Bacterial Expression and Characterization of Catalytic Loop Mutants of Src Protein Tyrosine Kinase†

David J. Kemble, Yue-Hao Wang, and Gongqin Sun*

Department of Cell and Molecular Biology, University of Rhode Island, Kingston, Rhode Island 02881

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ABSTRACT: Protein tyrosine kinase Src is a key enzyme in mammalian signal transduction and an important target for anticancer drug discovery. Although recombinant expression in bacterial cells offers a convenient and rapid way for producing several other protein tyrosine kinases, active Src is difficult to produce in bacterial systems. However, a kinase-defective Src mutant (due to a single point mutation, Lys295Met) is expressed strongly in bacteria. We hypothesize that the difficulty with expressing active Src in bacteria is due to toxicity caused by Src kinase activity. To test this hypothesis, we generated a series of Src mutants by altering certain residues, especially His384, in the catalytic loop and examined their expression in the bacteria and their kinase activity. The results demonstrate that Src mutants with kinase activity above a certain threshold could not be purified from a bacterial expression system, while a variety of mutants with a kinase activity below this threshold could indeed be expressed and purified. These observations support the conclusion that Src activity is toxic to the bacteria, which prevents high-level expression of fully active Src. We further demonstrated that His384, a universally conserved residue among protein tyrosine kinases, is not essential for Src catalysis or its inactivation by C-terminal tail Tyr phosphorylation. Interestingly, His384 mutants undergo autophosphorylation on Tyr416 like wild-type Src but are not activated by autophosphorylation. The potential role of His384 in Src activation by autophosphorylation is discussed in the context of Src structure.

Src protein tyrosine kinase (PTK)[1] is a key enzyme in numerous signal transduction pathways initiated by a variety of cell surface receptors, such as receptor tyrosine kinases (*1*), cytokine receptors and antigen receptors (*2*), and G-protein-coupled receptors (*3*). Constitutive activation of Src results in cell transformation (*4*) and is associated with numerous human cancers (*5*), making Src an important proto-oncogene and target for drug discovery (*6*). Src catalyzes phosphorylation of specific Tyr residues in more than 30 protein substrates to regulate their functions (*4*). The diverse roles of Src in signal transduction depend on two important properties: its complex regulation and substrate specificity.

The foundation for the complex Src regulation and substrate specificity is its complex domain structure. It has an N-terminal myristoylation motif, a unique region, an SH3 domain, an SH2 domain, a catalytic domain, and a C-terminal regulatory tail (*4*). The catalytic domain is composed of two lobes, the N-terminal lobe and the C-terminal lobe (*7*). Although catalysis is mainly carried out by the catalytic domain, the other structural domains may also contribute to this function, as the SH2 and SH3 domains have been identified as aids in Src substrate recruitment (*8*). An important function of the noncatalytic domains is to regulate Src kinase activity. The C-terminal tail contains a Tyr residue (Tyr527), and phosphorylation of Tyr527 leads to Src inactivation (*9*). The inactivation is triggered by binding of the phosphorylated Tyr527 to the SH2 domain (*7*). The SH3 domain is also required for this inactivation (*10*). Additionally, Src can be activated by several mechanisms such as binding of the SH2 (*11*) or SH3 domain (*12*) to their respective ligands, dephosphorylation of Tyr527 (*13*), autophosphorylation on Tyr416 (*14*), and binding to an activated α-subunit of G-proteins (*15*). The structural and mechanistic basis of these regulatory mechanisms is not fully understood.

Src phosphorylates more than 30 protein substrates, although how Src recognizes these diverse substrates is still poorly understood (*4*). Accumulating evidence suggests that the SH3 and SH2 domains are involved in recruiting Src substrates containing binding motifs for the Src SH2 and SH3 domains (*8*). Recently, we have demonstrated a docking-based substrate recognition mechanism for the catalytic domain of protein tyrosine kinase Csk (*16*, *17*). It is not clear if the Src catalytic domain uses a similar mechanism for substrate recognition.

