The phosphorylating and transforming activities of c-Src are negatively regulated by phosphorylation at Tyr-527 near its carboxyl terminus. Previous studies have indicated that c-Src preferentially autophosphorylates Tyr-416, a residue in the middle of the catalytic domain, in vitro, and that Tyr-527 is phosphorylated by the carboxyl-terminal Src kinase, Csk. However, indirect evidence suggests that c-Src may also autophosphorylate Tyr-527 as part of a negative feedback loop. While some in vivo evidence suggests that Tyr-527 can be autophosphorylated in an intermolecular interaction, it has not previously been possible to directly demonstrate significant autophosphorylation in vitro. Here we show that c-Src purified from recombinant bacteria can autophosphorylate Tyr-527 to high levels in vitro when incubated with sufficiently high concentrations of ATP (K_m(Mg^2+/ATP) ~ 20 μM) that are well above those that have been used previously. In vitro Tyr-527 autophosphorylation can occur both as an intra- and intermolecular interaction; higher enzyme concentrations are required for intermolecular Tyr-527 phosphorylation than for Tyr-416 autophosphorylation. These results support the possibility that, like G-proteins, c-Src can switch itself off in vivo by its own enzymatic activity.

c-Src activity is negatively regulated in vivo primarily by phosphorylation of Tyr-527 (located near the carboxyl terminus) to high levels (~90%) (1). Tyr-527 can be phosphorylated by Csk, and its phosphorylation is reduced 2–5-fold in transgenic Csk knock-out mice (2, 3). However, some evidence points to a role for Tyr-527 phosphorylation in mitosis (5, 6). These observations suggest the existence of a negative feedback loop that might be mediated by Tyr-527 dephosphorylation of Src prior to immunoprecipitation was achieved by incubation with digitation-permeabilized cells in the presence of EDTA, as described previously (12). Src kinase was inactivated by including 1 mM FSBA during this incubation. Cells were lysed by the addition of 2 × 3T3 lysis buffer (1 × 1% Nonidet P-40, 0.25% sodium deoxycholate, 25 mM HEPES, pH 7.5, 150 mM NaCl, 25 mM NaF, 1 mM EDTA, 1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Overexpressed Src was immunoprecipitated from cleared lysates with anti-Src monoclonal antibody EC10 (13), which recognizes chicken, but not mouse, c-Src. Immune complexes were collected on protein A-Sepharose beads and washed sequentially once with 3T3 lysis buffer; once with 10 mM Tris-HCl, pH 7.2, 1 × NaCl, 0.5% Triton X-100; once with 3T3 lysis buffer; once with kinase buffer (200 μM ATP (unless otherwise specified), 50 mM HEPES, pH 7.5, 0.2 mM sodium vanadate); and then resuspended in kinase buffer. Autokinase assays were started by the addition of 5 μL of 50 mM MgCl_2 to 45 μL of immune complex bound to beads. Reactions were performed at 30 °C for 20 min and terminated by the addition of 50 μL of 2 × SDS-PAGE sample buffer. Equal volumes were subjected to SDS-PAGE (10% acrylamide) and Western blotting. Matched blots were probed with anti-phosphotyrosine (4G10; UBI) or anti-Src monoclonal 327 (14) antibodies, rabbit anti-mouse IgG secondary antibody and 125I-protein A and visualized by autoradiography.

Some indirect evidence has been presented to suggest that c-Src might autophosphorylate Tyr-527 (8), but direct evidence for autophosphorylation of Tyr-527 in vitro is lacking. When incubated with ATP in vitro, c-Src preferentially autophosphorylates Tyr-416, a residue in the center of the catalytic domain, and not Tyr-527 (9, 10). However, all in vitro Src-autophosphorylation studies of which we are aware have used ATP concentrations in the low micromolar range, presumably because of technical limitations imposed by the use of radioactive isotopes. Physiological ATP concentrations are significantly higher (>1 mM), and it is possible that Tyr-527 autophosphorylation might proceed efficiently at these concentrations. To unambiguously resolve this issue, we have investigated the ability of c-Src to autophosphorylate Tyr-527 in vitro using both immunoprecipitated proteins and proteins purified from recombinant bacteria (which are believed to contain no other protein-tyrosine kinases). We demonstrate that c-Src can extensively phosphorylate Tyr-527 by intra- as well as intermolecular mechanisms, implying that isolated c-Src molecules can down-regulate their own activity.

