Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10696–10700, December 1991
Biochemistry

Selective binding of activated pp60$^{c\text{-}src}$ by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60$^{c\text{-}src}$

(Rous sarcoma virus/transformation/phosphotyrosine/SH2 domain)

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Communicated by John M. Buchanan, September 4, 1991

ABSTRACT **Phosphorylation of the carboxyl terminus of pp60$^{c\text{-}src}$, the product of the c-*src* protooncogene, at Tyr-527 suppresses its tyrosine kinase activity and transforming potential. It has been proposed that the phosphorylated carboxyl terminus of pp60$^{c\text{-}src}$ inhibits kinase activity by binding to the SH2 (src homology 2) domain. We have synthesized peptides corresponding to the carboxyl-terminal 13 residues of pp60$^{c\text{-}src}$ phosphorylated and nonphosphorylated at Tyr-527. A highly transforming mutant, pp60$^{c\text{-}src(F527)}$, in which Tyr-527 is mutated to Phe, bound to the phosphorylated peptide immobilized to Affi-Gel 10. Binding of the phosphorylated peptide was abolished by deletion of residues 144–175 in the SH2 domain but not by deletion of residues 93–143, which removes most of the SH3 domain. The phosphorylated peptide also bound to pp60$^{v\text{-}src}$, the transforming protein of Rous sarcoma virus. Only traces of pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src(F527)}$ bound to the corresponding nonphosphorylated c-src peptide. Normal pp60$^{c\text{-}src}$ bound much less efficiently to the phosphorylated peptide than did pp60$^{c\text{-}src(F527)}$. A phosphorylated peptide corresponding to the carboxyl terminus of the c-fgr protein also bound to pp60$^{c\text{-}src(F527)}$, but with weaker affinity. Furthermore, the phosphorylated synthetic carboxyl-terminal pp60$^{c\text{-}src}$ peptide markedly inhibited phosphorylation of pp60$^{c\text{-}src(F527)}$ during cytoskeletal kinase assays. These results provide direct evidence for models in which the phosphorylated carboxyl terminus of pp60$^{c\text{-}src}$ binds intramolecularly or intermolecularly to the SH2 domain of the c-src protein.**

The product of the c-*src* protooncogene, pp60$^{c\text{-}src}$, is the normal cellular counterpart of the transforming protein of Rous sarcoma virus, pp60$^{v\text{-}src}$ (1, 2). The major site of tyrosine phosphorylation of pp60$^{c\text{-}src}$ is located near its carboxyl terminus (3, 4). The phosphorylated residue, Tyr-527 (3), has been implicated as a site of negative regulation (3–15). Phosphorylation of pp60$^{c\text{-}src}$ at Tyr-527 suppresses its tyrosine kinase activity and transforming potential (5–15), whereas phosphorylation at Tyr-416 contributes to the enhanced kinase activity and transforming properties of activated pp60$^{c\text{-}src}$ mutants (8, 9, 14, 16, 17). Evidence in support of a regulatory role for Tyr-527 phosphorylation includes the observation that the kinase activity of pp60$^{c\text{-}src}$ is stimulated by enzymatic dephosphorylation or by mutation of Tyr-527 (5, 6, 8–14). The mechanism by which carboxyl-terminal phosphorylation suppresses pp60$^{c\text{-}src}$ kinase activity and its transforming ability is not known. It has been proposed that the phosphorylated carboxyl terminus of pp60$^{c\text{-}src}$ may regulate its activity by binding to other regions of the c-*src*-encoded protein, possibly acting as an allosteric effector, pseudosubstrate inhibitor, or product analog inhibitor (6, 15, 18–23).

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pp60$^{c\text{-}src}$ contains two domains, SH2 (src homology 2, residues 140–250) and SH3 (src homology 3, residues 88–139), that are highly conserved among nonreceptor tyrosine kinases (boxes A, B, and C) (23–25). The finding that the v-*crk*-encoded protein SH2 domain, as well as bacterially expressed SH2 constructs, binds to phosphotyrosine-containing proteins (19, 26–29) has suggested that the SH2 domain may regulate the activity of normal pp60$^{c\text{-}src}$ by binding to its phosphorylated carboxyl terminus (19–22).

We have now synthesized phosphorylated and nonphosphorylated peptides modeled on the carboxyl terminus of pp60$^{c\text{-}src}$ and tested them for the ability to bind to normal pp60$^{c\text{-}src}$ and to a highly transforming activated mutant protein, pp60$^{c\text{-}src(F527)}$, in which Tyr-527 is replaced by phenylalanine. We found that the phosphorylated peptide preferentially bound to activated pp60$^{c\text{-}src(F527)}$ but not to a similar protein in which residues 144–175 of the SH2 domain were deleted. In addition, the phosphorylated peptide inhibited phosphorylation of pp60$^{c\text{-}src(F527)}$ during kinase assays conducted on the detergent-insoluble cytoskeletal matrix.