One essential tool for detailed dissection of molecular mechanisms is site-specific mutagenesis. To fully understand the mechanisms of regulation and substrate recognition for Src, it is helpful to have a rapid and convenient method for recombinant expression and purification. Although bacterial expression has been a workhorse in dissecting the molecular

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* To whom correspondence should be addressed: Department of Cell and Molecular Biology, 117 Morrill Hall, 45 Lower College Rd., University of Rhode Island, Kingston, RI 02881. Telephone: (401) 874-5937. Fax: (401) 874-2202. E-mail: gsun@uri.edu.

[1] Abbreviations: INAC, immobilized Ni^{2+} affinity chromatography; kdSrc, kinase-defective Src; PTK, protein tyrosine kinase(s).

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mechanisms of numerous enzymes, its utility in Src research has been limited by technical problems. When active Src is expressed in various bacterial systems (*18*, *19*), the yield is invariably extremely low, because of the low expression level, incorrect folding, or a lack of stability. The impurity and low yield negate the advantages of the bacterial expression systems in speed and convenience. Recently, a kinase-defective Src mutant (kdSrc) has been successfully expressed in *Escherichia coli*, and up to 15 mg of protein can be purified from 1 L of a bacterial cell culture (*20*). kdSrc is different from fully active Src in a single point mutation (Lys295Met). kdSrc can be phosphorylated well by the C-terminal Src kinases (Csk) and retains the ability to undergo conformational changes triggered by this phosphorylation. The success of expressing kdSrc in bacteria and the conformational fidelity of kdSrc demonstrate that bacterial cells are fully capable of expressing and folding Src and suggest that the difficulty in expressing active Src in bacteria is likely associated with the kinase activity.

In this paper, we report that Src mutants with a kinase activity below a certain threshold can be expressed strongly and purified from the bacteria, while others exceeding a specific activity are plagued by poor solubility, low yield, and extensive degradation. We further characterize a group of Src mutants by varying a residue in the catalytic loop, His384. His384 is universally conserved among protein tyrosine kinases; however, it is not an essential residue for catalytic activity and not required for inactivation of Src by the C-terminal tail Tyr phosphorylation. Interestingly, His384Tyr retains the ability to autophosphorylate on Tyr416, but unlike wild-type Src, the mutant is not activated by autophosphorylation. These results provide insights into the difficulty in expressing PTKs in bacteria and demonstrate the utility of recombinant bacterial expression for biochemical studies of Src regulation.

MATERIALS AND METHODS

Reagents and Chemicals. All reagents used for bacterial culture and protein expression were purchased from Fisher. The chromatographic resin, iminodiacetic acid−agarose, was purchased from Sigma. DNA primers were synthesized by Integrated DNA Technologies. [γ-^{32}P]ATP (6000 Ci/mol) was purchased from Perkin-Elmer.

Plasmid Construction. The pRSET-a-kdSrc plasmid was constructed as previously described (*20*). Src mutants were generated by introducing specific residue substitutions employing QuikChange (Stratagene). Using pRSET-a-kdSrc, specific substitutions were introduced into the catalytic domain first, followed by mutating Met 295 to Lys. The pRSET-a-Src plasmids were then introduced into *E. coli* BL21(DE3) cells harboring the pREP4groESL plasmid by electroporation. The pREP4groESL plasmid contains the genes for the GroES/EL chaperone, which helps with the correct folding of Src (*20*).

Expression and Purification of the Fusion Proteins. In a 2 L flask, a single bacterial colony containing recombinant plasmids was inoculated into 400 mL of Luria-Bertani (LB) culture medium containing kanamycin (35 μg/mL) and ampicillin (100 μg/mL). The culture was grown to an OD_{600} of 2.5 and brought to an OD_{600} of 1.2 via the addition of 400 mL of fresh LB medium containing the antibiotics. The cultures were air-cooled to approximately 25 °C, and 0.4 mM isopropyl β-D-thiogalactopyranoside was added to induce the production of the fusion protein. Cultures were allowed to induce for 4 h at 25 °C while being shaken at 250 rpm. Bacterial cultures were harvested by centrifugation at 5000 rpm and stored at −20 °C.

