE, polyacrylamide gel electrophoresis; hnRNP, heterogeneous nuclear ribonucleoprotein.

Btk has been identified as the gene defective in murine X-linked immunodeficiency (xid) (18, 19) and human X-linked agammaglobulinemia (XLA) (20, 21). In xid mice, B cell numbers are reduced to one-half of normal and the titers of specific immunoglobulin isotypes are significantly reduced; in addition, xid B cells are insensitive to a number of mitogenic stimuli (22, 23). For reasons which have yet to be elucidated, the human disorder is much more severe, resulting in nearly complete elimination of the B cell compartment and dramatically reduced immunoglobulin levels (24). Biochemical studies have supported multiple roles for Btk in B cell activation. Btk kinase activity and tyrosine phosphorylation are increased after cross-linking either the B cell receptor on B cells (13–15) or the high affinity IgE receptor, FcεRI, on mast cells (12). Interleukin-5 (25) and interleukin-6 (26) treatment have also been shown to lead to the activation of Btk.

Itk, like Btk, is tyrosine-phosphorylated upon antigen receptor cross-linking (27). In addition, peripheral T cells from mice lacking functional Itk are refractory to stimulation by antibodies to CD3 plus antigen presenting cells (28). These Itk-deficient T cells can be stimulated by phorbol ester and calcium ionophore, demonstrating that Itk acts in signaling pathways proximal to the TCR. However, the manner in which Itk activity is regulated by, and integrated with, other TCR-derived signals is not well understood. Unlike the related Src family tyrosine kinases, the Tec family kinases lack the negative regulatory tyrosine phosphorylation site at the carboxyl terminus. Furthermore, the amino-terminal myristylation site crucial for the membrane localization of Src family kinases is not present in Tec family kinases.

The conserved noncatalytic domains of many nonreceptor tyrosine kinases are required for intermolecular interactions with activators and effectors, as well as intramolecular regulatory interactions (8, 29). The PH domains present in Itk and Btk have been shown to bind phosphatidylinositol 4,5-phosphate (30) and the Btk PH domain has also been shown to bind heterotrimERIC G protein βγ subunits (31). Furthermore, the xid mutation lies within the Btk PH domain (18, 19). These properties suggest that the PH domains of Itk and Btk mediate the membrane association of these kinases and may further be required to stabilize interactions between these kinases and components of signal transducing pathways. Several studies have demonstrated that the proline-rich motifs in Btk and Itk are recognized by SH3 domains of the Src family kinases Fyn, Hck, Lyn, and Btk in vivo (11, 32). Recent experiments have also indicated that Btk can be phosphorylated and activated by these Src family kinases; however, these kinases have not been shown to interact directly with Btk in vitro (32–34).

The specific roles played by the SH3 and SH2 domains of Btk

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family kinases are less well understood. Recent studies have demonstrated that these domains are required for the activation of Btk by Src family kinases (33). However, direct binding partners for the Btk and Itk SH2 domains have not been reported, and only one binding partner of the Btk SH3 domain, the proto-oncogene product Cbl (35), has been identified. As SH3 domains are important for regulating enzymatic activity (36, 37), recruiting substrates (38, 39), and directing the subcellular localization of proteins (40), we elected to study the binding properties of the Itk SH3 domain. In this report we demonstrate that synthetic peptides interact with the Itk SH3 domain with high affinity and selectivity, and describe the identification of four proline-rich ligands of the Itk SH3 domain: Sam 68, hnRNPK, WASP, and Cbl. We further demonstrate that the Itk and Src SH3 domains interact with Sam 68 and WASP via overlapping but distinct sets of proline-rich binding sites, and suggest that these ligands may act as scaffolds enabling the formation of signal transducing complexes.

EXPERIMENTAL PROCEDURES

GST-SH3 Fusion Matrices—The Itk SH3 domain was amplified from the murine cDNA clone (2) by polymerase chain reaction. The product, encoding amino acids 171 to 232, was cloned into the BamHI site of pOEX-2T (Pharmacia). The W250K mutation was generated by polymerase chain reaction using the enriched deletion subcloned shuttle vector and the mutagenic oligonucleotide P13K, and Abl SH3 domain constructs have been previously described (41). The Lck SH3 domain and the Grb2 amino-terminal SH3 domain construct were kindly provided by Stuart Schreiber (Harvard University, Cambridge, MA). GST fusion proteins were produced in Esche- richia coli (DH5α) and purified on glutathione-Sepharose beads as described (42); the fusion proteins were greater than 95% pure, as assayed by Coomassie Blue staining. The resulting affinity matrices were used directly in binding assays.

Preparation of Lysates—Jurkat cells, of subclone E6.1 (43), were maintained in RPMI (Cellgro, Mediatech) supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine, 50 μg/ml β-mercaptoethanol, and 25 mM HEPES, pH 7.4. Jurkat cells to be stimulated were starved overnight in media containing 1% fetal calf serum. Before stimulation, the cells were rinsed twice with ice-cold media lacking fetal calf serum, and resuspended at 5 × 10⁶ cells/ml. Stimulations were performed by incubating Jurkat cells on ice for 15 min with 3 μg/ml OKT3 (ATCC), quickly pelleting the cells, and resuspending in 15 μg/ml rabbit anti-mouse Ig (Sigma) at 37°C for the indicated times. Stimula- tions were terminated by diluting the cells 3-fold into ice-cold buffered saline supplemented with 1 mM sodium orthovanadate and 20 mM sodium fluoride. Cells were pelleted and resuspended in ice-cold lysis buffer at 5 × 10⁶ cells/ml. After 10 min on ice, lysates were cleared by spinning 15 min at 16,000 rpm. Cleared lysates were adjusted to the desired detergent conditions; all salts were maintained at the levels present in lysis buffer. Lysis buffer contained 1% Triton X-100, 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM diithothreitol, 20 mM sodium pyrophosphate, 20 mM sodium fluoride, and 1 mM sodium orthovanadate; this buffer was supplemented with phenylmethylsulfo- nyl fluoride, peptatin A, aprotinin, and leupeptin before use. Thymo- cytes were freshly isolated from B10.BR mice, rinsed in serum-free RPMI, and lysed immediately at 5 × 10⁶ cells/ml. Thymocyte lysates were cleared as described above.