MATERIALS AND METHODS

Peptide Synthesis. The *O*-phosphotyrosine-containing peptide Thr-Ser-Thr-Glu-Pro-Gln-Tyr(*P*)-Gln-Pro-[^{14}C]Gly-Glu-Asn-Leu, which corresponds to the carboxyl terminus of pp60$^{c\text{-}src}$ phosphorylated at Tyr-527, was synthesized by standard Merrifield solid-phase procedures (30, 31). The corresponding nonphosphorylated carboxyl-terminal peptide of pp60$^{c\text{-}src}$ (Thr-Ser-Thr-Glu-Pro-Gln-Tyr-Gln-Pro-[^{14}C]-Gly-Glu-Asn-Leu), and an analog with a tyrosine → phenylalanine substitution (Thr-Ser-Thr-Glu-Pro-Gln-Phe-Gln-Pro-[^{14}C]Gly-Glu-Asn-Leu) were similarly prepared. Phosphorylated and nonphosphorylated peptides modeled on the carboxyl terminus of the c-*fgr*-encoded protein, Thr-Ser-Ala-Glu-Pro-Gln-Tyr(*P*)-Gln-Pro-[^{14}C]Gly-Asp-Gln-Thr and Thr-Ser-Ala-Glu-Pro-Gln-Tyr-Gln-Pro-[^{14}C]Gly-Asp-Gln-Thr were also synthesized. Phosphotyrosine was incorporated as the *tert*-butoxycarbonyl-*O*-(dibenzylphosphono)-L-tyrosine derivative (Peninsula Laboratories). A complete description of the synthetic procedures will be published elsewhere.

Isotopic Labeling of Cells and Preparation of Cell Extracts. Plasmid-transfected NIH 3T3 cells that overexpress wild-type chicken pp60$^{c\text{-}src}$ [NIH(pMcsrc/foc)B] cells or pp60$^{c\text{-}src(F527)}$ containing a Tyr-527 → Phe mutation [NIH-(pcsrc527/foc/EP)B1] cells have been previously described (8, 32). NIH 3T3 cells expressing pp60$^{c\text{-}src(F527)}$ with deletion of residues 93–143 (D11) and residues 144–175 (D12) (33, 34) were provided by Harold Varmus (University of California, San Francisco). Cells were labeled for 3 hr, as described (35), with [^{35}S]methionine (0.5 mCi/ml; 1 Ci = 37 GBq). Each 10-cm plate of cells was washed twice with ice-cold phos-

Abbreviation: NP-40, Nonidet P-40.

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phate-buffered saline (PBS) and extracted with 2 ml of NP-40 buffer [1% Nonidet P-40/140 mM NaCl/20 mM Tris·HCl, pH 7.5/1 mM $CaCl_2$/1 mM $MgCl_2$/leupeptin (25 μg/ml, Sigma, freshly added)/aprotinin (10 μg/ml, Sigma, freshly added)/0.5 mM phenylmethylsulfonyl fluoride (Sigma, freshly added from a 100 mM stock solution in absolute ethanol)/0.1 mM sodium orthovanadate] by scraping with a rubber policeman at 4°C.

Binding of $pp60^{c\text{-}src(F527)}$ and $pp60^{v\text{-}src}$ to Affi-Gel-10-Linked Peptides. ^{14}C-labeled peptides were covalently coupled to Affi-Gel-10 (Bio-Rad). The degree of substitution, measured by scintillation counting, was 5.1 μmol/ml for both the phosphorylated and nonphosphorylated carboxyl-terminal peptides of the c-src protein, 9.4 μmol/ml for the nonphosphorylated carboxyl-terminal peptide of the c-fgr protein, and 5.6 μmol/ml for the phosphorylated carboxyl-terminal peptide of the c-fgr protein. NP-40 extracts were normalized to contain comparable levels of immunoprecipitable [^{35}S]methionine-labeled $pp60^{c\text{-}src}$ and $pp60^{c\text{-}src(F527)}$. Similarly, NP-40 extracts of [^{35}S]methionine-labeled NIH 3T3 cells expressing high levels of $pp60^{c\text{-}src(F527)}$ with deletions of residues 93–143 (D11) or residues 144–175 (D12) were normalized to contain approximately equal quantities of each mutant protein. NP-40 extracts of [^{35}S]methionine-labeled cells or [γ-^{32}P]-ATP-labeled cytoskeletal preparations (see below) were precleared batchwise with Affi-Gel-10 blocked with ethanolamine in NP-40 buffer essentially as described (4). Aliquots of the precleared lysates, 200 μl, were shaken end-over-end for 30 min with 200 μl of Affi-Gel-10/peptide, 50% (vol/vol) in NP-40 buffer, at 4°C. Competitive binding studies were conducted with 50 μl of Affi-Gel-10/peptide in the presence or absence of 1.3 mM phosphorylated or nonphosphorylated peptides. The pellets were washed four times with 1 ml of NP-40 buffer. Washed pellets were boiled for 3 min in 0.1 ml of SDS sample buffer [10% (vol/vol) glycerol/2.3% (wt/vol) SDS/0.0625 M Tris·HCl, pH 6.8/5% (vol/vol) 2-mercaptoethanol)] and analyzed by electrophoresis on SDS/9% polyacrylamide gels as described (35).

