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Association of p62, a Multifunctional SH2- and SH3-Domain-Binding Protein, with src Family Tyrosine Kinases, Grb2, and Phospholipase Cγ-1

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src family tyrosine kinases contain two noncatalytic domains termed src homology 3 (SH3) and SH2 domains. Although several other signal transduction molecules also contain tandemly occurring SH3 and SH2 domains, the function of these closely spaced domains is not well understood. To identify the role of the SH3 domains of src family tyrosine kinases, we sought to identify proteins that interacted with this domain. By using the yeast two-hybrid system, we identified p62, a tyrosine-phosphorylated protein that associates with p21^{ras} GTPase-activating protein, as a src family kinase SH3-domain-binding protein. Reconstitution of complexes containing p62 and the src family kinase p59^{fyn} in HeLa cells demonstrated that complex formation resulted in tyrosine phosphorylation of p62 and was mediated by both the SH3 and SH2 domains of p59^{fyn}. The phosphorylation of p62 by p59^{fyn} required an intact SH3 domain, demonstrating that one function of the src family kinase SH3 domains is to bind and present certain substrates to the kinase. As p62 contains at least five SH3-domain-binding motifs and multiple tyrosine phosphorylation sites, p62 may interact with other signalling molecules via SH3 and SH2 domain interactions. Here we show that the SH3 and/or SH2 domains of the signalling proteins Grb2 and phospholipase Cγ-1 can interact with p62 both in vitro and in vivo. Thus, we propose that one function of the tandemly occurring SH3 and SH2 domains of src family kinases is to bind p62, a multifunctional SH3 and SH2 domain adapter protein, linking src family kinases to downstream effector and regulatory molecules.

The src family of protein tyrosine kinases includes the polypeptide products of the *blk*, c-*fgr*, *fyn*, *hck*, *lyn*, *lck*, c-*src*, c-*yes*, and *yrk* genes (for a review, see reference 9). Although v-*src* was one of the first oncogenes identified, the exact physiological function of p60^{src} and its related proteins is still not known. Nonetheless, these proteins have been implicated in the regulation of cell growth, differentiation, transformation, and signal transduction.

Although much progress has been made recently in understanding signalling by receptor tyrosine kinases (67), much less is known about the signalling pathways utilized by the src family tyrosine kinases. Although one pathway, activation of p21^{ras}, has been shown to be critical for p60^{src} signalling (49, 70), the proteins that link src family kinases with p21^{ras} activation have not been identified. Because src family kinases can associate with phospholipase Cγ-1 (PLCγ-1) (54, 82), the phosphatidylinositol (PI) 3-kinase (11, 26), and the p21^{ras} GTPase-activating protein (GAP) (1, 5, 7, 61), it is possible that src family kinases mediate some of their effects through these proteins. However, except for the interaction of p59^{fyn}, p56^{lck}, and p60$^{v\text{-}src}$ with PI 3-kinase (43, 59, 60, 80), little is known about the molecular basis of these interactions or their importance in src family kinase signal transduction.

As first noted by Pawson and coworkers, the src family of tyrosine kinases have similar structural organizations with four domains (66). The src homology 1 (SH1) domain encompasses the catalytic domain at the C terminus of the molecule and is the most conserved of the four domains, having high sequence identity among src family kinases as well as with other types of tyrosine kinases. The other three domains, the SH2 and SH3 domains and the unique amino-terminal domain, are noncatalytic and thought to have important regulatory and effector functions. The exact functions of these three domains are not known.

The SH2 domain, conserved among all of the src family kinases, is found in a variety of other signalling molecules (for a review, see reference 57). Because mutations in the SH2 domain can activate these kinases and because src family kinase activity is regulated by a conserved carboxy-terminal phosphotyrosine residue, current models propose that src family SH2 domains interact intramolecularly with the regulatory phosphotyrosine residue, thus repressing kinase activity (35, 45, 55, 68, 75). Because mutations in the SH2 domain also impair downstream signalling by src family tyrosine kinases (37, 63, 79), it has been suggested that the SH2 domain may have an effector role as well.

In contrast, much less is known about the function of src family SH3 domains or why in many cases they are arranged in close proximity to SH2 domains. SH3 domains are found in a variety of signalling molecules as well as in some cytoskeletal proteins (for reviews, see references 46, 52, and 57). SH3 domains have been shown to mediate subcellular localization of proteins (2) and to mediate specific protein-protein interactions via the recognition of proline-rich sequences (6, 62). As mutations in the SH3 domain can activate src family kinases, it has been proposed that the SH3 domain, like the SH2 domain, may play a role in kinase regulation (38, 51, 56, 63, 68, 75, 79)

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or with protein-protein interactions. Although several proteins have been shown to associate with src family SH3 domains, such as the p85 subunit of the PI 3-kinase (43, 59, 60, 80), paxillin (83), and AFAP-110 (24), the physiological significance of these interactions is not known.

To fully understand the function of src family SH3 domains, it will be important to identify additional interacting proteins. To this end, we used the yeast two-hybrid system to identify proteins that interact with the SH3 domain of $p59^{fyn}$. One of the proteins that we identified by using this strategy was the $p21^{ras}$ GAP-associated protein p62. Because it represented a possible link between src family kinases and the $p21^{ras}$ signalling pathway, we focused on defining the interaction between $p59^{fyn}$ and p62 and its functional relevance.

MATERIALS AND METHODS

Antibodies. The anti-myc monoclonal antibody 9E10 (20) was obtained from the American Type Culture Collection (Rockville, Md.). The anti-$p60^{src}$ monoclonal antibody 327 was purchased from Oncogene Sciences Inc. (Manhassett, N.Y.) and was also the gift of Joan Brugge. The mouse control antibody was purchased from Sigma Chemical (St. Louis, Mo.). The mixed monoclonal antibodies for PLCγ-1, the antiphosphotyrosine monoclonal antibody 4G10, and the rabbit polyclonal antibodies to Grb2 were purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). The $p59^{fyn}$ rabbit polyclonal antibody used for immunoblotting was a generous gift from André Veillette. The $p59^{fyn}$ monoclonal antibody (immunoglobulin G1, clone 4.2) was generated by immunizing BALB/c mice with a bacterial fusion protein containing the first 200 amino acids of $p59^{fyn}$ linked to the C terminus of glutathione *S*-transferase (GST) expressed in *Escherichia coli*. Rabbit polyclonal antibodies to p62 and Grb2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.). p62 polyclonal antibodies were generated in rabbits by using GST-p62 (amino acids 331 to 443) as an antigen.

Isolation of the mouse p62 cDNA by using the yeast two-hybrid system. The first 200 amino acids of $p59^{fyn}$ were amplified by PCR with oligonucleotides 5′-GGA CCA TGG GCT GTG TGC AAT GT-3′ and 5′-AGT TGC TGA GTT TCA-3′ and the mouse $p59^{fyn}$ cDNA in Bluescript SK+ as the template (29). The DNA fragment was digested with *Nco*I and subcloned in the *Nco*I and *Sma*I sites of plasmid pAS1-CYH2 (15). This plasmid was transformed into *Saccharomyces cerevisiae* Y190, and the expression of the GAL4BD-SH3fyn fusion protein was verified by immunoblotting with anti-$p59^{fyn}$ antibodies. The GAL4BD-SH3fyn protein did not induce expression of the *GAL1-lacZ* reporter gene, as determined by a colony lift assay. To identify binding proteins, *S. cerevisiae* Y190 containing the $p59^{fyn}$ pAS1-CYH2 plasmid was transformed with a mouse T-cell cDNA library in the pACT vector as described previously (15, 17). Yeast colonies were scored positive by virtue of their ability to transactivate the *GAL1-lacZ* reporter gene, as assessed by the colony lift assay. The yeast cells containing the library plasmid were cured of the $p59^{fyn}$ pAS1-CYH2 plasmid by growing the yeast cells in the presence of cycloheximide. The cells containing the library plasmids were then mated to a series of strains expressing the GAL4 activation domain fused to SNF1, human immunodeficiency virus (HIV) Tat, HIV Rev, p53, lamin, N-terminal MEK kinase, or full-length MEK kinase (3, 15). Library clones were discarded if they resulted in *lacZ* expression when coexpressed with any of the unrelated GAL4 activation domain fusion proteins. On the basis of this analysis, 12 clones appeared to encode proteins that interacted specifically with the GAL4BD-SH3fyn fusion protein; these clones were sequenced. One of the positives was the $p21^{ras}$ GAP-associated protein p62 (86), and the partial DNA sequence of p62 enabled us to synthesize two oligonucleotides, 5′-GTA ACG AAT TCG CTC ATA ACT GAT A-3′ and 5′-TAC GAA TTC ACC GGG CCT CGC AAC ACC CAG ACC GCC-3′, that were used to amplify the coding region of p62 from the pACT library plasmid. The amplified fragment was digested with *Eco*RI and subcloned in myc-Bluescript KS+. myc-Bluescript KS+ was constructed by annealing two oligonucleotides (5′-CGC GGA TCC ACC ATG GCG TCT ATG GAA CAA AAG CTG ATT AGC G-3′ and 5′-GCG CTC GAG AAT TCC CGT TGT TCA GGT CCT CTT CGC TAA TCA GCT TTT-3′) and filling in the ends with DNA polymerase I (Klenow fragment). This DNA fragment was subcloned into the *Bam*HI and *Xho*I sites in Bluescript KS+ (Stratagene, La Jolla, Calif.).

Full-length p62 was cloned from a day 13 thymocyte cDNA library by using a DNA probe encoding amino acids 98 to 294 of murine p62 generated by PCR. A single 2.5-kb insert was sequenced by using Sequenase (U.S. Biochemical, Cleveland, Ohio).