EXPERIMENTAL PROCEDURES

**Auto-kinase Assay of Immunoprecipitated Src—**Cell lines NIH(pcsrc16)A, NIH(pcsrc527/fox/EP)B1, and NIH(pcs16577/fox3A, overexpressing c-SrcY416F, c-SrcY527F, and c-SrcY416F/Y527F), respectively, have been described previously (11). In situ dephosphorylation of Src prior to immunoprecipitation was achieved by incubation of digitonin-permeabilized cells in the presence of EDTA, as described previously (12). Src kinase was inactivated by including 1 mM FSBA during this incubation. Cells were lysed by the addition of 2 × 3T3 lysis buffer (1 × 1% Nonidet P-40, 0.25% sodium deoxycholate, 25 mM HEPES, pH 7.5, 150 mM NaCl, 25 mM NaF, 1 mM EDTA, 1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Overexpressed Src was immunoprecipitated from cleared lysates with anti-Src monoclonal antibody EC10 (13), which recognizes chicken, but not mouse, c-Src. Immune complexes were collected on protein A-Sepharose beads and washed sequentially once with 3T3 lysis buffer; once with 10 mM Tris-HCl, pH 7.2, 1 × NaCl, 0.5% Triton X-100; once with 3T3 lysis buffer; once with kinase buffer (200 μM ATP (unless otherwise specified), 50 mM HEPES, pH 7.5, 0.2 mM sodium vanadate); and then resuspended in kinase buffer. Autokinase assays were started by the addition of 5 μL of 50 mM MgCl_2 to 45 μL of immune complex bound to beads. Reactions were performed at 30 °C for 20 min and terminated by the addition of 50 μL of 2 × SDS-PAGE sample buffer. Equal volumes were subjected to SDS-PAGE (10% acrylamide) and Western blotting. Matched blots were probed with anti-phosphotyrosine (4G10; UBI) or anti-Src monoclonal 327 (14) antibodies, rabbit anti-mouse IgG secondary antibody and 125I-protein A and visualized by autoradiography.

**Recombinant DNA Manipulations—**Restriction endonucleases and T4-DNA ligase were purchased from New England Biolabs and used as recommended by the supplier. Escherichia coli DH10B (F’ mcrA Δ(mrr hsd Rms-mcrBC) Δ(lacZΔM15 lacY17Δ) de3 recA1 endA1 araD139 ara- leu7697 galK galU rpsL tnl5 napG) was transformed with plasmid pSC416F and used for expression of c-Src(Y416F). Western blots of lysates from transformed E. coli with anti-Src MAb 327 showed that c-Src(Y416F) was, as expected, expressed only in cells that had been induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (data not shown). About 10% of total expressed c-Src(Y416F) was soluble and found in the cytoplasmic fraction that was used as the starting material for purification.

**Purification of Wild-type and Mutant c-Src—**Cultures of E. coli DH10B carrying pSC416F were grown at 37 °C in LB medium contain-
Autophosphorylation of c-Src

Extensive Tyr-527 Autophosphorylation at 200 μM ATP Concentration—To avoid the limitations on ATP concentrations imposed by the use of radioactive isotopes, we measured Tyr-527 autophosphorylation in immunoprecipitates from Src overexpresser cells using 200 μM nonradioactive ATP and Western blots with anti-phosphotyrosine antibody (Fig. 1). Autophosphorylation was detected in c-Src(Y416F)/Y527F, a mutant that lacked intramolecular Tyr-527 phosphorylation (Fig. 1, lanes 1–4). This indicated that the tyrosine phosphorylation that was observed in other c-Src mutants could be ascribed to phosphorylation at one of these sites. Cyaagogen bromide digests of autophosphorylated 32P-labeled c-Src(Y416F) directly confirmed that Tyr-527 was the site of tyrosine phosphorylation (data not shown).

