Detection of Src Homology 3-Binding Proteins, Including Paxillin, In Normal and v-Src-transformed Balb/c 3T3 Cells*

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Zhiqiang Weng‡, Jennifer A. Taylor‡, Christopher E. Turner§, Joan S. Brugge‡, and Cynthia Seidel-Dugan‡†

From ARIAD Pharmaceuticals, Cambridge, Massachusetts 02139 and Howard Hughes Medical Institute, Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 and the ‡Department of Anatomy and Cell Biology, State University of New York Health Science Center at Syracuse, Syracuse, New York 13210

The Src homology 3 (SH3) domain, located in the amino-terminal, noncatalytic half of pp60<sup>Src</sup> is highly conserved among members of the Src family of tyrosine kinases. SH3 domains have also been identified in a variety of proteins otherwise unrelated to protein-tyrosine kinases. The presence of SH3 domains in proteins with diverse functions suggests this domain may be important for directing protein-protein interactions necessary for protein function or cellular localization. To explore possible interactions between the SH3 domain and cellular proteins, we have established conditions for the isolation of proteins that bind in solution to the Src SH3 domain. A 67-amino acid fragment of c-Src containing either the entire glutathione S-transferase-SH3 domain (GST-SH3) or the SH3 domain from the neuronal form of c-Src (GST-SH3*) was expressed as a glutathione S-transferase fusion protein. The GST fusion proteins were incubated with lysates from [35S]methionine-labeled Balb/c 3T3 cells or v-Src-transformed Balb/c 3T3 cells. We found that GST-SH3, but not wild-type GST, specifically interacted with multiple cellular proteins, whereas GST-SH3* only weakly associated with a small subset of these proteins. The majority of the SH3-binding proteins were found in particulate and detergent-insoluble cell fractions. Anti-phosphotyrosine immunoblots of the SH3-binding proteins revealed that several of the SH3-binding proteins are phosphorylated on tyrosine in vivo.

In addition, a number of the SH3-binding proteins were phosphorylated on serine and/or threonine in <i>in vitro</i> kinase assays, suggesting that one or more of the SH3-binding proteins has kinase activity. We identified paxillin, a vinculin-binding protein, as one of the Src SH3-binding proteins. This finding strongly supports the hypothesis that SH3 domains may be involved in subcellular localization of proteins to cytoskeleton and/or cellular membranes.

The Src protein is a 60-kilodalton membrane-bound protein tyrosine kinase that serves as the prototype for a family of at least eight related proteins (1). Src and related kinases are composed of multiple domains that are responsible for catalytic activity, localization of the protein within the cell, interaction with other cellular proteins, regulation of its own kinase activity, and regulation by other cellular kinases. A structural and functional analysis of these domains has defined an important role of many of these domains. The catalytic protein tyrosine kinase domain is contained within the carboxy-terminal half of the protein (2–4). The regulatory site of tyrosine phosphorylation, Tyr-527, is located just outside the catalytic domain in the 11 carboxy-terminal amino acids of c-Src (5–8). The first 15 amino-terminal residues of c-Src are required for myristylation and membrane association (9–12). The amino acid sequences immediately downstream are not conserved within the Src family of protein tyrosine kinases and have been termed the unique domain.

The remaining portion of the amino-terminal half of c-Src contains the Src homology 2 (SH2) and the Src homology 3 (SH3) domains (13, 14). These two noncatalytic regions are highly conserved among members of the Src family of protein tyrosine kinases. In addition, SH2 and SH3 domains have been identified in a number of other proteins otherwise unrelated to protein tyrosine kinases including phospholipase C-γ1 and 2, a Ras GTPase-activating protein, and the p85 subunit of phosphatidylinositol 3'-kinase (13). Another group of SH2- and SH3-containing proteins such as c-Crk, Nck, Sem-5, and GRB2 have no apparent catalytic activity and has been proposed to function as adapter molecules that regulate other proteins (13, 15, 16). SH2 domains specifically interact with sequences containing phosphotyrosine (17–21); the resulting oligomeric complexes formed by binding of an SH2-containing protein to a tyrosine-phosphorylated target may be involved in signal transduction events initiated by protein tyrosine kinases (13, 22). The SH2 domain of Src-like kinases is also believed to be involved in substrate recognition (23–26), and there is evidence that the SH2 domain of c-Src may specifically interact with phosphorylated Tyr-527 to repress the kinase activity of this molecule (27).

