Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 12338–12342, December 1995
Cell Biology

Phosphotyrosine-independent binding of a 62-kDa protein to the src homology 2 (SH2) domain of p56^{lck} and its regulation by phosphorylation of Ser-59 in the lck unique N-terminal region

(serine/threonine kinase/allosteric regulation)

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Contributed by Jack L. Strominger, September 8, 1995

ABSTRACT **A previously undescribed 62-kDa protein (p62) that does not contain phosphotyrosine but, nevertheless, binds specifically to the isolated src homology 2 (SH2) domain of p56^{lck} has been identified. The additional presence of the unique N-terminal region of p56^{lck} prevents p62 binding to the SH2 domain. However, phosphorylation at Ser-59 (or alternatively, its mutation to Glu) reverses the inhibition and allows interaction of the p56^{lck} SH2 domain with p62. Moreover, p62 is associated with a serine/threonine kinase activity and also binds to ras GTPase-activating protein, a negative regulator of the ras signaling pathway. Thus, phosphotyrosine-independent binding of p62 to the p56^{lck} SH2 domain appears to provide an alternative pathway for p56^{lck} signaling that is regulated by Ser-59 phosphorylation.**

In many cell types, mitogenic signals seem to be transduced from cell surface to nucleus by initial activation of plasma-membrane-proximal tyrosine kinases and subsequent activation of down-stream transducers including second messenger production systems and serine/threonine kinase cascades. Studies of receptors for insulin (1) and platelet-derived growth factor (2) showed that structural features that could mediate specific protein–protein interactions, in addition to the receptor tyrosine kinase activity, are crucial in those signal transduction pathways.

p56^{lck} is a member of the c-src family of cytoplasmic tyrosine kinases that is found predominantly in cells of lymphoid origin (3, 4). Multiple functions of p56^{lck} have been described in hematopoietic cell differentiation and activation (for review, see ref. 5). As a src family tyrosine kinase, in addition to a C-terminal catalytic domain [src homology I domain (SH1)], p56^{lck} has a src homology 3 (SH3) domain and a src homology 2 (SH2) domain. SH2 and SH3 domains are small modules that mediate protein–protein interactions between signaling proteins through direct binding to regions containing phosphotyrosine and proline-rich sequences, respectively (6). Thus, multiple functions of p56^{lck} may also be exerted by specific association with certain proteins in different cellular environments utilizing SH2 and SH3 domains of p56^{lck}.

A critical role for the p56^{lck} SH2 domain in T-cell signaling has been demonstrated in that catalytically inactive p56^{lck} is still able to transmit signals for interleukin 2 secretion as long as its SH2 domain remains intact (7). While there is some controversy, *in vitro* binding studies using recombinant proteins showed direct binding of the p56^{lck} SH2 domain to a tyrosine phosphatase CD45 (8) and a tyrosine kinase ZAP-70 (9) through their phosphotyrosine residues. Binding of p56^{lck} with tyrosyl-phosphorylated phospholipase C-γ (10) and p95-vav (11) was also reported although involvement of the SH2 domain in these interactions was not clear. However, whether these associations represent all of the p56^{lck} SH2-domain-mediated protein interactions and how they are engaged in the multiple functions of p56^{lck} are still in question.

In the present studies, a specific and phosphotyrosine-independent interaction of the p56^{lck} SH2 domain with a 62-kDa protein has been identified and its regulation is discussed.

MATERIALS AND METHODS

Cell Culture, Transfection, and Metabolic Labeling. HeLa and CD4$^+$ HeLa cells (12) and Jurkat T cells were maintained in 10% (vol/vol) fetal bovine serum supplemented Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium, respectively. For v-src expression, HeLa cells were transiently transfected with 20 μg of cDNA per 10-cm plate by using the calcium phosphate precipitation method (13). For metabolic labeling, cells were incubated with [^{35}S]methionine (100 μCi/ml; 1 Ci = 37 GBq) in methionine-free DMEM for 1 h.