Tyr-527 autophosphorylation was observed in the presence of 200 μM Mg2+/ATP, immunoprecipitated, dephosphorylated c-Src(Y416F) autophosphorylated at Tyr-527 to levels similar to the steady-state levels observed in vivo (Fig. 1, lanes 1–4). This level has been shown by different methods to be ≥90% (4, 6, 20). Autophosphorylation at Tyr-527 by c-Src(Y416F) was completely abolished by pretreatment with FBSA (lanes 5 and 6). However, stoichiometric phosphorylation of FBSA-treated, kinase-inactive c-Src(Y416F) was observed in the presence of co-immunoprecipitated kinase-active c-Src(Y416F)/Y527F (lanes 7 and 8). This indicated that c-Src(Y416F)/Y527F was able to phosphorylate Tyr-527 of c-Src(Y416F) in an intermolecular interaction (in trans). The level of intermolecular Tyr-527 phosphorylation was similar to the level of Tyr-527 autophosphorylation observed with kinase-active c-Src(Y416F) alone (cf. lanes 4 and 8). We conclude that the in vitro reaction can phosphorylate most of the available Tyr-527 residues.

Tyr-527 autophosphorylation of the c-Src(Y416F) mutant exhibited a K_m for Mg2+/ATP of approximately 20 μM (data not shown). In agreement with previous results, little autophosphorylation was detected at ATP concentrations below 10 μM. Autophosphorylation of Tyr-527 (in c-Src(Y416F)) was at least 2-fold slower than autophosphorylation of Tyr-416 (in c-Src(Y527F)) at 200 μM ATP (data not shown). These results suggest that c-Src can autophosphorylate Tyr-527 to high levels at physiological ATP concentrations, and that this can occur by an intermolecular interaction. However, in spite of stringent immune complex washing conditions, as with prior studies, the possibility that the immunoprecipitates contained Csk or another contaminating exogenous Tyr-527 kinase could not be formally excluded.

Tyr-527 Autophosphorylation by c-Src(Y416F) Purified from Recombinant Bacteria—To unambiguously exclude the possibility that the Tyr-527 phosphorylation had been catalyzed by an associated kinase, c-Src(Y416F) was purified from recombinant E. coli, which carried pLS416F, a c-src(Y416F) expression plasmid. E. coli was appropriate for this purpose since it appears to lack any endogenous protein-tyrosine kinase activity that could confound the interpretation of the experiments. Plasmid pLS4, which contains a lac UV5 promoter linked to the v-src gene, has previously been used for expression of v-Src in bacteria (21, 22). pLS416F was constructed by replacing the v-src gene with the c-src(Y416F) gene (from plasmid pRLC416F, generously provided by B. C. Cobb, University of Virginia). The Nael-MluI and MluI-Thil1111 fragments containing the c-src(Y416F) coding region were isolated from pRLC416F and ligated into the homologous Nael and Thil1111 sites in pLS4. c-Src(Y416F) was purified from lysates of pLS416F-transformed DH10B cells as described under “Experimental Procedures.” Western blots with anti-phosphotyrosine antibody showed that the purified protein contained negligible phosphotyrosine, possibly because of dephosphorylation during purification in the absence of phosphatase inhibitors (data not shown). The specific activity of the purified protein, using polyE4Y1 random oligomers of glutamate and tyrosine as substrate, was approximately 230 nmol 32P incorporated/min/nmol of c-Src(Y416F).

Autophosphorylation assays were conducted using radioac-
Autophosphorylation of c-Src

1. Analysis of autophosphorylated c-Src(Y416F) purified from recombinant bacteria. Purified, dephosphorylated c-Src(Y416F) (5 ng) was autophosphorylated by incubation in 50 μl of a buffer containing 20 μM [γ-32P]ATP (8,600 cpm/pmol) and 10 mM MgCl2 (30 min at room temperature). Wild-type c-Src was used in parallel for comparison. Autophosphorylated 32P-labeled Src proteins were subjected to subsequent analyses as described under “Experimental Procedures.”