The bacterial cell pellet was resuspended with ice-cold lysis buffer [50 mM Hepes (pH 8.0), 200 mM NaCl, 5 mM imidazole, and 0.1% Triton X-100]. Cells were lysed by sonication and clarified by centrifugation at 20 000 rpm for 30 min at 4 °C. The supernatant was added to 1.2 mL of iminodiacetic acid−agarose beads charged with $NiCl_2$ and gently mixed by rotation at 4 °C for 30 min. The beads were loaded into a column and then washed with wash buffer 1 [50 mM Hepes (pH 8.0) and 10 mM imidazole], wash buffer 2 [50 mM Hepes (pH 8.0) and 20 mM imidazole], and wash buffer 3 [50 mM Hepes (pH 8.0) and 30 mM imidazole]. Proteins were eluted using 200−500 mM imidazole in 50 mM Hepes (pH 8.0). Enzymes were stored at −20 °C in 40% glycerol. Protein concentrations were determined with the Bradford Reagent (Bio-Rad) standardized with BSA (0.2−1.0 mg/mL). The purity of protein fractions was determined with a 12% SDS−PAGE gel (Bio-Rad) stained with Coomassie Blue.

Kinase Assays. For quantifying PTK activities, we measured the phosphorylation of $polyE_4Y$ using the acid precipitation assay (*14*). Standard assay reaction mixtures (50 μL) contained 75 mM EPPS (pH 8.0), 1 mg/mL $polyE_4Y$, 200 μM [^{32}P]ATP (∼1000 dpm/pmol), 12 mM $MgCl_2$, 5% glycerol, and 0.005% Triton X-100. K_m and k_{cat} values were determined with a double-reciprocal plot. When $polyE_4Y$ was used as the variable substrate (from 20 to 200 μg/mL), the ATP concentration in the assay was maintained at 0.2 mM. When ATP was the variable substrate (from 20 to 200 μM), 1 mg/mL $polyE_4Y$ was used as the phosphate-accepting substrate. To assess the inactivation of Src by Csk phosphorylation, Src mutants were preincubated with varying amounts of Csk in the presence of 0.2 mM ATP in the kinase assay buffer for 30 min at 30 °C. At the end of the preincubation, $polyE_4Y$ and [γ-^{32}P]ATP (∼1000 dpm/pmol) were added to the reaction mixture. After incubation at 30 °C for an additional 30 min, the reaction mixtures were spotted onto 1 cm × 2 cm filter paper strips, which were then washed in 5% trichloroacetic acid (three times for a minimum of 10 min each). Phosphorylated and unphosphorylated $polyE_4Y$ was precipitated onto the filter paper, and the phosphate that precipitated onto the filter paper was assessed by liquid scintillation counting. The Src activity was determined by subtracting the Csk activity from the total kinase activity.

RESULTS

Bacterial Expression of Inactive Src. Although numerous attempts to express Src protein tyrosine kinase in bacteria have been reported (*18*, *19*), such efforts have been hindered by low protein yield and extensive degradation. Recently, a kinase-defective form of chicken Src (kdSrc), which is inactivated by a single point mutation (Lys295Met), is expressed in a bacterial system (*16*, *20*). kdSrc lacks the myristoylation motif and the unique region and is expressed as a fusion protein with a His_6 tag using the pRSET-a vector.