SH3 domains have also been identified in a variety of proteins that lack SH2 domains, suggesting that the two domains are not obligate partners. SH3-containing proteins include nonerythroid c-spectrin, two neutrophil NADPH oxidase-associated proteins p47 and p67, cytoskeleton-associated proteins p80/p85, myosin 1B, CDC25, FUS1, IRA1, Bem1p, ABP-1, an erythrocyte palmitoylated membrane protein p55, the lethal (1) discs large-1 tumor suppressor gene product, and human HS1 (28). The presence of the SH3 domain in such a wide variety of unrelated proteins leads to...

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†To whom correspondence should be addressed: ARIAD Pharmaceuticals, 26 Landsdowne St., Cambridge, MA 02139. Tel.: 617-494-0400; Fax: 617-494-0208.

‡The abbreviations used are: SH2, Src homology 2; SH3, Src homology 3; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; RIPA, radioimmunoprecipitation assay; MAP, mitogen-activated protein.
the speculation that this motif might play an important role in protein-protein interactions essential to cellular functions and/or subcellular localization (13, 29).

Consistent with this hypothesis, three possible functions of the SH3 domain have been suggested. First, like the SH2 domain, the SH3 domain might serve as a regulatory domain in Src-like kinases since deletions or mutations in this domain increase catalytic activity and oncogenic potential (30-35). In addition, the Ab1 kinase has been proposed to be partially regulated by noncovalent associations with an intracellular inhibitor through the SH3 domain (33). Therefore, the SH3 domain may mediate binding to regulatory ligands that down-regulate the kinase activity and block the transforming ability of various tyrosine kinases (14, 35). Second, the SH3 domains may be involved in substrate recognition. For example, the SH3 domain is required for association and phosphorylation of the Src substrate p110 (26, 32). Finally, SH3 domains may be important for subcellular localization since most of the proteins containing SH3 domains associate with the cortical actin cytoskeleton and/or cellular membranes (29, 36, 37). One possibility is that SH3 domains are involved in modifying cell shape, adhesion, or motility by recruiting signaling molecules or their effectors to the membrane cytoskeleton. The recent identification of point mutations in the SH3 domain of Sem-5 that severely impair Sem-5 function in Caenorhabditis elegans vulva development first provides the developmentally specific evidence that SH3 domains are essential for cellular signaling (16).

Despite the potential importance of SH3 domains, little is known about proteins that interact with these domains. Recently, Cicchetti and co-workers (38) identified two c-Ab1 SH3-binding proteins, 3BP-1 and 3BP-2, using a GAT11 phage expression method. However, SH3-binding proteins have not yet been isolated using a solution binding assay similar to that used for the detection of SH2-binding proteins (20, 21). We have established conditions that allow detection of the Src SH3-binding proteins in solution. The c-Src SH3 domain and a neuronal form of the c-Src SH3 domain, which contains a six-amino acid insertion, were expressed as glutathione S-transferase (GST) fusion proteins (39) and used to affinity purify SH3-binding proteins from radiolabeled cell extracts. Several candidate Src SH3-binding proteins were isolated from lysates of Balb/c 3T3 cells and v-Src-transformed Balb/c 3T3 cells. Most of these SH3-binding proteins are associated with cytoskeleton-rich cell fractions; there is a serine/threonine kinases activity associated with the binding proteins, and a few proteins were phosphorylated on tyrosine in v-Src-transformed cells. Interestingly, the neuronal Src SH3 fusion protein weakly associated with only a small subset of these cellular proteins. In addition, we identified paxillin, a vinculin-binding protein associated with cytoskeleton, as one of the Src SH3-binding proteins. The identification of paxillin as one of the Src SH3-binding proteins provides evidence that the Src SH3 domain may be involved in subcellular localization.

**EXPERIMENTAL PROCEDURES**

**Generation of Recombinant pGEX-2T Plasmids**—The pGEX-2T vector was constructed by H. Wu (Northwestern University) (39). A CDNA containing the SH3 domain of chicken c-Src (representing amino acids 81-147) was amplified by polymerase chain reaction and cloned into the pGEX-2T vector at the BamHI site. The recombinant plasmids were subjected to double-stranded DNA sequencing to check for orientation and sequence integrity of the insertion.

