Two activated transforming mutants of human pp60<sup>src</sup> were found to possess single point mutations within the regulatory carboxyl terminus (E527K in CY CST201) and the kinase domain (E381G in WO CST1), respectively, that do not directly interfere with either the regulatory c-Src kinase (CSK) phosphorylation site (Tyr<sup>530</sup>) or the SH2/3 domains. In vivo, both mutant proteins are hypophosphorylated on their carboxyl-terminal regulatory tyrosines and are hyperactive. In an in vitro Src kinase inactivation assay, both mutant Src proteins exhibited resistance to inactivation by CSK relative to wild-type Src. Under these in vitro conditions, E381G c-Src was found to be phosphorylated by CSK to wild-type levels, while E527K c-Src was not detectably phosphorylated. The ability of CSK to phosphorylate a carboxyl-terminal peptide modelled against E527K c-Src was also impaired, suggesting that CSK is unable to recognize E527K c-Src as an efficient substrate. In the case of E381G c-Src, examination of whether its SH2/3 domains were accessible to the carboxyl-terminal regulatory phosphotyrosine revealed a highly reduced ability of autophosphorylated E381G c-Src to bind to a synthetic phosphopeptide modelled from the SH2-binding region of polyoma middle-T antigen which binds to Src SH2 with high affinity. This suggests that the E381G c-Src mutation results in an altered or reduced accessibility of the SH2 domain of the autophosphorylated form of E381G c-Src and may represent a previously undescribed mode of Src activation. Further study of these and other Src mutants may offer additional new insights into the regulation of “Src family” kinases.

pp60<sup>src</sup>, or Src, is a membrane-associated tyrosine kinase and the cellular homologue of the highly oncogenic form, pp60<sup>v-src</sup>, or v-Src, that is encoded by Rous sarcoma virus. pp60<sup>src</sup>, and the other related members of the “Src family” which includes Src, Lck, Fyn, Lyn, Hck, Fgr, Blk, Yrk, and Yes, are thought to be key components in signal transduction pathways that relay signals received at the cell membrane to the cytoplasm and nucleus (1). Current investigations have identified the particular importance of Lck in T-cell receptor (2) and Src in platelet-derived growth factor receptor (3) pathways. Src family members have the potential to become transforming proteins as a result of regulatory defects, and pp60<sup>src</sup>, in particular, has been implicated in the development of human breast and colon cancer (4, 5).

pp60<sup>src</sup> kinase activity is tightly controlled in vivo, and various studies have elucidated the importance of a highly conserved carboxyl-terminal tyrosine regulatory site (Tyr<sup>527</sup>) in chicken pp60<sup>src</sup>, Tyr<sup>530</sup> in human pp60<sup>src</sup>). Src proteins which lack this tyrosine due to mutation exhibit elevated kinase activity in vivo and the ability to cause cellular transformation and tumor formation (6, 7). Activation has also been observed in other Src family members with similar mutations (8–10), emphasizing the importance of this region and suggesting the presence of a common regulatory mechanism.

The mechanism by which phosphorylation at Tyr<sup>527</sup> regulates Src activity has been addressed in several laboratories (11–16). The currently accepted theory proposes an intramolecular interaction of the phosphorylated carboxyl-terminal tyrosine with the SH2/SH3 domains of Src such that the enzyme activity is inhibited. Recently, a protein tyrosine kinase, CSK,<sup>1</sup> that can phosphorylate Tyr<sup>527</sup> and inactivate Src activity has been identified (17–21). CSK can also regulate other Src family members, including Lck, Fyn, and Lyn, by phosphorylation of the corresponding carboxyl-terminal tyrosine residue (17, 22). Src, Fyn, and Lyn activity is elevated in CSK<sup>−/−</sup> mice (23, 24), supporting the in vivo importance of CSK and implying a common mode of regulation for several Src family members.

Interestingly, activating mutations in the Src family of tyrosine kinases are not limited to mutations that remove the CSK tyrosine phosphorylation site. Activating mutations have been found throughout Src, with the exception of the unique region (25). In our laboratory, we have used a retroviral-mediated selection procedure to isolate and characterize a number of activated transforming mutants of human Src that do not directly alter Tyr<sup>530</sup> (7) and have begun biochemical studies.
using highly purified forms of Src to examine how these mutations alter Src tyrosine kinase activity and make Src refractory to normal regulation by CSK.