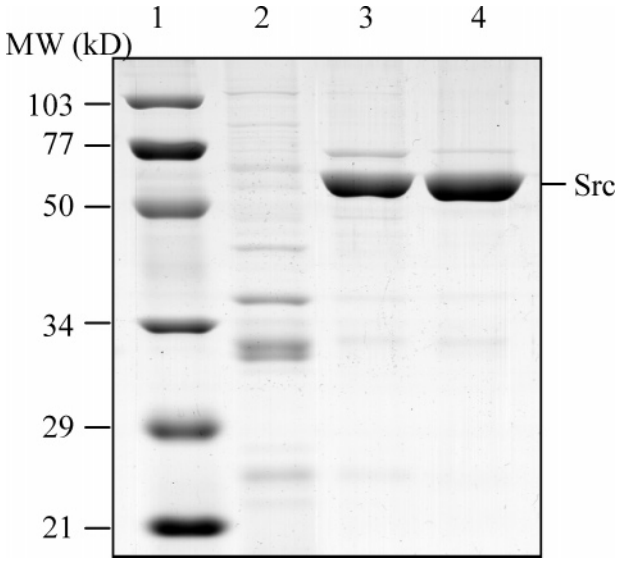

FIGURE 1: Expression of active Src and Src mutants. Active Src or Src mutants were expressed using the pRSET plasmid and purified as described in the text. Purified proteins (1 μg in each lane) were fractionated by SDS−PAGE. Lane 1 contained molecular weight markers. The sizes are shown at the left. Lane 2 contained wild-type Src, lane 3 kdSrc, and lane 4 the Src-Asn391Asp mutant. The size of intact Src and Src mutants is indicated at the right.

The fusion protein was coexpressed with the GroES-EL chaperone introduced by a separate pREP4groESL plasmid. Up to 15 mg of kdSrc can be purified from 1 L of bacterial culture under the conditions described previously (*20*). Although kdSrc lacks the kinase activity, it retains the conformational integrity required for regulation by domain−domain interaction (*20*−*22*) and C-terminal Tyr phosphorylation (*16*, *17*). We investigated if this expression system could be extended to produce active Src.

kdSrc was mutated back into the active form (by mutating Met295 of kdSrc to Lys) and electroporated into bacterial cells coexpressing the GroES-EL chaperone. Numerous attempts to purify the active Src protein from this system resulted in an impure and very low yield of protein mixtures. A typical purification of active Src is represented by the experiment depicted in Figure 1 (lane 2). Although the protein that eluted displayed detectable protein tyrosine kinase activity, the activity was inconsistent and not reproducible (data not shown). In contrast, purification of kdSrc from the same expression system resulted in predominantly kdSrc, with yields consistently ranging from 4 to 5 mg/L of culture induced for 4 h (lane 3). During the attempts to express active Src, we also identified one mutant of Src that was expressed strongly and readily purified with a yield similar to that of kdSrc (lane 4). DNA sequencing identified a single point mutation of Asn391Asp in this mutant. Asn391Asp displayed readily detectable kinase activity, with a specific kinase activity of 0.08 min^{-1} using $polyE_4Y$ as the substrate under standard assay conditions. This activity is approximately 0.03−0.1% of that of wild-type Src purified from insect cell expression systems (*23*). Asn391 is a residue located on the Src catalytic loop, which has the HRDLRAAN^{391} sequence. Although there are variations to the sequence in the catalytic loop among protein kinases, Asn391 is universally conserved in all protein tyrosine kinases, as well as protein Ser/Thr kinases. The facts that Asn391 is highly conserved and that it can be only marginally replaced with an Asp residue indicate that Asn391 is an important residue for Src catalytic activity. The good expression level of Asn391Asp also suggested that Src mutants with reduced kinase activity could be expressed well, while the fully active wild-type Src could not.

