MOLECULAR AND CELLULAR BIOLOGY, June 1989, p. 2648–2656
0270-7306/89/062648-09$02.00/0

Vol. 9, No. 6

Structural Differences between Repressed and Derepressed Forms of p60$^{c\text{-}src}$

ALASDAIR MACAULEY* AND JONATHAN A. COOPER

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104

Received 23 November 1988/Accepted 13 March 1989

The kinase activity of p60$^{c\text{-}src}$ is derepressed by removal of phosphate from Tyr-527, mutation of this residue to Phe, or binding of a carboxy-terminal antibody. We have compared the structures of repressed and active p60$^{c\text{-}src}$, using proteases. All forms of p60$^{c\text{-}src}$ are susceptible to proteolysis at the boundary between the amino-terminal region and the kinase domain, but there are several sites elsewhere that are more sensitive to trypsin digestion in repressed than in derepressed forms of p60$^{c\text{-}src}$. The carboxy-terminal tail (containing Tyr-527) is more sensitive to digestion by pronase E and thermolysin when Tyr-527 is not phosphorylated. The kinase domain fragment released with trypsin has kinase activity. Relative to intact p60$^{c\text{-}src}$, the kinase domain fragment shows altered substrate specificity, diminished regulation by the phosphorylated carboxy terminus, and novel phosphorylation sites. The results identify parts of p60$^{c\text{-}src}$ that change conformation upon kinase activation and suggest functions for the amino-terminal region.

p60$^{c\text{-}src}$ is a membrane-associated, protein-tyrosine kinase that is expressed in most mammalian and avian cell types examined (13). Its oncogenically activated form, p60$^{v\text{-}src}$, is the transforming protein of Rous sarcoma virus. In vitro mutagenesis experiments on v-*src* and sequence comparisons with other protein kinases suggest that p60$^{c\text{-}src}$ has a catalytic domain extending from about residue 260, close to a nucleotide-binding motif (Gly-Xaa-Gly-Xaa-Xaa-Gly), to Leu-516 (20, 36, 42, 52). The region carboxy terminal to Leu-516 does not appear to be directly involved in catalysis (39, 53, 54). The amino-terminal 250 residues appear to be unimportant for catalysis but are probably involved in other p60$^{c\text{-}src}$ functions, since deletions in the amino-terminal region of p60$^{v\text{-}src}$ can affect the phenotype of transformed cells (24, 48).

The repressed form of p60$^{c\text{-}src}$ has two major sites of phosphorylation in vivo: Ser-17 (33) and Tyr-527 (14, 27). Ser-17 can be phosphorylated by the cyclic AMP-dependent protein kinase (33, 41), and Tyr-527 can be phosphorylated by p60$^{c\text{-}src}$ and perhaps by other protein-tyrosine kinases (16, 17, 21, 43). Transformation by p60$^{c\text{-}src}$ is generally associated with the replacement of carboxy-terminal sequences or the loss of phosphate from Tyr-527 and the stimulation of p60$^{c\text{-}src}$ kinase activity. Site-directed mutagenesis experiments have shown that either truncation of p60$^{c\text{-}src}$ between Leu-516 and Tyr-527 or replacement of Tyr-527 by Phe or Ser is oncogenic (7, 9, 26, 34, 39, 53). Transformation of cells by polyomavirus middle T antigen involves formation of a complex between middle T antigen and p60$^{c\text{-}src}$, dephosphorylation of Tyr-527, and stimulation of p60$^{c\text{-}src}$ kinase activity (3, 8, 18). Dephosphorylation of Tyr-527 by cellular phosphatases or by exposure to acid phosphatase in vitro stimulates the kinase activity of p60$^{c\text{-}src}$ (15, 18, 43).

These results suggest that the phosphorylated Tyr-527 in p60$^{c\text{-}src}$ represses kinase activity. The mechanism of repression is not known, but the kinetics of the repressed state are consistent with models in which the phosphorylated tail serves as a product analog inhibitor or in which the active site undergoes a conformation change (15). The activation of p60$^{c\text{-}src}$ by mutations in the amino-terminal domain suggests that this region may be required for repression of the kinase domain by phosphorylated Tyr-527 (23, 35). Point mutations in the amino-terminal region, in the vicinity of Arg-95, can partially activate the transforming potential of c-*src* while retaining phosphorylation of Tyr-527 at unknown stoichiometry. These mutations may exert their effects by either altering the accessibility of Tyr-527 to kinases or phosphatases or affecting enzymatic activity more directly, for example, by abrogating the ability of the phosphorylated carboxy terminus to inhibit.

p60$^{c\text{-}src}$ molecules become phosphorylated upon incubation with ATP in vitro, in a reaction that appears to be intermolecular (16). The site phosphorylated by p60$^{c\text{-}src}$ in vitro is Tyr-416 (32, 44). This residue is also a major site of phosphorylation in vivo in all active forms of p60$^{c\text{-}src}$, including p60$^{v\text{-}src}$, various activated mutants, and p60$^{c\text{-}src}$ complexed with middle T antigen (8, 18). Both genetic and biochemical evidence suggest that phosphorylation of Tyr-416 is important for full activity (25, 26, 45). A Phe-416, Phe-527 double-mutant form of p60$^{c\text{-}src}$ is less oncogenic and has a lower kinase activity than the Phe-527 single mutant (26). Incubation of cells expressing wild-type p60$^{c\text{-}src}$ in media containing *ortho*-vanadate, a phosphotyrosine-phosphatase inhibitor, raises the level of phosphorylation of Tyr-416 in p60$^{c\text{-}src}$ and increases p60$^{c\text{-}src}$ kinase activity (25). A homologous residue is found in all protein-tyrosine kinases, and in some other cases (e.g., P140$^{gag\text{-}fps}$ and the insulin receptor) phosphorylation at this position is stimulatory (19, 40, 49, 51).

The regulation of enzymatic activity is generally associated with conformational changes that can be probed by using chemical reagents or proteases. Proteases cleave native proteins at the exposed regions between tightly folded domains, which can vary as a result of changes in conformation. Two protein-tyrosine kinases have been mapped by partial proteolysis to define structural domains. Digestion of the epidermal growth factor receptor with trypsin resulted in cleavage to produce a 42-kilodalton (kDa) fragment that binds 5′-*p*-fluorosulfonylbenzoyl adenosine and retains ki-

* Corresponding author.

nase activity (1, 10). Partial proteolysis of p60$^{c\text{-}src}$ by membrane-associated proteases clips off the amino-terminal 8 kDa, releasing the bulk of the protein from the membrane as a 52-kDa fragment (28). Further proteolysis with trypsin produces a 30-kDa fragment that retains the protein-tyrosine kinase activity (4, 28). This fragment has a three- to fivefold-increased specific kinase activity, relative to that of intact p60$^{v\text{-}src}$, when assayed with angiotensin as a substrate (4). We used proteases to map exposed regions in repressed (wild-type) and derepressed (dephosphorylated wild-type and Phe-527 mutant) p60$^{c\text{-}src}$ molecules and found that the phosphorylation state of residue 527 directly affected the conformation of p60$^{c\text{-}src}$. Repressed p60$^{c\text{-}src}$ had a protease-sensitive site in the kinase domain, but the tail was relatively protected. Derepressed p60$^{c\text{-}src}$ had a protease-resistant kinase domain, but the tail was exposed. Furthermore, the activity of the kinase domain was modified in a substrate-specific fashion by the amino-terminal region of p60$^{c\text{-}src}$.