Site-Directed Mutagenesis, Glutathione *S*-Transferase (GST) Fusion Protein Production, and Protein Precipitation. Site-directed mutagenesis was performed on uracil-containing phage DNA (14) by using the M13 Muta-Gene kit (Bio-Rad). GST fusion proteins were produced as described (15, 16). HeLa cell lysate was prepared and used for GST fusion protein binding as described (15). Phosphatase inhibitors were added as indicated. For the competition assay, the stated amounts of phosphotyrosyl peptides were added to the lysates during incubation. After washing three times with lysis buffer, bound proteins were eluted by boiling in SDS/PAGE loading buffer. After SDS/PAGE, [^{35}S]methionine-labeled proteins on the gel were fluorographed, dried, and visualized by autoradiography. For Western blot analysis, proteins were electrotransferred to nitrocellulose and examined by immunoblot analysis using 4G10 monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse antibody. Signals were developed with enhanced chemiluminescence (Amersham).

RESULTS

Phosphotyrosine-Independent Protein Binding to the p56^{lck} SH2 Domain. [^{35}S]Methionine-labeled CD4$^+$ HeLa cell lysates were used for precipitation with GST fusion proteins of p56^{lck} subdomains (Fig. 1*A*). Each subdomain of p56^{lck} can specifically bind to proteins from this HeLa cell lysate (Fig. 1*B*). Particularly, GST.119–224 (the SH2 domain alone) uniquely

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Abbreviations: GAP, GTPase-activating protein; SH2 and SH3 domains, src homology 2 and 3 domains, respectively; GST, glutathione *S*-transferase; MBP, myelin basic protein.

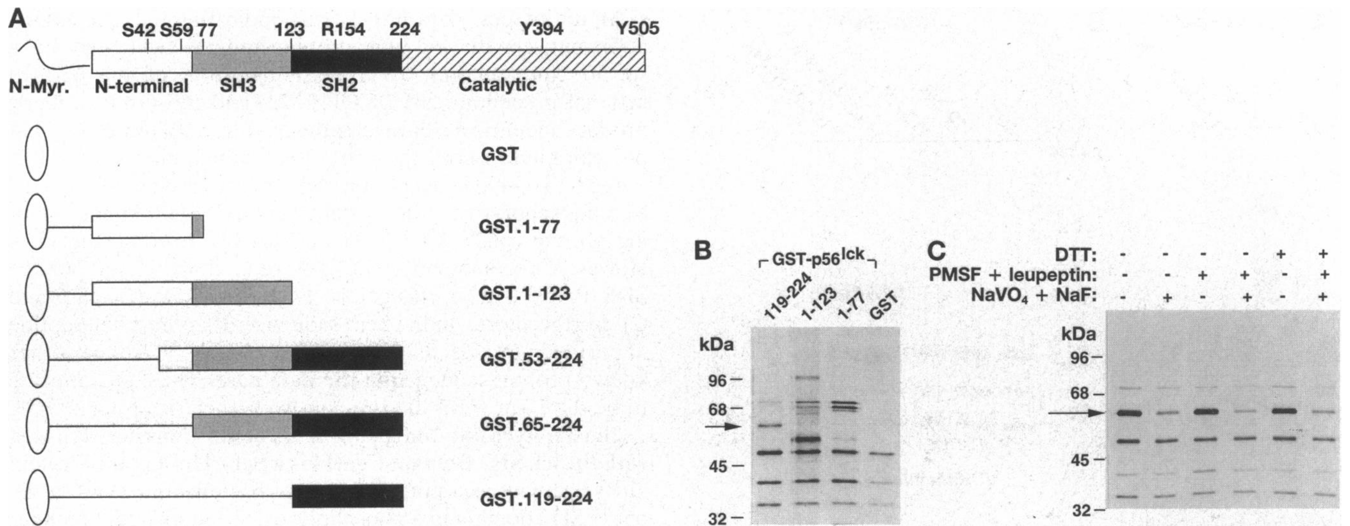

FIG. 1. Phosphotyrosine-independent binding of p62 to lck SH2 domain. (*A*) Construction of GST fusion proteins containing regions of $p56^{lck}$. Myr, myristic acid. (*B*) GST and GST fusion proteins containing the unique N-terminal region (amino acids 1–77), the unique N-terminal region and SH3 domain (amino acids 1–123), and the SH2 domain (amino acids 119–224) were incubated with lysates from [^{35}S]methionine-labeled HeLa cells. Bound proteins were separated on 9% SDS/PAGE gels, fluorographed, and detected by autoradiography. A 62-kDa protein (p62) that bound specifically to the SH2 domain is marked with an arrow. (*C*) [^{35}S]Methionine-labeled HeLa cells were lysed in the presence or absence of phosphatase inhibitors ($NaVO_4$ and NaF), protease inhibitors [phenylmethylsulfonyl fluoride (PMSF) and leupeptin], or reducing reagent [dithiothreitol (DTT)]. The lysates were incubated with GST.119–224, and bound proteins were analyzed by SDS/PAGE.

precipitated a 62-kDa protein (p62) that was not precipitated by any of the other proteins. The binding of p62 to the $p56^{lck}$ SH2 domain was also observed in cell lysate of nonactivated Jurkat T cells (data not shown).

