Multiple Proline-rich Regions of GAP-associated Phosphoprotein p62 Bind with Different Affinities to the Src Homology 3 Domains of Fyn and Src[1]

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ABSTRACT

Several proteins of Jurkat cells were identified on SDS-PAGE gels by Coomassie Blue staining that bound specifically to affinity matrices made of five different Src homology 3 (SH3) domains fused to glutathione S-transferase (GST). Purification of the major specific band of approximately 70kDa with affinity beads of the SH3 domain of Fyn tyrosine kinase resulted in an identification of a GAP-associated p62-related protein as a ligand to the Fyn and Src SH3 domains. Indeed, from a lysate of a Rous sarcoma virus-transformed rat fibroblast line, Src co-precipitated with the 70kDa and also bound to a putative SH3 binding sequence of p62. Bacterially expressed GST fusion proteins containing sequences encompassing each of the proline-rich putative SH3 binding sites of p62 bound to a subset of SH3 domains with different affinities. Phospholipase Cγ2-SH3 also revealed strong binding to the bacterially expressed p62 fusion proteins *in vitro* but did not show primary binding to the cellular 70kDa. The multiple SH3 binding sequences with different affinities to various SH3 molecules together with their phosphorylation on tyrosine residue(s) suggest a role of p62 as a foothold on which signal transduction proteins, including Src-family kinases, link together.

Key words: GAP-associated p62, Proline-rich, Protein-protein interaction, Protein tyrosine kinase, Src homology 3.

Abbreviations: SH2, src homology 2; SH3, src homology 3; GST, glutathione S-transferase; PTK, protein tyrosine kinase; PI 3'-kinase, phospatidylinositol 3'-kinase; PLC, phospholipase C.

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INTRODUCTION

Cicchetti *et al.* (1) were the first to prove that Src homology 3 (SH3) domain participates in a specific protein-protein interaction. This finding opened a path for answering the following questions on this domain: why SH3 regions are found in a variety of proteins involved in signal transduction and often next to Src homology 2 (SH2) regions; why disrupting the SH3 region converts protein-tyrosine kinases (PTK) like Src ($pp60^{src}$) and Abl to the transforming type (2-6); and why SH3 regions are found in some proteins of the cytoskeleton and submembranous structure (7-9). To date, one major consensus on the SH3-mediated interaction is that the target sequences of SH3 have proline-rich motifs (10, 11). Association of SH3 domains with proline-rich sequences of target proteins has been shown in 3BP1 and 3BP2, which were found to be ligands for Abl SH3 (1), and in dynamin, which was identified as a ligand for the SH3 domains of phosphatidylinositol (PI) 3'-kinase p85 subunit and other proteins (12, 13). Proline-rich sequences were also implicated in the binding of mSos1 to the Grb2 SH3 domain (14-16) and of C3G to the Crk and Grb2 SH3 domains (17). Binding of PI 3'-kinase and paxillin to the SH3 domains of Src-family PTKs (18-23) was also reported. However, natural ligands for most SH3 domains still remain unknown. Identification of the ligands for these SH3 domains will certainly help to elucidate the process of signal transduction.

In the present study, we found a prominent association of a 70kDa protein with the Fyn SH3 domain. Affinity purification using immobilized fusion protein of the Fyn SH3 domain and subsequent characterization of the protein showed that GAP-associated phosphoprotein p62 was a possible ligand of the Fyn and Src SH3 domains. Binding affinities of p62 fragments to different SH3 domains were compared, and the intracellular association of the 70kDa with v-Src kinase was demonstrated.

MATERIALS AND METHODS

Cells——A human leukemic T cell line, Jurkat (clone E6-1) (24), was obtained from ATCC (Rockville, MD) and cultured in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated (56℃ for 30 min) fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 units/m*l* penicillin, and 50 μg/m*l* streptomycin. A rat fibroblast line transformed with Rous sarcoma virus, SR-3Y1-1 (25, 26), and its parent 3Y1 were obtained from Riken Cell Bank (Wakoh-shi, Japan) and cultured in Dulbecco's medium supplemented with 10% fetal bovine serum, 100 units/m*l* penicillin, and 100 μg/m*l* streptomycin.