MATERIALS AND METHODS

Antisera and immunoprecipitations. The monoclonal antibody 327 (30) was kindly supplied as a hybridoma by J. Brugge (State University of New York, Stony Brook, N.Y.). Ascites fluid from nude mice was used for immunoprecipitation. The antiserum aZ was from rabbits immunized with a synthetic peptide corresponding to amino acids 519 to 533, the extreme carboxy terminus of chicken p60$^{c\text{-}src}$, conjugated to bovine serum albumin (15). The immunoglobulin fraction was partially purified from the serum on a DEAE Affigel Blue column (Bio-Rad Laboratories, Richmond, Calif.) according to the recommended procedure of the manufacturer. Dishes (50 mm) of NIH 3T3 cells expressing high levels of p60^{WT} or p60^{F527} were lysed with 1 ml of modified RIPA buffer (15), and the lysates were clarified by centrifugation at 20,000 × *g* at 4°C for 30 min. p60$^{c\text{-}src}$ was then immunoprecipitated from the clarified lysates either with 327, rabbit anti-mouse immunoglobulin G (IgG), and Pansorbin (Calbiochem-Behring, La Jolla, Calif.) or with aZ and Pansorbin (15). The immunoprecipitates were washed three times with RIPA buffer and once with 100 mM NaCl–10 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.0)–40 μg of aprotinin per ml. The immunoprecipitates were then stored at −70°C until use.

Phosphatase treatment. For phosphatase treatment of p60$^{c\text{-}src}$, the immunoprecipitates were pelleted from 100 mM NaCl–10 mM PIPES (pH 7.0)–40 μg of aprotinin per ml and suspended in the same volume of 40 mM PIPES (pH 6.0)–1 mM dithiothreitol–10 μg of aprotinin per ml–20 μM leupeptin. One-tenth of a volume of potato acid phosphatase (PAP; 1 mg/ml) was added, and the samples were incubated at 30°C for 5 min (15). After PAP treatment, samples were washed twice with RIPA and once with 100 mM NaCl–10 mM PIPES (pH 7.0) before kinase assays. Treatment without PAP did not affect kinase activity.

Kinase reactions. In vitro phosphorylation reactions of p60$^{c\text{-}src}$ were performed, using the following proportions. One-fortieth of an immunoprecipitate from a 50-mm dish of cells was incubated in 10 μl of 20 mM PIPES (pH 7.0)–10 mM $MnCl_2$–5 μCi [γ-^{32}P]ATP for 10 min at 30°C. Reactions were terminated by 50-fold dilution with trypsin digestion buffer (TDB, defined below) and centrifugation.

Partial proteolysis. After immunoprecipitates were incubated in an in vitro kinase reaction, 0.5 ml of TDB (containing 1 mM $CaCl_2$ and 10 mM Tris hydrochloride [pH 8.1]) was added, and the immunoprecipitates were collected by centrifugation. The pellets were suspended in TDB to 2.5% (wt/vol) final concentration of Pansorbin. The proteases used and their final concentrations are indicated below. The analytical digests were done for 5 min at 25°C, and the preparative digests used to produce the 29.5-kDa fragment for kinase assays were done with a final concentration of 100 μg of pronase E per ml for 10 min at 25°C. After partial proteolysis, the fragments that remained bound to the immunoprecipitate were assayed for kinase activity by being washed twice with RIPA buffer and once with 10 mM PIPES (pH 7.0)–100 mM NaCl–40 μg of aprotinin per ml before being suspended in the same kinase mix used with the intact p60$^{c\text{-}src}$. For kinase reactions with exogenous substrates, the substrates were included in the kinase reaction mix at the concentrations indicated in the legends to the figures, and the reactions were at 30°C for 5 min and were terminated by adding EDTA to a final concentration of 0.025 M. The reaction products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (enolase and tubulin) or by thin-layer electrophoresis at pH 3.5 (angiotensin). The regions containing the phosphorylated products were removed and counted by Cerenkov radiation.

Tryptic peptide mapping. Intact p60$^{c\text{-}src}$ and 29.5-kDa fragment labeled in in vitro kinase reactions were isolated from gel pieces. Extracted phosphoproteins were trichloroacetic acid precipitated with 10 μg of RNase A as a carrier and oxidized with performic acid (2). After lyophilization, the samples were digested at 37°C in 50 mM ammonium bicarbonate with two additions of 5 μg of TPCK (tolysulfonyl phenylalanyl chloromethyl ketone)-trypsin over 16 h. The samples were lyophilized and analyzed on thin-layer cellulose plates by electrophoresis at pH 8.9 and chromatography (butanol:H_2O:acetic acid:pyridine, 15:12:3:10).

RESULTS

The structure of p60$^{c\text{-}src}$ was probed by proteolysis of material labeled in in vitro kinase reactions. Tyr-416 is the major site of phosphorylation in vitro, so only fragments that contain Tyr-416 are detectable in this type of experiment. Furthermore, since the only digestion products detected were phosphorylated at Tyr-416, it was not possible to draw any conclusions regarding possible effects of the Tyr-416 phosphorylation state on protease sensitivity. p60^{WT} (wild-type chicken p60$^{c\text{-}src}$) and p60^{F527} (an activated form of chicken p60$^{c\text{-}src}$ with Phe at residue 527) were immunoprecipitated from NIH 3T3 cells expressing high levels of these proteins from introduced genes. Two antibodies specific for p60$^{c\text{-}src}$ were used for immunoprecipitation. Monoclonal antibody 327 recognizes an epitope contained between residues 92 and 128 (W. Potts and T. Parsons, personal communication). Antiserum aZ was raised to a synthetic peptide corresponding to residues 519 to 533 at the carboxy terminus of p60$^{c\text{-}src}$ (15). The p60^{WT} immunoprecipitated by antibody 327 is in a repressed state but can be activated in vitro by either treatment with PAP or binding of the aZ antibody (15). The p60^{WT} immunoprecipitated by aZ is derepressed, p60^{F527} is constitutively derepressed.

Digestion with trypsin. Immunoprecipitates of p60^{WT} and p60^{F527} were prepared with antibody 327, incubated with or without PAP (15), washed to remove PAP, and then incubated with [γ-^{32}P]ATP. The immunoprecipitates were then incubated with TPCK-treated trypsin (100 μg/ml final concentration) for 5 min at 25°C. The digestion products were centrifuged to separate products that were bound to the antibody from those that were released. Equal fractions of each sample were analyzed by SDS-PAGE.