Interestingly, p62 could not be detected by immunoblot analysis using 4G10 anti-phosphotyrosine antibody (see Fig. 4). Furthermore, p62 binding to the SH2 domain was enhanced in cell lysates prepared in the absence of phosphatase inhibitors, sodium vanadate and sodium fluoride, while the binding was insensitive to the lack of protease inhibitors and reducing reagents (Fig. 1*C*). These data suggested that p62 binding to the $p56^{lck}$ SH2 domain is phosphotyrosine-independent.

Cross-Talk Between Phosphotyrosine-Dependent and -Independent Binding to the $p56^{lck}$ SH2 Domain. Two phosphotyrosyl peptides, pY324 and pY505 [derived from polyoma middle-sized tumor antigen (EPQpYEEIPIYL) and from the C-terminal negative regulatory region of $p56^{lck}$ (TEGQpYQPQPA), respectively] bind strongly and specifically to the $p56^{lck}$ SH2 domain (16). These two specific peptides competed with p62 for binding to GST.119–224 at 1 μM pY324 peptide and 15 μM pY505 peptide (Fig. 2). Phosphotyrosyl peptides that bind poorly [pY771 (SSNpYMAPYDNY) and pY536 (ESEpYGNITYPP)], however, did not affect p62 binding to GST.119–224. Thus, phosphotyrosine-independent binding of p62 to the $p56^{lck}$ SH2 domain is interrupted by binding of the phosphotyrosyl peptide to the SH2 domain.

An arginine residue (Arg-154 of $p56^{lck}$) that is conserved in all SH2 domains and is a part of the phosphotyrosine binding pocket (17, 18) was mutated to lysine (GST.119–224.R154K). The mutant did not bind to phosphotyrosyl proteins (Fig. 3*A*). The binding of p62, however, was unaltered in the GST.119–224.R154K protein and was not inhibited by high concentration of pY324 (Fig. 3*B*). These data suggest that p62 binds to a specific site other than the phosphotyrosine-dependent binding site of the SH2 domain.

Effects of Ser-59 Modification on Phosphotyrosine-Independent p62 Binding to the lck SH2 Domain. The Ser-59 phosphorylation site in the unique N-terminal region affects the binding affinity and specificity of the SH2 domain of $p56^{lck}$ for phosphotyrosyl proteins (15, 19). The effect of the Ser-59 phosphorylation site on p62 binding to the $p56^{lck}$ SH2 domain was, therefore, examined by comparing protein binding to GST.119–224 and to GST.53–224, which contains the Ser-59 phosphorylation site (amino acids 53–64). Bound proteins were detected by autoradiography to detect [^{35}S]methionine (Fig. 4*A*) or by Western blot analysis with a 4G10 anti-phosphotyrosine antibody (Fig. 4*B*). As expected, GST.119–224 precipitated a unique set of phosphotyrosyl proteins (pp130 and pp80) from v-src-transfected cell lysate in the presence of phosphatase inhibitors, whereas GST.53–224 precipitated phosphotyrosyl proteins pp70, pp130, and pp80 (15). However, in the absence of phosphatase inhibitors, GST.119–224, but not GST.53–224 or GST alone, strongly bound to ^{35}S-labeled p62 in both v-src-transfected and untransfected cell lysates (Fig. 4*A*). Interestingly, binding of the SH2 domain in GST.53–224 to p62 was restored by truncation of the unique N-terminal region (using GST.65–224, which contains SH3 and SH2 domains only) or by mutation of Ser-59 → Glu (by