Metabolic Labeling—Jurkat cells were incubated in medium lacking methionine and cysteine for 15 min at 37°C. Cells were then labeled by incubating for 3 h at 37°C in labeling medium supplemented with EXPRE-2SS (DuPont NEN). 2 mM l-cysteine was used per 10⁶ Jurkat cells. Labeling medium contained 1% dialyzed fetal calf serum, penicil- lin, streptomycin, and glutamine, 50 μg/ml β-mercaptoethanol, and 25 mM HEPES, pH 7.4, in RPMI lacking methionine and cysteine (Life Tech- nologies, Inc./BRL).

Binding Assays—To deplete lysates of GST-binding proteins, aliquots of lysate (5 × 10⁶ cells equivalents) were incubated for 30 min at 4°C with 10 μg of GST affinity matrix. The GST-bound beads were washed three times and the lysates were deproteinated again as described. Binding assays were then performed for 1 h at 4°C using 2 μg of GST or GST-SH3 affinity matrix per aliquot of lysate. Bound complexes were washed three times with ice-cold lysis buffer. Samples were resolved by SDS-PAGE. 35S-Labeled proteins were detected by autoradiography; all other proteins were transferred to Immobilon-P for Western analysis. ENLIGHTENING (DuPont NEN) was used to enhance autoradio- graphic detection. 5 × 10⁶ cell equivalents of thymocyte lysate were used for each binding assay; depletion and binding were as described for Jurkat cells.

For peptide competition experiments 2 μg of GST-SH3 matrices were preincubated in 100 μl of lysis buffer containing peptides at twice the desired final concentration. After 30 min at 4°C, 100 μl of lysis (5 × 10⁷ cell equivalents) was added. After 1 h at 4°C bound complexes were washed and analyzed as above.

For reciprocal depletion experiments aliquots of 35S-labeled lysate (5 × 10⁶ cell equivalents) were twice depleted of GST-binding proteins as described. Lysates were depleted of SH3-binding proteins by incubating two times at 4°C for 30 min with 30 μg of the depleting affinity matrix. To confirm the depletion, lysates were incubated another hour at 4°C with 2 μg of the depleting affinity matrix; these beads were washed twice above and analyzed by SDS-PAGE analysis.

Binding assays were then performed and analyzed as described above.

Blotting—Filters were incubated in blotting buffer (150 mM NaCl, 50 mM Tris, pH 7.6, 0.1% Tween 20) supplemented with 5% bovine serum albumin or 5% nonfat dry milk and 0.02% sodium azide for at least 1 h at room temperature. Filters were then incubated with primary anti- bodies for 1 h at room temperature or overnight at 4°C. Sam 68 was detected using antisera to the protein encoded by the clone for GAP-106, which was kindly provided by Brian Druker (Oregon Health Science University, Portland, OR). After rinsing, filters were incubated for 15 min at room temperature with horseradish peroxide-conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham) in blotting buffer supplemented with 5% bovine serum albumin or 5% nonfat dry milk. After extensive rinsing, immunoreactive proteins were detected by ECL (Amersham).

To generate labeled fusion protein probes, GST and GST-Itk SH3 were eluted from glutathione-agarose using reduced glutathione. The fusion proteins were dialyzed into 0.1 mM sodium bicarbonate and adjusted to 1 mg/ml. Biotin N-hydroxysuccinimide (BRL) was resuspended in dimethyl sulfoxide at 1 mg/ml immediately before use. Ali- quots of fusion protein were incubated for 4 h at room temperature with 30, 60, or 120 μg/ml biotin N-hydroxysuccinimide. Biotinylation reac- tions were terminated by the addition of 100 mM Tris, pH 8.0, followed by overnight dialysis against phosphate-buffered saline. Probes were tested on total lysates and the optimally biotinylated probes were used at 5 μg/ml to blot for direct binding proteins. The bound probes were detected using ECL and then horseradish peroxide-conjugated streptavidin (Amasham) and ECL.

Phage Display and Peptide Synthesis—The Xc-PIP, RSLRPLLP, and GAAPPLPPP-Xc phage display libraries have been previously de- scribed (41, 48). The Xc-YSKKPPPP and Xc, libraries were generated by a similar cloning strategy. All libraries were screened as described previously (41). The number of recombinants in each unamplified li- brary ranged from 2 × 10⁶ to 2 × 10⁷. The SH3 domain binding peptides VSLARRPLPP, GWYSKPPPPP, and AYSKKPPPPP were synthe- sized by Chiyoshi Kasahara as described (49).

In Vitro Translation—All constructs were translated using the Pro- mega TNT Reticulocyte Lysate System, using either T3 or T7 polymer- ase. Labeled mature [35S]methionine was obtained from DuPont NEN. Equal amounts of each in vitro translation product, as assayed by autoradiography, were diluted into lysis buffer; binding assays were performed as described above. The myc-tagged Sam 68 deletion con- structs were originally generated by S. Richard (50) and were kindly provided by Andrey Shaw.