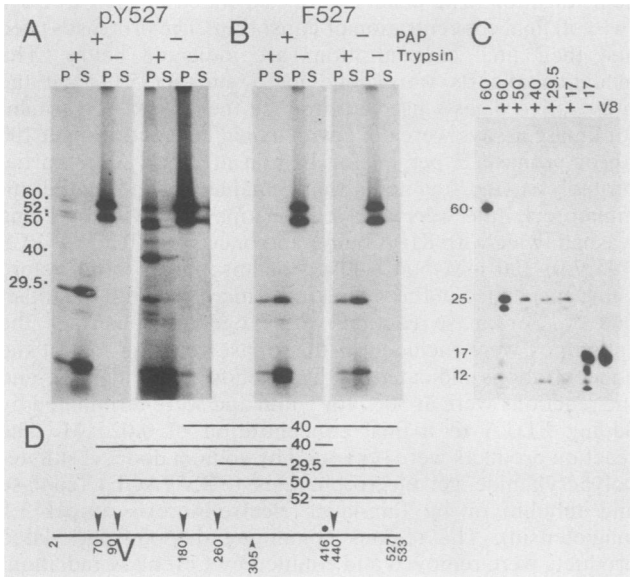

FIG. 1. Partial trypsin digestion of derepressed and repressed forms of $p60^{c\text{-}src}$. (A and B) $p60^{WT}$ and $p60^{F527}$ were immunoprecipitated with monoclonal antibody 327, incubated with (+) or without (−) PAP, labeled in an in vitro kinase reaction, and incubated with 0 (−) or 100 (+) μg of trypsin per ml. Aprotinin was then added to a final concentration of 200 μg/ml, and the reactions were centrifuged. Each pellet was washed once with TDB, and this wash was pooled with the first supernatant. The pooled supernatants (S) were mixed with an equal volume of twice-concentrated SDS-PAGE sample buffer. The washed pellets (P) were suspended in SDS-PAGE sample buffer, and equal fractions of the samples were analyzed by SDS-PAGE (15% acrylamide, 0.087% bisacrylamide). Gel lanes containing $p60^{WT}$ that had not been treated with PAP were exposed six times as long as the other lanes to allow for lower incorporation. The locations of $p60^{c\text{-}src}$ and the 50-, 40-, and 29.5-kDa proteolytic products are indicated on the left. (C) Fragments (60, 50, 40, 29.5, and 17 kDa) generated by trypsinolysis of $p60^{WT}$ were excised from a polyacrylamide gel and digested with 0 (−) or 50 (+) ng of *S. aureus* V8 protease during SDS-PAGE (11). Major digest products of about 25 and 12 kDa are marked. (D) Putative structures of the fragments. The heavy line indicates $p60^{c\text{-}src}$. The 327-binding site (V), the position of labeling by in vitro phosphorylation (●), the approximate sites of trypsin cleavage (▾), and the position where *S. aureus* V8 protease cleaves $p60^{c\text{-}src}$ under denaturing conditions (K. Beemon, personal communication) (▴) are shown.

Trypsin digestion of derepressed forms of $p60^{c\text{-}src}$ (PAP-treated $p60^{WT}$ and PAP-treated or control $p60^{F527}$) released major products of approximately 29.5 and 16 to 18 kDa (Fig. 1A and B). The 29.5-kDa fragment was released from antibody 327 but could be reprecipitated by aZ (data not shown). When incubated with [γ-^{32}P]ATP, this fragment became labeled and could transfer phosphate to exogenous substrates (see below). These data identify the 29.5-kDa fragment as the kinase domain of $p60^{c\text{-}src}$, comparable to the 30-kDa kinase fragment of $p60^{v\text{-}src}$ (4, 28). Immunoprecipitation of the 29.5-kDa fragment by aZ suggests that the carboxy terminus is intact, since there are no Lys or Arg residues (sites for trypsin cleavage) within the 15-residue sequence recognized by aZ. Therefore, the cleavage site that produces the 29.5-kDa fragment must lie close to residue 260, at the start of the kinase domain (Fig. 1D). Treatment of $p60^{F527}$ with PAP prior to labeling and digestion did not alter the pattern of fragments that was generated (Fig. 1B). Since $p60^{F527}$ is phosphorylated at Ser-17 in the cell and the phosphate is removed by PAP treatment (15), this suggests that the phosphorylation state of Ser-17 does not affect the protease-sensitive sites.

In contrast to the pattern produced from derepressed forms of $p60^{c\text{-}src}$, digestion of the phosphorylated, repressed form of $p60^{WT}$ produced two major fragments of 40 and 50 kDa, as well as the 29.5- and 16- to 18-kDa fragments (Fig. 1A). The 29.5-, 40- and 50-kDa bands were digested much more slowly than intact $p60^{c\text{-}src}$ and did not appear to be related to each other as precursors and products. Presumably, individual molecules of $p60^{WT}$ are cleaved initially at different sites, and the fragments released resist further digestion. Phosphorylated $p60^{WT}$ appeared to be more resistant to proteolysis than either dephosphorylated $p60^{WT}$ or $p60^{F527}$. The 29.5-kDa fragment produced by trypsin digestion of $p60^{WT}$ resembled that from $p60^{F527}$, on the basis of immunoprecipitation by aZ and intrinsic kinase activity (data not shown). The lower yield of 29.5-kDa fragment from $p60^{WT}$ suggests that the proteolysis site at about residue 260 is less accessible in repressed forms of $p60^{c\text{-}src}$.

Several pieces of information allow tentative mapping of trypsin-sensitive sites in $p60^{WT}$ to residues 90 to 100, 170 to 180, and 440 to 450 (Fig. 1D). The 50-kDa fragment was immunoprecipitated by aZ, suggesting that its carboxy terminus was intact. This was confirmed by digestion of the SDS-denatured fragment with V8 protease (11, 12). Under these conditions, $p60^{c\text{-}src}$ is cleaved at residues 305 to 310 (K. Beemon, personal communication) to give a 25-kDa band. V8 digestion of SDS-denatured 50- and 29.5-kDa bands gave the same size fragment as was obtained from $p60^{c\text{-}src}$ (Fig. 1C), showing that the entire region between residues 305 to 310 and the C terminus was present. Therefore, a site of trypsin sensitivity falls in the vicinity of residues 90 to 100 (Fig. 1D).

The 40-kDa band often appeared as a doublet, with variable resolution and yields of the two bands. The two fragments are thought to represent two distinct digestion products, as illustrated in Fig. 1D. One of the fragments is released from antibody 327 and can be reprecipitated by aZ, suggesting that it has an intact C terminus and is cleaved in the vicinity of amino acids 170 to 180. This should give rise to the usual 25-kDa V8 protease fragment. The other was at least partially retained by 327 and cannot be precipitated with aZ, suggesting that it lacks the carboxy terminus and should give a smaller V8 protease fragment. Indeed, digestion of a mixture of the 40-kDa fragments with V8 protease in the presence of SDS gave both the 25-kDa band and a 12-kDa band (Fig. 1C). A labeled fragment of 12 kDa could be produced by cleavage by trypsin in the vicinity of residues 440 to 450 and by V8 at residues 305 to 310. The origin of the small tryptic fragments of 16 to 18 kDa is not clear; they may be a heterogeneous mixture of peptides. They were cleaved by V8 to yield several subfragments, including one of 12 kDa (Fig. 1C). This is consistent with the possibility some of the fragments were derived by cleavage near amino acid 260, at the amino-terminal boundary of the kinase domain, and near amino acid 450 in the kinase domain (Fig. 1D).

Digestion of immunoprecipitates of $p60^{WT}$ or $p60^{F527}$ prepared with aZ yielded products similar to those generated from 327 immunoprecipitates of derepressed forms of $p60^{c\text{-}src}$ (data not shown). This result is consistent with the ability of aZ to derepress $p60^{c\text{-}src}$.

Digestion with pronase. Antibody 327 precipitates of $p60^{WT}$ and $p60^{F527}$ were exposed to four proteases besides

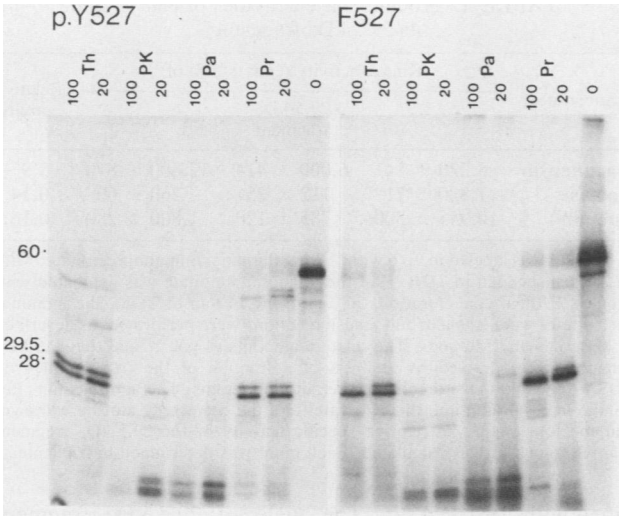

FIG. 2. Partial digestion of p60^{WT} and p60^{F527} with various proteases. p60^{WT} (left) and p60^{F527} (right) were immunoprecipitated with antibody 327, labeled at Tyr-416 in vitro, washed with TDB, and digested with no protease (0) or 20 or 100 μg of thermolysin (Th), proteinase K (PK), papain (Pa), or pronase E (Pr) per ml. Digestion products were separated by SDS-PAGE. The reactions containing p60^{WT} were exposed for three times longer than those containing p60^{F527}. Molecular sizes (in kilodaltons) are shown at the left.