2. Effect of protein concentration on c-Src autophosphorylation at Tyr-416 and Tyr-527. 1 ng of wild-type c-Src (open circles) or mutated c-Src(Y416F) (solid circles) was incubated at room temperature in buffer containing 50 μM [γ-32P]ATP (45,000 cpm/pmol) with 10 mM MgCl2 in a total volume of 7, 20, 70, or 200 μl to obtain the indicated enzyme concentrations. After 5 (c-Src) or 10 min (c-Src(Y416F)), the autophosphorylated proteins were trichloroacetic acid-precipitated and separated by SDS-PAGE, and the amounts of 32P incorporated into the labeled Src bands were measured using a Betascope (Betagen). Points are averages from two experiments; errors are approximately 10%.

3. Inter- and Intramolecular c-Src Autophosphorylation—Dilution experiments with v-Src (22) and platelet c-Src (16) purified by immunoaffinity chromatography have indicated that autophosphorylation at Tyr-416 is intramolecular. On the other hand, in vivo experiments with c-Src mutants expressed in yeast indicate that autophosphorylation at both Tyr-416 and Tyr-527 in c-Src can be intermolecular (8). Intermolecular Tyr-416 autophosphorylation was also demonstrated by mixing immunoprecipitates of kinase-defective and kinase-active c-Src mutants (8); this result was extended to Tyr-527 autophosphorylation in the experiment shown in Fig. 1.

To investigate the nature of the autophosphorylation reactions in more detail, we performed autophosphorylation dilution experiments with purified wild-type c-Src and c-Src(Y416F). Autophosphorylation of fixed amounts of these proteins was assayed in different volumes of a buffer containing 50 μM [γ-32P]ATP/10 mM Mg2+ along with 0.3% sodium deoxycholate (to minimize the possibility of aggregation). Both protein preparations contained no detectable phosphotyrosine and had similar specific activities for phosphorylation of polyE4Y1 synthetic random oligomers (3,000–4,000 n mole/min/mg). If autophosphorylation were intramolecular, we would expect the level to be independent of concentration; if it were intermolecular, we would expect it to increase linearly with concentration.

Quantitative analysis of the autophosphorylation levels by SDS-PAGE and autoradiography yielded the curves shown in Fig. 3. Incorporation of radioactive 32P into wild-type c-Src increased roughly linearly with concentrations in the range of 5–143 ng/ml. This indicates that much of the Tyr-416 autophosphorylation was intermolecular. The residual autophosphorylation in the (extrapolated) limit of zero c-Src concentration is attributable to an inevitable inefficiency of digestion and recovery of the tryptic peptides, the relative intensities of the spots do not necessarily correspond to the relative phosphorylation levels.) We conclude that c-Src(Y416F), purified from a source containing no other protein-tyrosine kinase activity, autophosphorylates itself primarily at Tyr-527 in the presence of sufficient (≈20 μM) ATP concentrations.
larger than the amount that can be explained by Tyr-527 autophosphorylation, which suggests, in agreement with previous results (16), that there is also an intramolecular Tyr-416 autophosphorylation activity. In contrast, autophosphorylation of c-Src(Y416F) was essentially independent of concentration. To exclude the possibility that this result was an artifact induced by c-Src(Y416F) aggregation, we analyzed the most diluted samples using Sephadex G-100 Superfine column chromatography in the presence of protein standards of known molecular weight (glucose oxidase, ~160 kDa, and bovine serum albumin, ~66 kDa). Column fractions were analyzed by SDS-PAGE and either Western blotting with anti-Src antibody MAb327 or silver staining (to locate the marker proteins). The Src proteins were detected in fractions containing proteins with molecular weights ≤66 kDa, indicating that they were present as monomers in the reaction. The concentration independence of Tyr-527 autophosphorylation indicates that most of this activity was intramolecular under these conditions.