The amount of $pp60^{v\text{-}src}$, $pp60^{c\text{-}src}$, or $pp60^{c\text{-}src(F527)}$ bound to the Affi-Gel-coupled peptides was quantified by reprecipitation with an antibody against the amino terminus of $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$. Pellets containing bound proteins in SDS sample buffer were diluted 10-fold with reprecipitation buffer (10 mM Tris·HCl, pH 7.4/0.3% SDS/10 mg of ovalbumin per ml/1% NP-40). The pellets were removed by brief centrifugation at 4°C. For reprecipitation of $pp60^{c\text{-}src}$ and $pp60^{c\text{-}src(F527)}$, 200 μl of the diluted supernatant solutions was immunoprecipitated with 10 μl of ascites fluid containing a monoclonal antibody directed against residues 2–17 of $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ (Microbiological Associates) and the immunoprecipitate was analyzed on SDS/9% polyacrylamide gels essentially as described (4, 35).

***In Situ* Kinase Assays.** *In situ* kinase assays were performed on the detergent-insoluble cytoskeletal matrix of NIH 3T3 cells transformed by $pp60^{c\text{-}src(F527)}$ [NIH(pcsrc527/foc/EP)B1 cells] (8), the Schmidt–Ruppin A v-src protein [NIHp(Mvsrc/foc/EP)A2 cells] (36), or the Schmidt–Ruppin D v-src protein [NIH(pMvsrcD/foc)A cells] (36) essentially as described (37, 38), in the presence and absence of 3.6 mM peptides. $pp60^{c\text{-}src(F527)}$ was reprecipitated from [γ-^{32}P]ATP-labeled cytoskeletal extracts in SDS sample buffer with a monoclonal antibody directed against residues 2–17, following a 10-fold dilution with reprecipitation buffer as described above. *In situ*-labeled cytoskeletal extracts and reprecipitated $pp60^{c\text{-}src(F527)}$ were analyzed by electrophoresis on SDS/9% polyacrylamide gels and were subjected to autoradiography at −70°C. Alkali gels were incubated with 1 M NaOH for 1 hr at 55°C to enhance detection of phosphotyrosine (39). [γ-^{32}P]ATP-labeled cytoskeletal preparations containing $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src(F527)}$ were also extracted with NP-40 buffer and analyzed for binding to the phosphorylated and nonphosphorylated c-src peptides as described above.

RESULTS

Activated $pp60^{c\text{-}src(F527)}$ Binds to the Phosphorylated Carboxyl-Terminal Peptide of the c-*src*-Encoded Protein. Synthetic peptides corresponding to the phosphorylated and nonphosphorylated carboxyl-terminal 13 residues of the c-src and c-fgr proteins immobilized on Affi-Gel-10 were tested for their ability to bind proteins from extracts of [^{35}S]methionine-labeled NIH 3T3 cells overexpressing a highly transforming c-src variant, $pp60^{c\text{-}src(F527)}$. The bound proteins were eluted by heating in SDS sample buffer and were resolved on an SDS/9% polyacrylamide gel. A 60-kDa protein was preferentially bound to the phosphorylated carboxyl-terminal peptide of $pp60^{c\text{-}src}$ (Fig. 1*A*, lane 2) and with weaker affinity to the phosphorylated carboxyl-terminal peptide of the c-fgr protein (Fig. 1*A*, lane 4). Only small amounts of the 60-kDa protein bound to the nonphosphorylated c-src peptide (Fig. 1*A*, lane 1) or to an analog with a tyrosine → phenylalanine substitution (Fig. 1*A*, lane 3).