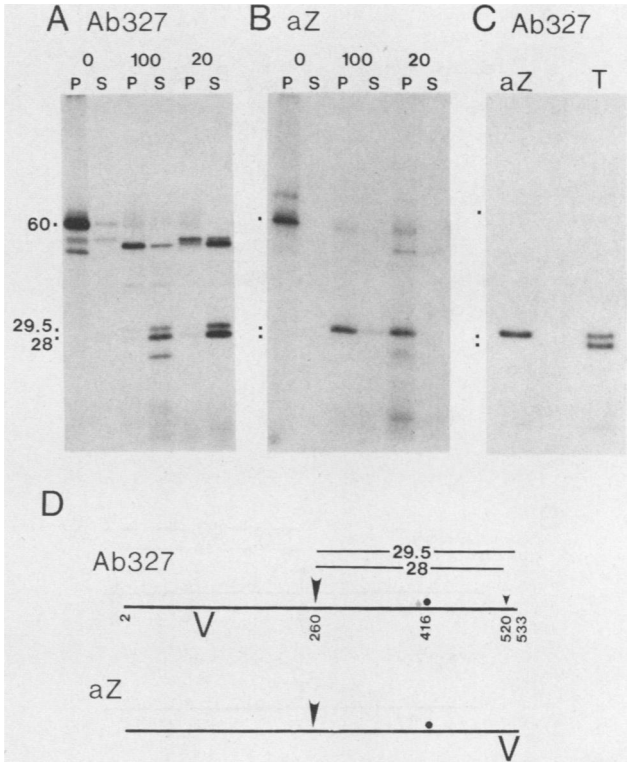

FIG. 3. Partial pronase E digestion of p60^{WT} in 327 and aZ immunoprecipitates. (A) p60^{WT} was immunoprecipitated with monoclonal antibody 327 (Ab327), labeled in an in vitro kinase reaction, and washed in TDB. Pronase E was added to a concentration of 20 or 100 μg/ml and incubated at 25°C for 5 min. The samples were centrifuged, and the supernatants (S) and pellets (P) were separated. An equal volume of twice-concentrated sample buffer was added to the supernatants, and the pellets were brought to the same final volume with sample buffer. Equal volumes of the samples were analyzed by SDS-PAGE. The positions of p60$^{c\text{-}src}$ and 29.5- and 28-kDa bands are indicated on the left of the gel. (B) p60^{WT} immunoprecipitated with aZ was labeled and prepared for digestion as described above. The sample was incubated with 0, 100, or 20 μg of pronase E per ml at 25°C for 5 min. The digested samples were fractionated as described for panel A and analyzed by SDS-PAGE. (C) p60^{WT} immunoprecipitated with 327 was labeled and digested with 100 μg of pronase E per ml as described for panel A. The supernatant from the digestion was diluted 25-fold with RIPA buffer and immunoprecipitated with aZ (lane aZ). A sample of the undiluted supernatant was retained, and an equal volume of twice-concentrated sample buffer was added (lane T). The samples were analyzed by SDS-PAGE. (D) Putative structures of fragments. Sites of cleavage (▼), the site of in vitro labeling (●), and the antibody-binding sites (V) are indicated.

trypsin (Fig. 2). Thermolysin and pronase E digestions gave two different sets of products according to whether the substrate was a repressed or derepressed form of p60$^{c\text{-}src}$. p60^{F527} was cleaved by either protease to 28- or 16- to 18-kDa fragments (Fig. 2). On the other hand, digestion of p60^{WT} with either protease resulted in major products of 50- and 29.5-kDa, in addition to the 28- and 16- to 18-kDa fragments detected with p60^{F527} (Fig. 2). The 50-kDa fragment resembled that produced by trypsin in its partial retention by antibody 327, suggesting cleavage at the trypsin-sensitive site (Fig. 3A). The sizes and kinase activities of the 29.5-kDa fragments released by pronase and thermolysin indicated that they were produced by cleavage at the trypsin-sensitive site at the beginning of the kinase domain. The 29.5-kDa pronase fragment could be precipitated by aZ, confirming that it contained most or all of the extreme carboxy terminus (Fig. 3C). In contrast, the 28-kDa fragment was not precipitated by aZ (Fig. 3C) and seemed to be derived from the 29.5-kDa fragment by further digestion (Fig. 4), suggesting that it lacked the extreme carboxy terminus. Since the sequence specificity of pronase E is broad, it is not clear where the carboxy-proximal cleavage may lie, but the size difference of 1.5 kDa allows tentative placement of a pronase-sensitive site close to Leu-516, the last residue in the kinase domain (Fig. 3D) (39, 52, 54).

Digestion with papain or proteinase K gave a subset of the fragments created by pronase E or thermolysin: mostly small fragments from both p60^{WT} and p60^{F527}, with low yields of a 28-kDa fragment (Fig. 2).

The pattern of pronase digestion depended on the antibody used for immunoprecipitation (Fig. 3A and B). The major pronase digestion product from an immunoprecipitate prepared with aZ appeared to be identical with the 29.5-kDa fragment produced by digestion of antibody 327 immunoprecipitates. The 28-kDa fragment was absent, consistent with masking of the proposed pronase cleavage site near Leu-516 when aZ was bound (Fig. 3D).

We tested whether the increased pronase sensitivity of the carboxy-terminal region of p60^{F527} (Fig. 2) was a general feature of derepressed forms of p60$^{c\text{-}src}$. Indeed, derepression of p60^{WT} by PAP treatment (15) also sensitized the site near Leu-516 (Fig. 4). At low doses of pronase, repressed p60^{WT} was cleaved to 52-, 50-, and 29.5-kDa fragments, but derepressed, PAP-treated p60^{WT} was cleaved to 29.5- and 28-kDa fragments. At higher doses of pronase, repressed p60^{WT} was cleaved further to give mostly the 29.5-kDa fragment and some of the 28-kDa fragment, but PAP-treated p60^{WT} or p60^{F527} was cleaved to give mostly the 28-kDa fragment (Fig. 2 and 4A). The enhanced cleavage of the dephosphorylated p60^{WT} or p60^{F527} by pronase E suggests

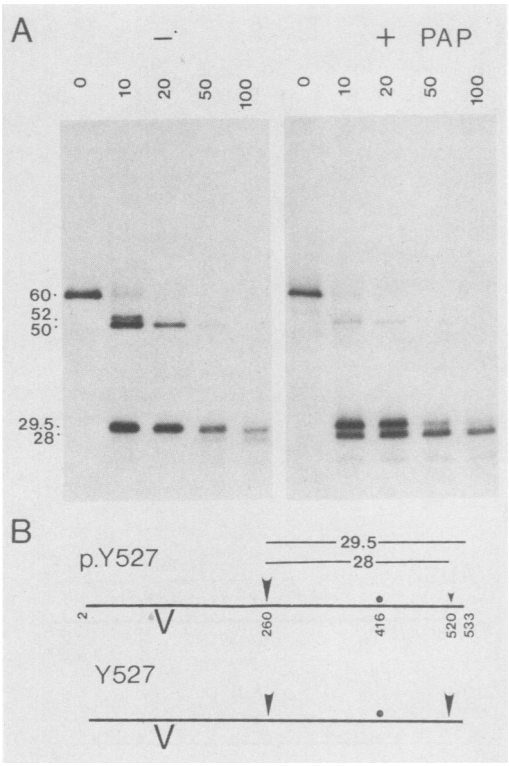

FIG. 4. Pronase E digestion of phosphorylated and dephosphorylated p60^{WT} immunoprecipitated by antibody 327. (A) Antibody 327 immunoprecipitates of p60^{WT} were treated without (−) or with (+) PAP, incubated in an in vitro kinase reaction, and prepared for digestion as described in Materials and Methods. Pronase E was added to the immunoprecipitates at final concentrations of 0, 10, 20, 50, and 100 μg/ml and incubated at 25°C for 5 min. An equal volume of twice-concentrated sample buffer was added, and the samples were analyzed by SDS-PAGE. Gel lanes containing phosphorylated p60^{WT} were exposed five times longer than lanes containing dephosphorylated p60^{WT}. The 50-, 29.5-, and 28-kDa bands are indicated on the left. (B) Sites of cleavage that are believed to give rise to the fragments are shown on the line drawing of p60$^{c\text{-}src}$. The sensitivity of the region of residue 520 to pronase is lower in phosphorylated p60^{WT} (▼) than in dephosphorylated p60^{WT} (▼). Antibody-binding sites (V) are shown.

that derepressed p60$^{c\text{-}src}$ is in a different conformation and that the carboxy-terminal region is more exposed in derepressed than in repressed forms of p60$^{c\text{-}src}$.