**Preparation and Purification of GST and Fusion Proteins**—The GST, GST-SH3, and GST-SH3* fusion proteins were expressed in Escherichia coli XL1 and purified by affinity chromatography. The 400-ml cultures were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at an A600 of 0.4 and incubated at 37 °C for an additional 3 h. The cells were pelleted, and the pellets were rinsed in cold buffer A (50 mM Tris (pH 8), 25% sucrose, 10 mM EDTA) and resuspended in 16 ml of buffer A. After 1 h of incubation with 1 ml of lysozyme (20 mg/ml in buffer A), the cells were pelleted at 8000 rpm for 10 min and resuspended in 10 ml of buffer B (10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin). The solution was quick frozen twice in liquid nitrogen to ensure complete bacterial lysis, and 20 ml of buffer C (20 mM HEPES pH 7.6, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml leupeptin) and 3 ml of 10% Triton X-100 were added. The fusion proteins were purified on glutathione-agarose (Sigma), eluted with 20 mM reduced glutathione in buffer C, dialyzed to remove the glutathione, and stored at −20 °C. Protein concentrations were determined by Lowry et al. (45).

**Affinity Binding Assay**—Balb/c 3T3 cells and v-Src-transformed Balb/c 3T3 cells (SRD 3T3) (2) were labeled overnight with 50 μCi of [35S]methionine/ml in methionine-free Dulbecco's modified Eagle's medium and lysed in buffer A (50 mM Tris (pH 7.2), 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100). After clarification at 28,000 × g, supernatants containing 0.5 mg of protein/ml (approximately 1010 trichloroacetic acid-precipitable [35S]counts/min) were used per reaction. Each reaction was preincubated by adding 50 μg of GST and 50 μl of a 1:1 slurry of glutathione-agarose at 4 °C for 2 h. The agarose was removed, and the supernatant was mixed with 30 μg of GST, GST-SH3, or GST-SH3* and 50 μl of a 1:1 slurry of glutathione-agarose. After incubation for 4 h at 4 °C, the pellets were washed four times in RIPA buffer four times, and the binding proteins were eluted with electroelution buffer (46) at 4°C for 3 h. The samples were fractionated on 10% SDS-polyacrylamide gels; the gels were soaked in sodium salicylate for 1 h, then dried and exposed to XAR-5 film (Kodak) at −70 °C.

**Cell Fractionation**—To prepare soluble and particulate fractions, a 100-mm dish of SRD 3T3 cells labeled with [35S]methionine was rinsed twice in buffer B (10 mM KCl, 20 mM Tris (pH 7.0), 0.1% β-mercaptoethanol, 1 mM EDTA), scraped into 1 ml of buffer B, and incubated on ice for 15 min. After homogenization and centrifugation at 45,000 × g for 30 min, the supernatant was collected, and the pellet was resuspended in 1 ml of KCl buffer (0.3 M KCl, 1 mM EDTA, 10 mM Tris (pH 7.0), 1% Nonidet P-40). After incubation on ice for 10 min and centrifugation at 28,000 × g for 30 min, the supernatant was collected, and the pellet was resuspended in buffer C for the particulate fraction. Each fraction was used in binding assays as described above. We also prepared detergent-soluble and -resistant fractions from SRD 3T3 cells labeled with [35S]methionine as described previously (47); these fractions were also used in the binding assays as described above.

**In Vitro Kinase Assay**—Lyastes were prepared from unlabeled Balb/c 3T3 and SRD 3T3 cells, and binding assays were performed as described above. After the third wash, the glutathione-agarose beads were subjected to a final wash in 1 ml of low salt buffer (100 mM NiCl, 10 mM Tris (pH 7.0), 5 mM MnCl). The beads were incubated in kinase buffer (10 mM Tris (pH 7.4), 2.5 mM MgCl, 2.5 mM MnCl, 1 μM ATP, 10 μCi of [γ-32P]ATP) for 30 min at room temperature. The reactions were fractionated on a 10% SDS-PAGE gel. The gel was dried and exposed at −70 °C to XAR-5 film (Kodak). To examine phosphorylation of exogenous substrates, binding assays were performed as described above, and GST-, GST-SH3-, and SH3-associated proteins were incubated with 1 mM ATP, 10 mM Tris (pH 7.4), 2.5 mM MgCl, 2.5 mM MnCl, 1 μM ATP, and 10 μCi of [γ-32P]ATP (10 μg of myelin basic protein) was added to each reaction. Protein kinase C inhibitor peptide (Upstate Biotechnology, Inc.) was added to the final concentration of 100 μM. To assay histone H1...
phosphorylation, 30 μl of reaction mixture (20 mM HEPES pH 7.3, 5 mM EGTA (pH 7.0), 10 mM MgCl₂, 5 μM ATP, 10 μCi of [γ-³²P]ATP, 25 μg of histone H1) was used for each reaction. To assay casein phosphorylation, 30 μl of reaction mixture (30 mM HEPES pH 7.0, 10 mM MgCl₂, 5 μM ATP, 10 μCi of [γ-³²P]ATP, 150 μg of casein) was added to each reaction. All the reactions were performed for 30 min at room temperature and fractionated on a 12.5% SDS-PAGE. The gel was dried and exposed at −70 °C to XAR-5 film (Kodak).