Antibodies and reagents——Commercial sources of antibodies were as follows: rabbit antiserum against the GAP-associated p62 (amino acid residues 103-281), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-human GAP (amino acid residues 171-448) rabbit serum, rabbit antisera against the p85 subunit of rat PI 3'-kinase, and anti-phosphotyrosine monoclonal antibody 4G-10, Upstate Biotechnology, Inc. (Lake Placid, NY); rabbit anti-Src sera, East-acres Biologicals (Southbridge, MA); anti-Src monoclonal antibody 327, Oncogene Science, Inc. (Uniondale, NY); anti-phosphotyrosine monoclonal antibody PT-66 and its agarose conjugate, anti-mouse IgG-agarose, monoclonal anti-rabbit immunoglobulins (clone RG-16) conjugated with alkaline phosphatase, and goat anti-mouse IgG (Fc specific) conjugated with alkaline phosphatase, Sigma (St.Louis, MO). ExtrAvidin conjugated with alkaline phosphatase, protein A-Sepharose, cyanogen bromide-activated Sepharose 4B, and glutathione-agarose were purchased from Sigma. Biotinylated probes were prepared by incubating 1mg GST fusion proteins and 200μg sulfo-NHS-biotin (Pierce, Rockford, IL) in 100mM sodium borate buffer (pH 8.8) for 4 h at room temperature followed by dialysis against phosphate-buffered saline (PBS).

Bacterial expression of GST fusion proteins——DNA fragments of target sequences were obtained by polymerase chain reaction. Each pair of oligonucleotide primers was tagged with BamHI and EcoRI sites to facilitate oriented, in-frame cloning into either the pGEX-2T or pGEX-3X expression vector (Pharmacia). Following SH3 domains were amplified from the indicated cDNA: human PLC-γ2 (amino acid residues 770-831) from a cDNA clone (27); human Lck (amino acid residues 61-125) from clone YT16 (28); human Fyn (amino acid residues 82-145) from clone pUC-Fyn (29); and human Vav (amino acid residues 773-845) from clone pSK8 (30). The SH3 domain of mouse c-Src (amino acid residues 81-142), and the p62-C fragment (amino acid residues 276-443) and the p62-CIII fragment (amino acid residues 336-382) of human p62 were amplified by reverse transcription PCR (RT-PCR). The p62-CII fragment (amino acid residues 315-349) of human p62 was amplified from the GST-p62C plasmid, pGEX-p62C. The p62-CI fragment (amino acid residues 276-331) of human p62 was made by cutting pGEX-p62C with XhoI and EcoRI followed by blunting with the Klenow fragment and ligation. RNA used for RT-PCR of p85 and p62 was isolated from Jurkat cells, and that of c-Src was isolated from BALB/3T3 clone A31 by Isogen (Nippongene, Japan) according to the manufacturer's protocol. Every DNA obtained by PCR was sequenced by the dideoxy method using Sequenase (US Biochemical) in order to check for possible misreadings by Taq polymarase. *E.coli* strain BL21 (DE3) was transformed with the pGEX recombinant plasmids. Each of the GST fusion proteins was induced

by 100μM IPTG for 4 h from 1 liter culture of the transformant and purified according to Smith and Johnson (31).

Purification of 70kDa and determination of its internal peptide sequences——Jurkat cells (10^9) were lysed with a lysis buffer (20 mM Tris-HCl pH 7.5, 1% NP-40, 150mM NaCl, 2.5mM EDTA, 50mM NaF, 1mM Na_3VO_4, 20μg/ml leupeptin, 100units/ml aprotinin and 1mM phenylmethylsulfonyl fluoride). The supernatant obtained by centrifuging the lysate at 15,000$\times g$ for 10min was mixed with 1mg GST-Fyn-SH3 bound to 150μl glutathione-agarose beads. The beads were then washed three times with a lysis buffer containing 0.1% NP-40. Bound proteins were eluted by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE according to the method of Laemmli (32) and blotted to Immobilon-P^{SQ} (Millipore, Bedford, MA). The 70kDa band was located by Coomassie Blue staining, excised, and digested *in situ* with TPCK-treated trypsin (Sigma) as described earlier (33). The released peptide fragments were separated by high performance liquid chromatography on a reverse-phase C18 microbore column (Applied Biosystems, Foster City, CA). The column was developed with a gradient of 0 to 35% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 50min followed by a 35% to 70% acetonitrile gradient in 0.1% TFA over 15min at a flow rate of 160μl/min. Optical density at 215nm was monitored and peaks were collected manually. Collected peptides were subjected to microsequencing using an Applied Biosystems model 477 A sequencer.

Affinity binding——GST-SH3 affinity matrices were made by incubating 30mg of GST-SH3 fusion proteins with 10ml glutathione-agarose beads for 5min at room temperature. The beads were then washed extensively with PBS. Affinity beads were also made by conjugating 3mg GST fusion proteins with 1ml of CNBr-activated Sepharose 4 B and 10μl, unless otherwise stated, of the beads was used for each assay. The affinity beads were incubated at 4℃ for 4 h, unless otherwise stated, with Jurkat, 3Y1 and SR-3Y1-1 cell extract, or, for *in vitro* binding assays, for 2 h with 3μg of GST fusion proteins in TBST (25mM Tris-HCl pH 7.5, 150mM NaCl and 0.05% Tween 20).