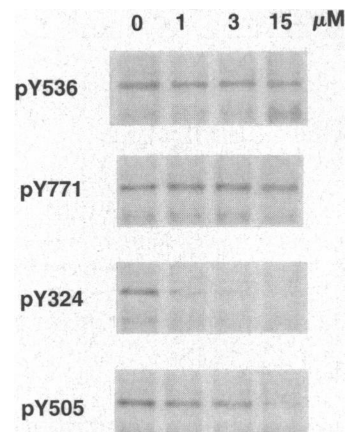

FIG. 2. Phosphotyrosine-independent binding of p62 to the $p56^{lck}$ SH2 domain is competed by specific phosphotyrosyl peptides. [^{35}S]Methionine-labeled HeLa cells were lysed in the presence of phosphatase inhibitors ($NaVO_4$ and NaF). The lysates were incubated with increasing concentrations of phosphotyrosyl peptides pY324, pY505, pY771, and pY536, as indicated. Bound p62 was separated on 9% SDS/PAGE gel, fluorographed, and detected by autoradiography.

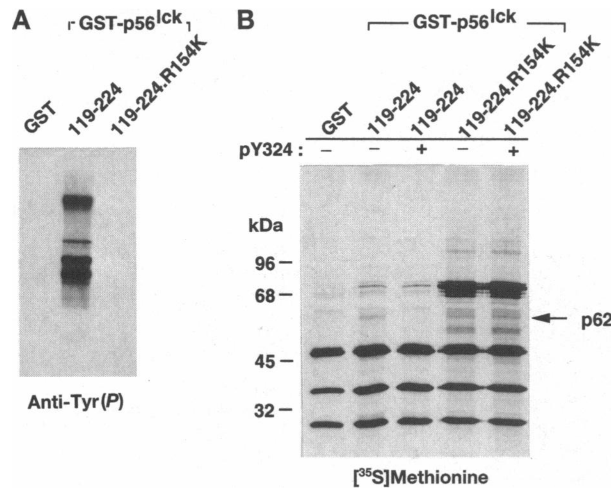

FIG. 3. Distinct mechanisms for phosphotyrosine-dependent and -independent protein binding to the SH2 domain. (*A*) GST alone, GST.119–224, and GST.119–224.R154K were incubated with v-src-transfected HeLa cell lysate in the presence of phosphatase inhibitors. Bound proteins were examined by immunoblot analysis with anti-phosphotyrosine [Tyr(*P*)] antibody. (*B*) GST alone, GST.119–224, and GST.119–224.R154K were incubated with [^{35}S]methionine-labeled HeLa cell lysate in the presence of phosphatase inhibitors. Competition of p62 binding to the SH2 domain by phosphotyrosyl peptide was measured by adding 10 μM pY324 peptide in the incubation mixture. Bound proteins were analyzed by SDS/PAGE.

using GST.53–224.S59E) (compare Fig. 4 *C* to *A*). These data suggest that the phosphotyrosine-independent binding of p62 to the p56^{lck} SH2 domain is also regulated by phosphorylation of Ser-59, for which the S59E mutation is a substitution.

p62 Binds to the p120 ras GTPase-Activating Protein (ras-GAP). A protein of the same molecular mass as p62 (62 kDa) was precipitated by an antiserum raised against p120 ras-GAP but not by control rabbit serum (Fig. 5*A*) or by antibodies against phosphatidylinositol 3-kinase, mitogen-activated protein (MAP) kinase, CD4, or phospholipase C-γ (data not shown). The 62-kDa protein was precipitated by two anti-ras-GAP antibodies (data not shown), indicating that the association between the 62-kDa protein and ras-GAP may be a specific interaction. V8 protease digestion of the 62-kDa proteins precipitated by GST.119–224 and anti-GAP antibody produced identical cleavage patterns (Fig. 5*D*), indicating that p62 can bind to both the p56^{lck} SH2 domain and ras-GAP.

A 62- to 68-kDa phosphotyrosyl protein has been recognized as a phosphotyrosine-dependent ras-GAP SH2 domain binding protein (p62^{GAPbp}) and its cDNA has been cloned (20). However, recombinant p62^{GAPbp} runs slower than p62 on SDS/PAGE and, in this gel, is closer to 68 kDa (Fig. 5 *B* and *C*). Furthermore, amino acid sequence of an internal peptide of purified p62 (Fig. 5*E*) does not match p62^{GAPbp} or any other known protein sequence in the data base. Thus, p62 appears to be different from the previously characterized pp62^{GAPbp}.