Anti-phosphotyroine Immunoblot—SH3-binding proteins containing phosphotyrosine were detected by immunoblotting (32). The affinity binding reactions were performed as described above, and the reactions were separated by electrophoresis on 10% polyacrylamide gels. The proteins were transferred to nitrocellulose, and the blot was incubated in blocking buffer (5% crystallized bovine serum albumin, 170 mM NaCl, 0.2% Nonidet P-40, 50 mM Tris (pH 7.5)) overnight at room temperature. Each filter was probed with anti-phosphotyroine antibody 4G10 (kindly provided by T. Roberts, Dana Farber Cancer Institute) followed by horseradish peroxidase-coupled secondary antibody. Immunoreactivity was detected by enhanced chemiluminescence (Amersham Corp.).

Paxillin Binding Assays—The GST-SH3 and GST-SH3⁺ fusion proteins were bound to glutathione-agarose (Sigma) and cleaved with thrombin (Enzyme Research Laboratories, Inc.) in thrombin cleavage buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 2.5 mM CaCl₂). The SH3 and SH3⁺ peptides were cross-linked to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions. Briefly, the Affi-Gel 10 was washed extensively with cold H₂O, and cross-linking was performed in the presence of 100 mM HEPES pH 3.5 overnight. A precautionary blocking of any active esters was performed by incubating with 1 M ethanolamine for 1 h. The Affi-Gel 10 beads were washed five times with RIPA buffer, and the cross-linking efficiency was monitored by Coomassie staining. Affi-Gel 10, Affi-Gel 10-SH3, and Affi-Gel 10-SH3⁺ were mixed with Balb/c 3T3 and SRD 3T3 lysates overnight at 4 °C. After five washes in RIPA buffer, the binding proteins were eluted with electrophoresis sample buffer (46), separated by electrophoresis on a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with a paxillin monoclonal antibody (Zymed) at a dilution of 1:5000.

For the direct binding assay, the GST fusion proteins were normalized by both Lowry et al. (45) and Coomassie staining, and duplicate samples of each protein were separated by electrophoresis on a 12.5% polyacrylamide gel and transferred to nitrocellulose. One filter was stained with India ink to visualize the relative amount of GST fusion proteins transferred, and the duplicate filter was incubated with glutathione-agarose and incubated with GST-SH3 bound to glutathione-agarose. After several washes in lysis buffer, the bound proteins were eluted from the beads with sample buffer and separated by SDS-PAGE. As shown in Fig. 2, GST-SH3 bound to multiple cellular proteins in Balb/c 3T3 (lane 2) or SRD 3T3 (lane 5) lysates, while no proteins were detected bound to wild-type GST (lanes 1 and 4). The SH3-binding proteins in the Balb/c 3T3 cells had apparent molecular masses of 290, 250, 220, 200, 170, 120, 100, 80–83, 67–73, 62, 48, 43, and 32–34 kDa. The

RESULTS

Isolation of SH3-binding Proteins in Balb/c 3T3 and SRD 3T3 Lysates—The pGEX expression vector was used to generate GST-SH3 and GST-SH3⁺ fusion proteins. Each of the proteins was expressed in E. coli and purified from lysates by binding to glutathione-agarose and elution with soluble glutathione. A sample of the purified proteins is shown in Fig. 1. To identify cellular proteins that are able to interact with the Src SH3 domain, lysates were prepared from [³⁵S]methionine-labeled Balb/c 3T3 or SRD 3T3 cells using RIPA buffer. Following preclearing with wild-type GST bound to glutathione-agarose, the lysates were divided into three aliquots and incubated with equal amounts of either GST, GST-SH3, or GST-SH3⁺ bound to glutathione-agarose. After several washes in lysis buffer, the bound proteins were eluted from the beads with sample buffer and separated by SDS-PAGE. As shown in Fig. 2, GST-SH3 bound to multiple cellular proteins in Balb/c 3T3 (lane 2) or SRD 3T3 (lane 5) lysates, while no proteins were detected bound to wild-type GST (lanes 1 and 4). The SH3-binding proteins in the Balb/c 3T3 cells had apparent molecular masses of 290, 250, 220, 200, 170, 120, 100, 80–83, 67–73, 62, 48, 43, and 32–34 kDa. The