Protein expression and protein analysis. HeLa cells plated at a density of 0.5×10^6/3.5-cm-diameter petri dish were infected with a recombinant vaccinia virus containing the T7 DNA polymerase (25) with a multiplicity of infection of 10 in 0.75 ml of Dulbecco's minimum essential medium (DMEM) as described previously (69). After 30 min at 37°C, 5 μg of plasmid DNA was added to 0.75 ml of DMEM containing 15 μl of Lipofectase (GIBCO/BRL, Gaithersburg, Md.). The cells were then incubated at 37°C for 8 to 12 h and lysed in 0.5 ml of 1× Laemmli buffer (41) (for whole-cell lysate) or 0.5 ml of lysis buffer (1% Triton, 150 mM NaCl, 20 mM Tris-HCl [pH 8.0], 50 mM NaF, 100 μM sodium vanadate, 0.01% phenylmethanesulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml). The cellular debris and nuclei were removed by a 10-min spin in a microcentrifuge at 4°C. The supernatant was incubated with the specified antibody for 1 h at 4°C. Then 20 μl of a 50% protein A-Sepharose slurry was added for 30 min with constant end-over-end mixing at 4°C. This step was omitted when the antibody was covalently conjugated to protein A-Sepharose. The beads were then washed three times with lysis buffer and dissociated in 2× Laemmli buffer. The samples were analyzed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with the specified antibody. The immunoblots were developed by using a goat anti-mouse or a goat anti-rabbit antibody conjugated to horseradish peroxidase (Organon Teknika-Cappel, Durham, N.C.), using a chemiluminescence kit (Dupont/New England Nuclear, Boston, Mass.) for detection.

For affinity chromatography using GST fusion proteins, HeLa cells expressing various p62 constructs were lysed as described above. Cell lysates were incubated at 4°C for 1 h with 20 μl of a 50% slurry consisting of the GST fusion protein covalently coupled to Affi-Gel 10 (Bio-Rad Laboratories, Hercules, Calif.) at a concentration of 2 mg/ml. As a positive control, 20 μl of a 50% slurry consisting of anti-myc antibodies covalently coupled to protein A-Sepharose with dimethylpimelimidate (33) was incubated with equal amounts of cellular lysate. The beads were washed three times with lysis buffer and once with phosphate-buffered saline. The bound proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and immunoblotted with anti-myc antibodies.

p62 and $p59^{fyn}$ constructs. P1/2/3/4 was constructed by digesting p62 in myc-Bluescript KS+ with *Sac*I and filling in the ends with the Klenow fragment of DNA polymerase I. The plasmid was redigested with *Bam*HI, and the DNA fragment was subcloned into *Bam*HI and *Kpn*I restriction sites of myc-Bluescript KS+. The *Kpn*I site was filled in with the Klenow fragment and was destroyed. P1/2/3 was generated by digesting p62 in myc-Bluescript KS+ with *Xho*I, filling in the ends, and ligating them together with T4 DNA ligase. This removed the DNA sequences C terminal of the *Xho*I site in p62, and a stop codon was created in the plasmid sequences. P1/2 was generated by digesting p62 in myc-Bluescript KS+ with *Bgl*II and *Xho*I, filling in the ends with the Klenow fragment, and ligating the ends with T4 DNA ligase. The sequences C terminal to the *Bgl*II site in p62 were deleted. P1/2/4/5 was constructed by inverse PCR (34) using the following oligonucleotides: 5′-TCT TGG CAG CTC CTC GTC CTC TCA C-3′ and 5′-TCT CTG GAC GTG GTG TTG GAC CAC C-3′. P1/2/4 was constructed similarly to P1/2/3/4 except that P1/2/4/5 was used as the starting plasmid rather than p62 in myc-Bluescript KS+. P1/2/5 was generated by inverse PCR with the following oligonucleotides: 5′-TCT TGG CAG CTC CTC GTC CTC TCA C-3′ and 5′-TCT CTG TGA GGG GTG CTC CAA CAC C-3′.

The construct encoding fyn:mSH3 contains a substituted leucine for a proline at amino acid 134 in the SH3 domain of the mouse $p59^{fyn}$ protein. This plasmid was generated by inverse PCR (34) with oligonucleotides 5′-GGT TAC ATT CTG AGC AAT TAC G-3′ and 5′-AGT TTC CCC GGT TGT CAA-3′ and the mouse $p59^{fyn}$ cDNA in Bluescript SK+ as the template (29). The plasmid encoding fyn:mSH2 contains a lysine at position 176 rather than an arginine in the SH2 domain of the mouse $p59^{fyn}$. This construct was generated by inverse PCR with oligonucleotides 5′-GAG AGC CAA ACC ACC AAA GGT-3′ and 5′-TTT GAT AAG AAA AGT ACC TCT TGG GT-3′. The plasmid encoding fyn:mSH3/2 was generated by sequentially mutating the SH3 domain and then the SH2 domain by inverse PCR.

Cloning of the mouse Grb2 cDNA. RNA was extracted from the brain of a BALB/c mouse by using 4 M guanidine thiocyanate and purified over a 5.7 M CsCl cushion. One microgram of total RNA was reverse transcribed and amplified essentially as described previously (64), using oligonucleotides based on the published mouse Grb2 sequence (72). The antisense Grb2 oligonucleotide that contained an *Xho*I restriction site (5′-ATC TCG AGT GCT TCT TAG ACG-3′) was used to generate first-strand cDNAs. The sense Grb2 oligonucleotide (5′-GCG AAT TCA ATG GAA GCC ATC GCC AAA TAT G-3′) used in the PCR contained an *Eco*RI site at its 5′ end. The amplified DNA fragment was digested with *Eco*RI and *Xho*I and subcloned into Bluescript KS+. Dideoxynucleotide sequencing was used to verify that the mouse Grb2 sequence was identical to the previously reported mouse Grb2 sequence (72).

GST fusion proteins. The GST-fynSH3 fusion protein contained amino acids 86 to 140 of the mouse $p59^{fyn}$ linked to the C terminus of GST. The oligonucleotides 5′-CAG GAA TTC CAC TGT TTG TGG CGC TT-3′ and 5′-TGA AAG CTT GGA GCC ACG TAA TTG CTG G-3′, which also contain *Eco*RI and *Hin*dIII sites (underlined), were used to amplify the DNA fragment encoding these amino acids, using the mouse $p59^{fyn}$ cDNA as a template. The amplified DNA fragment was digested with *Eco*RI and *Hin*dIII and subcloned into pGEX-KG (32). The GST-fynSH2 bacterial fusion protein contained amino acids 145 to 247 of the mouse $p59^{fyn}$. The plasmid encoding GST-fynSH2 was generated similarly to the plasmid encoding GST-fynSH3 except that *Eco*RI and *Xho*I restriction sites were used. The oligonucleotides used to generate GST-fynSH2 had the following sequences: 5′-CAG GAA TTC AGG CAG AAG AGT GGT ACT-3′ and 5′-GTA CTC GAG GAT GAA ACC ACA GTT AAG-3′.

The plasmid encoding the GST-Grb2 fusion protein was constructed by digesting the Grb2 cDNA from Bluescript KS+ with *Xba*I and *Xho*I and subcloning the DNA fragment in pGEX-KG. The plasmid encoding the Grb2–N-SH3 protein, which contains the N-terminal SH3 domain of Grb2 linked to GST, was constructed by digesting GST-Grb2 with *Sty*I and *Xho*I, filling in the ends with the Klenow fragment of DNA polymerase I, and ligating the ends with T4 DNA ligase. This removed the Grb2 DNA sequences C terminal to the *Sty*I restriction site which is located C terminal to the N-terminal SH3 domain. The Grb2-SH2 fusion protein corresponded to amino acids 54 to 164 of Grb2 linked to the C terminus of the GST protein. The DNA fragment encoding these amino acids was amplified by PCR using oligonucleotides 5′-TTA TCT AGA AAT GAA ACC ACA TCC-3′ and 5′-TCC TCG AGC GCC TGG ACG TAG GTT G-3′ and Grb2 in Bluescript KS+ as a template. The fragment was digested with *Xba*I and *Xho*I and subcloned in the corresponding sites in pGEX-KG. The DNA fragment encoding the C-terminal SH3 domain of Grb2 (Grb2–C-SH3) was amplified by using the two oligonucleotides 5′-GAT TCT AGA GCA GCC AAC CTA CGT C-3′ and the M13 reverse sequencing primer of Bluescript KS+. The DNA fragment was digested with *Xba*I and *Xho*I and subcloned in pGEX-KG. The PI 3-kinase C-terminal SH2 domain linked to GST was a gift from Tony Pawson. The GST-rat PLCγ-1SH3 (amino acids 790 to 850), GST-rat PLCγ1-SH2/2 (amino acids 548 to 760), and GST-human GAPSH3 (amino acids 277 to 346) bacterial fusion proteins were purchased from Santa Cruz Biotechnology Inc.

All DNA constructs were verified by dideoxynucleotide sequencing using the Sequenase DNA sequencing kit from U.S. Biochemical or by automated sequencing.

Cells. The murine CD4-positive, class II-restricted T-cell hybridoma derived from the T cells of the DO. 10 T-cell receptor transgenic mouse (50) was maintained in DMEM supplemented with 10% fetal bovine serum. HeLa, v-*src*-transformed NIH 3T3, and parental NIH 3T3 cells were maintained in DMEM supplemented with 10% bovine calf serum.

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been assigned GenBank accession number U17046.