MATERIALS AND METHODS

Isolation and Cloning of Mutant Retroviruses—CEF cells were infected with a replication-competent retroviral vector containing normal human pp60^c-src DNA (WO CS) (26). After 1 month in culture, the cells in some plates became phenotypically transformed, and WO CST1 and other Src mutants were derived from these cultures (7). Other plates of infected cells were treated with 5-azacytidine for 24 h, and CY CST201 was isolated from these cultures. Mutant viruses were recovered from these plates and plated on normal chicken embryo cells in a focus-forming assay (27). Isolated foci displaying a transformed phenotype were identified by hybridization of nylon filter plaque lifts with a Src-specific DNA probe. Bluescript plasmid containing the CY CST201 DNA insert was recovered by in vivo excision, and the 3.2-kilobase EcoRI insert containing WO CST1 was transferred to a pBR322 subclone containing a 3-Rous sarcoma virus vector derived from pAT CS (26). BAC Src insert was subjected to deoxyribonuclease I digestion.

Baculovirus Constructs—Bluescript plasmids (Stratagene) containing wild-type or E381G c-Src or an EcoRI insert were initially digested with NcoI, treated with the Klenow fragment of DNA polymerase I to create blunt ends, and partially digested with KpnI to excise a fragment containing the entire Src coding region. The purified insert was directionally ligated into Smal/KpnI-digested baculovirus transfer vector pVL941 (kindly provided by M. Summers). pVL941 containing the Src insert was cotransfected with wild-type baculovirus DNA into Sf9 cells and recombinant baculoviruses isolated by visual screening for occurrence negative plaques. Src production by baculovirus-infected Sf9 cells was confirmed by immunoblots with 327 anti-Src antibody (29).

Isolation of Src Mutants Resistant to CSK—Sf9 cells (approximately 10^9 cells) grown in TNM medium (Life Technologies, Inc.) were infected with recombinant baculoviruses. Sf9 cells were scraped and resuspended in lysis buffer containing 60 mM Hepes, pH 7.5, 5 mM EDTA, 1%, Nonidet P-40, 50 μg/ml leupeptin, 0.86 mg/ml benzamidine-HCl, 0.07 mM phenylmethylsulfonyl fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10% (v/v) glycerol, and 50 mM NaCl. The lysate was then clarified by centrifugation at 15,000 × g for 15 min. The clarified lysate was then incubated with 327 anti-Src antibody-coupled Hg agarose (Bio-Rad) for 2 h at 4°C. The agarose beads were washed sequentially in buffers containing 25 mM Hepes, pH 7.4, 0.15 M NaCl, 0.1% Nonidet P-40 (Buffer A), 25 mM Hepes, pH 7.4, 1 mM DTT, 0.1% Nonidet P-40 (Buffer B), and 1 M Hepes, pH 7.4, 1 mM DTT, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 50 mM NaCl. The beads were then washed 3 times with binding buffer. The resin was then resuspended in 1.5 × 1.5 cm square of p81 phosphocellulose paper. The filter papers were washed five times for 3 min each with 0.43% phosphoric acid, rinsed once with acetone, air-dried, and the counts incorporated into the peptide were quantitated by liquid scintillation counting.

CNBr Cleavage Analysis—The Src band was visualized by autoradiography or excised and incubated in solution of 50 mg/ml CNBr in 70% formic acid for 1 h at 20°C as described previously (31). The cleaved product protocol.

Preparation of Synthetic Peptides—Synthetic peptides were synthesized with the Applied Biosystems Model A431 automated peptide synthesizer using Fmoc chemistry. The middle-T antigen phosphopeptide (YEEIPIYL) was added to the growing GTPase chain using the procedure of Ottinger et al. (32). The resulting peptides were purified by gel filtration followed by C_{18}-reverse phase HPLC. Purity and authenticity was confirmed by analytical reverse phase HPLC, amino acid analysis, and mass spectrometry.