The human WASP cDNA (51) was obtained from S.-P. Kwan (Rush Medical School, Chicago, IL), and was subcloned into the BamHI and NotI sites of pBluescriptII SK− (Stratagene). The WASP-ADH con- struct was generated by deleting WASP regions B and C (amino acids 218–386) by cutting with PmJ, generating blunt ends with T4 polymer- ase, and religating. The WASP-ADH construct was generated by cloning the BamHI-BglII fragment of WASP (region A) into the BamHI site of pBluescriptII SK−, truncating WASP at amino acid 266. The WASP- ABCD and WASP-AB constructs were generated by subcloning WASP fragments encoding regions ABCD and AB, respectively, into a variant of pBluescriptII SK− in which stop codons were introduced in all three frames between the EcoRI and HindIII sites of pBluescriptII SK−. The fragment encoding WASP-ABCD was released with EcoRI and cloned into the EcoRI site in the stop vector, truncating WASP at amino acid
442. The fragment encoding WASP:AB was released with BamHI and partially digested with SfiI. Blunt ends were generated with T4 polymerase and the fragment was cloned into the EcoRV site in the stop vector, truncating WASP at amino acid 364. Region A was deleted from the WASP:AB construct by cutting with XcmI and BglII, generating blunt ends with T4 polymerase, and recircularizing the construct. The resulting construct, WASP:B, lacks amino acids 117–265. Region A was deleted from the WASP:ABCD construct by cutting with XcmI and NolI, generating blunt ends with T4 polymerase, and recircularizing the construct. The resulting construct, WASP:BCD, lacks amino acids 117–308. The WASP:CD construct was generated by ligating the NotI-Stul fragment of WASP:ABCD into the NotI, Smal cut WASP:ABCD vector, deleting amino acids 137–367. Full-length WASP:ABCD, WASP:A, and WASP:ADE were translated using T7 polymerase to generate transcripts. The WASP:ABCD, WASP:BCD, WASP:CD, WASP:AB, and WASP:B constructs were translated using T3 polymerase to generate transcripts.

## RESULTS

**Itk SH3 Domain Selects Specific Ligands from Biased Phage Display Libraries**—Recently, phage display and peptide bead library technologies have simplified the identification of high affinity peptide ligands for SH3 domains (48, 52, 53). In order to screen phage display libraries for an Itk SH3 domain binding motif we generated a GST-Itk SH3 domain fusion protein and verified that it was functional (see below). Src family SH3 domains have been shown to interact with ligands in either of two orientations, referred to as class I and class II (52). The first library screened was degenerate at six positions amino-terminal to the proline-rich core, biasing this X₆-PPPPIP library toward the identification of class I SH3 domain ligands. After four rounds of selection using the Itk SH3 domain, phage were isolated and sequenced (Fig. 1A). All phage sequenced had a proline residue at position 6, immediately amino-terminal to the minimal core. Tyrosine and lysine residues were strongly preferred at positions 2 and 4, respectively; only one phage had residues at positions 1, 3, and 5. No strong amino acid preferences, although position 5 demonstrated a weak preference for threonine or proline. The resulting consensus, YKxPPPPIP, is distinct from all previously reported core binding motifs for SH3 domains.

To determine if residues amino-terminal to the core binding motif could enhance peptide binding, we constructed another library based on the Itk-specific core binding motif. Phage were enriched from the X₅-YSKPPPPIP library by repeated rounds of selection on the Itk SH3 domain. The resulting phage were isolated and sequenced (Fig. 1B). No residues were strongly selected at positions 1, 2, and 3. The most consistently observed feature was the selection of tryptophan residues at position 5. In addition, glycine was present at position 4 in more than half of the sequenced phage, yielding the extended motif GWYxKPPPPIP. The selection of glycine and tryptophan at positions 4 and 5 clearly suggests that residues amino-terminal to the core binding site are important for Itk SH3 domain binding.

Three additional libraries were tested. No amplification was observed with any of these libraries. The first was the RSL-RPL-X₅ library used to “back-select” for the exact composition of the proline-rich cores preferred by Src family SH3 domains. This failure was probably due to the incompatibility of the library with the amino-terminal flanking residues preferred by the Itk SH3 domain. A second library tested was a class II-biased library, GAAAPLPRP–X₅; it is possible that this library is also incompatible with the Itk SH3 domain, or alternatively, that the class II mode of binding to this GST-SH3 domain fusion protein is sterically hindered in the phage display system. Finally, we were unable to enrich phage from an Xₐ₋ₐ random library. The reasons for this failure are unclear; however, it is possible that the library is not extensive enough to contain phage binding to the Itk SH3 domain with sufficiently high affinity to be enriched (41). The overall results of these phage display screens indicated that the binding specificity of the Itk SH3 domain was significantly different from previously reported SH3 domain specificities.

**Phage Display Consensus Peptides Demonstrate Itk SH3 Domain Binding Specificity**—To confirm that the GST-Itk SH3 domain fusion protein used above would bind cellular ligands, an affinity matrix was generated by binding the fusion protein to glutathione-Sepharose beads. This affinity matrix was used to purify Triton-soluble Itk SH3 domain binding proteins from [³⁵S]methionine-labeled Jurkat T cells. Fig. 2A shows the broad range of cellular proteins specifically bound by the Itk SH3 domain (center lane) as compared to GST alone (left lane). Although the binding pattern appears extremely complex, it does not represent nonspecific binding, as the pattern of bands eluted from the beads does not reflect the abundance of proteins in total lysate (data not shown). More conclusively, a single point mutation in the predicted binding pocket of the SH3 domain (54, 55), converting tryptophan 208 to lysine, eliminated all binding of cellular proteins (Fig. 2A, right lane). This confirms that the binding pocket characterized in other SH3 domains is essential for the specific binding mediated by the Itk SH3 domain.