We suspected that the 60-kDa protein bound by the phosphorylated peptide was activated $pp60^{c\text{-}src(F527)}$. We therefore tested the binding of various peptides to cell extracts containing $pp60^{c\text{-}src(F527)}$ and reprecipitated the bound proteins with antibodies specific for the amino terminus of $pp60^{c\text{-}src}$. The antibodies detected $pp60^{c\text{-}src(F527)}$ bound to the phosphorylated $pp60^{c\text{-}src}$ carboxyl-terminal peptide (Fig. 1*B*, lane 3). Only slight traces of $pp60^{c\text{-}src(F527)}$ bound to the nonphosphorylated carboxyl-terminal peptides of the c-src and c-fgr proteins (virtually undetectable in Fig. 1*B*, lanes 1 and 5). Immunoprecipitation of $pp60^{c\text{-}src(F527)}$ bound to phosphoryl-

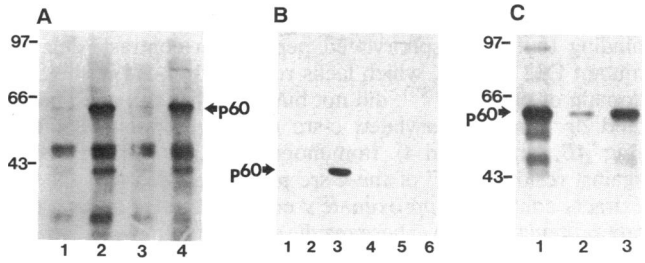

FIG. 1. Binding of $pp60^{c\text{-}src(F527)}$ to the phosphorylated carboxyl-terminal peptides of the c-src and c-fgr proteins. (*A*) NP-40 extracts of [^{35}S]methionine-labeled NIH 3T3 cells expressing $pp60^{c\text{-}src(F527)}$ were bound to immobilized 13-residue peptides modeled on the nonphosphorylated carboxyl terminus of the c-src protein (lane 1), the phosphorylated carboxyl terminus of the c-src protein (lane 2), the carboxyl terminus of the c-src protein with a phenylalanine substitution at position 527 (lane 3), and the phosphorylated carboxyl terminus of the c-fgr protein (lane 4). (*B*) NP-40 extracts of [^{35}S]methionine-labeled NIH 3T3 cells expressing $pp60^{c\text{-}src(F527)}$ were bound to immobilized peptides modeled on the nonphosphorylated carboxyl terminus of the c-src protein (lanes 1 and 2), the phosphorylated carboxyl terminus of the c-src protein (lanes 3 and 4), and the nonphosphorylated carboxyl terminus of the c-fgr protein (lanes 5 and 6), eluted, and immunoprecipitated with antibodies directed against the amino terminus of the c-src protein (lanes 1, 3, and 5) or with antibodies against the amino terminus of the c-src protein blocked by prior incubation with the immunizing peptide (lanes 2, 4, and 6). (*C*) Inhibition of binding of $pp60^{c\text{-}src(F527)}$ by competition with soluble phosphopeptide. NP-40 extracts of [^{35}S]methionine-labeled NIH 3T3 cells expressing $pp60^{c\text{-}src(F527)}$ were bound to the immobilized $pp60^{c\text{-}src}$ carboxyl-terminal 13-residue phosphopeptide in the presence of the corresponding soluble nonphosphorylated (lane 1) and phosphorylated (lane 2) peptides (1.3 mM) or in the absence of any competing peptides (lane 3). Proteins were analyzed on an SDS/9% polyacrylamide gel. The electrophoretic mobility of $pp60^{c\text{-}src(F527)}$ is indicated. Molecular masses of marker proteins are indicated in kDa.

ated c-src peptide was blocked by the immunizing peptide (Fig. 1*B*, lane 4). Binding of pp60$^{c\text{-}src(F527)}$ to the immobilized phosphorylated peptide was markedly reduced by competition with the soluble phosphorylated peptide (Fig. 1*C*, lane 2) but not by the nonphosphorylated peptide (Fig. 1*C*, lane 1).

We then compared the binding of each peptide to activated pp60$^{c\text{-}src(F527)}$ and normal pp60$^{c\text{-}src}$. Extracts of [^{35}S]methionine-labeled NIH 3T3 cells overexpressing either normal pp60$^{c\text{-}src}$ or activated pp60$^{c\text{-}src(F527)}$ were adjusted to contain approximately equal amounts of the corresponding c-src proteins. Each extract was allowed to react with Affi-Gel-coupled peptides corresponding to the phosphorylated and nonphosphorylated carboxyl termini of the c-src protein and the nonphosphorylated carboxyl terminus of the c-fgr protein. Bound proteins were eluted and reprecipitated with antibodies specific for residues 2–17 of pp60$^{c\text{-}src}$. A 60-kDa protein was reprecipitated from extracts of pp60$^{c\text{-}src(F527)}$-transformed cells bound to the phosphorylated carboxyl terminus of the c-src protein (Fig. 2, lane 5). Only small amounts of normal pp60$^{c\text{-}src}$ bound to the phosphorylated carboxyl-terminal pp60$^{c\text{-}src}$ peptide (barely detectable in Fig. 2, lane 2).