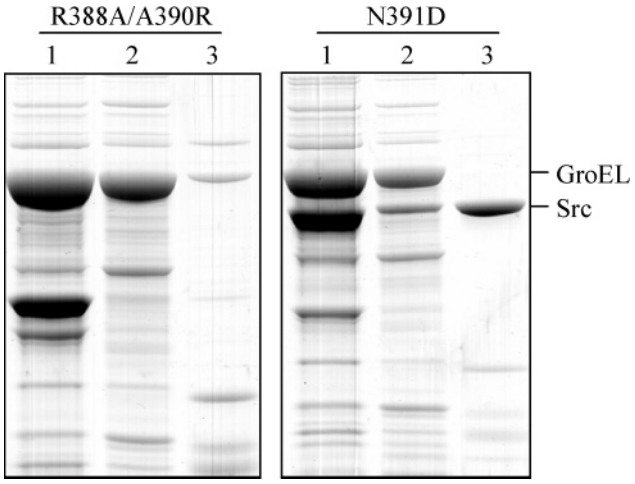

FIGURE 2: SDS−PAGE analysis of expression and purification of Arg388Ala/Ala390Arg and Asn391Asp mutants. Cell lysates were separated into insoluble and soluble fractions by centrifugation at 35000*g* for 15 min. The insoluble fraction (lane 1), soluble fraction (lane 2), and purified fraction (lane 3) were analyzed by SDS−PAGE and Coomassie blue staining. The insoluble and soluble fractions represented ~10 μL of culture in lanes 1 and 2. Approximately 1 μg of purified protein was loaded in lane 3.

The Src-Arg388Ala/Ala390Arg Mutant Is Not Express Strongly in Bacteria. To further test the suggestion given above and determine if we can obtain Src mutants with reasonable activity for biochemical and biophysical studies, we sought to create a series of Src mutants with variable kinase activity. In published studies (*24*, *25*) and ongoing studies of the Csk protein tyrosine kinase, it is observed that mutations in the catalytic loop often resulted in mutants with varying levels of kinase activity. We sought to use this strategy to produce active Src mutants that can be expressed in bacteria. The first mutant we generated was a double mutant switching an Arg and Ala at positions 388 and 390, respectively. The catalytic loop of Src has the H^{384}RDLRAAN sequence, while most other PTK catalytic loops have the HRDLAARN sequence, as if residues Arg388 and Ala390 are switched in Src relative to other protein tyrosine kinases. Switching these two residues in Csk to match those of Src generated a mutant retaining ~25% of the wild-type Csk kinase activity (*25*). By switching these two residues in Src, we hoped to generate a Src mutant with similarly reduced activity that might be expressed in bacteria. However, the mutant Src-Arg388Ala/Ala390Arg did not accumulate to detectable levels in either the insoluble fraction or the soluble fraction. Consequently, no protein of the size corresponding to that of Src was purified (Figure 2). In contrast, Src-Asn391Asp accumulated to a predominant level in the insoluble fraction and a significant level in the soluble fraction. Upon purification by immobilized nickel affinity chromatography (INAC), Src-Asn391Asp was the predominant protein in the purified sample. Recently, the same Src-Arg388Ala/Ala390Arg mutant was generated and purified from insect cells, and the mutant has a kinase activity approximately twice that of wild-type Src (*26*). This high

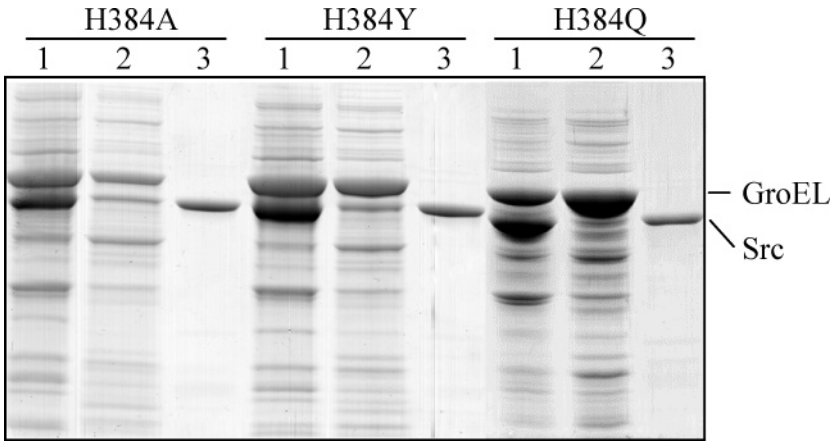

FIGURE 3: SDS−PAGE analysis of expression and purification of His384Ala, His384Tyr, and His384Gln. Sample processing was carried out as described in the legend of Figure 2.