To assess the selectivity of binding by the Itk SH3 domain, we compared the ability of peptides based on the Itk or Src SH3 domain binding motifs to compete for binding to the Itk SH3 domain. Two peptides based on the phage display consensus binding motifs for the Itk SH3 domain were synthesized (GW-YSKPPPPIP and AYSKPPPPIP, referred to as Itk-long and Itk-short, respectively). In addition, a high affinity Src SH3-
specific peptide (VSLARRPLPPLP (48, 49)) was synthesized. As a means of assessing the specificity of Itk SH3 domain interactions, we compared the concentrations of each peptide necessary to eliminate binding of $^{35}$S-labeled Jurkat proteins to Itk SH3 domain affinity matrices in $1\%$ Triton X-100. We also assessed the ability of these peptides to compete for binding to the Src and Abl SH3 domains.

As can be seen in Fig. 2B, the long Itk peptide half-maximally inhibited binding of labeled proteins to the Itk SH3 domain at $2\ \mu M$. The short Itk peptide appeared slightly less effective at inhibiting binding. The Src peptide was substantially less effective at competing for binding to the Itk SH3 domain (Fig. 2B). In comparison, the Itk peptides were inefficient competitors of binding to the Src and Abl SH3 domains (Fig. 2, C and D), whereas the Src peptide completely inhibited binding to the Src SH3 domain at $2\ \mu M$ (Fig. 2C). This is consistent with studies demonstrating a $K_d$ of 450–860 nM for the binding of this Src peptide to the Src SH3 (48, 49). Interestingly, high concentrations of the long Itk peptide, but not the Src peptide, were able to compete for binding to the Abl SH3 domain (Fig. 2D). This may result from similarities between the consensus binding motifs for the Itk and Abl SH3 domains; for instance, both SH3 domains preferred proline residues at positions 1 and 2 amino-terminal to the PXPF core (41). Reciprocal depletion experiments with the Itk and Abl SH3 domains support the possibility that these two domains share some binding specificity (see below). The inability of the Src peptide to bind to the Abl SH3 domain is consistent with previous work (38, 41). Therefore, these data demonstrate that the Itk-specific phage display-derived peptides are very selective in inhibiting interactions with the Itk SH3 domain, confirming that the Itk SH3 domain has unique and specific binding properties for cellular ligands.

**Differential Association of the Itk, Src, and Abl SH3 Domains with TCR-induced Tyrosine Phosphoproteins**—Functional analyses have indicated that T lymphocytes derived from Itk-deficient mice are impaired in their responses to TCR stimulation (28), implicating Itk in the TCR signaling pathway. We therefore examined the ability of the Itk SH3 domain to interact with tyrosine phosphoproteins induced by TCR cross-linking. As shown in Fig. 3, the Itk SH3 domain was observed to bind several tyrosine phosphoproteins from Triton X-100 lysates of TCR-stimulated Jurkat cells. The set of tyrosine phosphoproteins bound to the Itk SH3 was very similar to that bound to the Src SH3. In particular, an intensely tyrosine-phosphorylated protein migrating at 115 kDa was bound to both SH3 domains. Abl SH3-bound proteins were virtually devoid of tyrosine phosphorylation on this timescale. When the same experiments were repeated in RIPA buffer (1% Triton-X, 1% deoxycholate, 0.1% SDS), no tyrosine phosphoproteins were bound to the Itk SH3 domain; whereas the 115-kDa tyrosine phosphorylated protein remained bound to the Src SH3 domain (data not shown).

The proto-oncogene product Cbl migrates at approximately 115 kDa and has been shown to be highly phosphorylated on tyrosine residues in response to TCR cross-linking (56). In addition, Cbl has been shown to bind to the SH3 domain of Btk (35). To test the ability of the Itk SH3 domain to interact with Cbl, the anti-phosphotyrosine blots described above were reprobed with antibodies to Cbl. We determined that Cbl comigrated exactly with the 115-kDa tyrosine phosphoprotein bound to the Itk SH3 in Triton X-100 (Fig. 3) and that Cbl binding to the Itk SH3 domain was eliminated by the addition...
of RIPA buffer (data not shown). Cbl binding to Src SH3 matrices was independent of the activation state of the cells (Fig. 3), and was maintained in the presence of RIPA buffer (data not shown). We also observed that the binding of Cbl to the Itk SH3 domain in Triton X-100 lysates was affected by the activation state of the cells; binding was increased from basal levels at 30 s to 2 min after TCR stimulation and was eliminated by 30 min after stimulation (Fig. 3). This precisely parallels the observed antigen receptor dependence of the interaction between the Btk SH3 domain and Cbl (35).

Since the Src family tyrosine kinase Fyn is known to interact with Cbl (57), we also tested for the possibility that Fyn is found in the Itk SH3-bound complex. The same bands were stripped and reprobed with antibodies to Fyn. Like Cbl, Fyn bound to Itk SH3 matrices in Triton X-100 (Fig. 3) but did not bind in RIPA (data not shown). The amount of Fyn bound to the Itk SH3 was slightly reduced at 30 min after activation. We were unable to detect Fyn bound to the Src SH3 domain in either Triton X-100 (Fig. 3) or RIPA lysates (data not shown), despite the presence of Cbl bound to these matrices. The absence of Fyn may result from the saturation of Fyn SH3 binding sites on Cbl by the Src SH3 domain fusion protein. These data demonstrated binding of the Itk SH3 domain to TCR-responsive phosphoproteins, including Cbl and Fyn. This is consistent with a role for the Itk SH3 domain in recruiting Itk to activated signaling complexes.

**The SH3 Domain-Ligand Interaction Is Detergent-sensitive**—In an attempt to identify additional tyrosine phosphoproteins interacting with the Itk SH3 domain, we probed Western blots of Itk SH3-bound proteins with a panel of antibodies to known signal transducing proteins. Vav, Zap-70, Jak1, c-Raf, and Erk1 were observed to bind to the Itk SH3 in Triton X-100 lysates of murine thymocytes (data not shown). Although Cbl and Vav contain proline-rich domains with partial matches to the Itk SH3 binding motif, the other proteins do not. For instance, while most of these proteins contain at least one PX motif, there are very few additional identities between these putative sites and the Itk SH3 binding motif. This suggested that these proteins may bind indirectly to the Itk SH3 domain, complicating the identification of direct Itk SH3 ligands.