The phosphorylated c-src peptide also bound to pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src(F527)}$ labeled with [γ-^{32}P]ATP in cytoskeletal preparations (Fig. 3, lanes 1, 3, and 5). In contrast, only traces of pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src(F527)}$ bound to the nonphosphorylated c-src peptide (Fig. 3, lanes 2, 4, and 6).

Deletion of Residues 144–175 in the SH2 Domain of pp60$^{c\text{-}src(F527)}$ Abolishes Binding to the Phosphorylated c-src Carboxyl-Terminal Peptide. Deletion mutant D11 (33, 34), which lacks residues 93–143 of pp60$^{c\text{-}src(F527)}$, bound efficiently to the phosphorylated carboxyl-terminal peptide of the c-src protein (Fig. 4*B*, lane 1) but not very well to the corresponding nonphosphorylated peptide (Fig. 4*B*, lane 2). These results indicated that residues 93–143, which include most of the SH3 domain, are not essential for binding to the phosphorylated peptide. In contrast, deletion mutant D12 (33, 34), which lacks residues 144–175 in the SH2 domain of pp60$^{c\text{-}src(F527)}$, did not bind to either the phosphorylated or nonphosphorylated c-src carboxyl-terminal peptides (Fig. 4*B*, lanes 3 and 4). Immunoprecipitation with antibodies against residues 2–17 of the c-src protein verified that the cell extracts contained approximately equal quantities of each mutant protein (Fig. 4*A*). These results are consistent with previous

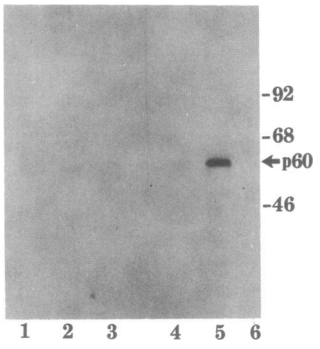

FIG. 2. Comparison of the binding of synthetic peptides to normal pp60$^{c\text{-}src}$ and activated pp60$^{c\text{-}src(F527)}$. NP-40 extracts of [^{35}S]methionine-labeled NIH 3T3 cells expressing normal pp60$^{c\text{-}src}$ (lanes 1–3) or activated pp60$^{c\text{-}src(F527)}$ (lanes 4–6) were bound to immobilized synthetic peptides modeled on the nonphosphorylated carboxyl terminus of the c-src protein (lanes 1 and 4), the phosphorylated carboxyl terminus of the c-src protein (lanes 2 and 5), and the nonphosphorylated carboxyl terminus of the c-fgr protein (lanes 3 and 6). Bound proteins were washed, eluted, and immunoprecipitated with antibodies directed against the amino terminus of pp60$^{c\text{-}src}$. Immunoprecipitates were analyzed on an SDS/9% polyacrylamide gel. The electrophoretic mobility of pp60$^{c\text{-}src}$ (p60) is indicated.

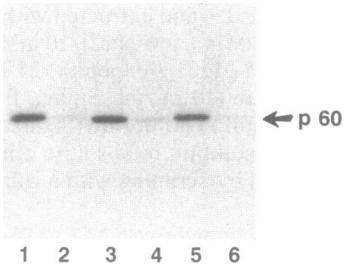

FIG. 3. Binding of phosphorylated pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src(F527)}$ to the c-src carboxyl-terminal peptide. Detergent-extracted cytoskeletal preparations of NIH 3T3 cells transformed by pp60$^{c\text{-}src(F527)}$ (lanes 1 and 2), plasmid p(Mvsrc/foc/EP)A2 (36) encoding the Schmidt–Ruppin A v-src protein (lanes 3 and 4), or plasmid (pMvsrcD/foc)A (36) encoding the Schmidt–Ruppin D v-src protein (lanes 5 and 6) were labeled *in situ* with [γ-^{32}P]ATP. NP-40 extracts were bound to immobilized synthetic peptides modeled on the phosphorylated carboxyl terminus of the c-src protein (lanes 1, 3, and 5) or the nonphosphorylated carboxyl terminus of the c-src protein (lanes 2, 4, and 6). Bound proteins were washed, eluted, and immunoprecipitated with antibodies directed against the amino terminus of pp60$^{c\text{-}src}$. Immunoprecipitates were analyzed on an SDS/9% polyacrylamide gel.

suggestions of an interaction of the phosphorylated carboxyl terminus of pp60$^{c\text{-}src}$ with the SH2 domain (19–22).