FIG. 1. Purification of GST and GST-SH3 fusion proteins. Bacterial lysates containing each protein were prepared as described under "Experimental Procedures" and incubated with glutathione-agarose. The glutathione-agarose was then washed, and bound proteins were eluted with 20 mM free glutathione. The purified GST (lane 3), GST-SH3 (lane 2), and GST-SH3⁺ (lane 1) fusion proteins were resolved by electrophoresis on a SDS-10% polyacrylamide gel and visualized by Coomassie Blue staining.

FIG. 2. Binding of cellular proteins to the SH3 domain of c-Src. [³⁵S]Methionine-labeled lysates of Balb/c 3T3 (lanes 1–3) and SRD 3T3 cells (lanes 4–6) were precleared by incubation with GST-agarose and incubated with glutathione-agarose bound with GST (lanes 1 and 4), GST-SH3 (lanes 2 and 5), and GST-SH3⁺ (lanes 3 and 6). Bound proteins were eluted with SDS sample buffer, subjected to electrophoresis in a SDS-10% polyacrylamide gel, and visualized by autoradiography.

profiles of the SH3-binding proteins in the Balb/c 3T3 and SRD 3T3 cells were very similar except that the 200- and 170-kDa proteins are absent in the SRD 3T3 profile. This result suggests that tyrosine phosphorylation is not required
for binding to the Src SH3 domain. The most prominent bands in both the Balb/c 3T3 and SRD 3T3 cells were 100, 83-80, and 62 kDa.

It is interesting that GST-SH3+, which has a neuronal insert in the SH3 domain, only weakly associated with some of the proteins bound to GST-SH3 (lanes 3 and 6). The cellular proteins that bound to GST-SH3+ had apparent molecular masses of 220, 73-83, 62, 48, and 43 kDa, and there were no differences in the profiles of SH3+–binding proteins detected in Balb/c 3T3 and SRD 3T3 cell lysates.

Subcellular Localization of the SH3-binding Proteins—To examine the detergent solubility of the SH3-binding proteins, we fractionated the [35S]methionine-labeled SRD 3T3 cells by solubilization in a buffer containing 1% Triton X-100 (47). The majority of the SH3-binding proteins was found in the detergent-insoluble fraction (Fig. 3, lane 8); only a small percentage of the SH3-binding proteins was solubilized in 1% Triton X-100 (lane 6). As a control for the fractionation procedure, we also examined the solubility of v-Src and MAP kinase (MAPK/ERK) in 1% Triton X-100 and confirmed that v-Src was found predominantly in the detergent-insoluble fractions (47), while MAP kinase was detected exclusively in the soluble fractions (data not shown). These results suggest that a majority of the SH3-binding proteins is associated with a nonionic detergent-resistant cellular structure that consists largely of cytoskeletal proteins.

Cell lysates were also separated into soluble and particulate fractions by homogenization in hypotonic buffer and centrifugation at 45,000 × g. As shown in Fig. 3, the SH3-binding proteins were recovered predominantly in the particulate fraction of SRD 3T3 cells (lane 4). Similar results were obtained with Balb/c 3T3 cell lysates were fractionated in the same manner (data not shown). The particulate fraction was not contaminated with whole cells since MAP kinase, a soluble protein marker (49, 50), was found exclusively in the soluble fraction of SRD 3T3 cells (data not shown).

A Serine/Threonine Kinase(s) Is Associated with the SH3-binding Proteins—To further characterize the SH3-binding proteins, we used an in vitro kinase assay to determine whether any of these proteins possessed protein kinase activities. SH3-associated proteins were incubated with [γ-32P]ATP to allow in vitro phosphorylation. As shown in Fig. 4, multiple SH3-binding proteins were phosphorylated in this assay (lanes 2 and 5), including the GST-SH3 fusion protein itself. No phosphorylated proteins were detected bound to GST alone (lanes 1 and 4). Little kinase activity was initially detected in association with SH3+ (lanes 3 and 6); however, it is evident on longer exposure of the autoradiogram that a number of proteins were phosphorylated and that many of the phosphorylated proteins have electrophoretic mobilities distinct from the in vitro phosphorylated SH3-binding proteins. The kinase(s) found associated with the SH3-binding proteins is likely to be serine and/or threonine kinase(s) since