Phosphotyrosine-Independent Protein Complex Formed with the lck SH2 Domain Contains a Ser/Thr Kinase. Protein kinase activity as a potential role of proteins that bind to the p56^{lck} SH2 domain in a phosphotyrosine-independent manner was examined. In addition to p62, three additional discrete ^{35}S-labeled protein bands including p160 and two high molecular weight protein bands were sometimes observed in HeLa cell lysate as p56^{lck} SH2 domain binding proteins (Fig. 6*A*, lane 6). When [γ-^{32}P]ATP and kinase reaction buffer were added, the protein complex containing the p56^{lck} SH2 domain and the bound proteins induced phosphorylation of p62, p160, and a few other binding proteins including a 100-kDa common GST binding protein (lane 5). This phosphorylation event was not observed in the GST–protein complex (lanes 1 and 3) or in the GST.SH2–protein complex formed in the presence of $NaVO_4$ and pY324 (lane 7). This kinase activity can also use MBP as an exogenous substrate (Fig. 6*B*) and the kinase activity can be eluted from the protein complex by $NaVO_4$ and pY324 (Fig. 6*C*). Phosphoamino acid analysis of phosphorylated MBP of Fig. 6*B* produced mostly phosphoserine and some phosphothreonine (Fig. 6*D*). The same phosphoamino acid composition was found for endogenous substrates such as p35, p62, p110, and p160 of Fig. 6*A*, lane 5 (data not shown). These results suggest that one of the phosphotyrosine-independent proteins binding to the p56^{lck} SH2 domain is a Ser/Thr kinase.