Table 1: Purification and Catalytic Parameters of Src His384 Mutants

		polyE$_4$Y			ATP		
	yield (mg/L)	K_m (μg/mL)	k_{cat} (min^{-1})	k_{cat}/K_m	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m
wild-type Src	*a*	105 ± 10	136 ± 5.5	1.3	75 ± 8	211 ± 11	2.8
H384A	2.0	36 ± 3.0	6.2 ± 0.3	0.2	340 ± 10	12.5 ± 0.1	0.04
H384Y	1.8	26 ± 3.3	13 ± 1.4	0.5	309 ± 20	32 ± 2	0.1
H384Q	0.3	23 ± 2.4	48 ± 2.3	2.1	327 ± 4	116 ± 5	0.4
H384Y/Y416F	1.8	24 ± 2.2	4.3 ± 0.2	0.2	335 ± 5	9.5 ± 0.5	0.03
H384Q/Y416F	2.1	26 ± 3.8	9.2 ± 0.5	0.4	345 ± 5	24 ± 0.5	0.07
H384Y/Y527F	0.5	24 ± 3.5	26 ± 1.5	1.1	253 ± 7	59 ± 3	0.2

[a] Wild-type Src is expressed and purified as described in ref *31*.

kinase activity explains the difficulty in expressing the Src-Arg388Ala/Ala390Arg mutant in bacteria.

Expression of Src Variants at His384. His384 of Src is another highly conserved residue in protein tyrosine kinases, but the corresponding residue is replaced with a Tyr in the Ser kinase, the cAMP-dependent protein kinase, suggesting that the His residue is not an essential residue for kinase activity (*27*). We mutated His384 individually to Ala, Tyr, or Gln to generate three Src mutants in the pRSET expression system (Figure 3). All three mutants accumulated to significant levels in the insoluble fraction, were detectable in the soluble fraction, and could be purified by INAC. His384Ala and His384Tyr had similar yields, resulting in ~1.8 mg of purified protein from each 800 mL culture induced for 4 h. For comparison, kdSrc expressed under the same conditions typically yields 4−5 mg of protein. His384Gln, however, consistently had a much lower yield of ~0.2−0.4 mg/L of culture, and this lower yield was consistent with the apparent lower level of accumulation of this mutant in the soluble bacterial lysate.

We performed kinetic analysis on the three purified His384 mutants (Table 1). With polyE$_4$Y as the variable substrate and ATP as the fixed substrate at 0.2 mM, all three mutants exhibited similar K_m values for polyE$_4$Y but different k_{cat} values ranging from 6 min^{-1} for H384A to 48 min^{-1} for His384Gln. With ATP as the variable substrate and polyE$_4$Y at 1 mg/mL as the fixed substrate, all three mutants exhibited K_m values of ~320 μM, but the k_{cat} values varied significantly, ranging from 12 min^{-1} for His384Ala to 116 min^{-1} for His384Gln. The lower k_{cat} values observed using polyE$_4$Y versus ATP as the variable substrate can be ascribed to the fact that the fixed concentration of 0.2 mM ATP was only approximately half the K_m for ATP while the fixed concentration of 1 mg/mL of polyE$_4$Y was nearly saturating. The k_{cat} trend using either variable substrate was identical for the three mutants, with His384Gln being the most active and His384Ala the least active. We also observed a general correlation between the purification yield and the kinase activity among the three mutants. The most active mutant, His384Gln, consistently had the lowest yield of purification, typically 7−10-fold lower than those of the other mutants. The general correlation between higher activity and lower yield is also observed with several other Src mutants used in later sections of the study (Table 1). The activity of His384Gln (at 116 min^{-1}) appears to approximate the upper limit of the kinase activity that is tolerated by the bacterial expression system. From these observations, we conclude that Src kinase activity is toxic to the bacteria and only mutants with activity below a threshold can be expressed and purified.

Characterization of His384Tyr and His384Gln. We then determined if these mutants were still subject to regulation by the same mechanisms that modulate wild-type Src activity. Src is regulated by phosphorylation on two Tyr residues (*4*). Tyr416 in the activation loop is the site of autophosphorylation, and Tyr527 on the C-terminal tail is phosphorylated by Csk. Autophosphorylation on Tyr416 activates Src, while phosphorylation of Tyr527 leads to Src inactivation.