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**Fig. 3.** Subcellular localization of the SH3-binding proteins. Proteins from [35S]methionine-labeled SRD 3T3 cells were separated into soluble (S) (lanes 1 and 2) and particulate fractions (P) (lanes 3 and 4) or detergent-soluble (DSF) (lanes 5 and 6) and detergent-resistant fractions (DRF) (lanes 7 and 8) as described under "Experimental Procedures." The fractions were precleared by incubation with GST-agarose and then incubated with glutathione-agarose bound with GST (lanes 1, 3, 5, and 7) or GST-SH3 (lanes 2, 4, 6, and 8). Bound proteins were eluted with SDS sample buffer, resolved by electrophoresis in a SDS-10% polyacrylamide gel, and visualized by autoradiography.

**Fig. 4.** In vitro kinase activity of the SH3-binding proteins. Balb/c 3T3 (lanes 1–3 and 7) and SRD 3T3 cells (lanes 4–6) were lysed and precleared with GST-agarose. The aliquots were incubated with glutathione-agarose bound with GST (lanes 1 and 4), GST-SH3 (lanes 2 and 5), and GST-SH3+ (lanes 3, 6, and 7). The proteins bound to the glutathione-agarose were subjected to an in vitro kinase assay in the presence of [γ-32P]ATP as described under "Experimental Procedures." The phosphorylated proteins were eluted with SDS sample buffer, fractionated on a SDS-10% polyacrylamide gel, and visualized by autoradiography. Lane 7 is a longer exposure of lane 3, which shows the kinase activity associated with GST-SH3+ in Balb/c 3T3 cells.
the phosphates were hydrolyzed by KOH treatment (data not shown) (51).

One possible explanation of these data is that a serine/threonine protein kinase bound nonspecifically to both the GST and GST-SH3 beads and that the enhanced detection of phosphorylated proteins in the GST-SH3-binding proteins reflected the increased number of proteins in this kinase reaction. To examine this possibility, we performed the kinase reactions in the presence of three exogenous substrates, which are myelin basic protein, histone H1, and casein. The GST- and GST-SH3-binding proteins were preincubated with a high concentration of unlabeled ATP to saturate the phosphorylation sites on GST, GST-SH3, and their associated proteins (since some of these proteins display electrophoretic mobilities similar to those of the exogenous substrates) before incubation with \( \gamma^{32} \)P\-ATP and exogenous substrates. The protein kinase C inhibitor peptide (52) was included in one set of reactions to examine whether the SH3-associated kinase is a protein kinase C isozyme. As shown in Fig. 5, myelin basic protein was specifically phosphorylated by GST-SH3-associated kinase(s) (lane 6), and this phosphorylation was not blocked by the protein kinase C inhibitor peptide (lane 2). In contrast, there was only a slightly higher level of phosphorylation of histone H1 (lanes 9 and 10) and casein (lanes 11 and 12) in the GST-SH3-binding protein reactions compared with that found in GST controls. Phosphorylation of casein was not stimulated by cAMP (data not shown). These results indicate that one or more of the SH3-binding proteins, or proteins associated with them, possess serine/threonine kinase activity capable of phosphorylating myelin basic protein.

**Tyrosine Phosphorylation of SH3-binding Proteins**—Since SH3 domains may be involved in the association of Src-like kinases with tyrosine-phosphorylated substrates (26), we examined whether any of the cellular proteins bound to GST-SH3 or GST-SH3* were phosphorylated on tyrosine in vivo. Fig. 6 is an immunoblot of the SH3-binding proteins probed with monoclonal antibodies to phosphotyrosine. Several of the SH3-binding proteins detected in SRD 3T3 cell lysates were phosphorylated on tyrosine (lane 5), the most prominent being a series of proteins with apparent molecular masses between 70 and 75 kDa. None of the SH3-binding proteins from Balb/c 3T3 cell lysates were phosphorylated on tyrosine (lane 2), and no tyrosine-phosphorylated proteins were detected bound to GST-SH3* (lanes 3 and 6).