Phosphorylation of Fyn Carboxyl-terminal Peptides by CSK—75 ng of purified CSK was incubated with either a peptide modelled against residues 505–537 of the human Fyn carboxyl terminus (fcp) containing the sequence of polyoma middle-T antigen derived from the sequence of polyoma middle-T antigen (THQE EEPQ YTEP IE FCP) and fcp containing a mutation at Giu^{522} V Lysis corresponding to the ES27K C-Src mutation (mtcp). Incubations of the peptides with CSK were carried out in 30-μl reaction volumes in buffer containing 50 mM Hepes, pH 7.4, 5 mM MgCl_{2}, 50 μM [γ^{32}P]ATP (approximately 1 μCi/250 pmol), 0.15 M NaCl, 1 mM DTT, and 0.02% Nonidet P-40 for 30 min at 30°C. 10 μl of 250 μM cd2 substrate peptide (5'Kcoc2) (30) was then added and incubated for an additional 15 min. The reaction was stopped by the addition of EDTA to a final concentration of 50 μM (v/v) glycerol, and 50 μM sodium-magnesium-phosphate-buffered saline containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG followed by detection using ECL immunoblot detection reagents (Amersham) and exposure to x-ray film as described in the product protocol.

Src Inactivation Assay—30 ng of each purified Src protein was incubated together with purified CSK (kindly provided by Dr. D. O. Morgan) in 40 μl of kinase reaction buffer containing 50 mM Hepes, pH 7.4, 5 mM MgCl_{2}, 62.5 μM [γ^{32}P]ATP (approximately 1 μCi/250 pmol), 0.15 M NaCl, 1 mM DTT, and 0.02% Nonidet P-40 for 40 min at 30°C. 10 μl of 250 μM cd2 substrate peptide (5'Kcoc2) (30) was then added and incubated for an additional 15 min. The reaction was stopped by the addition of EDTA to a final concentration of 50 μM (v/v) glycerol, and 50 μM sodium-magnesium-phosphate-buffered saline containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG followed by detection using ECL immunoblot detection reagents (Amersham) and exposure to x-ray film as described in the product protocol.

Phosphorylation of Src by CSK—100 ng of Src was incubated with either a peptide modelled against residues 505–537 of the human Fyn carboxyl terminus (fcp, WK D PEERPFTF YQ SQFL ED YAT E P T QY QP G N E L) or fcp containing a mutation at Giu^{522}→Lys corresponding to the ES27K C-Src mutation (mtcp). Incubations of the peptides with CSK were carried out in 30-μl reaction volumes in buffer containing 50 mM Hepes, pH 7.4, 5 mM MgCl_{2}, 50 μM [γ^{32}P]ATP (approximately 1 μCi/1500 pmol), 0.15 M NaCl, 1 mM DTT, and 0.02% Nonidet P-40 for 45 min at 30°C. After stopping the reaction, the incorporated radioactivity was quantitated as described in the Src inactivation assay.

Binding of Src to a Phosphopeptide Derived from Polyoma Middle-T Antigen—A synthetic phosphopeptide derived from the sequence of polyoma middle-T antigen (THQ E EPQ Y TEP IE FCP) was coupled to Affi-Gel 35 (Bio-Rad) using the enclosed product protocol. The concentration of phosphopeptide per ml of washed resin following the coupling reaction was approximately 100 μl. For carrying out Src binding experiments, 1 volume of phosphopeptide-coupled resin was diluted with 4 volumes of Affi-Gel 15 that contained no bound peptide, but whose reactive groups had been blocked with ethanolamine.

To measure the effects on phosphorylation of Src on its ability to bind to the middle-T phosphopeptide resin, 100 ng of Src was incubated in the absence or presence of 25 μM ATP and 300 ng of CSK in 50 μl of kinase reaction buffer containing 50 mM Hepes, pH 7.4, 5 mM MgCl_{2}, 0.15 M NaCl, 1 mM DTT, and 0.02% Nonidet P-40 for 30 min at 30°C. EDTA was then added to a final concentration of 25 μM and the ability of Src to bind to the middle-T phosphopeptide resin was measured by adding one-fifth of each kinase reaction (10 μl) to 10 μl (packed volume) of middle-T phosphopeptide beads that had been washed and resuspended in 30 μl of binding buffer containing 50 mM Hepes, pH 7.4, 1 mM DTT, 0.1% Nonidet P-40, 10% glycerol, 0.1 M NaCl, and 0.1% (w/v) bovine serum albumin. Following a 1-h incubation at 4°C on a rotator, the supernatant was removed and the beads were washed three times with buffer. The resin was then washed three times in SDS-PAGE sample buffer, and an aliquot was subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with 2-17 anti-Src antibody.
RESULTS