Western blotting——Eluted proteins from the affinity beads were resolved by SDS-PAGE and blotted onto PVDF membranes (Immobilon-P, Millipore, Bedford, MA). The memblane filters were blocked with 3% BSA in TBST for 30 min at 60℃ and then probed in TBST containing 1% BSA for 2 h at 4℃. Probes used were 1μg/ml biotinylated GST-SH3 fusion proteins, and properly diluted monoclonal antibodies and rabbit antisera. The primary antibodies used in the detection were indicated in the figure legends. The membranes were washed with TBST three times and probed again with ExtrAvidin alkaline phosphatase or second antibodies conjugated with alkaline phosphatase in TBST for 1h, foll-

owed by three washes in TBST. Positive bands were detected by CSPD (Tropix, Bedford, MA), or nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma, St.Louis, MO).

RESULTS

Specific binding of proteins in Jurkat cell extract to SH3 domains——Our first interest was whether proteins which bind to an SH3 domain, if any, can be made visible by Coomassie blue staining on SDS-PAGE gels. A series of glutathione S-transferase (GST)-SH3 domain fusion proteins, GST-Fyn-SH3, GST-c-Src-SH3 (GST-Src-SH3), GST-Lck-SH3, GST-Vav-C-terminal SH3 (GST-Vav-SH3), and GST-phospholipase C $\gamma 2$ (GST-PLC$\gamma 2$-SH3), was constructed using pGEX expression vectors. Each fusion protein was affinity-purified and immobilized on glutathione-agarose beads. These matrices along with GST control beads were incubated with NP-40 extract of Jurkat cells and bound proteins

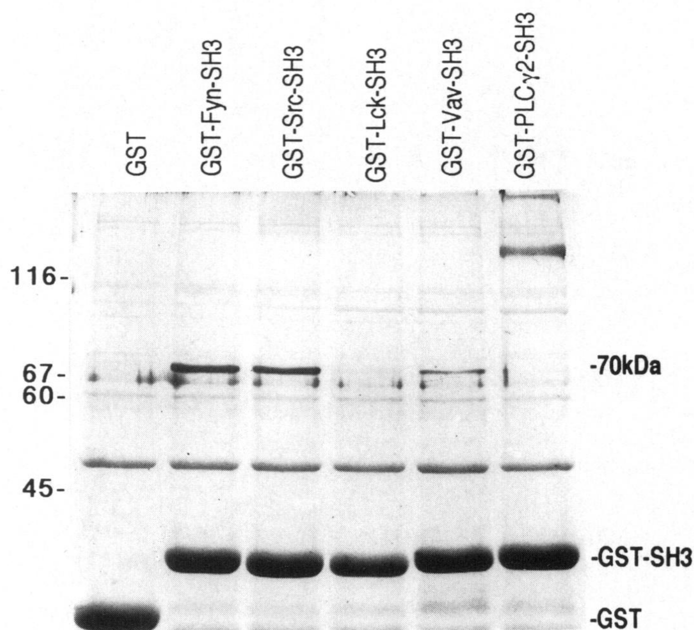

Fig. 1. Binding of cellular proteins to different SH3 domains. GST-SH3 fusion proteins were bound to glutathione-agarose beads and incubated with the Jurkat cell extract in the lysis buffer. The beads were washed extensively and bound proteins were eluted by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE in a 10% gel followed by staining with Coomassie Blue. Numbers marked at left indicate the positions of molecular weight standards.

were analyzed by SDS-PAGE. On the background of nonspecific bands seen also in GST control, several proteins bound specifically to different SH3 domains (Fig. 1) Among them, an approximately 70kDa band is notable and is seen to bind abundantly to GST-Fyn-SH3 and GST-Src-SH3, and in a lesser amount, to GST-Vav-SH3. Another notable fraction is a 170kDa band that associates to GST-PLCγ2-SH3. Since PI 3'-kinase p85α subunit has been shown to bind SH3 domains of Fyn (18), Src (20) and Lck (21), we tried anti-p85α sera to examine those associations. An immunoblot of the bound proteins revealed specific binding of p85 to Fyn-SH3, Src-SH3 and PLCγ2-SH3 (Fig. 2a). Binding of p85 to Lck-SH3 was not detected in our system.