We first determined if His384Tyr is subject to Csk inactivation. To fully evaluate the effect of Csk phosphorylation of Tyr527 on the activity of His384Tyr, we also generated a double mutant (His384Tyr/Tyr527Phe). The Tyr527Phe mutation increased the kinase activity of His384Tyr ~1-fold (Table 1). His384Tyr and double mutant His384Tyr/Tyr527Phe were separately incubated with increasing amounts of Csk in the presence of ATP and $MgCl_2$ for 30 min, and

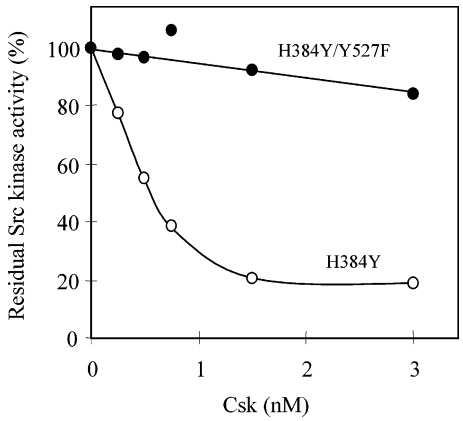

FIGURE 4: Inactivation of His384Tyr and His384Tyr/Tyr527Phe. The two Src mutants (119 nM for His384Tyr and 19 nM for His384Tyr/Tyr527Phe) were preincubated with varying concentrations of Csk in the presence of ATP and $MgCl_2$ in the kinase assay buffer for 30 min as described in the text. At the end of the preincubation, the kinase activity was determined using $polyE_4Y$ as a substrate. Src activity was calculated by subtracting Csk activity from the total kinase activity. The residual Src activity was plotted as a function of Csk concentration in the preincubation.

the residual Src kinase activity was determined using 1 mg/mL $polyE_4Y$ as the substrate. As shown in Figure 4, the presence of an increasing Csk concentration resulted in a progressive decrease in Src activity for His384Tyr to approximately 20% of that in the absence of Csk. In contrast, His384Tyr/Tyr527Phe was not inactivated by this treatment, indicating that the inactivation was indeed due to the phosphorylation of Tyr527 by Csk. This result demonstrated that the His384Tyr mutant was subject to inactivation by Csk phosphorylation on Tyr527, similar to wild-type Src.

To evaluate the ability of the mutant to undergo autophosphorylation and the associated activation, we determined the time course of Src autophosphorylation (Figure 5A). The level of autophosphorylation increased with time of incubation with ATP, increasing to an ~40% stoichiometry at 40 min. To determine the effect of autophosphorylation, the activity of Src before and after incubation with ATP for 1 h was compared. Wild-type Src activity increased ~150% upon incubation, consistent with previous reports (*28*, *29*). However, the activity of His384Tyr did not change with preincubation, indicating that this mutant is not activated by autophosphorylation. This experiment was repeated more than three times with consistent results.

We generated Tyr416Phe mutants in both His384Tyr and His384Gln. Kinetic analysis revealed that the mutation reduced Src activity ~3- and ~6-fold (Table 1) for His384His and His384Gln, respectively. While the reduction in activity was consistent with the possibility that Tyr416 phosphorylation stimulated Src kinase activity in the His384 mutants, it is also possible that the mutation of Tyr to Phe itself, rather than the resultant lack of autophosphorylation, is responsible for the reduction in activity.