**Identification of Paxillin as One of the Src SH3-binding Proteins**—Since most of the SH3-binding proteins are associated with the cytoskeleton, we probed blots containing SH3-binding proteins with antibodies that recognize a number of different cytoskeletal proteins. Neither vinculin, \( \alpha \)-actinin, nor p80/p85 (53) was detected among the Src-SH3-binding proteins; however, a monoclonal antibody against paxillin specifically reacted with a Src SH3-binding protein with an electrophoretic mobility similar to that of paxillin (Fig. 7). In this assay, the SH3 domains of GST-SH3 and GST-SH3* fusion proteins were isolated after cleavage with thrombin and cross-linked to Affi-Gel 10, and the Affi-Gel 10-SH3 beads were used to affinity purify SH3-binding proteins. During the course of these experiments, we found that the use of this matrix allowed more efficient binding to paxillin without losing specificity. Paxillin detected in total cell lysates

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**Fig. 5.** In vitro phosphorylation of exogenous substrates by the SH3-binding proteins. SRD 3T3 cells were lysed and precleared with GST-agarose. The aliquots were incubated with glutathione-agarose bound with GST (odd numbered lanes) or GST-SH3 (even numbered lanes). The proteins bound to the glutathione-agarose were prephosphorylated with unlabeled ATP and then the exogenous substrates myelin basic protein (lanes 1, 2, 5, and 6), histone H1 (lanes 9 and 10), or casein (lanes 13 and 14) were added to the reactions in the presence of \( \gamma^{32} \)P\-ATP as described under "Experimental Procedures." Protein kinase C inhibitor peptide was included (lanes 1 and 2) in the kinase assay with myelin basic protein. Arrowheads denote the positions of myelin basic protein, histone H1, and casein.

The bound proteins were eluted with SDS sample buffer, fractionated in a SDS-10% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-phosphotyrosine monoclonal antibody 4G10 as described under "Experimental Procedures."
Identification of SH3-binding Proteins

**DISCUSSION**

In this study, we have detected multiple cellular proteins that bind to the SH3 domain of c-Src. No binding proteins were detected with GST alone, and the six-amino acid insertion in the SH3* domain dramatically disrupted the association of these proteins, providing evidence for the specificity of these interactions. Although it is not clear from this study whether all of these proteins bind directly to the SH3 domain or if some are associated with the SH3 domain indirectly through other proteins, the direct binding of paxillin to the Src SH3 in a blotting assay suggests that in this particular case binding to the SH3 domain may be a direct interaction.

In cell fractionation studies, the majority of the SH3-binding proteins was associated with membrane and cytoskeleton-rich cell fractions. Since most of the known SH3-containing proteins are associated with the membrane cytoskeleton, it is not surprising that the SH3-binding proteins were localized in the same subcellular structures. Recently, Bem1p, a yeast protein necessary for cell polarization, was found to have two SH3 domains (54). Since Bem1p has a direct role in organizing the actin cytoskeleton, it was proposed by the authors that the SH3 domain might be involved in binding actin or some other component of the cortical cytoskeleton. Although little is known about the nature of these c-Src SH3-binding proteins or the basis for their association with Triton-insoluble cell fractions, our findings support the possibility that the SH3 domain of c-Src may interact with components of the cytoskeleton. Most notably, several lines of evidence indicate that paxillin, a 68-kDa vinculin-binding protein, is one of the Src SH3-binding proteins. This finding provides the first evidence supporting the hypothesis that SH3 domains may be involved in subcellular localization.

Paxillin was originally identified as a component of focal adhesion contacts (48, 55) where the actin cytoskeleton is linked to the extracellular matrix via integrin receptors (56) and cytoskeleton proteins such as talin, vinculin, α-actinin, fibrin, and tensin (57). Since paxillin can bind to the carboxy-terminal region of vinculin in vitro (48), vinculin could couple paxillin to focal adhesion sites. It has been shown that Src is associated with the cytoskeleton in transformed cells (47). Our finding raises the possibility that this association may be mediated, at least in part, by paxillin binding to the Src SH3 domain.

Binding of integrins to extracellular matrix ligands or clustering of integrins on the cell surface induces tyrosine phosphorylation of paxillin and the focal adhesion kinase p125FAK, while inhibition of tyrosine phosphorylation of these proteins abolishes the formation of focal adhesions and stress fibers (58). This suggests that integrin-induced tyrosine phosphorylation of paxillin may be involved in the formation of cytoskeleton-membrane attachment sites (58). Paxillin is also a major substrate of pp60c-Src in v-Src-transformed cells as well as a major tyrosine kinase substrate in developing tissues (55, 59). Localization of Src via its SH3 domain to focal adhesions through paxillin may be one mechanism for linking tyrosine phosphorylation and focal adhesion formation.