Kinase activity of the 29.5-kDa fragment. Digestion of p60$^{v\text{-}src}$ with trypsin releases a 30-kDa fragment with three- to fivefold-increased specific activity, relative to that of undigested p60$^{v\text{-}src}$, when assayed on [Val^5]-angiotensin II (4). We determined the specific activity of the 29.5-kDa fragment of p60$^{c\text{-}src}$ on angiotensin, enolase, and tubulin substrates. To permit comparison with intact p60$^{c\text{-}src}$, we used immunoprecipitates prepared with aZ, incubated with or without pronase E, so that the 29.5-kDa fragment and intact p60$^{c\text{-}src}$ were both assayed as immune complexes. This approach avoided the necessity of comparing antibody-bound intact p60$^{c\text{-}src}$ with soluble 29.5-kDa fragment that would have arisen if antibody 327 immunoprecipitates were used, but introduced the complication that only derepressed activities could be measured (aZ derepresses the activity of p60$^{c\text{-}src}$). The yield of 29.5-kDa fragment was estimated by digesting in parallel an aZ immunoprecipitate of p60$^{c\text{-}src}$ that had been labeled either in vivo with [^{35}S]methionine or in vitro with [γ-^{32}P]ATP. The yield of 29.5-kDa fragment relative to intact p60$^{c\text{-}src}$ was then used to correct the measured kinase activities.

TABLE 1. Protein kinase activities of p60$^{c\text{-}src}$ and its 29.5-kDa fragment[a]

Substrate	Kinase activity (cpm ± SE) of:			Relative activity
	p60$^{c\text{-}src}$	29.5-kDa fragment	Corrected	
Angiotensin	3,220 ± 141	6,000 ± 414	12,600 ± 870	3.9
Enolase	1,820 ± 21	112 ± 25	260 ± 60	0.14
Tubulin	10,955 ± 500	785 ± 120	1,800 ± 280	0.16

[a] p60^{WT} was labeled in vivo with [^{35}S]methionine, immunoprecipitated with aZ, and suspended in TDB. Half the immunoprecipitate was incubated with pronase E (final concentration, 20 μg/ml) for 10 min at 25°C. The immunoprecipitates were washed, and kinase reactions were performed, as described in Materials and Methods. The relative amounts of p60^{WT} and the 29.5-kDa fragment were derived by analysis of a fraction of the two samples by SDS-PAGE and densitometry of the autoradiograms. The actual counts per minute incorporated into the substrates and the corrected values are shown, and the fold change in relative specific activity of the 29.5-kDa fragment relative to p60^{WT} was calculated. Each point was determined in triplicate.

Table 1 shows that the specific activity of the 29.5-kDa fragment relative to that of intact p60^{WT} was elevated fourfold (three- to fivefold in six different experiments) with angiotensin as the substrate but decreased sixfold in this experiment with tubulin as the substrate and decreased sevenfold (7- to 15-fold over four experiments) with enolase as the substrate. Since a fraction of the p60^{WT} was undigested after pronase E treatment, as much as 30 to 50% of the phosphorylation of enolase or tubulin detected with the 29.5-kDa fragment may be due to intact p60^{WT}, suggesting that cleavage of p60^{WT} reduces phosphorylation of protein substrates as much as 15- to 20-fold.

Phosphorylation of exogenous substrates by p60^{WT} is stimulated by aZ (15). To test whether the 29.5-kDa fragment from p60^{WT} was similarly stimulated by aZ, soluble 29.5-kDa fragment was released with trypsin from a 327 immunoprecipitate of p60^{WT}, and phosphorylation of angiotensin was assayed in the presence of various concentrations of aZ (Fig. 5A, lower panel). The ^{32}P incorporated into angiotensin fell within 10% of a mean of 2,220 cpm, independent of aZ concentration, suggesting that the 29.5-kDa fragment from p60^{WT} is not regulated by its phosphorylated carboxy terminus.

Phosphorylation of the 29.5-kDa fragment. Intact p60$^{c\text{-}src}$ becomes phosphorylated at Tyr-416 when incubated with [γ-^{32}P]ATP in vitro, apparently due largely to an intermolecular reaction (16). In contrast, when soluble 29.5-kDa fragment was released with trypsin from 327 immunoprecipitates of p60^{WT} or p60^{F527} and incubated with [γ-^{32}P]ATP, the rate of phosphorylation of the fragments was very low. If purified aZ IgG was added to the phosphorylation reaction, phosphorylation of the 29.5-kDa fragment was stimulated (Fig. 5A). This was observed for 29.5-kDa fragments derived from both p60^{WT} (Fig. 5A, upper panel) and p60^{F527} (data not shown). The degree of stimulation depended on the concentration of aZ: with increasing concentrations of aZ (up to 1% of the reaction volume) the incorporation increased up to 100-fold, but increasing the aZ concentration further (to 10% the reaction volume) gave only 15- to 30-fold stimulation of autophosphorylation over that of the unstimulated reaction. It is possible that aZ stimulates phosphorylation of the 29.5-kDa fragment because it increases the frequency of intermolecular collisions between 29.5-kDa molecules.