The Phosphorylated Carboxy-Terminal Peptide of pp60$^{c\text{-}src}$ Inhibits *in Situ* Phosphorylation of Activated pp60$^{c\text{-}src}$. Since the phosphorylated carboxyl terminus of pp60$^{c\text{-}src}$ negatively regulates its kinase activity, it was possible that binding of the synthetic phosphopeptide to pp60$^{c\text{-}src(F527)}$ might have a similar effect. *In situ* kinase assays were performed on the detergent-insoluble cytoskeletal matrix of NIH 3T3 cells expressing pp60$^{c\text{-}src(F527)}$ after incubation with phosphorylated or nonphosphorylated carboxyl-terminal peptides. A 60-kDa protein was one of the major proteins phosphorylated in the absence of peptides (Fig. 5*A*, lane 2). Reprecipitation of the phosphorylated extracts with antibodies specific for the amino terminus of pp60$^{c\text{-}src}$ demonstrated that activated pp60$^{c\text{-}src(F527)}$ was the 60-kDa protein phosphorylated in cytoskeletal kinase assays (Fig. 5*B*, lane 2). The radioactivity incorporated into pp60$^{c\text{-}src(F527)}$ was stable to alkali treatment of gels, consistent with the presence of phosphotyrosine. The

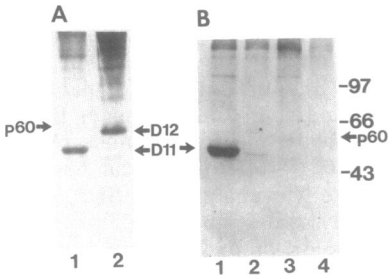

FIG. 4. Analysis of binding of pp60$^{c\text{-}src(F527)}$ SH3 and SH2 deletion mutants to the phosphorylated carboxyl-terminal c-src peptide. NP-40 extracts of [^{35}S]methionine-labeled NIH 3T3 cells D11 (expressing pp60$^{c\text{-}src(F527)}$ with a deletion of residues 93–143) and D12 (expressing pp60$^{c\text{-}src(F527)}$ with a deletion of residues 144–175) were adjusted to contain equal quantities of each mutant. (*A*) Equal volumes of the D11 (lane 1) and D12 (lane 2) extract were immunoprecipitated with antibodies against residues 2–17 of the c-src protein to verify that the extracts contained the same level of each mutant. (*B*) The equalized D11 (lanes 1 and 2) and D12 (lanes 3 and 4) extracts were mixed with immobilized peptides modeled on the phosphorylated (lanes 1 and 3) and nonphosphorylated (lanes 2 and 4) carboxyl termini of the c-src protein. Bound proteins were eluted and immunoprecipitated with antibodies directed against the amino terminus of the c-src protein. The reprecipitated c-src mutant proteins were analyzed on an SDS/9% polyacrylamide gel.

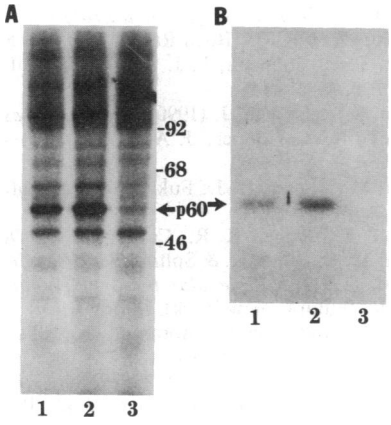

FIG. 5. Influence of carboxyl-terminal c-src peptides on *in situ* phosphorylation of pp60$^{c\text{-}src(F527)}$. (*A*) The detergent-insoluble cytoskeletal matrix of NIH 3T3 cells expressing activated pp60$^{c\text{-}src(F527)}$ was analyzed for *in situ* kinase activity with [γ-^{32}P]ATP in the presence of synthetic peptides modeled on the nonphosphorylated (lane 1) and phosphorylated (lane 3) carboxyl termini of pp60$^{c\text{-}src}$, and in the absence of peptide additives (lane 2). (*B*) An aliquot of the phosphorylation products of each kinase assay in *A* was immunoprecipitated with antibodies against the amino terminus of pp60$^{c\text{-}src}$. Lanes are numbered as in *A*.