The 70kDa fraction was revealed to be GAP-associated phosphoprotein p62

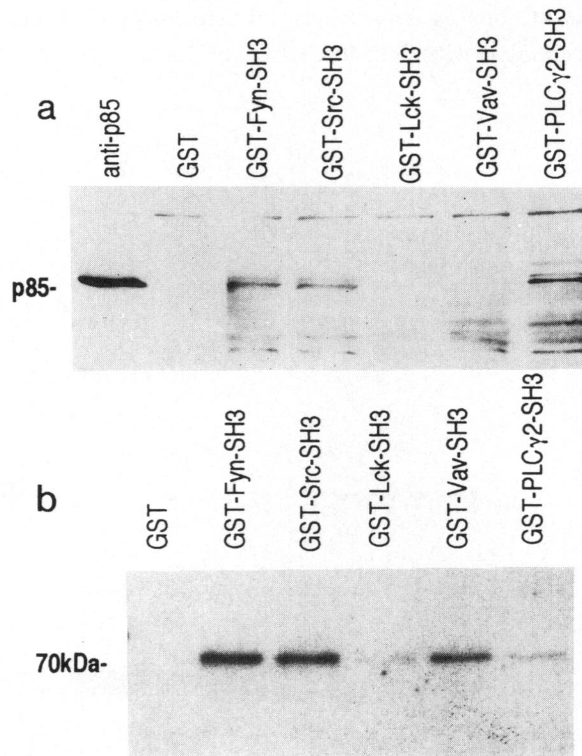

Fig. 2. SH3 domain specificity in the binding of p85 and p62. The same samples as in Fig. 1 were separated by SDS-PAGE in a 7.5% gel (a) and a 10% gel (b), transferred to PVDF membrane, and probed with anti-p85 sera (a) and anti-p62 serum (b) followed by binding the RG-16 antibody conjugated with alkaline phosphatase. In (a), an immunoprecipitate obtained with anti-p85 sera coupled to protein A Sepharose beads is also applied on the same gel to locate the p85 band (leftmost).

homologue——The Coomassie Blue stained 70kDa fraction remained single spot after resolving by urea-Triton gel electrophoresis in the second dimension (34) and was also able to be probed with biotinylated GST-Fyn-SH3 after the electrophoresis (data not shown). This fraction was collected for partial determination of the amino acid sequence. A large scale purification of the 70kDa band was performed using the affinity matrix of GST-Fyn-SH3 protein coupled to glutathione-agarose beads. The bound proteins from the Jurkat cell extract were fractionated by SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane. The 70kDa band was cut out after Coomassie Blue staining and digested *in situ* with trypsin. The proteolytically released fragments were sepa-

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Peak 1  LQEETGAK
        PDDPAA
Peak 2  AKEEEL
         LMA
Peak 3  DSLDPSF
        QPPL
Peak 4  AXPATQPPPLLPPXATG
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MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGG
Peak1 Peak3

SRGGARASPATQ**PPPLLPP**SATGPDATVGG**PAPTPLLPP**SATASVKMEPE
Peak4 NI NII

NKYLPELMAEKDSLDPSFTHAMQLLTAEIEKIQKGDSKKDDEENYLDLFS
Peak3

HKNMKLKERVLIPVKQYPKFNFVGKILGPQGNTIKRLQEETGAKISVLGK
Peak1

GSMRDKAKEEELRKGGDPKYAHLNMDLHVFIEVFGPPCEAYALMAHAMEE
Peak2 Peak2

VKKFLVPDMMDDICQEQFLELSYLNGVPEPSRGRGVPVRGRGAA **PPPPPV**
CI

PRGRGVGPPRGALVRGTPVRGAITRGATVTRGV **PPPPTVRGAPAPR**ARTA
CII

GIQRI **PLPPPPAP**ETYEEYGYDDTYAEQSYEGYEGYYSQSQGDSEYYDYG
CIII

HGEVQDSYEAYGQDDWNGTRPSLKAPPARPVKGAYREHPYGRY

Fig. 3. Location of the 70kDa tryptic peptide sequences in the GAP-associated phosphoprotein p62 and identification of proline-rich sequences in p62. Peptide sequences obtained from HPLC peaks of the 70kDa tryptic digests are shown in Peaks 1-4. They are positioned (Peak 4) and tentatively assigned (other Peaks) to the amino acid sequence of human p62 (36) as indicated by underlines. The proline-rich sequences in p62 are marked with bold letters and designated NI, NII, CI, CII, and CIII.

rated by HPLC and the peaks were subjected to a protein sequence analysis.

The resulting sequences were used to search protein sequence databases for homologous proteins by BLAST program at NCBI (35). As shown in Fig. 3, the sequence from Peak 4 exhibited a complete match to the amino acid sequence of GAP-associated phosphoprotein p62 (36). Peaks 1-3 were also coherent with the p62 sequence except for one amino acid in Peak 1; the first residue of the tentatively assigned N-terminal counterpart seems to be proline instead of arginine. Anti-p62 immunoblot of the proteins bound to GST-SH3 domains showed that the 70kDa band was indeed probed with anti-p62 (see Fig. 2b). As shown in the later experiments (Fig. 6), the same 70kDa in Rous sarcoma virus-transformed rat fibroblasts, SR-3Y1-1 cells, was highly phosphorylated. We thus concluded the 70kDa was GAP-associated p62 or a variant form. The origin of the apparent p62 heterogeneity in size is not certain; it could result from modificational/conformational change of the protein, or from divergency in the primary structure.