RESULTS

Isolation of p59^{fyn} SH3-domain-binding proteins by using the yeast two-hybrid system. To identify proteins that interact with the SH3 domain of the src family tyrosine kinase p59^{fyn}, we used the yeast two-hybrid system developed by Fields and Song (22) and modified by Durfee et al. (15). The chimeric protein that we used contained the DNA-binding domain of the GAL4 protein fused to the unique amino-terminal and SH3 domains of p59^{fyn} (GAL4BD-SH3fyn). The chimeric protein was expressed in yeast cells but did not activate transcription of a reporter gene containing GAL4 protein-binding sites fused to the *lacZ* gene. Coexpression of the GAL4BD-SH3fyn fusion protein with a chimeric protein containing the GAL4 transcriptional activation domain fused to a protein that binds the SH3 domain of p59^{fyn} might reconstitute the GAL4 protein function and result in activation of the *lacZ* reporter gene.

A mouse T-cell cDNA expression library was introduced by transformation into yeast cells expressing the GAL4BD-SH3fyn fusion protein. When the library is introduced in yeast cells, cDNAs are expressed as chimeric proteins containing the GAL4 transcriptional activation domain. From 5×10^5 transformants, 98 colonies that expressed the *lacZ* gene were identified. Of these 98 colonies, 19 expressed proteins that activated *lacZ* expression only when coexpressed with GAL4BD-SH3fyn and not with other unrelated chimeric proteins fused to the GAL4 DNA-binding domain. Twelve clones expressing the highest levels of β-galactosidase were selected for DNA sequencing.

Seven of the twelve clones encoded novel proteins. Two of these contained proline-rich sequences and were homologous to RNA-binding proteins. Of the five clones representing known proteins, one was the p21^{ras} GAP-associated protein p62. p62 was first identified as a tyrosine-phosphorylated substrate in v-*src*-, v-*fms*-, v-*abl*-, and v-*fps*-transformed cells and shown to interact with p21^{ras} GAP (19, 47, 48). However, the function of p62 is unknown. Because p62 might represent a potential link between src family tyrosine kinases and the p21^{ras} signalling pathway, we examined the p62-p59^{fyn} interaction further.

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Mouse p62   ........S. LT......C. K.......... .L....PS.. ...P......  50
Human p62   ........A. MS......G. M.......... .Q....QP.. ...S......
Consensus   MQRRDDPA.R ..RSSGRS.S .DPSGAHPSV R.TPSR..PL PHR.RGGGGG

Mouse p62   P......... .......... TP......V. S......... ...A......  100
Human p62   S......... .......... AT......G. P......... ...S......
Consensus   .RGGARASPA TQPPPLLPPS ..GPDATV.G .APTPLLPPS ATA.VKMEPE
                          P1                    P2

Mouse p62   ...P...... .......... .....SV... .....E.... ..........  150
Human p62   ...L...... .......... .....TA... .....D.... ..........
Consensus   NKY.PELMAE KDSLDPSFTH AMQLL..EIE KIQKG.SKKD DEENYLDLFS

Mouse p62   .......... .......... .......... .......... ..........  200
Human p62   .......... .......... .......... .......... ..........
Consensus   HKNMKLKERV LIPVKQYPKF NFVGKILGPQ GNTIKRLQEE TGAKISVLGK

Mouse p62   .......... .......... .......... .......... ..........  250
Human p62   .......... .......... .......... .......... ..........
Consensus   GSMRDKAKEE ELRKGGDFKY AHLNMDLHVF IEVFGPPCEA YALMAHAMEE

Mouse p62   .......... .......... .......... ......S... ..........  300
Human p62   .......... .......... .......... ......P... ..........
Consensus   VKKFLVPDMM DDICQEQFLE LSYLNGVPEP SRGRGV.VRG RGAAPPPPPV
                                                                P3

Mouse p62   .......... .......... .S........ .......... ...T......  350
Human p62   .......... .......... .A........ .......... ...A......
Consensus   PRGRGVGPPR GALVRGTPVR G.ITRGATVT RGVPPPPTVR GAP.PRARTA
                                              P4

Mouse p62   .........T .......D.. .......... .......... ..E.......  400
Human p62   .........P .......E.. .......... .......... ..D.......
Consensus   GIQRIPLPP. PAPETYE.YG YDDTYAEQSY EGYEGYYSQS QG.SEYYDYG
              P5

Mouse p62   ...L...... .......... .......... .......... ...  443
Human p62   ...V...... .......... .......... .......... ...
Consensus   HGE.QDSYEA YGQDDWNGTR PSLKAPPARP VKGAYREHPY GRY
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FIG. 1. Amino acid sequence alignment of the mouse and human p62 proteins. The amino acid differences are shown. The sequence identities are represented by periods, and the consensus sequence is shown below the human and mouse sequences. The sequences of the five putative SH3-domain-binding proline motifs are underlined and designated P1 through P5.

The mouse p62 cDNA that we cloned was missing only the sequences encoding the N-terminal 62 amino acids. We obtained a full-length clone from a day 13 mouse thymus library (Fig. 1). It had a greater-than-95% sequence identity with the previously cloned human p62 sequence (86). Importantly, the amino acid sequence of p62 contains five proline-rich sequences, designated P1 through P5, that could provide binding sites for the SH3 domain of p59^{fyn} (Fig. 1, underlined sequences).

Interaction of p62 with p59^{fyn} in mammalian cells. To determine whether p62 interacts with p59^{fyn} in vivo, we first generated a rabbit polyclonal antibody against the C-terminal region of p62. Immunoblotting demonstrated that the antiserum was specific and detected a single band with an apparent molecular mobility of 68 kDa in whole-cell lysates from human and mouse T cells (Fig. 2A, lane 1, and data not shown). As reported by others, the 68-kDa protein likely represents either p62 or a p62-related protein (27, 77). Anti-fyn immunoprecipitates were prepared from T-cell lysates and then immunoblotted with the p62 antiserum. A 68-kDa immunoreactive protein was detected in the p59^{fyn} immunoprecipitates (Fig. 2A, lanes 2 and 3). Similar results were obtained when immunoprecipitates of p56^{lck} were immunoblotted with the p62 antiserum (data not shown). p62-p59^{fyn} complexes are therefore present in unstimulated T lymphocytes.

To facilitate analysis of the interaction, we next sought to determine whether the p62-p59^{fyn} complexes could be reconstituted by overexpression in HeLa cells in the vaccinia virus

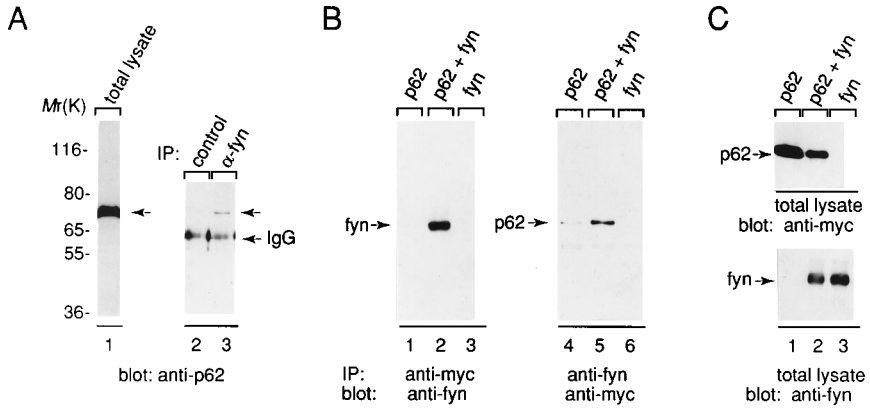

FIG. 2. Association of p62 with p59^{fyn} in T cells and HeLa cells. (A) Whole-cell extracts from a T-cell hybridoma were separated by SDS-PAGE and immunoblotted with anti-p62 antibodies (lane 1). The position of p62 is indicated by the arrow on the right. T cells (10^7) were immunoprecipitated (IP) with a control antibody (immunoglobulin G1 [IgG]) or an anti-fyn monoclonal antibody (α-fyn) as described in Materials and Methods. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-p62 antibodies (lane 2 and 3). (B) HeLa cells were transfected with plasmids encoding p62-myc (lane 1), p62-myc plus p59^{fyn} (lane 2), or p59^{fyn} (lane 3). The cells were lysed as described in Materials and Methods and immunoprecipitated with an anti-myc monoclonal antibody covalently cross-linked to protein A-Sepharose. The bound proteins were separated on SDS–10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with rabbit polyclonal anti-p59^{fyn} antibodies. The same lysates as in lanes 1 to 3, containing p62-myc (lane 4), p62-myc plus p59^{fyn} (lane 5), and p59^{fyn} (lane 6), were also immunoprecipitated with an anti-p59^{fyn} monoclonal antibody covalently cross-linked to protein A-Sepharose. The bound proteins were separated on SDS–10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-myc antibodies. (C) Anti-p59^{fyn} and anti-myc immunoblots of total lysates from cultures prepared in duplicate with those used for panel B. The cells were lysed in 0.5 ml of 1× Laemmli buffer, and 20 μl of each lysate was resolved on SDS–10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antibodies to p59^{fyn} and myc.

T7 expression system (25). To distinguish transfected p62 from endogenous p62, the p62 cDNA was appended with sequences encoding an epitope tag myc, which reacts with monoclonal antibody 9E10 (20). Expression of the epitope-tagged protein, p62-myc, in HeLa cells demonstrated that the protein had an apparent mobility of 62 kDa on SDS-polyacrylamide gels and could be recognized by both the myc antibody and our anti-p62 antiserum.