We attempted to circumvent this difficulty by using more stringent detergent conditions to remove weakly or indirectly associated proteins from the Itk SH3 domain bound complexes. In order to preclude complications resulting from the differential solubilization of cellular proteins, lysates were prepared in 1% Triton X-100, adjusted to more stringent conditions by the addition of increasing amounts of deoxycholate and SDS, and depleted of GST-binding proteins. The lysates were then incubated with Itk SH3 affinity matrices. To assess the binding of ligands that interact directly with the Itk SH3 domain, we biotinylated the Itk-SH3 domain fusion protein and used it as a probe. The results of this “far Western” blot are shown in Fig. 4. The Itk SH3 domain bound complexes from Triton X-100 lysates contained many Itk SH3 domain binding proteins. A severe threshold effect was observed as the deoxycholate concentration rose from 0.3 to 1%, resulting in the elimination of all but six major Itk SH3 domain binding proteins. Two of these deoxycholate-resistant ligands were gradually eliminated as the SDS concentration was increased. Although many direct SH3 domain ligands were removed by the addition of 1% deoxycholate, we decided that the elimination of indirectly bound proteins was of greater importance, and chose to screen 1% Triton X-100, 1% deoxycholate lysates for direct SH3 domain ligands.

**SH3 Domains Bind Similar but Distinct Sets of Cellular Proteins**—We attempted to circumvent this difficulty by using more stringent detergent conditions to remove weakly or indirectly associated proteins from the Itk SH3 domain bound complexes. In order to preclude complications resulting from the differential solubilization of cellular proteins, lysates were prepared in 1% Triton X-100, adjusted to more stringent conditions by the addition of increasing amounts of deoxycholate and SDS, and depleted of GST-binding proteins. The lysates were then incubated with Itk SH3 affinity matrices in Triton X-100/deoxycholate lysates was substantially reduced, and underlying similarities were revealed. Specifically, all SH3 domains tested bound a 60-kDa protein and at least one member of a doublet at 48/50 kDa that were not bound to the other SH3 domain affinity matrices (white arrows). In contrast, the number of proteins bound to SH3 affinity matrices in Triton X-100/deoxycholate lysates was substantially reduced, and underlying similarities were revealed. Specifically, all SH3 domains tested bound a 60-kDa protein and at least one member of a doublet at 48/50 kDa (dark gray arrows), and all SH3 domains except that of Abl bound a 68-kDa protein (light gray arrow). A 115-kDa band bound by the Itk SH3 domain in Triton X-100 was absent in Triton X-100/deoxycholate; this band remained bound to the Src, Fyn, Lck, P13K, and Grb2N SH3 domains in Triton X-100/deoxycholate (striped arrows). The Itk SH3 domain also bound proteins at 105 and 150 kDa in Triton X-100/deoxycholate; proteins migrating at approximately the same sizes bound both the Src and Abl SH3 domains (black arrows).

The Itk SH3 domain appeared to share ligands with both the Src and Abl SH3 domains. In order to distinguish between specific and shared SH3 domain binding proteins, reciprocal depletion experiments were performed. Lysates were extensively depleted with one SH3 domain before binding to a second
plexes (that had been observed in both Itk and Abl SH3 bound complexes). The 90-kDa band which had appeared Itk specific was eliminated the binding of these bands to Itk SH3 affinity matrix; based on the strength of binding in Triton X-100 and Triton X-100/deoxycholate, except for Abl SH3 domain; in addition, the 68-kDa proteins bound by the Src and Itk SH3 domains were shown to be identical by reciprocal depletion experiments. Sam 68 migrates at 68 kDa, has extensive proline-rich domains, and is known to interact directly with the SH3 domains of both Src and Fyn, but not Abl (38, 50). We tested for Itk SH3-bound Sam 68 by Western blotting (Fig. 6A). Consistent with previous reports, Sam 68 bound to all SH3 domains tested in Triton X-100 and Triton X-100/deoxycholate, except for Abl. Specifically, Sam 68 bound the Itk SH3 domain in Triton X-100 and Triton X-100/deoxycholate lysates, confirming that Sam 68 is an Itk SH3 domain ligand. To verify that the Itk SH3-Sam 68 interaction was mediated by the predicted ligand binding pocket, we tested the ability of the Itk and Src SH3-specific peptides to compete with Sam 68 for binding to the Itk and Src SH3 domains. As shown in Fig. 6B, low levels of Itk SH3 domain-specific peptides were highly effective at eliminating Sam 68 binding to the Itk SH3 domain; similarly the Src peptide eliminated Sam 68 binding to the Src SH3 domain. Each peptide was inefficient at competing with Sam 68 for binding to the mismatched SH3 domain.

HNRP-K is related to Sam 68 (47). Like Sam 68, hnRNPK also contains proline-rich regions and is known to interact with SH3 domains via these proline-rich sequences (58–60). As shown in Fig. 6A, Western blotting with the anti-hnRNPK mAb 3C2 indicates that hnRNPK binds to the Itk SH3 domain in Triton X-100 and Triton X-100/deoxycholate. Although related to Sam 68, the SH3 domain binding profile of hnRNPK differed significantly from that of Sam 68. In particular, hnRNPK bound strongly to fewer of the SH3 domains tested and hnRNPK binding to all SH3 domains tested was proportionately reduced by Triton X-100/deoxycholate. These data confirm that hnRNPK is a ligand of the Itk SH3 domain.