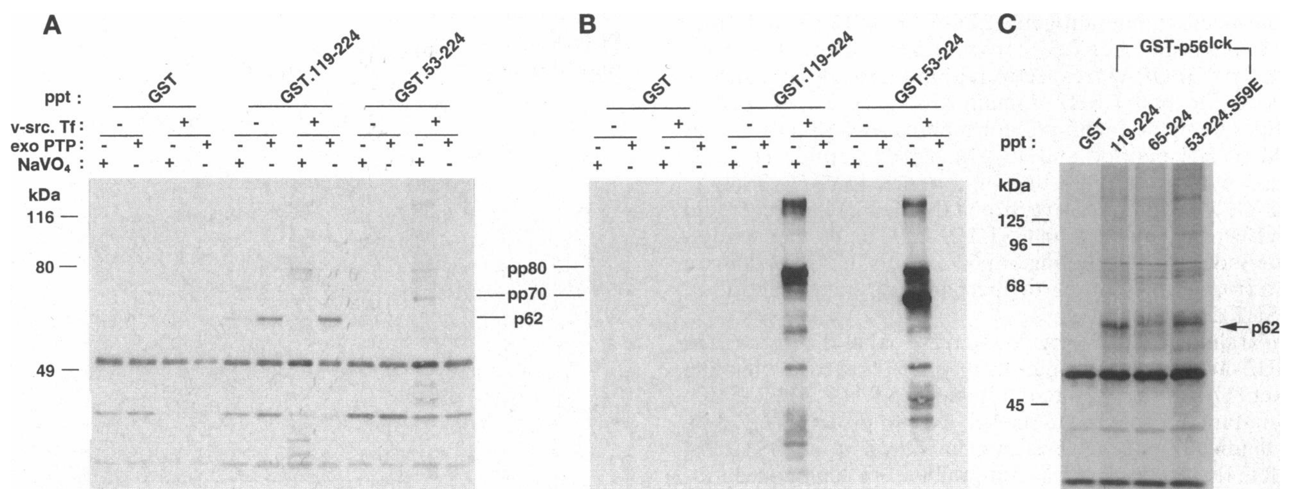

FIG. 4. Regulation of p62 binding to SH2 domain by Ser-59 phosphorylation site of the p56^{lck}. (*A*) HeLa cells transfected with v-src (v-src Tf.) or vector alone were labeled with [^{35}S]methionine and lysed in the presence or absence of phosphatase inhibitors. Samples that were lysed in the absence of phosphatase inhibitors were treated with exogenous recombinant phosphatase mixture (exo PTP, recombinant catalytic fragments of the tyrosine phosphatases LAR, CD45, and SHPTP-1). The lysates were incubated with GST alone, GST.119–224, and GST.53–224. Bound proteins were separated on 8% SDS/PAGE gels, electrotransferred to nitrocellulose, and detected by autoradiography. (*B*) Membrane was examined by immunoblot analysis with anti-phosphotyrosine antibody. p62 and two phosphotyrosyl proteins (pp70 and pp80) are marked. (*C*) Truncation of the Ser-59 region or mutation of Ser-59 → Glu restores p62 binding to the SH2 domain. HeLa cells were labeled with [^{35}S]methionine, lysed in the absence of phosphatase inhibitors, incubated with GST alone, GST.119–224, GST.65–224, and GST.53–224.S59E. Bound proteins were separated on 9% SDS/PAGE gels, fluorographed, and detected by autoradiography.

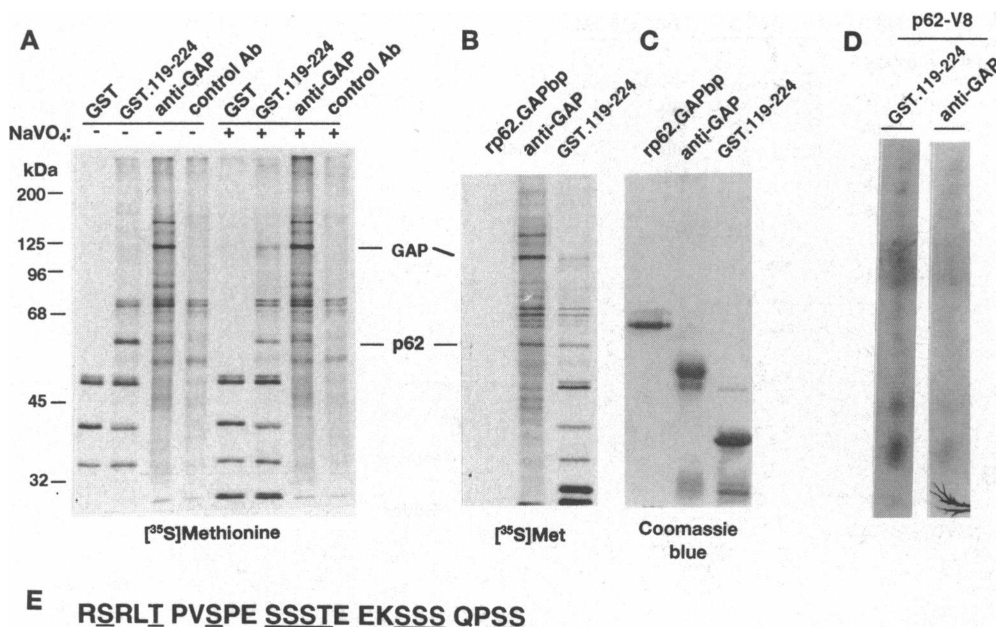

FIG. 5. p62 binds to p120 ras-GAP. (*A*) A protein that comigrates with p62 is coimmunoprecipitated by ras-GAP. [^{35}S]Methionine-labeled HeLa cells were lysed in the presence or absence of phosphatase inhibitors. The lysates were incubated with GST alone or with GST.119–224. Alternatively, the lysates were immunoprecipitated with anti-GAP antibody or with a preimmune serum. Bound proteins were separated on 9% SDS/PAGE gels, fluorographed, and detected by autoradiography. (*B* and *C*) Recombinant p62 GAP binding protein (rp62^{GAPbp}) was electrophoresed on SDS/PAGE gels along with GST.119–224 and ras-GAP binding proteins of Fig. 6*A*. Proteins were detected both by autoradiography (*B*) and by Coomassie blue staining (*C*). The prominent bands in *C* are rp62^{GAPbp} (lane 1), antibody (lane 2), and fusion protein (lane 3). (*D*) V8 partial digestions of p62 bound to GST.119–224 and ras-GAP. [^{35}S]Methionine-labeled p62 protein bands from Fig. 6*B* were excised and partially digested in the second-dimensional 15% SDS/PAGE gel. (*E*) Amino acid sequence of a Lys-C-digested peptide of purified p62. p62 was purified from a 200-liter HeLa cell culture by using GST.119–224 affinity column, separated on 8% SDS/PAGE gels, and electrotransferred to poly(vinylidene difluoride) membrane. The p62 band was excised from the blot. The p62 was digested with Lys-C. The sequence of one of the separated peptides is shown; underlined residues are hydroxyamino acids.