By using the vaccinia virus T7 expression system, HeLa cells were cotransfected with the cDNAs encoding p62-myc and p59^{fyn}. Immunoprecipitates prepared from cell lysates with the monoclonal antibody that recognizes p62-myc or with a monoclonal antibody that recognizes p59^{fyn} were analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to p59^{fyn} and myc, respectively. p59^{fyn} and p62-myc formed stable complexes when coexpressed in HeLa cells (Fig. 2B). p59^{fyn} immunoprecipitates contained p62-myc, and p62-myc immunoprecipitates contained p59^{fyn} (Fig. 2B). Immunoblotting of whole-cell lysates confirmed that p62-myc and p59^{fyn} expression levels were equivalent in all of the samples (Fig. 2C). Interestingly, when p62-myc was expressed alone, a small amount of p62-myc was observed in p59^{fyn} immunoprecipitates (Fig. 2B, lane 4). Since HeLa cells express p59^{fyn} (40), these findings suggest that p62-myc associates with endogenous p59^{fyn}.

The SH3 and SH2 domains of p59^{fyn} bind p62. Our ability to reconstitute p62-p59^{fyn} complexes in transfected cells allowed us to map the sites of interaction between these two proteins. We first confirmed that the SH3 domain of p59^{fyn} was involved in binding to p62-myc by testing the ability of a mutated p59^{fyn} protein, fyn:mSH3, to bind p62. This construct encodes a proline-to-leucine substitution at position 134 in the SH3 domain of p59^{fyn}. Substitution of proline 134, a residue conserved in all SH3 domains, is known to abolish SH3 domain function (8). Mutation of the p59^{fyn} SH3 domain reduced its ability to bind p62-myc more than fivefold but did not abolish it entirely (Fig. 3A; compare lanes 1 and 2). These results suggested that although the interaction was primarily mediated by the p59^{fyn}

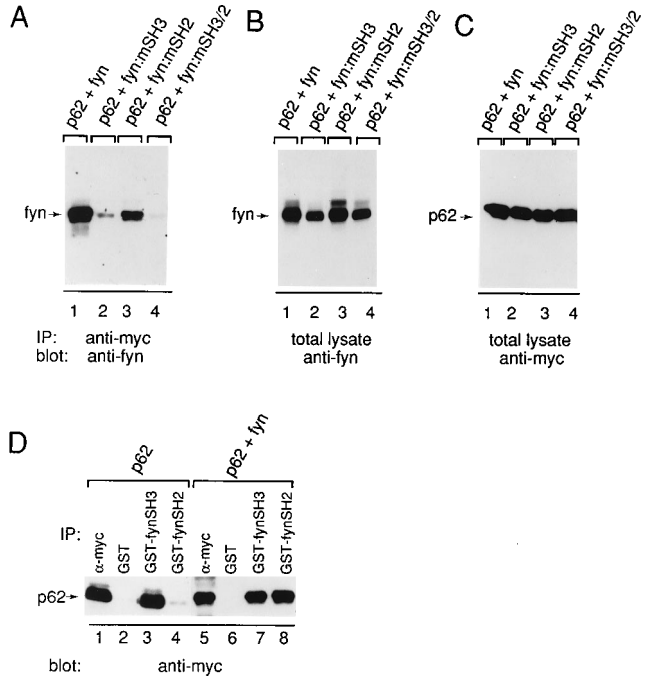

FIG. 3. The SH3 and SH2 domains of p59^{fyn} mediate p62 binding. (A) p62-myc was cotransfected in HeLa cells with p59^{fyn} (lane 1), p59^{fyn} containing a mutation in the SH3 domain (lane 2), p59^{fyn} containing a mutation in the SH2 domain (lane 3), or p59^{fyn} containing both SH3 and SH2 domain mutations (lane 4). Cell lysates were immunoprecipitated (IP) with anti-myc antibodies, analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit polyclonal anti-p59^{fyn} antibodies. (B and C) Anti-p59^{fyn} and anti-myc immunoblots of total lysates from cultures prepared in duplicate with those used in panel A. (D) HeLa cells expressing p62 in the presence or absence of cotransfected p59^{fyn} were lysed in lysis buffer, and the lysate was divided equally and incubated with anti-myc antibody (lanes 1 and 5), GST (lanes 2 and 6), GST-fynSH3 (lanes 3 and 7), or GST-fynSH2 (lanes 4 and 8) covalently coupled to beads. Bound proteins were immunoblotted with anti-myc antibodies.

SH3 domain, other domains of p59^{fyn} might also interact with p62.

We suspected that the SH2 domain of p59^{fyn} might also play a role in binding p62-myc because p62 can be phosphorylated on tyrosine residues. This possibility was tested by generating an additional p59^{fyn} mutant, fyn:mSH2, that contains a substitution at a critical residue of the SH2 domain, arginine 176. This arginine residue, which is in the FLVRES region of the p59^{fyn} SH2 domain, is known to make important contacts with the phosphate group on phosphotyrosine (81). Substitution of this residue with lysine abolishes phosphotyrosine-SH2 domain binding. p62-myc immunoprecipitates contained an approximately twofold reduction in levels of the fyn:mSH2 (Fig. 3A; compare lanes 1 and 3), confirming that the SH2 domain contributed to complex formation. A p59^{fyn} construct containing both the SH2 and SH3 domain point mutations (fyn:mSH3/2) did not bind to p62-myc (Fig. 3A, lane 4). Immunoblotting of whole-cell lysates demonstrated that the expression of fyn:mSH3/2 and p62-myc was comparable to that observed in experiments using wild-type p59^{fyn} (Fig. 3B and C). These results demonstrate that the association of p62-myc with p59^{fyn} is mediated by both the SH2 and SH3 domains of p59^{fyn}.

The interaction between p62 and the SH3 and SH2 domains of p59^{fyn} was confirmed by using an in vitro binding assay. The p59^{fyn} SH3 and SH2 domains were individually expressed as GST fusion proteins in *E. coli* and covalently coupled to Sepharose beads. Cell lysates containing p62-myc or p62-myc coexpressed with p59^{fyn} were divided equally and incubated either with the p59^{fyn} SH3 domain fused to GST (GST-fynSH3), GST fused to the p59^{fyn} SH2 domain (GST-fynSH2), GST alone, or anti-myc antibodies. When p62-myc was expressed alone, it bound strongly to the GST-fynSH3 fusion protein, whereas little or no binding to the GST-fynSH2 fusion protein was detected (Fig. 3D, lanes 1 to 4). However, when p62-myc was coexpressed with p59^{fyn} (which results in tyrosine phosphorylation of p62-myc), similar amounts of p62-myc bound to both the GST-fynSH3 and GST-fynSH2 fusion proteins (Fig. 3D, lanes 5 to 8). Therefore, when p62-myc is not tyrosine phosphorylated, it binds to p59^{fyn} via its SH3 domain, and when it is tyrosine phosphorylated, p62 binds p59^{fyn} via its SH3 and SH2 domains.

Determination of the SH3 and SH2-domain-binding sites in p62. A series of p62-myc deletion constructs was generated to identify the SH3 and SH2-domain-binding sites in p62-myc. Each construct was coexpressed in HeLa cells with p59^{fyn} and tested for its ability to bind the GST-fynSH3 and/or the GST-fynSH2 bacterial fusion proteins in vitro. p59^{fyn} was coexpressed to ensure that p62-myc was tyrosine phosphorylated (Fig. 4A, lane 4). A mutated p62 construct, P1/2/3/4, which lacks the fifth proline motif and the tyrosine-rich carboxy-terminal domain, bound the SH3 domain but not the SH2 domain of p59^{fyn} (Fig. 5). No phosphotyrosine immunoreactivity was detected when P1/2/3/4 was coexpressed with p59^{fyn} (Fig. 4A, lane 2), which indicated that the SH2 domain of p59^{fyn} was probably interacting with a phosphotyrosine residue within the carboxy-terminal domain of p62-myc.

The domain of p62 that binds the p59^{fyn} SH3 domain was defined by using two additional p62 constructs. The construct P1/2/3 lacks the fourth and fifth proline motifs, and the construct P1/2 lacks the third, fourth, and fifth proline motifs. P1/2/3 bound to the p59^{fyn} SH3 domain with moderate apparent affinity, and P1/2 did not bind to the p59^{fyn} SH3 domain (Fig. 5). These results indicate that the first and second proline motifs do not participate in binding to the SH3 domain of p59^{fyn} and that the third proline motif of p62 is necessary for binding to the SH3 domain of p59^{fyn}.

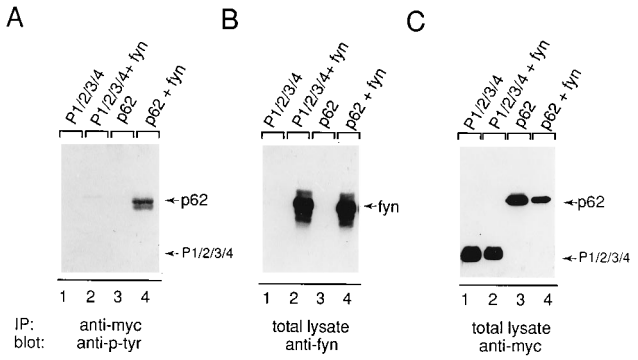

FIG. 4. The C-terminal tyrosine-rich region of p62 is the site of tyrosine phosphorylation in vivo. (A) HeLa cells were transfected with the plasmids encoding p62-myc or P1/2/3/4 in the presence or absence of p59^{fyn}. The cellular lysates were immunoprecipitated (IP) with anti-myc antibodies, and the bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine (anti-p-tyr) antibodies. The positions of P1/2/3/4 and p62-myc are shown on the right. (B and C) Anti-p59^{fyn} and anti-myc immunoblots of total lysates from cultures prepared in duplicate with those used for panel A.