A number of SH3-binding proteins were phosphorylated on serine or threonine in vitro, while no tyrosine kinase activity was detected. This result suggests that a serine/threonine kinase is associated with the SH3 domain, either by direct binding to the Src SH3 domain or by association with other proteins that bind to the SH3 domain.
interaction with the SH3 domain or by association with another protein that binds to the SH3. The SH3-associated kinase(s) could specifically phosphorylate myelin basic protein, and the phosphorylation of myelin basic protein could not be blocked by protein kinase C inhibitor peptide. To examine whether any of the known MAP kinases (MAPK/ERK family) are SH3-binding proteins, we probed immunoblots of the SH3-binding proteins with a monoclonal antibody that recognizes the p42 and p44 forms of ERK1 and ERK2. Neither form of the MAPK/ERK was detected in this assay (data not shown). These results suggest that a serine/threonine kinase that associates with the Src-SH3 phosphorylates myelin basic protein more efficiently than histone H1 or casein. Although the significance of this finding is not clear, the identification of a serine/threonine kinase associated with the SH3 domain is intriguing. One possibility is that the SH3 domain of c-Src may be a functional link to a serine/threonine phosphorylation pathway involved in cytoskeletal rearrangements.

Several of the SH3-binding proteins were phosphorylated on tyrosines in 14-Src-transformed cells. Kannor et al. (28) have previously shown that the SH3 domain is necessary for the association of c-Src with its substrate p110. Although p110 may be present in our affinity-purified SH3-binding protein preparations, we have not detected a 110-kDa protein that reacts with a monoclonal antibody to p110 (60). The results presented here provide further evidence that the SH3 domain of Src may be involved in the recognition of a subset of Src substrates besides p110. Although it is possible that paxillin may be one of the tyrosine-phosphorylated proteins bound to the Src SH3 with electrophoretic mobilities of 70–75 kDa, this phosphorylation does not seem to be necessary for SH3 association since the non-tyrosine-phosphorylated paxillin found in Balb/c 3T3 cells bound to the SH3 with similar efficiency as tyrosine-phosphorylated paxillin from SRD 3T3 cells.

Recently, Cicchetti and workers (38) identified a protein, 3BP-1, which binds to the Abl SH3 and with slightly lower affinity to the Src SH3 domain. In preliminary experiments, one of the SH3-binding proteins identified in this study has a similar electrophoretic mobility as 3BP-1 and is recognized in an immunoblot assay by antibodies prepared against the SH3 domain. We do not know whether our other SH3-binding proteins have a similar motif.

It is interesting that in most of the experiments presented here, we did not detect many proteins bound to the neuronal Src SH3. This result suggests that the hydrophilic six-amino acid insertion (RKVDVR) dramatically alters the binding specificity of this SH3 domain. This result is consistent with the weak binding of SH3 to 3BP-1 in blotting assays (38). The recently derived structure of the Src SH3 domain reveals that the location of the neuronal insertion is adjacent to the proposed ligand binding site (61). The binding site is a hydrophobic pocket on the surface of the domain that is lined with the side chains of conserved aromatic amino acids. The sequence, ALYDY, which is highly conserved among the SH3 domains, comprises one end of the receptor site, and the other end of the binding site is defined by the loop containing Thr-113 and Glu-114 adjacent to the site of the neuronal insertion at Glu-114. Since the neuronal insert is in the loop that is part of the ligand binding site, it is not surprising that the insert may disrupt the association of binding proteins with the SH3 domain. In the Src SH3 domain, we did detect several SH3-binding proteins, many of which were different from the proteins bound to SH3. This result supports a model suggesting that the neuronal insert confers specificity to the interaction of the SH3 to different subsets of proteins (40, 41). A second variant of Src containing a 17-amino acid insertion have been detected in human and mouse neuronal cells (42); it is likely that this insertion will also determine binding specificities. We are in the process of characterizing SH3-binding proteins in other tissues and are particularly interested in identifying cellular proteins in neuronal tissues that will bind to SH3.

The SH3-binding proteins identified in this work were isolated under stringent binding conditions using a buffer containing 0.1% SDS, 1% deoxycholate, and 1% Triton X-100. As mentioned above, attempts to isolate the SH3-binding proteins using solution binding conditions similar to those used for the detection of SH2-binding proteins have not been successful (20, 21). It is possible that the more stringent buffers used in this study may be critical to solubilize SH3-binding proteins (since most are insoluble in nonionic buffers) and to unmask SH3-binding sites.

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