Proline-rich sequences of p62 selectively binds to different SH3 domains—— The ligand proteins for SH3 domains have been shown to have proline-rich sequences at their binding sites. Analysis of p62 sequence revealed the presence of proline-rich sequences that can work as binding sites for the SH3 domain. To elucidate whether these proline-rich regions of p62 actually bind to SH3 domains, proline-rich C-terminal regions, CI, CII and CIII (see Fig. 3), of p62 were expressed as GST fusion proteins, coupled to CNBr-activated Sepharose beads and used in an *in vitro* binding assay. Despite a rather protracted attempt, we were not successful in amplifying the N-terminal proline-rich regions by PCR. The C-terminal proline-rich domains used in this experiment do not match the consensus sequence of Ren *et al.* (10), XPXXPPPΨXP, where X is any amino acid and Ψ denotes hydrophobic amino acids, nor have the core sequence of Sosl, PPΨPPR, that targets Grb2-SH3 (14-16). None of the three sequences of p62 have much similarity to the reported Src- and Fyn-SH3 binding sequences of p85, either. As shown in Fig. 4, all of the three sequences bound to the SH3 domains used, but with different specificities. GST-p62-CI exhibited approximately equal binding to all GST-SH3 domains tried (Fig. 4a), except for GST-Lck-SH3. With GST-p62-CII, strong binding was only seen to GST-PLCγ2-SH3; associations to the rest of GST-SH3 domains were weaker (Fig. 4b, lane 6 versus lanes 2-5). In contrast, strong associations of GST-Src-SH3 and GST-Fyn-SH3 and moderate association of GST-Vav-SH3 were seen with CIII (Fig. 4c), and to a lessor extent with CI, showing a good correlation with the binding specificity of the cellular 70kDa. There was, however, an apparent contradiction in the case of GST-PLCγ2-SH3, which exhibited rather strong association to all of the

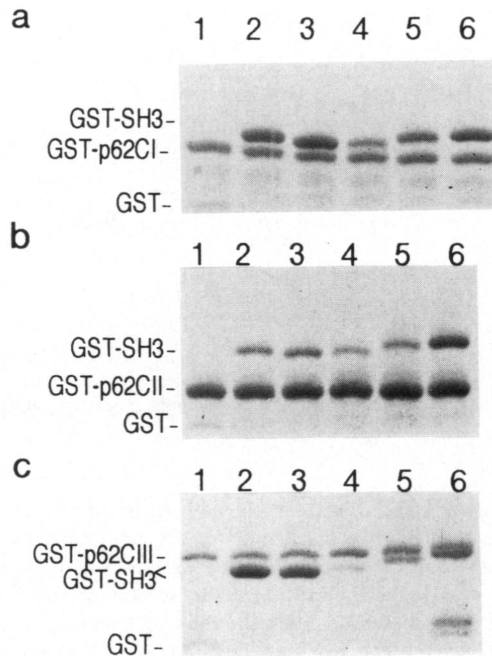

Fig. 4. Binding of various GST-SH3 fusion proteins to immobilized proline-rich sequences of p62. Sequences encompassing C-terminal proline-rich regions of p62, CI, CII and CIII, were expressed as GST fusion proteins and the proteins were coupled to CNBr-activated Sepharose beads. These affinity beads, which bind 30mg each of GST-p62-CI (a), GST-p62-CII (b) and GST-p62-CIII (c) were incubated with 3mg each of GST (lane 1) , GST-Fyn-SH3 (lane 2), GST-Src-SH3 (lane 3), GST-Lck-SH3 (lane 4), GST-Vav-SH3 (lane 5), and GST-PLCγ2-SH3 (lane 6). After extensive washing of the beads, bound proteins were eluted in SDS-PAGE sample buffer, resolved by SDS-PAGE in a 15% gel and stained with Coomassie Blue.

proline-rich stretches of CI, CII and CIII, while showing weak binding to the cell lular 70kDa. This could be explained by the existence of other proteins in the cell extract which may bind more strongly to GST-PLCγ2-SH3 than to the 70kDa. To assess this, we collected the unbound supernatant from the Jurkat cell lysate after the first binging to GST-PLCγ2-SH3 beads and incubated it with new GST-PLCγ2-SH3 beads. The second unbound supernatant was again collected for a third binding and this was repeated a total of four runs. The 70kDa was not seen in the first fraction but appeared after the second (Fi. 5, lane 1 versus lanes 2-4). There were also several other bands which were found only in the second and/or third bindings. The specific bands which were caught in the earlier bindings but not in the later ones include 74kDa, 95kDa, 100kDa, and