Human WASP is 512 amino acids in length and comigrates exactly with the 60-kDa Triton X-100/deoxycholate-resistant SH3-binding protein described above (data not shown). To verify that WASP was actually binding to our panel of SH3 affinity matrices, SH3 domain bound proteins were analyzed by Western blotting with anti-WASP rabbit sera (Fig. 6A). Every SH3 domain tested bound WASP in Triton X-100 and Triton X-100/deoxycholate; this is consistent with the possibility that WASP and the 60-kDa protein are identical, and suggests that all the SH3 domains tested bind WASP directly.

We also tested for the presence of the tyrosine phosphoprotein Cbl, which we had shown binds the Itk SH3 domain in Triton X-100, but not in FPA. We confirmed that Cbl bound the Itk SH3 domain in Triton X-100, and were unable to show this interaction in Triton X-100/deoxycholate (Fig. 6A). The Abl SH3 did not bind Cbl in either of the detergent conditions tested. These data are consistent with a previous report that Cbl binds in vivo to Fyn, Grb2-N, and phosphatidylinositol 3'-kinase (57). The results of these Western blots demonstrate that Sam 68, hnRNPK, WASP, and Cbl are ligands of the Itk SH3 domain; based on the strength of binding in Triton X-100/
deoxycholate, Sam 68 and WASP appear to be the most tightly bound of these ligands.

**Mapping of the Itk SH3 Domain Binding Site in Sam 68**—We sought to identify the binding site(s) for the Itk SH3 domain in Sam 68. This information would provide a basis for determining if Itk and Src family kinases could simultaneously bind to Sam 68. Previous work had identified five proline-rich regions, P1 through P5, in Sam 68. To map the Itk SH3 binding site on Sam 68 a set of deletion constructs was used to generate in vitro translated products (Fig. 7A). The binding of these proteins to Itk SH3 matrices was then assessed in the presence of 1% Triton-X (Fig. 7B). For comparison, the binding of these proteins to the Src SH3 and Abl SH3 matrices was also analyzed. The P12 translation product did not bind to any of these SH3 domains, indicating that neither P1 nor P2 was capable of mediating binding to the ITK, Src, and Abl SH3 domains. The Itk SH3 domain bound weakly to the P123 and P124 translation products, and undetectably to the P125 translation product. The amount of the P1245 translation product bound was comparable to the amount of P124 translation product bound, further demonstrating that the P5 proline-rich region had no affinity for the Itk SH3 domain. In contrast, the P1234 translation product bound to the Itk SH3 domain much more efficiently than either the P123 or P124 translation product, suggesting that the P1234 translation product may bind cooperatively to ITK SH3 matrices (Fig. 7B).

The binding profile of the Src SH3 was consistent with previous reports (38). The Src SH3 domain bound the P1234 and full-length translation products, but did not bind detectably to any other Sam 68 translation product.

These results suggest that the binding of each of these SH3 domains to Sam 68 may be enhanced by the presence of multiple SH3 domain binding sites. It is likely that this is contingent upon the dimeric nature of the GST-SH3 domain fusion proteins (61, 62); as the tyrosine kinases ITK, Src, and Abl are not known to function as dimers, this raised the possibility that the binding of these SH3 domains to Sam 68 might represent artifacts of the fusion protein system. In order to address this issue, maltose-binding protein fusion proteins containing the ITK SH3 domain were generated. These fusion proteins bound to the P123, P124, and P1234 translation products approximately as well as the GST-ITK SH3 fusion protein binds to the P123 and P124 translation products (data not shown). While this
confirmed that the extremely strong binding of the P1234 translation product to the GST-Itk SH3 fusion protein is an artifact, it also demonstrates that both P3 and P4 are capable of interacting with monovalent Itk SH3 domains.

Mapping of the Itk SH3 Domain Binding Site in WASP—In order to identify Itk SH3 binding sites in WASP we generated a set of deletion constructs spanning the PXXP motifs present in WASP. The regions of WASP were designated A through E (Fig. 8A). The extremely proline-rich domain of WASP falls into regions B, C, and D. Itk, Src, Lck, and Abl SH3 domain binding sites were mapped by testing the ability of these SH3 domains to bind $^{35}$S-labeled WASP in vitro translation products, as described above. As shown in Fig. 8B, the Itk SH3 domain bound strongly to the full-length, ABCD, and BCD translation products. Surprisingly, we could not detect binding of either the B or CD translation products to the Itk SH3 domain. Similarly, we could not detect binding of either the A or B translation product to the Itk SH3 domain, despite the fact that the AB translation product bound, albeit weakly, to the Itk SH3 domain. This suggests that the interaction between the BCD translation product and the Itk SH3 domain results either from binding to one site that is split between regions B and C, or from divergent interactions involving one site in region B and one site in region C or D. Malto-secting fusion proteins containing the Itk SH3 domain bound to the ABCD translation product (data not shown), suggesting that at least one monovalent site of intermediate affinity exists in this region. As A, B, and CD do not bind to the GST-Itk SH3 fusion protein, it is likely that an Itk SH3 domain binding site is split between two regions; if this is the case, the site probably lies at the junction of regions B and C, as the junction between regions A and B does not contain proline-rich motifs.

Region CD is sufficient for high affinity binding of the Abl SH3 domain to WASP. As the ADE translation product bound extremely weakly to the Abl SH3 domain, this suggests that either one high affinity site for the Abl SH3 exists in region C, or that independent sites in regions C and D synergize to yield high affinity binding. The binding of the WASP translation products to the Src SH3 domain could be completely explained by the presence of two high affinity binding sites, one in region B and one in region D. Alternative explanations invoking divalent binding are possible. Together, these data demonstrate that the Itk SH3 domain binds WASP in a manner qualitatively different from the Src and Abl SH3 domains.