Isolation and Sequencing of Src Mutants—Our laboratory has isolated several spontaneously derived and chemically induced transforming viruses harboring mutant forms of pp60^src from chicken embryo fibroblasts infected with a retroviral construct harboring the normal human pp60^src oncogene (WO CS (26)). Spontaneous mutants of WO CS arose during prolonged culture of WO CS-infected chicken embryo fibroblasts. Isolation and initial characterization of several of the mutant viruses that were capable of inducing distinct transformed morphologies in chicken embryo fibroblasts and tumors in chickens has been presented previously (7). Additional mutant viruses were obtained by treating WO CS-infected chicken embryo fibroblasts with 5-azacytidine for 24 h. Mutant viruses were biologically selected on the basis of their ability to form foci in chicken embryo fibroblast monolayers (Ref. 7 and results not shown).

Cloning and sequencing of the src gene of two of the viruses, WO CST1 and CY CST201, that were isolated either in the absence or presence of 5-azacytidine, respectively, revealed that CY CST201 (which will subsequently be referred to as E527K src) possessed only a single nucleotide mutation (A1142 → G) that caused a single amino acid change (Glu^527 → Lys) near the carboxyl terminus and 3 amino acids amino-terminal from the CSK phosphorylation site (Fig. 1). WO CST1 (which will subsequently be referred to as E381G src) was also found to contain only a single nucleotide mutation (A1424 → G) that caused a single amino acid change (Glu^381 → Gly), but it was located within the kinase domain.

Cyanogen bromide cleavage of in vivo[^2P]orthophosphate-labeled Src immunoprecipitated from virally infected CEF cells was used to generate Src fragments that contain the regulatory phosphorylation sites of Src (31). Fig. 2A shows that both E381G src and E527K src were hypophosphorylated on the 5.2-kDa peptide containing Tyr^530 and hyperphosphorylated on the 7.8-kDa peptide containing Tyr^419 relative to wild-type Src. In addition, the two mutant proteins had 10- and 29-fold elevations (E527K src and E381G src, respectively) in tyrosine kinase activity relative to wild-type Src when immunoprecipitated from CEF cells under conditions which preserve the regulatory phosphorylation sites (Fig. 2B).

Purification and Characterization of Src Protein—To facilitate biochemical studies, Src protein was isolated from baculovirus-infected Sf9 cells overexpressing wild-type or E381G src or from CEF cells infected with retroviruses encoding E527K c-src. All purifications were carried out using immunoaffinity chromatography in the absence of phosphatase inhibitors to facilitate purification of dephosphorylated Src protein in the "active" conformation. Some preparations of wild-type and E381G src were purified further by Mono Q anion exchange affinity chromatography in the absence of phosphatase inhibitors from normal human foreskin fibroblasts and which have been shown to be tyrosine-phosphorylated primarily at Tyr^530 (33). All of our purified Src preparations contained <10% of the phosphorysine found in human foreskin fibroblast pp60^src (by scanning densitometry of the bands).

The purified Src protein was next examined for tyrosine kinase activity by its ability to phosphorylate a highly Src family-specific synthetic peptide substrate modelled against residues 6–20 of cdc2 ([^32P]cdc2 (30)). Wild-type and E527K src demonstrated equivalent activities against the cdc2 peptide at all concentrations tested up to 1100 μM (Fig. 4). Interestingly, E381G src was approximately 2–4-fold more active at phosphorylating the peptide relative to the other two purified proteins. This difference was observed in several Src preparations and was observed also when using Src immunoprecipitated directly from crude Sf9 cell lysates (results not shown).

Regulation of Src Activity by CSK—We examined the ability of purified human CSK to inactivate purified wild-type, E381G src, or E527K c-src protein in a soluble in vitro inactivation
assay. CSK was incubated with each Src protein to allow for potential phosphorylation of Src by CSK, and then cdc2 peptide was added to measure Src kinase activity. As shown in Fig. 5, it was apparent that CSK inactivated wild-type Src, with half-maximal inactivation observed using 60 ng of CSK. Both E381G c-Src and E527K c-Src exhibited resistance to inactivation by CSK, and only a small amount of inactivation (approximately 10–15%) was observed at the highest level of CSK tested (300 ng). We estimate that wild-type Src is at least 80-fold more sensitive to inactivation by CSK than either of the mutant Src kinases. In control experiments (results not shown), CSK was unable to phosphorylate the synthetic cdc2 peptide. All assays were carried out under conditions in which Src phosphorylation of the synthetic cdc2 peptide was linear. The resistance of the mutants to inactivation by CSK was reproducibly observed under a variety of experimental conditions, including conditions utilizing Src in immunoprecipitates (results not shown).