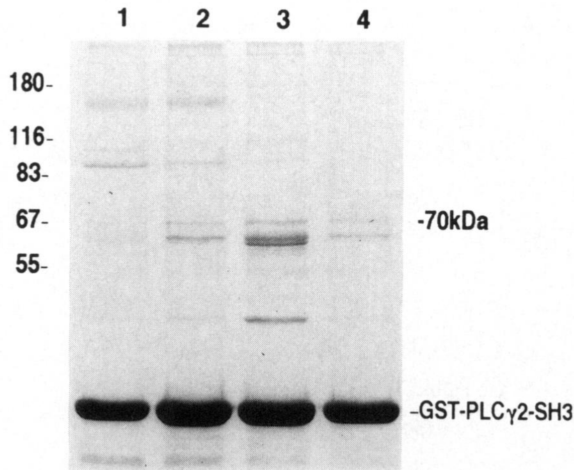

Fig. 5. Priority in binding of ligand proteins in Jurkat cell extract to GST-PLCγ2-SH3. NP-40 extract of Jurkat cells was incubated for 2 h with GST-PLCγ2-SH3 coupled to glutathione-agarose beads. The unbound supernatant was further incubated with the second GST-PLCγ2-SH3 beads for 2 h. The second unbound supernatant was incubated again with the third beads, and the third unbound supernatant with the fourth beads in the same way. The resulting first-run to fourth-run beads (lanes 1 to 4) were washed extensively and boiled in SDS-PAGE sample buffer, and the eluted proteins were resolved by SDS-PAGE in a 10% gel.

170kDa. As expected, excess amount of PLCγ2-SH3 beads in the first fraction resulted in the binding of all the bands above, including the 70kDa which was recognized by Fyn-SH3 and anti-p62 affinity blots (data not shown). The observed weak bindings of the 70kDa and p62 proline rich sequences to GST-Lck-SH3 implies that p62 could positively select its partner among Src family PTKs. Alternatively, it could conceivably reflect some conformational problem intrinsic to our FGST-Lck-CH3. Although we have tried several independent clones and obtained similar results, this possibility cannot be completely excluded. However, we belive that conformational problems contingent to each fusion protein including GST-Lck-SH3 are minimal, because in the binding assay with cellular lysate (Fig. 1), each recombinant exhibited its specific binding pattern.

Association of the 70kDa with v-Src in SR-3Y1-1 cell lysate——p62 is reported to be highly tyrosine-phosphorylated in v-*src*-transformed cells. In our experiment, antiphosphotyrosine monoclonal antibody immunoprecipitated a large number of tyrosine-phosphorylated proteins including the 70kDa (Fig. 6, lane 4) from the lysate of SR-3Y1-1 (Fig. 6, lane 2) but not from that of parent 3Y1 (lane 1) cells. Also, GST-Src-SH3 coupled to CNBr-activated Sepharose beads