The GST.SH2–protein complex (the same as Fig. 6*A*, lane 5) was separated by SDS/PAGE on a gel that was polymerized in the presence of MBP. Proteins on the gel were renatured and the location of kinase activity was measured (Fig. 6*E* and ref. 21). GST itself did not bring down any MBP kinase activity. However, GST.SH2 associated with an MBP kinase activity with migration the same as p62, and the kinase activity at the 62-kDa position was partially eluted by the competing peptide pY324. Thus p62 itself or a protein with similar molecular mass appears to be a Ser/Thr protein kinase, indicative of its potential role in a kinase cascade distinct from pathways initiated by binding of phosphotyrosine proteins.

DISCUSSION

The phosphotyrosine-independent binding of proteins to the p56^{lck} SH2 domain suggests another class of protein–protein interactions mediated by SH2 domains. This phenomenon may be related to the binding of the serine-rich regions of the N-terminal BCR domain intramolecularly with the SH2 domain of BCR–abl (22) and of serine-rich regions of Raf-1 and p130PITSLRE with the SH2 domains of src/fyn and blk, respectively (23, 24). These bindings were independent of phosphotyrosine but required phosphorylation of serine residues (22–24). However, p62 interaction with the p56^{lck} SH2 domain does not appear to require serine phosphorylation, as shown by reduced binding in the presence of phosphatase inhibitors (Fig. 1*C*).

The binding of the SH2 domain, a small module composed of ≈100 amino acids (6), to proteins in two different ways requires efficient use of the accessible surface. Competition between p62 and specific phosphotyrosyl-peptide binding to the p56^{lck} SH2 domain (Fig. 2) indicates that occupation of one of these protein binding sites excludes binding to the other site. Mechanisms for this exclusion include (*i*) the use of a single binding site or two adjacent sites for these two types of protein interaction resulting in steric hindrance induced by the binding of one ligand or (*ii*) the allosteric alteration of one site by the occupation of the other. Although the possibility of a single binding site has not been excluded, the observation that GST.53–224 binds tightly to phosphotyrosyl proteins but not to p62 (Fig. 4) indicates that phosphotyrosine-independent binding may use a site other than the phosphotyrosine binding pocket. Binding of GST.SH2.R154K, which has a dysfunctional phosphotyrosine binding pocket, to p62 (Fig. 3) suggests that these two binding modes of the SH2 domain have different binding mechanisms if not separate binding sites. In any case, competition between phosphotyrosyl peptides and p62 for the p56^{lck} SH2 domain permits only one of these two binding sites to be used at any given time, thus allowing the maintenance of two binding sites on such a small domain.

The C-terminal pY505 suppresses the catalytic activity through intramolecular interaction with the SH2 domain of p56^{lck} (3, 5). During T-cell activation, the C-terminal Tyr-505 is dephosphorylated, freeing the phosphotyrosine binding pocket of the SH2 domain, and Ser-59 undergoes transient phosphorylation after the activation of mitogen-activated protein (MAP) kinase (unpublished results). Since the binding of p62 to the p56^{lck} SH2 domain is sensitive both to Ser-59 phosphorylation (Fig. 4) and to phosphotyrosyl peptide binding (Fig. 2), interaction of p62 and SH2 domain in full-length p56^{lck} would be likely to occur at the time when Tyr-505 is dephosphorylated and Ser-59 is phosphorylated. Since MAP kinase activation precedes Ser-59 phosphorylation, the phosphotyrosine-independent binding of the p56^{lck} SH2 domain may be involved in regulation of later stages of signal transduction.