synthetic phosphotyrosine-containing carboxyl-terminal peptide of pp60$^{c\text{-}src}$ greatly reduced the *in situ* phosphorylation of pp60$^{c\text{-}src(F527)}$ (Fig. 5*A*, lane 3; Fig. 5*B*, lane 3). In contrast, the nonphosphorylated carboxyl-terminal peptide of the c-src protein had significantly weaker inhibitory activity (Fig. 5*A*, lane 1; Fig. 5*B*, lane 1). Since a nonphosphorylated peptide corresponding to the carboxyl terminus of the c-src protein is a substrate for pp60$^{c\text{-}src}$ (3), the weak inhibition of kinase activity by the nonphosphorylated c-src peptide was probably due to competitive inhibition. The marked inhibition of *in situ* kinase activity by the phosphorylated c-src peptide was probably a consequence of its binding to activated pp60$^{c\text{-}src(F527)}$. In accord with this premise, the phosphorylated peptide also inhibited autophosphorylation of pp60$^{c\text{-}src(F527)}$ during immune complex kinase assays (data not shown).

DISCUSSION

In this study we have shown that a synthetic phosphopeptide modeled on the carboxyl-terminal phosphoregulatory site of pp60$^{c\text{-}src}$ binds to pp60$^{c\text{-}src}$ activated by mutation of the Tyr-527 phosphorylation site to Phe. In contrast, the nonphosphorylated carboxyl-terminal peptide of the c-src protein did not bind very well to activated pp60$^{c\text{-}src(F527)}$. The inability of normal pp60$^{c\text{-}src}$ to bind efficiently to the phosphorylated c-src peptide suggests that it is in a conformation in which the binding sites are less accessible. One possibility is that the binding sites for the phosphorylated peptide may already be occupied as a result of self-interaction with the phosphorylated carboxyl terminus of normal pp60$^{c\text{-}src}$ and may therefore not be available for interacting with the synthetic phosphopeptide (Fig. 6*B*). This observation is consistent with proposed models in which the phosphorylated carboxyl terminus of pp60$^{c\text{-}src}$ regulates its activity by interacting with other sites on the c-src protein (6, 15, 18–23). These sites would presumably be unable to bind efficiently to the carboxyl terminus of pp60$^{c\text{-}src(F527)}$, since the carboxyl terminus is not phosphorylated. The vacant binding sites would therefore be free to bind the synthetic phosphopeptide (Fig. 6*A*). The inability of the nonphosphorylated c-src peptide and an analog with a Tyr → Phe substitution to bind efficiently to activated pp60$^{c\text{-}src(F527)}$ (Fig. 6*C*) is consistent

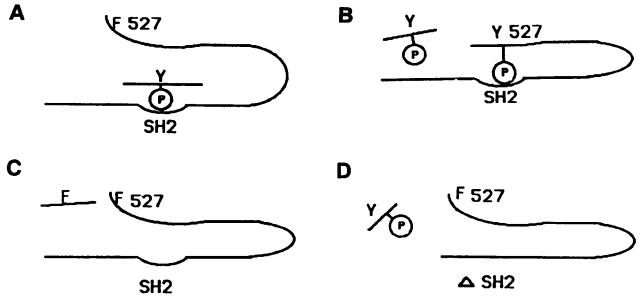

FIG. 6. Proposed model illustrating differential binding of carboxyl-terminal synthetic c-src peptides to normal and mutant c-src proteins. (*A*) pp60$^{c\text{-}src(F527)}$ interacts with the phosphorylated synthetic peptide because its SH2 domain is not bound tightly to its nonphosphorylated carboxyl terminus. (*B*) High-affinity binding of the phosphorylated carboxyl terminus of normal pp60$^{c\text{-}src}$ with its complementary binding site in the SH2 domain inhibits binding of the synthetic phosphopeptide. (*C*) pp60$^{c\text{-}src(F527)}$ does not bind with high affinity to a nonphosphorylated synthetic peptide with a Tyr-527 → Phe substitution. (*D*) Deletion of the putative binding site in the SH2 domain of pp60$^{c\text{-}src(F527)}$ prevents binding of the phosphorylated synthetic peptide.

with the deregulation associated with loss of the carboxyl-terminal phosphorylation site. The inhibition of phosphorylation observed during kinase assays on cytoskeletal preparations and immunoprecipitates containing activated pp60$^{c\text{-}src(F527)}$ is consistent with models in which binding of the phosphorylated carboxyl terminus of pp60$^{c\text{-}src}$ negatively regulates its kinase activity (6, 15, 18–23). The mechanism by which the phosphorylated peptide inhibits kinase activity is unclear.