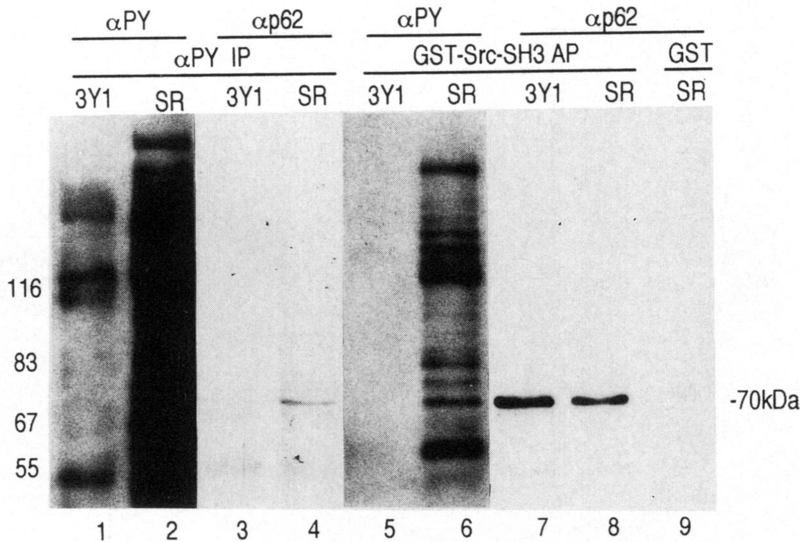

Fig. 6. Tyrosine-phosphorylation of the 70kDa protein in SR-3Y1-1 cells. NP-40 lysate of 3Y1 cells (lanes 1, 3, 5, 7) and SR-3Y1-1 cells (lanes 2, 4, 6, 8, 9) were precleared and mixed with 20μl of anti-phosphotyrosine beads (lanes 1-4), and 45μg each of GST-Src-SH3 (lanes 5-8) and GST (lane 9) which had been coupled to CNBr Sepharose beads. Bound proteins were washed and heated in SDS-PAGE sample buffer. Eluted proteins were resolved by SDS-PAGE, blotted on a PVDF membrane, and probed with anti-phosphotyrosine antibody 4G-10 (lanes 1, 2, 5, 6) and anti-p62 serum (lanes 3, 4, 7, 8, 9). The samples applied on each lane were from one 9cm dish of subconfluent SR-3Y1-1 cells and corresponding protein quantity of 3Y1 cells.

bound the 70kDa in both SR-3Y1-1 and 3Y1 cells, which was detected by anti-p62 (Fig. 6, lane 7 and 8). The 70kDa of SR-3Y1-1 cells was one of the major tyrosine-phosphorylated proteins bound to GST-Src-SH3 (Fig. 6, lane 6). Similar results as in Fig. 6 were obtained by the use of GST-Fyn-SH3 in place of GST-Src-SH3 (data not shown). The following results (shown in Fig. 7) indicate that the 70kDa associates *in vivo* with Src through the SH3 domain. From SR-3Y1-1 lysate, both anti-Src monoclonal antibody and GST-Src-SH3 coupled to Sepharose beads precipitated the 70kDa, which was detected by anti-p62 (lane 2 and 3) and by biotinylated GST-Src-SH3 as well (data not shown). Forty mM phospho-L-tyrosine, when included as a competitive inhibitor of the SH2-phosphotyrosine interaction (37), still left the p62-Src association unaffected (lane 4 compared to lane 3). Binding of v-Src to a putative SH3 binding sequence of p62 was directly demonstrated by affinity precipitation using GST-p62CIII coupled to CNBr Sepharose beads (lane 7). Finally, as a converse

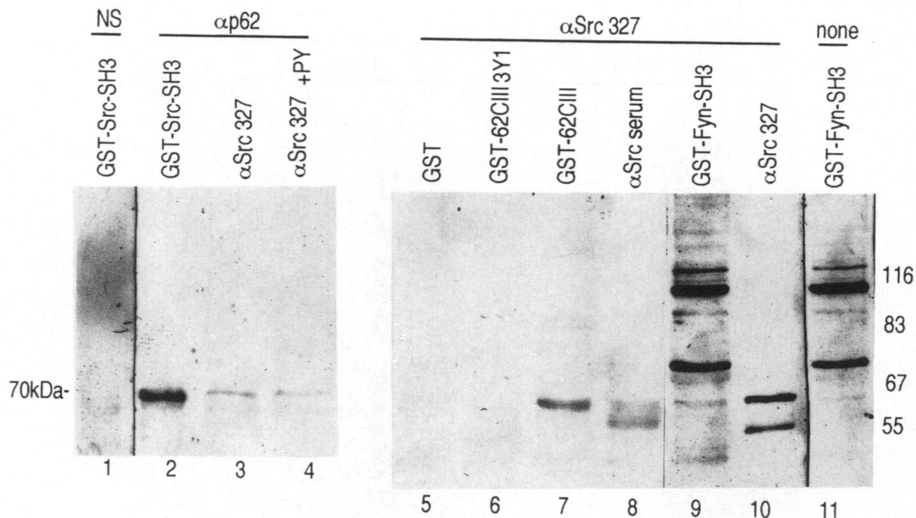

Fig. 7. Co-purification of p62 and Src in immunoprecipitation and affinity-isolation. Precleared cell extracts from 3Y1 in lane 6 and from SR-3Y1-1 in all other lanes were mixed with GST-Src-SH3 (for lanes 1, 2), GST (lane 5), GST-62CIII (lanes 6, 7) and GST-Fyn-SH3 (lanes 9, 11), all of which had been coupled to CNBr-activated Sepharose (15μl each), with 0.8 μg of anti-Src monoclonal antibody 327 plus 10μl anti-mouse Ig agarose (lanes 3, 4, 10), and with 4μl of anti-Src antiserum bound to 10μl protein A Sepharose (lane 8). For lane 4, phospho-L-tyrosine was added at 40mM during immunoprecipitation. The beads were washed and eluted proteins were resolved by SDS-PAGE in a 7.5% gel. Blots on a PVDF membrane were probed with normal rabbit serum (lane 1), anti-p62 monoclonal antibody (lanes 2-4), and with (lanes 5-10) or without (lane 11) anti-Src antibody 327. The lower band in lane 10 represents the heavy chain of 327 antibody. The upper band of the doublet around 60kDa in lane 9 which is not seen in lane 11 is considered to represent Src. Two (lanes 3, 4, 9, 11), one (lanes 5, 7, 8, 10), and a quarter (lanes 1, 2) plate(s), and 3Y1 cells corresponding to one plate (lane 6), of subconfluent SR-3Y1-1 cells were processed and applied to each lane.