Src Phosphorylation by CSK—We next examined whether the resistance to CSK inactivation was due to the inability of CSK to phosphorylate these mutant Src proteins or the inability of CSK phosphorylation to inactivate these Src proteins. Src was treated with 300 ng of CSK under conditions identical with those used in Fig. 5 with the exception of the use of higher specific activity ATP to facilitate the visualization of CNBr-generated phosphopeptides containing the various phosphorylation sites of Src. Results in Fig. 6 show the ability of CSK to phosphorylate a 5.2-kDa fragment of normal human Src containing Tyr530. CSK appeared equally able to phosphorylate E381G c-Src, but showed a marked inability to phosphorylate E527K c-Src. A very small amount of autophosphorylation on Tyr530 was evident in the wild-type and E381G c-Src lanes when the reactions were carried out in the absence of CSK, but was not observed with E527K c-Src. Low levels of autophosphorylation on Tyr530 have also been observed by other investigators (34). Small amounts of autophosphorylation and CSK-mediated phosphorylation of Tyr530 in E527K c-Src have been observed under different incubation conditions (results not shown), but the relative phosphorylation patterns of the three Src proteins have remained the same.

CSK Phosphorylation of Normal and Mutant Peptides Modelled against the Src Family Carboxyl Terminus—To further examine the importance of Glu527 in CSK phosphorylation, we synthesized a Fyn carboxyl-terminal peptide containing the E527K c-Src mutation (mfc) and compared the ability of CSK to phosphorylate the mutant and wild-type peptides. Phosphorylation assays using CSK indicated that there was a marked inability of CSK to phosphorylate the mutant peptide (mfc) compared to the normal peptide (fc) (Fig. 7). At the
highest concentration of peptide employed in the assay (1200 μM), there was a 10-fold difference in the velocity of the reaction. This result is consistent with our observation showing the inability of CSK to phosphorylate E527K c-Src protein.

Binding of Normal and E381G c-Src to a Synthetic Peptide

We have isolated a number of mutant viruses harboring the Src oncogene that, as a result of mutations within Src which activate the tyrosine kinase activity of the enzyme, are now capable of morphological transformation of CEF cells. These activating mutations were found throughout the Src molecule, with the exception of the unique domain (results not shown), and we are currently examining how these mutations activate Src in hopes of furthering our understanding of Src regulation.

In this report, two mutant forms of Src were examined and were found to be resistant to inactivation by CSK. Cloning and dideoxy-DNA sequencing revealed that one mutant, WO CST1, possessed only a single point mutation within the kinase domain resulting in a single amino acid substitution (E381G). The other, CY CST201, possessed only a single point mutation resulting in a single-amino acid substitution (E527K) that was localized near the carboxyl terminus and only 3 amino acids amino-terminal to the CSK phosphorylation site. Both mutations resulted in vivo hypophosphorylation of the regulatory phosphorylation site of Src accompanied by an elevation in Src tyrosine kinase activity. Previously, a mutation corresponding to the mutation in WO CST1 and mutations corresponding to the mutation in CY CST201 have been observed in the context of chicken Src (36–38). In both cases, the mutations were also thought to be responsible for the observed Src activation.

To further characterize the defects in Src regulation, we have purified both normal and mutant Src to homogeneity and have examined their susceptibility to both inactivation and phosphorylation by the important regulatory enzyme CSK. In activation assays, we found that both mutants were resistant to inactivation by CSK relative to wild-type Src. Interestingly, E381G c-Src became phosphorylated to wild-type levels in the absence of significant changes in tyrosine kinase activity. E527K c-Src could not be detectably phosphorylated by CSK under our soluble in vitro conditions.