Cbl Interacts Weakly with the Itk SH3 Domain—In order to determine whether Cbl could interact with the Itk SH3 domain in the absence of other T cell-specific proteins, the full-length Cbl cDNA clone was subjected to in vitro transcription/translation in the presence of $^{35}$S-methionine. The labeled protein was incubated with Itk and Src SH3 affinity matrices in the presence of both Triton X-100 and Triton X-100/deoxycholate; the bound protein was analyzed by SDS-PAGE followed by autoradiography (Fig. 9). The in vitro translated Cbl bound to the Src SH3 domain in both Triton X-100 and Triton X-100/deoxycholate, but bound to the Itk SH3 domain only in Triton X-100. In addition, Cbl bound to the Src SH3 matrix more efficiently than to the Itk SH3 matrix. This behavior is fully consistent with the binding of Cbl to the Itk and Src SH3 matrices in Jurkat lysates (Fig. 6A), and suggests that the interaction between Cbl and the Itk SH3 domain is direct, although qualitatively different from the interaction between Cbl and the Src SH3 domain.

Cbl contains multiple PXXP motifs, including putative Src family SH3 domain binding sites, as defined by phage display consensus motifs (41, 56). The proline-rich and tyrosine-phosphorylated regions of Cbl have been shown to interact with many SH3 and SH2 domain-containing proteins involved in T cell activation, including Grb2, Crk, Nck, P38, Fyn, and Zap-70 (56, 57, 63–66). This suggests that Cbl may function as a scaffold allowing TCR-responsive kinases to couple to their downstream effectors. We observed that Cbl was eliminated from the Itk SH3 domain by stringent detergent conditions (Triton X-100/deoxycholate and RIPA) that concurrently eliminated most of the tyrosine phosphoproteins bound to the Itk SH3 domain. To determine whether or not these tyrosine phosphoproteins had been recruited to the Itk SH3 domain via Cbl, we completely depleted Cbl from Triton X-100 lysates before performing Itk SH3 binding assays. Under these conditions the 115-kDa tyrosine phosphoprotein was completely eliminated from the Itk SH3 domain without affecting the binding of the remainder of the Itk SH3 domain-bound tyrosine phosphoproteins (data not shown), demonstrating that these tyrosine phosphoproteins bind the Itk SH3 domain independently of Cbl.
Previously identified binding partners of the noncatalytic domains of Btk and Itk (e.g., Cbl and the Src-family kinases Fyn, Hck, Lyn, and Blk) have not been reported to co-immunoprecipitate with Btk or Itk. After identifying a number of ligands for the Itk SH3 domain in vitro, we attempted to co-immunoprecipitate these proteins with native Itk. Neither Cbl nor the deoxycholate-resistant SH3 domain ligands Sam 68, hnRNP-K, and WASP could be detected in Itk immunoprecipitates from Jurkat T cells (data not shown).

**DISCUSSION**

In these studies, we have identified Cbl, Sam68, hnRNP-K, and WASP as ligands for the Itk SH3 domain. To date, we have been unable to co-immunoprecipitate these ligands with native Itk. This suggests a number of possibilities. First, only a small fraction of Itk may be complexed with a given protein in vivo. Second, our anti-Itk antibodies may interfere with interactions between Itk and these ligands. Third, Itk interactions mediated by the SH3 domain may be otherwise unstable to co-immunoprecipitation. For example, the binding of the Itk SH3 domain to ligands could be suppressed by intramolecular interactions analogous to those involved in the regulation of Src family kinases. Also, we cannot rule out the possibility that bona fide ligands for the Itk SH3 domain are recognized with low affinity in order to facilitate regulated and reversible interactions.

The Itk-specific peptides derived from the phage display consensus motifs bind to the Itk SH3 domain with extremely high affinity and selectivity. Although the proline-rich core of the Itk SH3 domain peptide ligands has not been optimized, the affinity with which these peptides bind to the Itk SH3 domain approaches the affinity with which the extensively optimized Src SH3 domain-specific peptide binds to the Src SH3 domain. The high affinity and selectivity of these Itk SH3 domain ligands will allow them to be used as tools to determine whether or not proteins co-immunoprecipitating with the native Itk from cells are bound by the SH3 domain of Itk.

The Itk SH3 domain binding site in a deoxycholate-resistant ligand has been precisely mapped in only one case: P4 in Sam 68. This region contains only one PXXP motif, therefore the register in which the Itk SH3 domain binds to this site is fixed. Working from this base, we were able to identify a putative Itk SH3 domain binding motif in the other two deoxycholate-resistant ligands and P3 of Sam 68. This motif, PPPPXRGG, is present exactly twice in each of the deoxycholate-resistant ligands we have identified. This is remarkable in the case of hnRNP-K, in which there are eight minimal binding motifs (PXXP), and more so in the case of WASP, in which there are 36 minimal binding motifs. The location of the PPPPXXRGG motifs in WASP is consistent with the mapping data in Fig. 8B; both motifs are located in region B, and one of these motifs is immediately adjacent to the junction of regions B and C. The presence of two PPPPPXXRGG motifs in each of the deoxycholate-resistant ligands clearly suggests that these ligands are all capable of making divergent interactions with the GST-Itk fusion protein. A less stringent variant of this motif, PPPXXXR, is present only twice in each of the deoxycholate-resistant ligands. It is interesting to note that Cbl contains one copy of this PPPXXXR motif. Point mutagenesis can be used to verify whether or not the PPPXXXR motifs we have identified are actually the Itk SH3 domain binding sites.