experiment of lane 3, GST-SH3 beads, which can bind the 70kDa, were also shown to co-precipitate Src (lane 9). Those results indicate that Src binds to p62 at some region(s) besides SH2, probably through the mediation of SH3 domain. This also suggests that there are more than one SH3 binding site on the 70kDa because GST-SH3 binding may still spare an association site for Src, a result consistent with the identification of multiple SH3 binding sequences in p62 described above.

DISCUSSION

Our *in vitro* binding experiments showed that the proline-rich stretches of

p62 fused to GST can bind to a series of SH3 domains. Surveying the p62 sequence, we found at least five proline-rich stretches which were candidates for the SH3 domain-binding sequence. Combination of the binding specificities seen among the C-terminal three proline-rich sequences, however, offer a plausible explanation for the overall binding specificity of the intact 70kDa to a series of SH3 domains tested. Multiple SH3-binding sites have also been found in other proteins as PI 3'-kinase p85 subunit (22), dynamin (12) and Sosl (15, 16). Interestingly, we were able to show in this study that each of the proline-rich stretches of p62 tested has a different binding specificity to a series of SH3 domains. Those stretches are aligned in juxtaposition. This could mean that several SH3 proteins can be brought together on p62, suggesting a role for p62 as a foothold on which signal trasduction molecules link. In this context, highly phosphorylated tyrosine residues of p62 could also contribute to the association of multiple SH2 bearing signal proteins on p62. Alternatively, side-by-side alignments of SH3- and SH2- binding sequences may strengthen the association of p62 with a single protein which has two or more SH2/SH3 domains, as has been suggested in GRB2-mSosl interaction (16).

There should be an unique consensus amino acid sequence that allows binding of a given SH3 domain with the strongest affinity. However, a certain proline-rich sequence seems in general to accept binding of a group of SH3 domains; this means that single proline-rich sequence could contain overlapped, multiple binding sites for different SH3 domains, allowing a group of SH3 proteins to share, or compete for, the association with the sequence. Among them, the SH3 domains of Fyn and Src may have a similar target ligand since binding specificity of GST-Src-SH3 to a series of p62 proline-rich sequences was shown comparable to that of GST-Fyn-SH3. PI 3'-kinase p85 has also been reported to contain the site which binds both Src- and Fyn-SH3 (18, 20-22).

Even though p62 is expressed abundantly in cells, not much is known concerning its functions. p62 seems at least to participate in the signal transduction of the PTK pathway because of its high phosphorylation. p62 was originally observed as a major tyrosine-phosphorylated protein in v-*src* transformed cells (38, 39). In this study we showed that the p62-related 70kDa protein co-precipitates with the *src* product. The meaning of the possible association of p62 with Src is not certain. Phosphotyrosine competition assay and evidence of Src binding to a GST-fused p62 proline-rich sequence indicate that p62-Src association involves SH3. This could mean that Src locates next to p62 prior to a cellular activation process and works upon stimulation as the very kinase that phosphorylates p62 tyrosine residues, or at least as a component that is directly involved in p62 phosphorylation cascade. In lymphocytes, Src expression is very low so

that Fyn, having a comparable ligand specificity in its SH3 domain, could play a similar role in p62 phosphorylation to that Src plays in fibroblasts.

The identification of those SH3-binding proteins as 3BP1 (1), dynamin (12, 13), Sos1 (14-16, 40) and hCDC42GAP (41) closely links the role of SH3 domain to the regulation of small G proteins. In this context, our finding of GAP-binding p62-related 70kDa to be a ligand of SH3 domains provides another example. On the other hand, paxillin is found associated with v-src SH3 domain (23), and α-spectrin (7) and myosin I (8) contain by themselves the SH3 domain. The presence of SH3 domains in the cytoskeletal proteins, however, is consistent with the above speculation because GTP binding proteins are closely related to cytoskeletal organizations (42). Binding of the p85 subunit of PI 3'-kinase to the SH3 domains of PTKs (14, 18, 19, 21, 22) is also reasonable in this context, because p85 indeed has a sequence related to Bcr (43) that has a GAP activity for Rac (44).

Recently, two papers have appeared concerning Src association of 68kDa protein that related to GAP associated p62 (45, 46). Although it is not certain their p68 and our 70kDa protein are identical, they share some characteristics including low (no) affinity to GAP and association to src product (data not shown). In any case, this apparent p62 heterogeneity and their functional shares, if any, remain to be addressed.

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