**DISCUSSION**

Previous studies showing that kinase-active but not kinase-defective c-Src is phosphorylated at Tyr-527 when expressed in yeast have suggested that c-Src can autophsyorylate this site (8). However, a clear demonstration of Tyr-527 autophosphorylation in the absence of potentially interfering cellular tyrosine kinases has been lacking. While autophosphorylation at Tyr-416 is readily observed in c-Src immunoprecipitates, significant Tyr-527 phosphorylation in vitro has not previously been observed. We have now shown that Tyr-527 can be autophosphorylated to high levels by c-Src in vitro. This was demonstrated with protein purified from recombinant E. coli, thereby removing the possibility that the phosphorylation was catalyzed by an associated protein-tyrosine kinase. The Tyr-527 autophosphorylation reaction has a \( K_m \) for ATP of about 20 \( \mu \)M in the presence of 5–10 mM MgCl\(_2\). This is significantly higher than the ATP concentrations used in previous published studies of c-Src autophosphorylation, which probably explains why this in vitro reaction has not previously been observed to occur at a significant level. However, the \( K_m \) is much lower than physiological ATP concentrations, so Tyr-527 autophosphorylation may well have physiological significance. In particular, it may account for the residual Tyr-527 phosphorylation observed in Csk-deficient mouse cells (2, 3) and for the decreased Tyr-527 phosphorylation level observed with kinase-defective c-Src during mitosis (5).

Even at high ATP concentrations, the Tyr-527 autophosphorylation rate is a few-fold slower than the Tyr-416 autophosphorylation rate. However, this still represents a significant activity that is capable of phosphorylating most of the available sites, at least in vitro. The ratio of autophosphorylation rates depends on the Src concentration since the Tyr-416 autophosphorylation rate is concentration-dependent, presumably a result of significant intermolecular autophosphorylation. The detection of intermolecular autophosphorylation in the dilution experiment is gratifying and consistent with earlier observation of intermolecular phosphorylation at this site in mixed immunoprecipitates and in cotransfected yeast (8). While the data suggest that some intramolecular autophosphorylation also takes place, our results contrast with earlier dilution studies with v-Src (23) and c-Src (16) (covering the same range of concentrations), which concluded that Tyr-416 autophosphorylation is strictly intramolecular. We do not know the reason for this difference.

In contrast with Tyr-416 autophosphorylation, Tyr-527 autophosphorylation was primarily intramolecular within the concentration range tested in the dilution experiments. While our immune complex mixing experiments and previous yeast cotransfection experiments (8) indicate that Tyr-527 can be autophosphorylated by an intermolecular interaction (presumably occurring at higher effective concentrations than those tested here), the dilution experiment results show that significant inhibition of Tyr-527 autophosphorylation can be achieved in a unimolecular interaction. This implies that negative feedback by Tyr-527 autophosphorylation need not depend on local accumulation of Src molecules.

The rate of unimolecular Tyr-527 autophosphorylation measured using purified protein was \( -0.2 \) mol PO\(_4\)/mol of Src, corresponding to a \( t_b \) for Tyr-527 autophosphorylation of ~35 min. Thus, c-Src, even in the absence of exogenous regulatory proteins like Csk, will only remain in an active state for a finite period of time after activation and Tyr-527 dephosphorylation. Although the Tyr-527 autophosphorylation rate is low, it may be functionally important if the competing dephosphorylation rate is also slow. This seems likely since dephosphorylation of Tyr-527 proceeds much more slowly in vitro than dephosphorylation of Tyr-416, presumably because of protection by intramolecular association of phosphotyrosine 527 with the c-Src SH12 domain. In this regard, c-Src autoregulation may be similar to that seen in G proteins that have an intrinsic mechanism for time-delayed down-regulation. It will be important to determine whether there are proteins that act analogously to GTPase-activating proteins to accelerate Tyr-527 autophosphorylation and c-Src kinase down-regulation. Such proteins would clearly possess tumor suppressor potential. Finally, our results place the model of a negative feedback loop involving direct c-Src autoregulation on firmer ground.

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