The mutation in E527K c-Src results in a rather drastic
charge change of +2 (Glu → Lys) only 3 amino acids amino-terminal to the CSK phosphorylation site and involves a residue that is highly conserved among all Src family members. We hypothesized that the change in charge interfered directly with the ability of CSK to phosphorylate the neighboring Tyr530 residue. In order to test this possibility directly, we synthesized a peptide modelled against the carboxyl terminus of Src family members. The carboxyl-terminal region of Src family members is highly conserved, and we have used a specific substrate peptide (fcp) derived from the carboxyl terminus of wild-type human Fyn (residues 503–537) to assay CSK during its purification from bovine thymus (results not shown). This region of Fyn is identical with the corresponding Src region with the exception of four conserved amino acid changes. We found that when the E527K c-Src mutation was incorporated into the peptide, this led to a dramatic decrease in its ability to be phosphorylated by CSK that was consistent with our results using the intact Src protein and suggests that the mutation causes an alteration in substrate recognition that can be localized to this 30-amino acid region of Src. The resistance of Tyr530 to phosphorylation by CSK in E527K c-Src also explains the in vivo hypophosphorylation at this site that we have observed (results not shown).

Supporting evidence for the role of Glu527 in efficient substrate recognition has been obtained through examination of mutations corresponding to the E527K c-Src mutation in the context of chicken Src, but in the presence of additional carboxyl-terminal mutations which may complicate interpretation (34, 37). Reduced in vivo phosphorylation was observed on the carboxyl-terminal regulatory tyrosine as well as resistance to CSK phosphorylation in vitro whenever the acidic residue amino-terminal to Tyr527 was mutated, suggesting that the presence of Glu524 in chicken appears to be essential for both efficient autophosphorylation and CSK phosphorylation of Tyr527 (34, 37).

In contrast to the putative mechanism of activation we have proposed with regard to E527K c-Src, an alternate mode of activation in an activated transforming mutant of chicken Src has been proposed (38). Interestingly, no obvious alteration in the in vivo phosphorylation status of the carboxyl-terminal regulatory tyrosine (Tyr527 in chicken) was observed by these investigators, suggesting that either the NIH 3T3 cells used to express Src contain additional tyrosine kinases able to phosphorylate Tyr527 or that CSK in these cells has enhanced activity or abundance relative to what we have observed with E527K c-Src in chicken embryo fibroblasts and were able to obtain in our in vitro assay. The investigators attributed the activation to a conformational change in the carboxyl terminus (38).

Other recent work may also be relevant to these findings. A synthetic phosphorylated Src carboxyl-terminal peptide that contains the same mutation as E527K c-Src binds much less efficiently to a Src SH2-GST fusion protein than the corresponding wild-type peptide (13). Thus, impairment of Src regulation by this single activating mutation may involve the combined effects of two distinct blockades. The first involves impairment of CSK’s ability to phosphorylate Src as suggested by our results. In the event that in vivo conditions might somehow allow CSK to overcome this blockade, as appears to be the case in the NIH 3T3 cells described above, the mutation would also impair the ability of the phosphorylated carboxyl terminus of Src to interact with its SH2 domain.

In contrast to E527K c-Src, the mechanism underlying the regulatory defect in E381G c-Src does not appear to involve resistance of Tyr530 to phosphorylation by CSK. We observed that E381G c-Src was phosphorylated by CSK in vitro to an extent similar to wild-type Src, but in the absence of any observed reduction in Src tyrosine kinase activity. The E381G c-Src mutation is in the kinase domain, is distant from the SH2/SH3 and carboxyl-terminal regulatory domains, and may be causing a conformational change such that: (a) the SH2/SH3 domains and carboxyl-terminal tyrosine interact in a nonproductive manner that does not inhibit the Src kinase domain or (b) the SH2/SH3 domains and carboxyl-terminal tyrosine can no longer interact.