Additional deletion constructs were generated to test whether P4 and/or P5 also contributed to the SH3 binding. P1/2/4/5, which lacks the third proline motif, bound to the p59^{fyn} SH3 domain, demonstrating that P4 and/or P5 are also capable of SH3 binding (Fig. 5). The individual contributions of P4 and P5 to this binding were tested by generating P1/2/4, which lacks both the third and fifth proline motifs, and P1/2/5, which lacks the third and fourth proline motifs. P1/2/5 efficiently bound the SH3 domain of p59^{fyn}, whereas P1/2/4 bound the SH3 domain less well (Fig. 5). These results taken together suggest that the association of p62 with the p59^{fyn} SH3 domain is mediated mainly by the fifth proline motif of p62, P5, but that the third and fourth proline motifs can also contribute to SH3 binding. Since binding to the SH2 domain of p59^{fyn} is mediated by the carboxy-terminal domain of p62, probably via a phosphotyrosine residue, it is interesting that the proximity of P5 to the carboxy-terminal tyrosine-rich domain might allow simultaneous contact of the SH3 and SH2 domains of p59^{fyn} with the same p62 molecule.

Role for the SH3 domain of p59^{fyn} in the tyrosine phosphorylation of p62. An inconsistency in the amount of p62 binding to the p59^{fyn} SH2 domain in vivo compared with the p59^{fyn} SH2 domain binding in vitro suggested that the SH3 domain of p59^{fyn} might play a role in facilitating tyrosine phosphorylation of p62. Experiments in vivo using the mutated p59^{fyn} proteins suggested that the association of p59^{fyn} and p62 was primarily mediated by the p59^{fyn} SH3 domains, with a small contribution from the p59^{fyn} SH2 domain (Fig. 3A, lanes 2 and 3). However, experiments in vitro demonstrated similar amounts of tyrosine-phosphorylated p62 binding to the SH3 and SH2 domains (Fig. 3D, lanes 7 and 8). One explanation for this discrepancy was that the mutated p59^{fyn} molecule, fyn:mSH3, not only was defective in its ability to bind via its SH3 domain but was also less effective in phosphorylating p62. Thus, both SH3 and SH2 domain interactions might have been impaired by this mutation. To investigate this possibility, p62-myc was coexpressed with fyn:mSH3, and anti-myc immunoprecipitates were analyzed for phosphotyrosine content by immunoblotting with antiphosphotyrosine antibodies. Although p62-myc was tyrosine phosphorylated in cells coexpressing p62-myc and p59^{fyn} (Fig. 6A, lane 2), coexpression of p62-myc with the fyn:mSH3 mutant resulted in little detectable p62 tyrosine phosphorylation

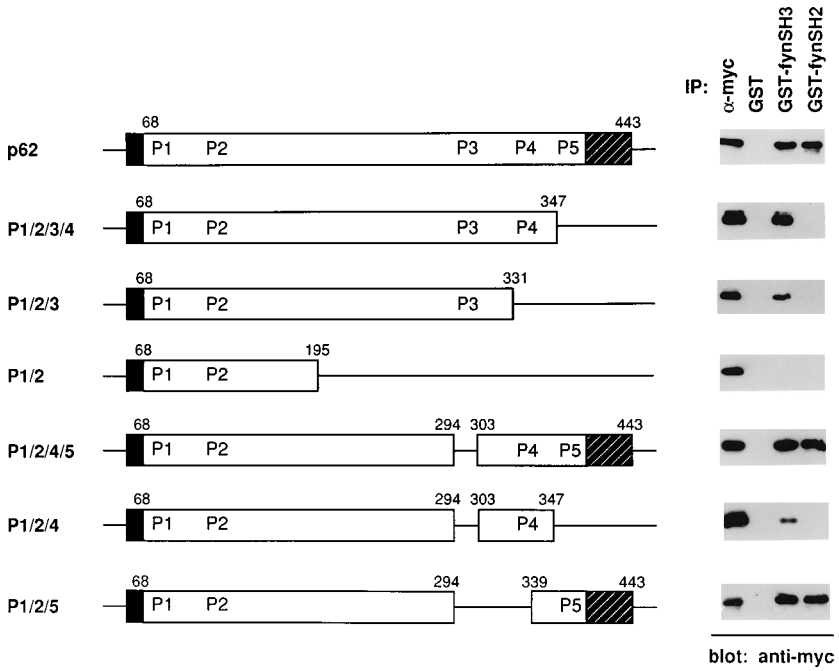

FIG. 5. Analysis of the SH3- and SH2-domain-binding sites in the p62 protein. A schematic diagram representing the various p62-myc constructs used is shown on the left. P1, P2, P3, P4, and P5 represent the five putative SH3-domain-binding proline motifs underlined in Fig. 1. The hatched box denotes the tyrosine-rich sequences, and the solid black box represents the myc tag. Each plasmid construct was cotransfected in HeLa cells with the plasmid encoding p59^{fyn}. The cells were lysed as described in Materials and Methods and immunoprecipitated (IP) with anti-myc antibodies (α-myc) or incubated with GST, GST-fynSH3, or GST-fynSH2 covalently coupled to Sepharose beads. The bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-myc antibodies.

(Fig. 6A, lane 3). This finding was not a result of lower kinase activity, since the mutation of the p59^{fyn} SH3 domain did not affect the tyrosine kinase activity in vitro (data not shown), nor was it due to lower levels of expression of p59^{fyn} or p62 (Fig. 6B and C). Apparently, interactions with the SH3 domain of p59^{fyn} are necessary and precede tyrosine phosphorylation of p62. The protein encoded by the fyn:mSH2 construct phosphorylated p62 to levels comparable with those of wild-type p59^{fyn} (data not shown). In agreement with our findings, other groups have also recently reported that mutations in the SH3 domain of src impaired phosphorylation of p62 (77, 84).

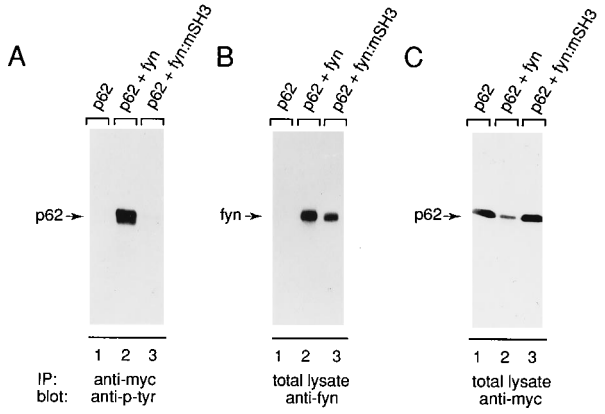

FIG. 6. The SH3 domain of p59^{fyn} is required for the tyrosine phosphorylation of p62 by p59^{fyn}. (A) DNA encoding p62-myc was transfected alone (lane 1) or cotransfected with p59^{fyn} (lane 2) or with p59^{fyn} containing a mutation in the SH3 domain (lane 3). The cells were lysed and immunoprecipitated with anti-myc antibodies. The bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine (anti-p-tyr) antibodies. The position of p62-myc is shown on the left. (B and C) Anti-p59^{fyn} and anti-myc immunoblots of total lysates from cultures prepared in duplicate with those used for panel A.

Association of Grb2 with p62. The sequence of the third proline motif (P3), PPPPPVPR, is similar to that of the Grb2 SH3-domain-binding site, PPPVPPR (42, 65), found in the guanine nucleotide exchange protein mSOS, which suggested that p62 might associate with Grb2. We were unable to detect any association between p62-myc and Grb2 in the absence of p59^{fyn} (data not shown). However, when p62-myc was coexpressed with p59^{fyn}, p62-myc was detected in Grb2 immunoprecipitates, demonstrating that tyrosine-phosphorylated p62-myc could associate with endogenous Grb2 molecules in HeLa cells (Fig. 7A, lane 4). Similar results were obtained when cDNAs for Grb2, p62-myc, and p59^{fyn} were simultaneously expressed in HeLa cells (Fig. 7A, lane 5). The converse was also true; anti-myc immunoprecipitates contained Grb2 (Fig. 7A, lane 11). Immunoblotting of whole-cell lysates confirmed that the expression levels of p62-myc, Grb2, and p59^{fyn} were equivalent (Fig. 7B). The requirement for p59^{fyn} to reconstitute the Grb2-p62 interaction suggested that the interaction was mediated by the SH2 domain of Grb2 rather than the SH3 domain as initially suspected.

To confirm this hypothesis and also to define the interaction between p62 and Grb2 in more detail, the Grb2 N-terminal SH3 domain, the Grb2 C-terminal SH3 domain, and the Grb2 SH2 domain were expressed as GST fusion proteins, purified, and covalently coupled to Sepharose beads. Each of the fusion proteins was incubated with cellular lysates prepared from HeLa cells coexpressing p62-myc and p59^{fyn}. p62-myc bound only to the bacterial fusion protein containing the SH2 domain of Grb2 (Fig. 7C, lanes 1 to 5), confirming our hypothesis that Grb2-p62 complexes were mediated by the Grb2 SH2 domain. This was a specific interaction, since the C-terminal SH2 domain of the 85-kDa subunit of PI-3 kinase could not bind