If the PPPXXXR motifs present in each of the deoxycholate-resistant ligands are actual Itk SH3 domain binding sites, they are likely to interact with the Itk SH3 in the class II orientation. As the Itk-specific peptides based on the phage display motif are unlikely to bind in a class II orientation, this could explain the discrepancy between the flanking sequences derived by phage display and those observed in actual SH3 domain ligands. The apparent incompatibility of this motif with the Src class II core binding motif may explain our inability to select phage from the GAAPPLPPRX₆ library. Even if the affinity of the PPPPXXR motif for the Itk SH3 domain is not as high as that of the phage display-derived peptide, this would not be inconsistent with a physiological role for related monovalent class II Itk SH3 domain binding sites; it is possible that a lower binding affinity may be required for regulated, reversible interactions with the Itk SH3 domain. Alternatively, the SH3 of Itk domain may function synergistically with neighboring domains of Itk in order to bind weaker ligands such as Cbl.

The deoxycholate-resistant Itk SH3 domain binding proteins Sam 68 and hnRNP-K are structurally and functionally related (44, 47, 67, 68). Both Sam 68 and hnRNP-K are known to interact with multiple proteins via their proline-rich domains. HnRNP-K interacts with SH3 domains present in Src and Vav (59), and Sam 68 interacts with SH3 domains present in phospholipase Cγ1, Src, Fyn, PI3K, and Crk (50, 69). Our results confirm that Sam 68 binds to the SH3 domains of PI3K, Src, and Fyn, and that Sam 68 binds weakly to the Abi SH3 domain in Triton X-100 (69). In addition, our results demonstrate the binding of Sam 68 to the Lck, Grb2-N-terminal, and Itk SH3 domains.

Sam 68 also appears to be a substrate of Src and Fyn; once these kinases phosphorylate the carboxyl-terminal tyrosine-rich region of Sam 68, the SH2 domains of these kinases, phospholipase Cγ1, Grb2, and GAP can bind (50). It has been proposed that Sam 68 and hnRNP-K can serve as scaffolds for the formation of signaling complexes (50, 59). Our binding site mapping experiments support this model by demonstrating that the Src and Itk SH3 domains can interact with distinct binding sites within Sam 68. Our results suggest that the Itk SH3 domain may bind P3 or P4 in Sam 68 while the SH3 domain of a Src family kinase may interact with P5. This arrangement could be important for the activation of Itk by Src family kinases, analogous to Btk activation (33, 34).

The discovery that the Itk SH3 domain binds directly to the Wiskott-Aldrich syndrome protein (WASP) provides an intriguing potential link between the TCR and the cytoskeleton. Wiskott-Aldrich syndrome (WAS), the disease associated with defects in WASP, affects multiple hematopoietic lineages, but has the most severe defects in platelets and lymphocytes. With respect to the function of Itk and Btk, the lymphocyte defect in WAS is most interesting. The cytoskeletal architecture of T cell lines derived from WAS patients is extremely defective. Most prominently, the microvilli are dramatically reduced in number and surface density (70). This is consistent with the recent demonstration that WASP interacts with the small GTP-binding protein, Cdc42 (46, 71, 72), which is involved in actin cytoskeletal organization (72) and also in the polarization of T cells toward their target cells (73). The function of WAS T cells is also impaired; in response to immobilized anti-CD3, T cell lines derived from WAS patients fail to proliferate or secrete interleukin-2 (74). This functional defect may also be related to the cytoskeletal defects, as reorganization of the actin cytoskeleton has been shown to be required for the response of T cells to CD3 (75). Finally, the proliferative defect in WAS T cells is highly restricted, as the WAS T cells will respond normally to all specific challenge, phytohemagglutinin, or phorbol 12-myristate 13-acetate plus ionomycin (74).

It is interesting to note that the functional defects of human WAS B and T lymphocytes are similar to those present in the B cells of xid mice and in the T cells of Itk-deficient mice. Both WAS (76) and xid (22, 23) B cells respond poorly to polysaccharides and other Type-II T cell-independent antigens. Similar to WAS T cell lines, the T cells of Itk-deficient mice have dramat-
ically reduced responses to immobilized CD3 plus antigen-presenting cells, but respond normally to PMA plus ionomycin (28). Itk-deficient T cells also proliferate, although at reduced levels, in response to allogeneic stimulation. These genetic data suggest a common signaling pathway involving WASP and Itk in T cells, or WASP and Btk in B cells. The physiological relevence of the WASP-Itk SH3 domain interaction will need to be examined by further co-immunoprecipitation experiments. We propose that WASP, in addition to its role in organizing the actin cytoskeleton, serves as a scaffold for the assembly of TCR-responsive signaling complexes containing Itk.

We have also demonstrated that Cbl, the protein product of the proto-oncogene c-cbl, interacts with the Itk SH3 domain. This is in agreement with a previous report demonstrating an interaction between Cbl and the Btk SH2 domain (35) and extends the functional analogies between Itk and Btk.

The results of our Itk SH3 binding assays using 

in vitro translated Cbl suggest that this interaction is direct, but weaker than the interaction between the Itk SH3 domain and Sam 68 or WASP. One explanation for this is that the interaction of the SH3 domain with Cbl might result from weak divalent interactions between the GST-Itk SH3 fusion protein and the multiple proline-rich motifs in Cbl. We have subsequently established that the ability of GST-Itk fusion proteins containing both the SH3 and SH2 domains to bind tyrosine-phosphorylated Cbl is significantly enhanced relative to either the GST-SH3 or GST-Itk SH2 fusion proteins.3 Therefore, although the individual Itk SH3 domain binding sites in Cbl may be of relatively low affinity, it is likely that the Itk SH3 domain synergizes with the Itk SH2 domain in order to bind Cbl in vivo.

The binding of the Itk SH3 domain, but not of the Src SH3 domain, to Cbl was regulated by TCR cross-linking. Shortly after activation by CD3 cross-linking, an increase in the amount of Cbl interacting with the Itk SH3 domain was detectable.

REFERENCES


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