Phosphorylation of tyrosine occurs at the carboxyl termini of other protein-tyrosine kinases, including the products of the *lck* (40, 41), *fyn* (35, 42), and c-*fgr* protooncogenes (43). Residues corresponding to the phosphorylated tyrosines are conserved in the c-yes, hck, and lyn proteins, suggesting that carboxyl-terminal tyrosine phosphorylation is a general feature of nonreceptor tyrosine kinases (15). These other tyrosine kinases may also bind to their phosphorylated carboxyl-terminal peptides. The ability of the lck carboxyl terminus to regulate pp60$^{c\text{-}src}$ (44) suggested that the phosphorylated carboxyl-terminal peptides of other src family members might also bind to activated pp60$^{c\text{-}src}$. In accord with this prediction, a phosphorylated 13-residue peptide modeled on the carboxyl terminus of the c-fgr protein bound to pp60$^{c\text{-}src(F527)}$. The binding of the c-fgr phosphopeptide was weaker than that of the corresponding c-src phosphopeptide. The c-fgr and c-src phosphopeptides differ at 4 of the 13 amino acids.

Deletion mutant D11 (33, 34), which is missing residues 93–143 of pp60$^{c\text{-}src(F527)}$, bound to the phosphorylated c-src carboxyl-terminal peptide. These results indicated that residues 93–143, which include most of the SH3 domain, are not essential for binding. On the other hand, deletion mutant D12 (33, 34), which lacks residues 144–175 in the SH2 domain of pp60$^{c\text{-}src(F527)}$, did not bind to the phosphorylated carboxyl-terminal c-src peptide (Fig. 6*D*). These results are consistent with previous suggestions that an interaction of the phosphorylated carboxyl terminus with the SH2 domain of pp60$^{c\text{-}src}$ regulates its activity (19–22).

We do not know whether the proposed interaction of the phosphorylated carboxyl terminus with pp60$^{c\text{-}src}$ is an intermolecular or intramolecular phenomenon. The inability of normal pp60$^{c\text{-}src}$ to bind efficiently to high concentrations of the immobilized phosphorylated peptide suggests that intramolecular binding predominates, with each molecule of pp60$^{c\text{-}src}$ binding its own carboxyl-terminal tail. Intramolecular binding could be favored as a consequence of proximity

or other stabilizing interactions. However, *in vivo* other proteins containing SH2 domains may bind to the phosphorylated carboxyl terminus of a subpopulation of $pp60^{c\text{-}src}$ molecules, as has been proposed for the v-crk protein (19).

Mutations in residues 144–175 of the SH2 domain are among those that suppress the transforming activity of $pp60^{c\text{-}src(F527)}$ in a host-dependent manner, indicating that this region may be crucial for interacting with specific host cell proteins (33, 34, 45). Indeed, residues 149–169 in this region of the SH2 domain have recently been found to be required for association of $pp60^{v\text{-}src}$ with the detergent-insoluble cytoskeletal matrix (46). $pp60^{v\text{-}src}$ and transforming variants of $pp60^{c\text{-}src}$ are associated with the detergent-insoluble matrix, whereas normal $pp60^{c\text{-}src}$ is not (37, 38, 47, 48). Binding of the phosphorylated carboxyl terminus of normal $pp60^{c\text{-}src}$ to the SH2 domain may repress its kinase activity and transforming potential, in part, by blocking interaction of the SH2 domain with specific cellular targets.

We thank Peter Drain for suggesting an interaction of $pp60^{c\text{-}src}$ with its phosphorylated carboxyl terminus, and Timothy Bestor, Frank Boschelli, and Thomas Laue for helpful discussions. We are grateful to Shubha Bagrodia for her generous assistance, and to Hisamaru Hirai, Kirsten Bibbins, and Harold Varmus for the D11 and D12 mutants. We thank Jessica Hiltebeitel, Kimberly Perry, Stephen Clark, Allison Curtis, Trisha Gushue, and Jamie Fox for assistance with peptide synthesis and Frank Boschelli for performing phosphopeptide binding studies and kinase assays on $pp60^{c\text{-}src}$ in yeast. We thank Harold Varmus, John Collins, Rick Cote, and Russell Doolittle for reading the manuscript. This work was supported by Grant MV-311 from the American Cancer Society (to A.P.L.), an Undergraduate Research Opportunities Program Award (to R.R.R.), Biomedical Research Support grants from the University of New Hampshire (to A.P.L.), and Grants CA32317 and CA47333 and a Research Career Development Award (CA01139) from the National Institutes of Health (to D.S.). R.R.R. was the recipient of a National Science Foundation Research Experiences for Undergraduates Fellowship. This is Scientific Contribution No. 1701 from the New Hampshire Agricultural Experiment Station.

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