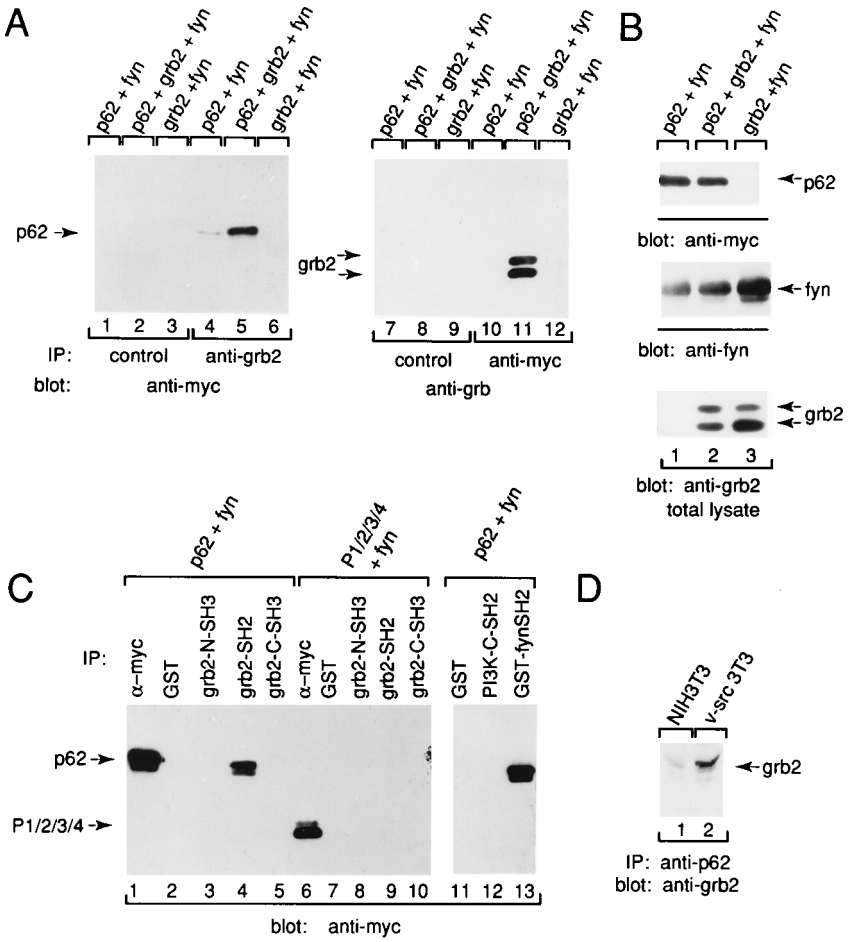

FIG. 7. p62 associates with Grb2. (A) HeLa cells were cotransfected with DNAs encoding $p59^{fyn}$ and p62-myc (lanes 1, 4, 7, and 10), p62-myc plus Grb2 (lanes 2, 5, 8, and 11), or Grb2 alone (lanes 3, 6, 9, and 12). Cells were lysed, and immunoprecipitates (IP) were prepared with normal rabbit antiserum (control; lanes 1 to 3), anti-Grb2 antibodies (lanes 4 to 6), a control antibody (mouse immunoglobulin G1; lanes 7 to 9), or anti-myc antibodies (lanes 10 to 12). The associated proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-myc (lanes 1 to 6) or anti-Grb2 (lanes 7 to 12) antibodies. The positions of p62-myc and Grb2 are indicated on the left. (B) Total lysates corresponding to the cell lysates from panel A were separated by SDS-PAGE and immunoblotted with anti-myc, anti-$p59^{fyn}$, and anti-Grb2 antibodies. The positions of p62-myc, $p59^{fyn}$, and Grb2 are indicated on the right. (C) The N-terminal SH3, the SH2, and the C-terminal SH3 domains of Grb2 were expressed as GST bacterial fusion proteins as described in Materials and Methods. HeLa cells expressing both p62-myc and $p59^{fyn}$ (lanes 1 to 5 and 11 to 13) or P1/2/3/4 and $p59^{fyn}$ (lanes 6 to 10) were lysed, divided equally, and immunoprecipitated with anti-myc antibodies (lane 1 and 6) or incubated with GST (lanes 2 and 7), Grb2–N-SH3 (lanes 3 and 8), Grb2-SH2 (lanes 4 and 9), and Grb2–C-SH3 (lanes 5 and 10) coupled to beads. In addition, cell lysates prepared from HeLa cells expressing p62-myc and $p59^{fyn}$ were divided equally and incubated with GST (lane 11), a GST fusion protein containing the C-terminal SH2 domain of PI 3-kinase (PI3K-C-SH2; lane 12), or GST-fynSH2 (as a positive control; lane 13) covalently coupled to Sepharose beads. Bound proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-myc antibodies. The positions of p62-myc and P1/2/3/4 are shown. (D) NIH 3T3 and v-*src*-transformed cells were immunoprecipitated with p62 antibodies. Immunoprecipitates were separated and analyzed for the presence of Grb2 by immunoblotting.

phosphorylated p62-myc (Fig. 7C, lane 12). Because the p62-myc mutant P1/2/3/4, lacking the tyrosine-rich carboxy-terminal domain, was unable to bind any of the Grb2 fusion proteins, the Grb2 SH2 domain likely interacts with a phosphotyrosine at the C terminus of p62 (Fig. 7C, lanes 6 to 10). Further mapping studies demonstrated that the SH2 domain of $p59^{fyn}$ and the SH2 domain of Grb2 interacted with distinct regions in the C-terminal region of p62 (63a). The interaction between the Grb2 SH2 domain and p62 would still allow the Grb2 SH3 domains to interact with the $p21^{ras}$ guanine nucleotide exchange factor SOS (16, 28, 42, 65).

We next sought to determine whether p62 and Grb2 interacted in vivo. As p62 becomes heavily tyrosine phosphorylated in NIH 3T3 cells after transformation with v-*src* (Fig. 9B, lane 4), we immunoblotted p62 immunoprecipitates from v-*src*-transformed and nontransformed NIH 3T3 cells with antibodies to Grb2. Although similar amounts of p62 were present in immunoprecipitates from the wild-type and transformed cells (Fig. 9B), only the p62 immunoprecipitate from the v-*src*-transformed cells contained Grb2 (Fig. 7D, lane 2). Immunoprecipitates with normal rabbit serum were negative for the presence of Grb2 (data not shown). Thus, the presence of Grb2-p62 complexes correlates with the phosphorylation of p62 and cellular transformation. It seems likely that the many stimuli known to cause p62 tyrosine phosphorylation will initiate the assembly of Grb2-p62 complexes.

To confirm that the Grb2-p62 interaction was direct, we expressed p62-myc alone, $p59^{fyn}$ alone, or both p62-myc and $p59^{fyn}$ in HeLa cells. Cell lysates were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with a purified fusion protein that contains the entire Grb2 molecule fused to GST. The membrane was then immunoblotted with polyclonal anti-Grb2 antibodies (Fig. 8A). Binding of Grb2 to p62-myc was detected only in lysates from

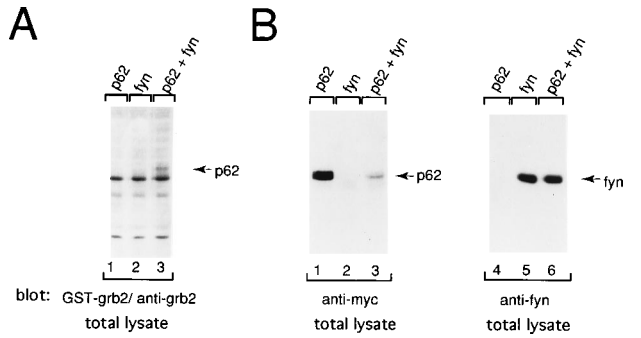

FIG. 8. Direct binding of Grb2 to phosphorylated p62. (A) HeLa cells expressing p62-myc alone (lane 1), p59^{fyn} alone (lane 2), and both p59^{fyn} and p62-myc (lane 3) were lysed in sample buffer. The total lysate was separated by SDS-PAGE, transferred to nitrocellulose, and incubated with GST-Grb2. Binding of Grb2 was detected by immunoblotting with polyclonal antibodies to Grb2. The position of p62 is indicated on the left. (B) Identical lysates were separated by SDS-PAGE and immunoblotted with anti-myc (lanes 1 to 3) and anti-p59^{fyn} (lanes 4 to 6) antibodies. The positions of p62-myc and p59^{fyn} are indicated on the right.

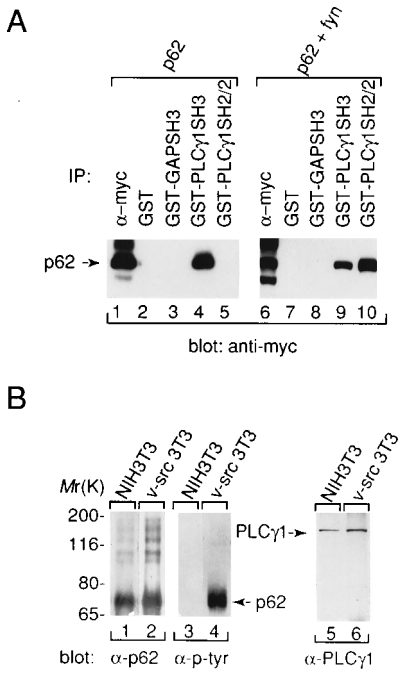

FIG. 9. p62 associates with PLCγ-1. (A) HeLa cells expressing p62-myc (lanes 1 to 5) or coexpressing p62-myc and p59^{fyn} (lanes 6 to 10) were lysed, and the lysates were divided equally and immunoprecipitated (IP) with anti-myc antibodies (lane 1 and 6) or incubated with GST (lanes 2 and 7), GST-GAPSH3 (lanes 3 and 8), GST-PLCγ1SH3 (lanes 4 and 9), and GST-PLCγ1SH2SH2 (lanes 4 to 10) covalently coupled to beads. The associated proteins were resolved on an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with anti-myc antibodies. (B) NIH 3T3 (lanes 1, 3, and 5) and v-*src*-transformed NIH 3T3 (lanes 2, 4, and 6) cells were lysed as described in Materials and Methods and immunoprecipitated with anti-p62 rabbit polyclonal antibodies. The bound proteins were separated on an SDS–10% polyacrylamide gel and immunoblotted with either anti-p62 (α-p62; lanes 1 and 2), antiphosphotyrosine (α-p-tyr; lanes 3 and 4), or anti-PLCγ-1 (α-PLCγ1; lanes 5 and 6) antibodies.

cells coexpressing p62 and p59^{fyn} (Fig. 8A). These findings demonstrate that the interaction of Grb2 with p62 is direct and requires phosphorylation by p59^{fyn}. Expression of p59^{fyn} and p62 was confirmed by anti-p59^{fyn} and anti-myc immunoblotting (Fig. 8B).