To examine these two possibilities, we tested the SH2 domain accessibility of both wild-type and E381G c-Src by examining whether they could bind to a synthetic phosphopeptide modelled against a region of polyoma middle-T antigen which binds unoccupied Src SH2 domain with high affinity (35). We found that, similar to binding experiments carried out by others with a carboxyl-terminal Src phosphopeptide (11), the middle-T phosphopeptide binds unphosphorylated wild-type Src. When the carboxyl terminus of wild-type Src was phosphorylated by CSK, the binding of Src to middle-T phosphopeptide resin was reduced dramatically. E381G c-Src also bound to the middle-T phosphopeptide resin to an extent similar to wild-type Src when unphosphorylated, but, unexpectedly, E381G c-Src exhibited reduced binding upon autophosphorylation alone, in the absence of high levels of phosphorylation at Tyr530. The reduced binding of autophosphorylated E381G c-Src to the middle-T phosphopeptide resin, whether in the presence or absence of the CSK phosphorylation at Tyr530, was not accompanied by any attenuation in E381G c-Src kinase activity (see Fig. 5 and results not shown). Although these results make it difficult to answer our original question of whether the SH2 domain of E381G c-Src is occupied by its phosphorylated carboxyl-terminal tail, they do suggest that, upon E381G c-Src autophosphorylation, the SH2 domain becomes effectively altered or conformationally blocked, resulting in our observed reduction in binding to both exogenously supplied binding sites (the synthetic phosphopeptide resin) as well as to the endogenously phosphorylated Tyr530. We have also observed similar reductions in the binding of a CSK-phosphorylated Fyn carboxyl-terminal peptide to E381G c-Src upon autophosphorylation (results not shown), suggesting that this phenomenon might have a role in play in the resistance of E381G c-Src to inactivation by CSK.

We do not feel that the difference in binding of the peptide to Src can be explained by autophosphorylation of Tyr530 because during our incubations as carried out in Fig. 8, the site undergoes ~10% of the phosphorylation that it would undergo in the presence of CSK, but its binding to the phosphopeptide was reduced by 66%. We are currently uncertain as to the role of Tyr419 phosphorylation. Results obtained from in vitro experiments (Fig. 6 and results not shown) demonstrating that the ability of CSK to inhibit wild-type Src activity appears independent of the extent of autophosphorylation on Tyr419, suggest that phosphorylation of Tyr419 in wild-type Src does not desensitize wild-type Src to CSK. This does not exclude the possibility that the E381G c-Src mutation alters Src in such a way that it now responds to Tyr419 phosphorylation.

In addition to the binding alterations in E381G c-Src we have measured in vitro using a phosphorylated peptide, it is possible that similar changes in the ability to bind tyrosine-phosphorylated molecules may also occur in vivo, resulting in reductions in the ability of E381G c-Src to bind proteins that have been reported to be found in association with Src, such as Fak, p130, and p110 (39–42). Even if there is impairment in vivo SH2 binding by E381G c-Src, it has not completely impaired the ability of E381G c-Src to transform chicken embryo fibroblasts;
Glu381 appears to be located only 6 amino acids amino-terminal (44) and Src family kinase domain homology with cAMP-de-

yntaphosphate (Tyr639) and to a regulatory region (residues 424–428 in pp60 src) in the kinase domain of Src (46) that it might interfere with several regulatory features of the protein.

Resistance of Src mutants to the effects of the regulatory phosphorysine have been reported by other investigators ex-
aminating activated Src mutants possessing mutations in either SH2 or SH3. It has been demonstrated recently that mutations in either the SH2 or SH3 domain leave Src resistant to CSK regulation without altering the ability of Src to be phosphorylated by CSK (12, 15, 16). The results suggest that mutations in the SH2/SH3 domains have the potential to interrupt interactions with the carboxyl-terminal phosphorysine, presumably through direct disruption of the SH2/3 domains, resulting in constitutive Src activation. The E381G c-Src mutation in Src is similar in that it also is resistant to inactivation by CSK even though it can become phosphorylated at Tyr639. What makes this mutation interesting and distinct from the SH2/3 domain leave Src resistant to CSK regulation and into the mechanisms responsible for mu-

tation. We have identified two new activated mutants of hu-

man Src exhibiting two different defects in Src regulation and represent an alter-

native mechanism for Src activation that has not currently been described or analyzed biochemically.

In this manuscript, we have demonstrated the resistance of two activated transforming mutants of pp60 src to regulation by CSK. It is apparent from these studies and others that mutations in the Src family act to usurp or modulate the ability of CSK to regulate tyrosine kinase activity. The mechanisms behind these regulatory defects appear to fall into specific categories depending upon the location and nature of the mu-
tation. We have identified two new activated mutants of hu-

man Src exhibiting two different defects in Src regulation and have noted important differences between the two mutant Srcs and wild-type Src. One of these alters the ability of CSK to phosphorylate Src, and the other inhibits the CSK phosphoryl-

ation from regulating Src activity through an apparent confor-
mational change induced by E381G c-Src autophosphorylation. These studies should provide interesting insights into Src enz-
yme regulation and into the mechanisms responsible for mu-
tational activation of Src.

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