The sites of aZ-stimulated 29.5-kDa fragment phosphory-

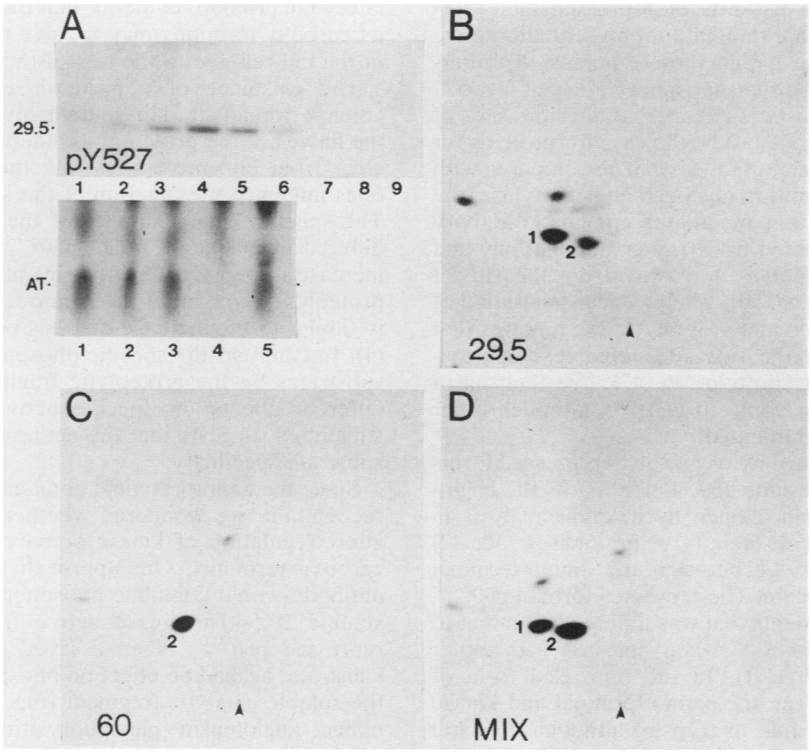

FIG. 5. Protein kinase activity and phosphorylation of the 29.5-kDa fragment. (A) p60^{WT} (upper panel) was immunoprecipitated with antibody 327, washed and suspended in TDB, and digested with 100 μg of TPCK-trypsin per ml at 25°C for 5 min. Aprotinin was added, the reaction was centrifuged, and the supernatant was reserved. The supernatant was split into nine aliquots, and IgG purified from aZ serum was added to the following relative concentrations: lane 1, 10^{-1}; lane 2, $10^{-1.5}$; lane 3, 10^{-2}; lane 4, $10^{-2.5}$; lane 5, 10^{-3}; lane 6, $10^{-3.5}$; lane 7, 10^{-4}; lane 8, $10^{-4.5}$; lane 9, 10^{-1} preimmune IgG. After incubation for 10 min at 0°C, an equal volume of 40 mM PIPES (pH 7.0)–20 mM $MnCl_2$–0.5 μCi of [γ-^{32}P]ATP per μl was added and the reactions were incubated at 30°C for 10 min. The reaction products were separated by SDS-PAGE and autoradiographed. In a parallel experiment (lower panel) the released 29.5 kDa was incubated with the purified aZ serum added to the following relative concentrations: lane 1, 10^{-1}; lane 2, $10^{-1.5}$; lane 3, 10^{-2}; lane 4, 10^{-1} (minus angiotensin); lane 5, 10^{-1} preimmune IgG. The reactions were performed as described above, except kinase reactions 1, 2, 3, and 5 included angiotensin at 1 mM. The reaction products were separated by thin-layer electrophoresis, and the ^{32}P incorporated into the angiotensin was determined by Cerenkov radiation. (B to D) The 29.5-kDa fragment derived from p60^{F527} was isolated from the gel and exhaustively digested with trypsin. A sample of p60^{WT} labeled in vitro was treated in parallel. The resultant phosphopeptides were separated by thin-layer electrophoresis and chromatography. (B) Tryptic phosphopeptides from the 29.5-kDa fragment. (C) Tryptic phosphopeptides of p60^{WT} phosphorylated in vitro. (D) Mixture of the tryptic phosphopeptides of p60^{WT} and the 29.5-kDa fragment. Peptide 1, Novel site of phosphorylation of the 29.5-kDa fragment; peptide 2, phosphopeptide containing Tyr-416. The arrowheads mark the origin.

lation were analyzed by exhaustive trypsin digestion (2, 31). Major and minor phosphopeptides were detected (spots 1 and 2, respectively, Fig. 5B). Both peptides contained only phosphotyrosine. Mixing the phosphopeptides with those released from p60$^{c\text{-}src}$ phosphorylated in vitro (Fig. 5C) showed that the minor site of phosphorylation (spot 2) contains Tyr-416 (Fig. 5D). The major site of phosphorylation (spot 1) is neither Tyr-416 nor Tyr-527. Partial digestion of the in vitro-labeled, denatured 29.5-kDa fragment with *Staphylococcus aureus* V8 protease followed by immunoprecipitation of the fragments with aZ showed that the major phosphorylation site lay in the carboxy-terminal 15 kDa of the kinase domain, but the site has not been localized further. It seems unlikely that the unusual phosphorylation in the 29.5-kDa fragment is an artifact of the reaction promoted by aZ, since aZ does not alter the site of phosphorylation of intact p60$^{c\text{-}src}$ (15). Instead, we suggest that removal of the amino-terminal region of p60$^{c\text{-}src}$ changes the conformation of the kinase domain to render Tyr-416 less accessible and reveal an alternative site for phosphorylation.

DISCUSSION

Partial proteolysis of p60$^{c\text{-}src}$ by pronase or trypsin revealed four or five sites of cleavage, respectively. Some of the sites are cleaved to different extents in repressed and derepressed forms of p60$^{c\text{-}src}$, presumably reflecting different conformations. The derepressed forms of p60$^{c\text{-}src}$ that were examined included p60^{F527}, p60$^{v\text{-}src}$, PAP-treated p60^{WT}, and p60^{WT} bound to aZ. All these proteins gave similar products after partial proteolysis with trypsin (Fig. 1 and unpublished data). The derepressed forms of p60$^{c\text{-}src}$ also gave similar patterns of fragments when digested with pronase E (Fig. 2 to 4). The sites of cleavage were mapped by further digestion of SDS-denatured fragments with *S. aureus* V8 protease and by testing for binding to antibodies that recognize different epitopes in p60$^{c\text{-}src}$. Some of the sites of cleavage coincide with boundaries between the three structural domains predicted from the amino acid sequence: the amino-terminal 250 to 260 residues, the kinase domain, and the carboxy-terminal regulatory tail.

The different cleavage patterns of repressed and derepressed forms of $p60^{c\text{-}src}$ are thought to represent alternative conformations. Others lie within these domains. An alternate explanation would be that phosphorylation of Tyr-527 results in steric or electrostatic effects that alter the sites of cleavage. This has not proven to be the case for proteins for which the results of partial proteolysis can be compared with other structural information. In glycogen phosphorylase, the site of phosphorylation that modulates enzymatic activity and structure as documented by X-ray crystallography and partial proteolysis is relatively far away from the site of proteolytic cleavage (37, 46, 50). The physical separation of the site of phosphorylation and proteolytic site requires that communication between the two sites involve conformational changes within the protein (46, 47). We feel that a similar model is likely to apply to $p60^{c\text{-}src}$, although other explanations cannot be eliminated.

The major site of cleavage by trypsin in derepressed forms of $p60^{c\text{-}src}$ lies close to residue 260, which marks the beginning of the kinase domain defined by deletion analysis in v-*fps*- and v-*abl*-encoded protein-tyrosine kinases (36, 42) and may represent a bridge between the amino-terminal region and the kinase domain. The repressed form of $p60^{c\text{-}src}$ showed a pattern of fragments that was different from that of the activated forms of $p60^{c\text{-}src}$ when digested with either trypsin or pronase E (Fig. 1). In the repressed form of $p60^{c\text{-}src}$, the bridge between the amino-terminal and kinase domains is not as accessible to trypsin, although it is still cleaved efficiently by pronase E and thermolysin. The increased accessibility of the bridge to trypsin upon activation may result from a shift of the amino terminus relative to the kinase domain.

A major site of trypsin cleavage in repressed phosphorylated $p60^{c\text{-}src}$ was mapped approximately to residues 440 to 450. This site falls within the kinase domain in the vicinity of mutations that modulate $p60^{v\text{-}src}$ activity (6, 23, 29). The exposure of this region implies that repression of $p60^{c\text{-}src}$ kinase activity by phosphorylation of Tyr-527 alters the conformation of the catalytic domain. Another major site of cleavage in the repressed $p60^{c\text{-}src}$ appears to fall in the SH2 region upstream of the kinase domain, which is conserved between *src* family kinases and also found in other cytoplasmic protein-tyrosine kinases (42). The function of this region is unknown, but mutations in this region in $p60^{v\text{-}src}$ or $P130^{gag\text{-}fps}$ can abrogate transforming activity (5, 42). The conformational changes in the SH2 region during activation of $p60^{c\text{-}src}$ implicate it in regulation of kinase activity.