Association of SH3 and SH2 domains of PLCγ-1 with p62. The fact that p62 was tyrosine phosphorylated and binds p59^{fyn} as well as Grb2 suggested that p62 might be acting as an adapter protein linking p59^{fyn} to multiple downstream effector molecules containing SH3 and SH2 domains. We reasoned that one function of p62 might be to act like IRS-1 (73) or the polyomavirus middle T protein (11, 13), which are tyrosine kinase substrates that control signalling pathways by recruiting molecules with SH2 domains. The presence of multiple potential SH3-domain-binding sites in p62 suggested that p62 might also function to recruit SH3-domain-containing signalling proteins.

We focused on GAP and PLCγ-1, since both are known to associate with p62 in vivo. Lysates prepared from HeLa cells expressing p62-myc or coexpressing p59^{fyn} and p62-myc were divided equally and incubated with anti-myc antibodies or with GST fusion proteins containing the SH3 domain of GAP or PLCγ-1, or the SH2 domains of PLCγ-1. p62-myc expressed alone bound to the fusion protein containing the SH3 domain of PLCγ-1 but not the SH3 domain of GAP or the SH2 domains of PLCγ-1 (Fig. 9A, lanes 1 to 5). These findings suggest that unphosphorylated p62 can interact with PLCγ-1 in an SH3 domain-dependent mechanism. When p62-myc was coexpressed with p59^{fyn} and tyrosine phosphorylated, the fusion protein containing both SH2 domains of PLCγ-1 also bound p62-myc but not fusion proteins containing either the N-terminal or C-terminal SH2 domain alone (Fig. 9A, lanes 6 to 10, and data not shown). These findings are in agreement with similar results reported by Maa et al. (44). The amino-terminal SH2 domain of GAP also bound to phosphorylated p62 as previously reported (reference 47 and data not shown). These data demonstrate that PLCγ-1 can interact with p62 via both its SH3 and SH2 domains and that GAP interacts with p62 only via its SH2 domain.

These results suggested that p62 complexes with PLCγ-1 might occur in vivo when p62 contains little or no detectable tyrosine phosphorylation. This hypothesis was tested by immunoblotting p62 immunoprecipitates from wild-type NIH 3T3 cells and v-*src*-transformed 3T3 cells with antibodies against PLCγ-1. As expected, PLCγ-1 was detected in p62 immunoprecipitates from both wild-type and transformed NIH 3T3 cells (Fig. 9B, lanes 5 and 6). The interaction was specific, as normal rabbit serum immunoprecipitates did not contain PLCγ-1 (data not shown). p62 immunoprecipitates from the transformed cells, however, contained approximately twice as much PLCγ-1 as did p62 immunoprecipitates from the wild-type cells (Fig. 9B; compare lanes 5 and 6). Immunoblotting with the p62 antiserum confirmed that both p62 immunoprecipitates contained equivalent amounts of p62, and immunoblotting with the antiphosphotyrosine antibody demonstrated that only the transformed cells contained detectable tyrosine-phosphorylated p62 (Fig. 9B, lanes 1 to 4). Therefore, PLCγ-1–p62 complexes are preassembled in the unstimulated cells and probably mediated via SH3 domain interactions. The avidity of these complexes is enhanced by the tyrosine phosphorylation of p62, which probably increases the affinity of the tandem SH3 and SH2 domains of PLCγ-1 for p62.

DISCUSSION

We used the yeast two-hybrid to identify p62 as a protein that binds the SH3 domain of p59^{fyn}. Analysis of p59^{fyn} immunoprecipitates from mammalian cells confirmed that p62 and p59^{fyn} form complexes in vivo. This interaction results in the tyrosine phosphorylation of p62, and complex formation is

mediated by both the SH3 and SH2 domains of $p59^{fyn}$. This interaction with p62 is likely to be a general property of all src family kinases, as we have also detected interactions with $p56^{lck}$ and $p60^{src}$ (data not shown). Interestingly, mutation of the $p59^{fyn}$ SH3 domain blocked tyrosine phosphorylation of p62 in vivo, suggesting that the SH3 domain of $p59^{fyn}$ may facilitate presentation of specific substrates to the kinase. In addition, we suspect that tyrosine phosphorylation of p62 enhances complex formation with $p59^{fyn}$ by binding to the SH2 domain as well as the SH3 domain of $p59^{fyn}$. Since the major SH3-domain-binding site of p62 is located near the carboxy-terminal tyrosine-rich domain, we suspect that p62 binds simultanously to the SH3 and SH2 domains of $p59^{fyn}$. It is likely, therefore, that at least one function of tandem SH3 and SH2 domains may be to target and enhance the affinities of specific protein-protein interactions.

Although little is known about the function of p62, tyrosine phosphorylation of p62 correlates with many biological phenotypes (5, 14, 18). For example, calcium-induced keratinocyte differentiation and stimulation of the T-cell membrane protein CD2, the insulin receptor, and the B-cell antigen receptor all result in the tyrosine phosphorylation of p62 (23, 31, 36, 74). In addition, p62 is phosphorylated after growth factor treatment and is constitutively phosphorylated in transformed cells. More recently, it has been shown that p62 (or a related protein) becomes tyrosine phosphorylated and associates with $p60^{src}$ during mitosis (27, 77). It seems likely that tyrosine phosphorylation of p62 plays an important role in many different signalling pathways.

The human p62 gene was recently cloned (86). Knowledge of the primary sequence, however, did not readily suggest the function of this molecule. Analysis of the deduced amino acid sequence revealed that p62 has sequence identity with a putative RNA-binding protein, GRP-33 (12), and belongs to a novel family of RNA-binding proteins containing what is known as the KH domain (30). p62 can bind RNA in vitro and like other RNA-binding proteins contains dimethylated arginine residues (53). The significance of the RNA-binding properties of p62 in signalling pathways is not known. Examination of the deduced amino acid sequence revealed that the carboxy terminus of p62 contains 16 tyrosines in the last 77 amino acids and is the site of tyrosine phosphorylation (86). In addition, we identified at least five proline-rich motifs in p62 that could represent SH3-domain-binding sites.

Role of tyrosine-phosphorylated p62 in mediating a simultaneous SH3- and SH2-domain-dependent interaction with other proteins. The function of p62 tyrosine phosphorylation may be to promote protein-protein interactions. Pawson and coworkers had shown previously that phosphorylated p62 can bind the SH2 domains of $p21^{ras}$ GAP, $p60^{src}$, and crk in vitro (39, 47). Here, we extend these observations and demonstrate that p62 interacts with the signalling molecules, $p59^{fyn}$, $p56^{lck}$, $p60^{src}$, Grb2, GAP, and PLCγ-1. We found that the association of PLCγ-1 with p62 was mediated by the SH3 domain of PLCγ-1 and that PLCγ-1 was constitutively associated with p62 in vivo. Tyrosine-phosphorylated p62 allows the SH2 domains of PLCγ-1 to interact with p62 and probably enhances the stability of these p62-containing complexes. Tyrosine-phosphorylated p62 can also bind the SH2 domains of Grb2 and GAP. Since Grb2 has been shown to link both growth factor receptors and the BCR-abl tyrosine kinase with $p21^{ras}$ (58), it seems likely that it plays a similar role in linking src family tyrosine kinases to $p21^{ras}$.

Analysis of candidate tyrosine phosphorylation sites in p62 did not reveal any obvious consensus sites as defined by Songyang et al. (71). Our ability to reconstitute interactions of p62 with the SH2 domains of $p59^{fyn}$ and PLCγ-1 or Grb-2 both in vivo and in vitro suggests that the affinities are high and probably physiologically significant. These interactions were also specific, as, for example, we could not demonstrate association of the SH2 domains of the p85 subunit of the PI 3-kinase with tyrosine-phosphorylated p62. It is possible that multiple closely spaced phosphotyrosines affect the recognition and affinity of SH2 domains for specific phosphotyrosine residues or that some of the true physiological ligands for SH2 domains differ from those described by Songyang et al. (71).

The SH3-domain-binding sites of p62. Current hypotheses favor a role for the SH3 domain in mediating protein-protein interactions. Using the abl SH3 domain, Baltimore and coworkers screened bacterial expression libraries and identified the first two SH3-domain-binding proteins, 3BP-1 and 3BP-2 (6, 62). Analysis of the 3BP-1 and 3BP-2 binding sites to the abl SH3 domain suggested that a proline-rich motif, PXXPPPψXP (X is any residue, and ψ is a hydrophobic residue), was the ligand for the abl SH3 domain (62). The first of the five p62 proline motifs, P1 (PRPPPLLPP), is the most similar to the proposed consensus sequence but was unable to bind the SH3 domain of $p59^{fyn}$. The $p59^{fyn}$ SH3 domain apparently has a binding specificity that is distinct from that of the abl SH3 domain. Specificity of the $p59^{fyn}$ SH3 domains is also supported by our inability to detect binding of p62 to either of the two Grb2 SH3 domains or the SH3 domain of GAP. Another SH3 domain that might bind p62 is the SH3 domain of the p85 subunit of PI 3-kinase, since the p85 subunit of PI 3-kinase has been demonstrated to bind p62 (74). The proline motifs that could bind the SH3 domain of $p59^{fyn}$ were P3 (PPPPPVPR), P4 (PPPPTVR), and P5 (PLPPTPAPE). Alignment of these three proline motifs revealed only the minimal sequence motif PPXP, which is consistent with the minimal motif (PXXP) recently reported by Yu et al. (87). Because P5 bound with the highest apparent affinity, some feature of the P5 proline motif or closely surrounding residues is probably important for $p59^{fyn}$ SH3 domain binding. We suspect that other SH3-domain-containing proteins with different specificities will bind to P1, P2, P3, and P4 with higher affinity. Indeed, the SH3 domain of PLCγ-1 binds only P4 (63a). It is interesting that proline motifs P3 and P4 are both flanked by the sequence arginine-glycine, which is the methylation site for a specific methyltransferase (53). As these arginines are probably methylated in vivo (86), it is possible that methylation of these residues regulates SH3 domain binding. Further analysis will be required to map the exact sequences in p62 necessary for binding the SH3 domain of $p59^{fyn}$.