We thank Drs. Insil Joung, David Winkler, and Lesley Stolz for helpful advice and Steven Shoelson for providing phosphotyrosyl peptides. This work was supported by National Institutes of Health

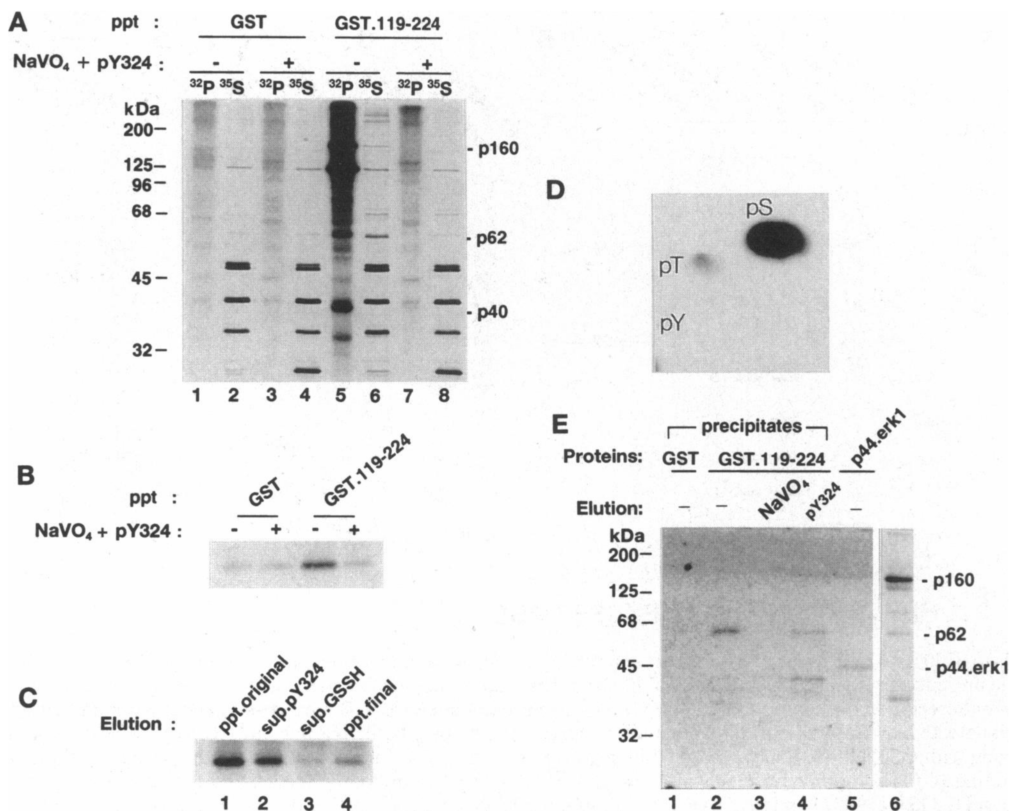

FIG. 6. Ser/Thr kinase associated with the lck SH2 domain by the phosphotyrosine-independent interaction. (*A*) [^{35}S]Methionine-labeled HeLa cells were lysed in the presence or absence of phosphatase inhibitors and competing peptide pY324. The lysates were incubated with GST alone or with GST.119–224. Bound proteins were separated on 9% SDS/PAGE gels, fluorographed, and detected by autoradiography (lanes 2, 4, 6, and 8). Kinase activity was also measured by incubating the bound proteins with kinase buffer and [γ-^{32}P]ATP (lanes 1, 3, 5, and 7). (*B*) Sample aliquots of *A*, lanes 2, 4, 6, and 8, were incubated with kinase buffer, [γ-^{32}P]ATP, and myelin basic protein (MBP) as exogenous substrate. MBP was separated on 12% SDS/PAGE gels, and its phosphorylation was visualized by autoradiography. (*C*) MBP kinase activity (lane 1) was sequentially eluted with competing pY324 peptide (lane 2) and then with glutathione (GSSH) (lane 3) from glutathione-agarose bound to GST.119–224 and its associated proteins (part of the sample shown in *A*, lane 6, was used). (*D*) Phosphoamino acid analysis of phosphorylated MBP of *B*. pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine. (*E*) GST and GST.119–224 bound proteins in HeLa cell lysates were prepared in the absence of $NaVO_4$ (lanes 1 and 2, respectively). GST.119–224-bound proteins were eluted with $NaVO_4$ (lane 3) or with pY324 peptide (lane 4). Samples were separated on a MBP-containing gel and were subjected to kinase assay (21). For a positive control, 0.5 μg of purified p44.erk1 (Upstate Biotechnology, Lake Placid, NY) was used (lane 5). The sample of an *in vitro* kinase assay as described in *A*, lane 5, was separately electrophoresed on a SDS/PAGE gel (lane 6) and compared with the in-gel kinase assay.

Research Grants GM48961 (J.S.), CA47554 (J.L.S.), and GM20011 (C.T.W.).

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