Digestions with pronase E and thermolysin suggest that the carboxy-terminal regulatory region exists as a separate domain. Mutant forms of $p60^{c\text{-}src}$ truncated at various residues distal to Leu-516 have catalytic activity (39, 53, 54), implying that the kinase domain folds independently of the carboxy terminus. The sequence immediately surrounding Tyr-527 can sustain some point mutations without abrogating the regulatory function (9, 31), but insertions or deletions between Asp-518 and Tyr-519 are activating (B. Cobb, M. Payne, and T. Parsons, personal communication). It appears that the contributions of individual residues to the structure of this region are relatively unimportant, but the spacing between Tyr-527 and the kinase domain may be important to allow interaction between the carboxy terminus and the body of the protein. The region may exist in a relatively extended conformation, hence its accessibility to proteases. Phosphorylation of Tyr-527 represses kinase activity and partially protects the tail from proteolysis by pronase E or thermolysin. The change in protease sensitivity in the regulatory tail provides evidence that the regulation of $p60^{c\text{-}src}$ by its carboxy terminus may involve a conformational change of the tail relative to the rest of the molecule.

The existence of a protease-sensitive bridge between amino-terminal and kinase domains of $p60^{c\text{-}src}$ suggests that the halves of the protein fold independently. However, it is clear from enzyme assays that the amino-terminal region does influence the function of the kinase domain (Table 1). The specific kinase activity of the isolated kinase domain differed from that of intact $p60^{c\text{-}src}$. The three- to fivefold increase in peptide (angiotensin) phosphorylation following proteolytic removal of the amino-terminal region of $p60^{c\text{-}src}$ is similar to the increase that has been reported for $p60^{v\text{-}src}$ (4). In contrast, the specific phosphorylation of two protein substrates by the proteolytic fragments was reduced. The different effects on specific activity toward the various substrates suggests that the amino-terminal region modifies substrate specificity.

Since the amino-terminal domain participates in substrate recognition, we wondered whether it was also required to allow regulation of kinase activity by the phosphorylated carboxy terminus. Our approach was to test whether aZ antibody would stimulate phosphorylation catalyzed by the soluble 29.5-kDa kinase active fragment produced from repressed $p60^{WT}$. When assayed with angiotensin as the substrate, aZ had no effect on phosphorylation catalyzed by the soluble catalytic fragment (Fig. 5), even though it stimulated angiotensin phosphorylation catalyzed by intact $p60^{WT}$ six- to eightfold. It appears that the amino-terminal domain is required for normal regulation of the kinase domain by the phosphorylated carboxy-terminal tail. If regulation involves the tail acting as a pseudosubstrate or pseudoproduct (15), then it is possible that the amino-terminal domain induces a conformation in the kinase domain that can interact with the carboxy terminus.

Curiously, aZ stimulated phosphorylation of the soluble catalytic domain up to 100-fold (Fig. 5). Since this effect was observed whether the fragment was derived from $p60^{WT}$ or $p60^{F527}$ and was not seen when an exogenous substrate was used, it is unlikely that aZ exerts its effect by increasing the specific activity of the fragment. We suspect that phosphorylation of the catalytic fragment is an intermolecular reaction that occurs at a negligible rate at the low enzyme concentrations employed but which is stimulated by cross-linking with aZ. The dose-response curve suggests that aZ is serving to promote intermolecular interactions between catalytic fragments. Thus, in antigen excess, at low doses of aZ, most antibody molecules bind two molecules of catalytic fragment, but in antibody excess the proportion of catalytic fragments that are bound with a partner to a single aZ molecule decreases.

The structural changes that result from proteolytic removal of the amino terminus are reflected in the change in the major site of phosphorylation as well as in changes in specific kinase activity. It is clear that the amino-terminal domain interacts with the kinase domain both to modify its substrate selectivity and to allow its regulation by the phosphorylated carboxy terminus. The complex effects of the amino-terminal domain on catalytic activity in vitro are consistent with the complex phenotypes of amino-terminal mutants of $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ (22, 24, 38, 48).

ACKNOWLEDGMENTS

We thank Tina Loucks for secretarial assistance and Janna Briscoe for technical assistance.

This work was supported by a grant from the National Cancer Institute of Canada (to A.M.) and by Public Health Service grant CA-41072 from the National Institutes of Health.