At least one role of the SH3 domain in src family kinases may be to facilitate substrate recognition by the kinase domain. Phosphorylation of p62 by $p59^{fyn}$ in vivo required the SH3 domain of $p59^{fyn}$. When the SH3 domain of $p59^{fyn}$ was mutated, few to no tyrosine-phosphorylated residues were detected in p62. This finding suggests that binding of the $p59^{fyn}$ SH3 domain to p62 is required and may facilitate presentation of the tyrosine rich C-terminal domain of p62 to the $p59^{fyn}$ kinase domain. This may explain why p62 is an excellent substrate for src family kinases in vivo and in vitro (data not shown). In addition, association of the SH3 domains of PLCγ-1 might enhance the ability of src family kinases to phosphorylate this protein when src family kinases are activated.

Another role of the SH3 domain may be to stabilize or fine-tune the specificity of SH2 domain binding. Studies of the affinity of specific SH2 domains toward a variety of different phosphotyrosine-containing peptides demonstrate a wide range of affinities ranging up to 10^{-9} M. In addition, these studies demonstrate that the dissociation rate of SH2 domains

from their specific ligands is extremely rapid (21). It is possible that associated SH3 domains may contribute to SH2 domain specificity by enhancing the stability of complexes by reducing the dissociation rates of SH2 domains or by enhancing lower-affinity interactions.

A model for p62 function. Activation of receptor tyrosine kinases such as the epidermal growth factor, platelet-derived growth factor, colony-stimulating factor 1, and fibroblast growth factor receptors results in the phosphorylation of multiple tyrosine residues in their cytoplasmic domains (67). Each phosphotyrosine is the site of assembly for a distinct SH2-domain-containing signalling molecule (78). Examples of such molecules include PLCγ-1, p21^{ras} GAP, PI 3-kinase, PTP1-d, p60^{src}, and Grb2. This system allows a single event, receptor autophosphorylation, to recruit a diverse number of signalling pathways that are related directly to the number and types of phosphorylated tyrosine residues present. The presence of similar and distinct tyrosine residues also allows for different receptors to transduce both common and unique signals. One exception appears to be the insulin receptor, since the receptor autophosphorylation sites are not thought to mediate significant recruitment of SH2-domain-containing molecules (85). Rather, this recruitment function appears to be fulfilled by IRS-1, the major tyrosine-phosphorylated substrate of the insulin receptor (73). The common theme, therefore, is that tyrosine kinase signalling is mediated by tyrosine phosphorylation of a scaffold protein that recruits various SH2-domain-containing signalling proteins. This scaffold may be provided either by the receptor cytoplasmic domain itself or by a substrate molecule like IRS-1.

src family kinases contain only two tyrosines which can be phosphorylated in vivo (10). Because phosphorylation of one of these tyrosines, at the C terminus of the molecule, represses kinase activity, this residue is likely to be dephosphorylated in the active kinase and therefore is unlikely to be the target of an SH2-domain-containing signalling molecule. The other tyrosine, the major site of autophosphorylation, is required for full activity of the kinase. Whether this is due to a local conformational effect or due to recruitment of SH2-domain-containing proteins is not known. The presence of only one phosphotyrosine suggests that signalling pathways stimulated by recruitment of SH2-domain-containing proteins to this site would be limited. It seems more likely, therefore, that a src family kinase substrate analogous to IRS-1 may play an important role in src family kinase signalling. One such protein may be the polyomavirus middle T antigen, which can associate with and be phosphorylated by p60^{src} (13, 76). Tyrosine-phosphorylated middle T antigen then allows the recruitment and assembly of PI 3-kinase (11) and Grb2/Shc (13). Although there is no known cellular homolog of polyomavirus middle T antigen, we believe that p62 may serve a similar function. The ability of p62 to bind both the SH2 and SH3 domains of src family kinases and PLCγ-1 and the close localization of these binding sites in p62 suggest that p62 is a physiological substrate for proteins containing tandem SH2 and SH3 domains.

Earlier observations of the elevated tyrosine phosphorylation of p62 and the ability of phosphorylated p62 to bind multiple SH2 domains led Koch et al. (39) to postulate that p62 may be a tyrosine-phosphorylated ligand that recruits multiple SH2-domain-containing proteins. In this report, we extend the previous data to include SH3 domains and propose the following model, depicted in Fig. 10. Inactive signalling molecules (e.g., src family tyrosine kinases and PLCγ-1) initially interact with p62 via their SH3 domains. Activation of the src family kinase then results in the tyrosine phosphorylation of p62 and associated substrates like PLCγ-1. Tyrosine phosphorylation of p62 then allows the recruitment of SH2-domain-containing signalling molecules like Grb2 and GAP and, in addition, can also stabilize the association of signalling molecules already associated with p62 like src family tyrosine kinases and PLCγ-1. It is intriguing to speculate that simultaneous engagement of the SH2 and SH3 domains might regulate the enzymatic activities of these proteins.

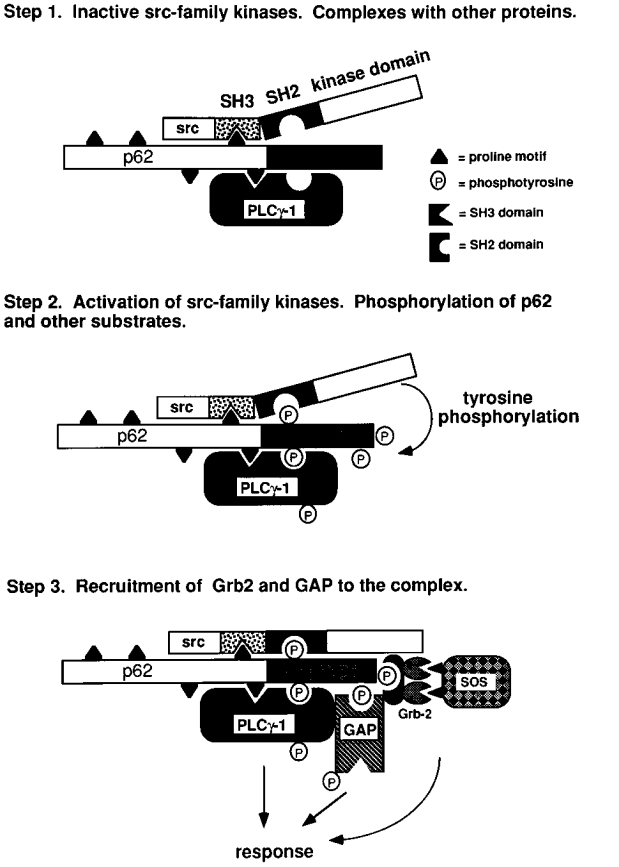

FIG. 10. Proposed model for p62 function. In step 1, p62 associates with the SH3 domains of src family tyrosine kinases in resting cells. This complex also contains PLCγ-1, and the association is mediated by the SH3 domain of PLCγ-1. In step 2, the activation of src family kinases results in tyrosine phosphorylation of p62 and other substrates, including PLCγ-1 and p21^{ras} GAP. In step 3, the phosphorylated p62 becomes the assembly site for Grb2 and p21^{ras} GAP. The SH3 domains of Grb2 are available to bind the guanine nucleotide exchange factor SOS, which ultimately leads to the regulation of p21^{ras}. The phosphorylated p62 can provide sites where several tandemly SH2- and SH3-domain-containing proteins such as PLCγ-1 and src family tyrosine kinases might bind in an SH3- and SH2-domain-dependent mechanism. The association of p62/src family kinase with PLCγ-1, GAP, and Grb2 would ultimately result in signal transmission.

In this report, we have presented data demonstrating that p62 may function as an adapter protein linking p59^{fyn} to PLCγ-1 and the p21^{ras} signalling pathway. The polyproline motifs and the tyrosine phosphorylation sites of p62 are the assembly sites for multiple signalling proteins with tandem SH3 and SH2 domains. Two such candidate proteins for binding in a tandem fashion are src family tyrosine kinases and PLCγ-1. We suspect that p62 serves as a docking site for other tandemly containing SH3- and SH2-domain-containing proteins. However, p62 is not the binding site for all SH3- and SH2-domain-containing proteins, as GAP and Grb2, two proteins known to contain SH3 and SH2 domains, bind p62 solely in an SH2-domain-dependent mechanism. Thus, p62 differs

from the known SH2-domain-recruiting proteins such as IRS-1, the cytoplasmic tail of growth factor receptors, and the polyomavirus middle T antigen in that p62 also recruits SH3-domain-containing proteins with its polyproline-rich motifs. It is important to note that since p62 contains an RNA-binding domain, the SH3 and SH2 domain adapter function that we ascribe to it in this report may not be the only function of this protein.

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