DISCUSSION

The inability to express some protein tyrosine kinases, including Src, in bacteria has been an intriguing problem. In most cases, PTKs are not soluble when expressed in bacteria, and a very low level of soluble protein can be purified. Several explanations for this phenomenon have been

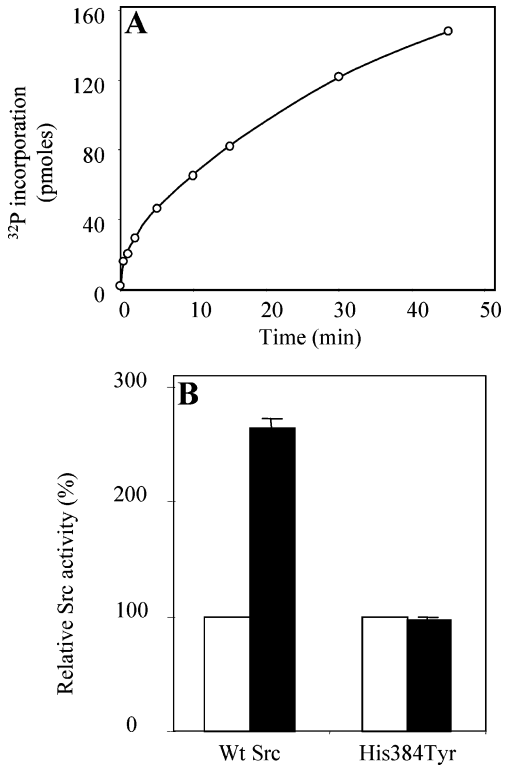

FIGURE 5: Autophosphorylation and activation of Src-His384Tyr. (A) His384Tyr (10 μM) was incubated with 200 μM ATP (10 000 cpm/pmol) and 12 mM $MgCl_2$ in the kinase assay buffer for the indicated period of time, and autophosphorylation of the mutant was assessed by acid precipitation and scintillation counting. (B) Comparison of Src kinase activity before and after autophosphorylation. Both wild-type Src and His384Tyr were incubated with ATP and $MgCl_2$ under standard phosphorylation conditions for 1 h, and the kinase activity of the wild type and the mutant before and after incubation was determined by the standard activity assay: white for the relative activity before autophosphorylation and black for the relative activity after autophosphorylation.

advanced, but there has been little systematic experimental examination. Here we document that the problem is associated with the kinase activity and only Src variants with activity above a certain threshold are difficult to express. This suggests that Src can phosphorylate certain bacterial proteins, which has detrimental effects on bacterial physiology. As a response to this toxicity, the bacteria somehow cause either degradation or low-level expression of the target kinase. The nature of the bacterial response is still unclear. Nevertheless, using Src mutants with activity below a certain threshold may provide a practical method for avoiding the toxicity associated with expressing active Src. Since overexpressed Src activity is responsible for the toxicity, the toxicity can also be overcome by coexpression of a protein tyrosine phosphatase activity (*30*−*32*). The phosphatase activity may dephosphorylate any protein phosphorylated by Src to minimize the toxic effect of Src.

Characterization of the Src mutants led to two interesting observations. First, His384 is a universally conserved residue in the catalytic loop of protein tyrosine kinases and highly conserved in protein kinases in general. However, significant variation is allowed at this position without abolishing the kinase activity. For example, Gln can substitute for this residue and retain a significant portion (~50% compared to wt Src activity) of the original kinase activity, and even the Ala variant retains a significant portion of the catalytic

activity. This suggests that the side chain of His384 plays no catalytic role. It is likely that this residue, by the interaction with its environment, helps position other residues in the catalytic loop that may be more directly involved in catalysis or regulation.

Second, even though His384Tyr retains the kinase activity and is inactivated by Csk phosphorylation on Tyr527, it is defective in the activation by autophosphorylation on Tyr416. While it is theoretically possible that His384 mutation globally and nonspecifically disrupted Src conformation to disable the activation, the fact that His384 mutants retained significant levels of kinase activity and the ability to respond to Csk inactivation argues against this possibility. It is more likely that some structural elements directly coupled to His384 are required for Src activation. An examination of the crystal structure of an activated Src family kinase, Lck (*33*), indicates that Arg385 directly coordinates with the phosphate on pTyr416. In the PTK v-Fps, the eqivalent Arg, Arg1042, is involved in a similar interaction, which appears to mediate the activation by autophosphorylation (*34*). Thus, it appears likely that the His384Tyr mutation disrupted the position of Arg385 and its interaction with pTyr416, eliminating the activation of Src by autophosphorylation. This proposal awaits experimental testing.

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