LITERATURE CITED

1. **Basu, M., R. Biswas, and M. Das.** 1984. 42,000-Molecular weight EGF receptor has protein kinase activity. Nature (London) **311**:477–480.
2. **Beemon, K., and T. Hunter.** 1978. Characterization of Rous sarcoma virus *src* gene products synthesized in vitro. J. Virol. **28**:551–566.
3. **Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A. Lipsich, and J. S. Brugge.** 1984. Enhancement of cellular *src* gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. Cell **38**:767–777.
4. **Brugge, J. S., and D. Darrow.** 1984. Analysis of the catalytic domain of phosphotransferase activity of two avian sarcoma virus-transforming proteins. J. Biol. Chem. **259**:4550–4557.
5. **Bryant, D., and J. T. Parsons.** 1982. Site-directed mutagenesis of the *src* gene of Rous sarcoma virus: construction and characterization of a deletion mutant temperature sensitive for transformation. J. Virol. **45**:1211–1216.
6. **Bryant, D. L., and J. T. Parsons.** 1984. Amino acid alterations within a highly conserved region of the Rous sarcoma virus *src* gene product pp60^{src} inactivate tyrosine protein kinase activity. Mol. Cell. Biol. **4**:862–866.
7. **Cartwright, C. A., W. Eckhart, S. Simon, and P. L. Kaplan.** 1987. Cell transformation by pp60$^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.
8. **Cartwright, C. A., P. L. Kaplan, J. A. Cooper, T. Hunter, and W. Eckhart.** 1986. Altered sites of tyrosine phosphorylation in pp60$^{c\text{-}src}$ associated with polyoma middle tumor antigen. Mol. Cell. Biol. **6**:1562–1570.
9. **Cheng, S. H., H. Piwnica-Worms, R. W. Harvey, T. M. Roberts, and A. E. Smith.** 1988. The carboxy terminus of pp60$^{c\text{-}src}$ is a regulatory domain and is involved in complex formation with the middle-T antigen of polyomavirus. Mol. Cell. Biol. **8**: 1736–1747.
10. **Chinkers, M., and J. S. Brugge.** 1984. Characterization of structural domains of the human epidermal growth factor receptor obtained by partial proteolysis. J. Biol. Chem. **259**:11534–11542.
11. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
12. **Collett, M. S., E. Erikson, and R. L. Erikson.** 1979. Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. J. Virol. **29**:770–781.
13. **Cooper, J. A.** 1989. The *src*-family of protein-tyrosine kinases. *In* B. Kemp and P. F. Alewood (ed.), Peptides and protein phosphorylation. CRC Press, Inc., Boca Raton, Fla.
14. **Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter.** 1986. Tyr^{527} is phosphorylated in p60$^{c\text{-}src}$: implications for regulation. Science **231**:1431–1434.
15. **Cooper, J. A., and C. S. King.** 1986. Dephosphorylation or antibody binding to the carboxy terminus stimulates pp60$^{c\text{-}src}$. Mol. Cell. Biol. **6**:4467–4477.
16. **Cooper, J. A., and A. MacAuley.** 1988. Potential positive and negative autoregulation of p60$^{c\text{-}src}$ by intermolecular autophosphorylation. Proc. Natl. Acad. Sci. USA **85**:4232–4236.
17. **Cooper, J. A., and K. Runge.** 1987. Avian pp60$^{c\text{-}src}$ is more active when expressed in yeast than in vertebrate fibroblasts. Oncogene Res. **1**:297–310.
18. **Courtneidge, S. A.** 1985. Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. EMBO J. **4**:1471–1477.
19. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45**:721–732.
20. **Hunter, T., and J. A. Cooper.** 1986. Viral oncogenes and tyrosine phosphorylation, p. 191–246. *In* P. D. Boyer and E. G. Krebs (ed.), The enzymes, vol. 17. Academic Press, Inc., Orlando, Fla.
21. **Jove, R., S. Kornbluth, and H. Hanafusa.** 1987. Enzymatically inactive p60$^{c\text{-}src}$ mutant with altered ATP-binding site is fully phosphorylated in its carboxy-terminal regulatory region. Cell **50**:937–943.
22. **Jove, R., B. J. Mayer, H. Iba, D. Laugier, F. Poirier, G. Calothy, T. Hanafusa, and H. Hanafusa.** 1986. Genetic analysis of p60$^{v\text{-}src}$ domains involved in the induction of different cell transformation parameters. J. Virol. **60**:840–848.
23. **Kato, J., T. Takeya, C. Grandori, H. Iba, J. B. Levy, and H. Hanafusa.** 1986. Amino acid substitutions sufficient to convert the nontransforming p60$^{c\text{-}src}$ protein to a transforming protein. Mol. Cell. Biol. **6**:4155–4160.
24. **Kitamura, N., and M. Yoshida.** 1983. Small deletion in *src* of Rous sarcoma virus modifying transformation phenotypes: identification of 207-nucleotide deletion and its smaller product with protein kinase activity. J. Virol. **46**:985–992.
25. **Kmiecik, T. E., P. J. Johnson, and D. Shalloway.** 1988. Regulation by the autophosphorylation site in overexpressed pp60$^{c\text{-}src}$. Mol. Cell. Biol. **8**:4541–4546.
26. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60$^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49**:65–73.
27. **Laudano, A. P., and J. M. Buchanan.** 1986. Phosphorylation of tyrosine in the carboxyl-terminal tryptic peptide of pp60$^{c\text{-}src}$. Proc. Natl. Acad. Sci. USA **83**:892–896.
28. **Levinson, A. D., S. A. Courtneidge, and J. M. Bishop.** 1981. Structural and functional domains of the Rous sarcoma virus transforming protein (pp60^{src}). Proc. Natl. Acad. Sci. USA **78**:1624–1628.
29. **Levy, J. B., H. Iba, and H. Hanafusa.** 1986. Activation of the transforming potential of p60$^{c\text{-}src}$ by a single amino acid change. Proc. Natl. Acad. Sci. USA **83**:4228–4232.
30. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma virus. J. Virol. **48**:352–360.
31. **MacAuley, A., and J. A. Cooper.** 1988. The carboxy-terminal sequence of p56^{lck} can regulate p60$^{c\text{-}src}$. Mol. Cell. Biol. **8**: 3560–3564.
32. **Patschinsky, T., T. Hunter, F. S. Esch, J. A. Cooper, and B. M. Sefton.** 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA **79**:973–977.
33. **Patschinsky, T., T. Hunter, and B. M. Sefton.** 1986. Phosphorylation of the transforming protein of Rous sarcoma virus: direct demonstration of the phosphorylation of serine 17 and identification of an additional site of tyrosine phosphorylation in p60$^{v\text{-}src}$ of Prague Rous sarcoma virus. J. Virol. **59**:73–81.
34. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of pp60$^{c\text{-}src}$. Cell **49**:75–82.
35. **Potts, W. M., A. B. Reynolds, T. J. Lansing, and J. T. Parsons.** 1988. Activation of pp60$^{c\text{-}src}$ transforming potential by mutations altering the structure of an amino terminal domain containing residues 90-95. Oncogene Res. **3**:343–355.
36. **Prywes, R., J. G. Foulkes, and D. Baltimore.** 1985. The minimum transforming region of v-*abl* is the segment encoding protein-tyrosine kinase. J. Virol. **54**:114–122.
37. **Raibaud, O., and M. E. Goldberg.** 1973. Characterization of two complementary polypeptide chains obtained by proteolysis of rabbit muscle phosphorylase. Biochemistry **12**:5154–5161.
38. **Raymond, V. W., and J. T. Parsons.** 1987. Identification of an amino terminal domain required for the transforming activity of the Rous sarcoma virus *src* protein. Virology **160**:400–410.
39. **Reynolds, A. B., J. Vila, T. J. Lansing, W. M. Potts, M. J. Weber, and J. T. Parsons.** 1987. Activation of the oncogenic potential of the avian cellular *src* protein by specific structural alteration of the carboxy-terminus. EMBO J. **6**:2359–2364.
40. **Rosen, O. M., R. Herrera, Y. Olowe, L. M. Petruzzelli, and M. H. Cobb.** 1983. Phosphorylation activates the insulin recep-

tor tyrosine protein kinase. Proc. Natl. Acad. Sci. USA **80:** 3237–3240.

41. **Roth, C. W., N. D. Richert, I. Pastan, and M. M. Gottesman.** 1983. Cyclic AMP treatment of Rous sarcoma virus-transformed Chinese hamster ovary cells increases phosphorylation of $pp60^{src}$ and increases $pp60^{src}$ kinase activity. J. Biol. Chem. **258:**10768–10773.
42. **Sadowski, I., J. C. Stone, and T. Pawson.** 1986. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus $P130^{gag\text{-}fps}$. Mol. Cell. Biol. **6:**4396–4408.
43. **Schuh, S. M., and J. S. Brugge.** 1988. Investigation of factors that influence phosphorylation of $pp60^{c\text{-}src}$ on tyrosine 527. Mol. Cell. Biol. **8:**2465–2471.
44. **Smart, J. E., H. Opperman, A. P. Gernilofsky, A. F. Purchiv, R. L. Erikson, and J. M. Bishop.** 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus ($pp60^{v\text{-}src}$) and its normal cellular homologue ($pp60^{c\text{-}src}$). Proc. Natl. Acad. Sci. USA **78:**6013–6017.
45. **Snyder, M. A., and J. M. Bishop.** 1984. A mutation at the major phosphotyrosine in $pp60^{v\text{-}src}$ alters oncogenic potential. Virology **136:**375–386.
46. **Sprang, S., and R. J. Fletterick.** 1979. The structure of glycogen phosphorylase a at 2.5Å resolution. J. Mol. Biol. **131:**523–551.
47. **Sprang, S. R., K. R. Acharya, E. J. Goldsmith, D. I. Stuart, K. Varvill, R. J. Fletterick, N. B. Madsen, and L. N. Johnson.** 1988. Structural changes in glycogen phosphorylase induced by phosphorylation. Nature (London) **336:**215–221.
48. **Stoker, A. W., S. Kellie, and J. A. Wyke.** 1986. Intracellular localization and processing of $pp60^{v\text{-}src}$ proteins expressed by two distinct temperature-sensitive mutants of Rous sarcoma virus. J. Virol. **58:**876–883.
49. **Tornquist, H. E., M. W. Pierce, A. R. Frackelton, R. A. Nemenoff, and J. Avruch.** 1987. Identification of insulin receptor tyrosine residues autophosphorylated in vitro. J. Biol. Chem. **262:**10212–10219.
50. **Weber, I. T., L. N. Johnson, K. S. Wilson, D. G. R. Yeates, D. L. Wild, and J. A. Jenkins.** 1978. Crystallographic studies on the activity of glycogen phosphorylase b. Nature (London) **274:**433–437.
51. **Weinmaster, G., M. J. Zoller, M. Smith, E. Hinze, and T. Pawson.** 1984. Mutagenesis of Fujinami sarcoma virus: evidence that tyrosine phosphorylation of $P130^{gag\text{-}fps}$ modulates its biological activity. Cell **37:**559–568.
52. **Wilkerson, V. W., D. L. Bryant, and J. T. Parsons.** 1985. Rous sarcoma virus variants that encode *src* proteins with an altered carboxy terminus are defective for cellular transformation. J. Virol. **55:**314–321.
53. **Yaciuk, P., M. T. Cannella, and D. Shalloway.** 1988. Comparison of the effects of carboxyl terminal truncation and point mutations on $pp60^{c\text{-}src}$ activities. Oncogene Res. **3:**207–212.
54. **Yaciuk, P., and D. Shalloway.** 1986. Features of the $pp60^{v\text{-}src}$ carboxyl terminus that are required for transformation. Mol. Cell. Biol. **6:**2807–2819.