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Bacterial expression of an active tyrosine kinase from a protein A/truncated c-*src* fusion protein

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The carboxy-terminal half of the c-*src* protein fused to the protein A moiety was expressed in bacteria. The protein A/truncated c-*src* fusion protein, which does not have SH2 and SH3 domains, is found in the periplasmic space allowing for a simple one-step purification and demonstrated high efficiency in autophosphorylation and exogeneous substrate phosphorylation The missense mutation at codon 294 (Ile → Thr), which is located in the ATP-binding domain of the c-*src*, resulted in dramatic reduction of tyrosine kinase activity of the fusion protein. Using the fusion protein, we also revealed that staurosporin, a well-known kinase inhibitor, directly affects autophosphorylation of the C-terminal half of the c-*src* protein. This truncated c-*src* expression system provides a good source of enzyme for diverse experiments and is an ideal model for understanding the implication of structural alterations in the catalytic activity of the c-*src* kinase by site-directed mutagenesis experiments.

c-*src*; Protein A; Protein tyrosine kinase; *Escherichia coli*

1. INTRODUCTION

Non-receptor protein tyrosine kinases (PTKs) of the c-*src* family are important intermediates in signal transduction pathways, coupling diverse signals mediated through tyrosine kinase receptors with a variety of downstream mediators. Additionally, activation of some of these kinases has been implicated as an important event(s) in the development and/or progression of several human tumors including chronic myelogenous leukemia, colon cancer, and neuroblastoma [1–7]. Thus, identifying the mechanisms by which these kinases interact with specific substrates, determining the aberrant processes that lead to their activation, and developing specific inhibitors of the tyrosine kinase activity of this class of molecules are active areas of research. Toward these goals, considerable effort has been devoted to expressing and purifying large quantities of individual tyrosine kinases in bacterial and insect cell systems. However, most bacterially expressed PTKs aggregate in inclusion bodies, and purification results in loss of enzymatic activity. Therefore, bacterially expressed PTKs have been used primarily to obtain immunogenic proteins for the production of antibodies [8,9]. Only a few bacterially expressed PTKs have enzymatic activity; these include v-*src* [10], v-*abl* [11,12], v-*fps* [13], and c-*abl* [14]. The expression of enzymatically active c-*src* protein in bacteria has not been described to date. The baculovirus system has been used for the successful purification of c-*lck* [15,16]. Although this system shows great promise, attempts to purify $pp60^{c\text{-}src}$ have been complicated by its toxicity, low specific activity, and low levels of expression [17].

Recently, alternate approaches have been used to study the kinase sequences responsible for enzymatic activity and specificity. The SH2 domain of $pp60^{c\text{-}src}$ has been expressed, purified and crystallized [18]. These fragments are providing important information about the role of these domains in binding specific substrates.

To purify the catalytic domain of the proteins, one successful approach has been to fuse the eukaryotic protein to a bacterial protein and to its expression in bacterial cells. Nilsson et al. [19] developed plasmid pRIT5, which contains the gene coding for staphylococcal protein A and is adapted for gene fusion. This vector allows fusion of any gene to the protein A moiety, giving rise to fusion proteins that can be purified in a one-step procedure using IgG affinity chromatography. Moreover, the vector was designed for secretion, giving a periplasmic fusion protein in *Escherichia coli* and an

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Abbreviations: PTK, protein tyrosine kinase; SH2, *src* homology region 2; PCR, polymerase chain reaction, EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; Glu, glutamic acid; Tyr, tyrosine; PBS, phosphate buffered saline; ECL, enhanced chemiluminescence, Ile, isoleucine; Thr, threonine; ATP, adenosine triphosphate; Lys, lysine; His, histidine; Arg, arginine.

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extracellular protein in Gram-positive hosts such as *Staphylococcus aureus*.

In the present study, we have established the bacterial expression of the carboxy-terminal half of the c-*src* protein using a protein A fusion protein expression system. The protein A/truncated c-*src* fusion protein has been detected to have tyrosine kinase activity, which indicates that the minimal kinase domain of pp60$^{c\text{-}src}$ is efficient for PTK catalytic activity. Furthermore, by using this recombinant protein, we determined that staurosporin, a well-known tyrosine kinase inhibitor, suppressed activity of this carboxyl-terminal half of c-*src* kinase in vitro.

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

Plasmid pRIT5 (Pharmacia, Piscataway, NJ), containing the gene coding for staphylococcal protein A and adapted for gene fusion [19], has been used for constructing the truncated pp60$^{c\text{-}src}$ expression plasmid. Plasmid pRSVc-*src*NP was kindly provided by Dr. J. Brugge [20] The carboxy-terminal half of the c-*src* cDNA fragment (c-*src*NP), which has a deletion of amino acids 15 to 225, was prepared by a polymerase chain reaction (PCR) using the pRSVc-*src*NP plasmid as a PCR template PCR was performed according to the method of Saiki et al. [21] with modifications. Target sequences were amplified in a 100 μl reaction volume containing 20 ng of plasmid (pRSVc-*src*NP) DNA, 1.25 mM deoxyadenosine triphosphate, 1.25 mM deoxycytidine triphosphate, 1.25 mM deoxyguanosine triphosphate, 1.25 mM deoxythymidine triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 50 pmol of the each primer (SR-1: 5′-CTGGAATTCCATTGAAGGCCGCATGGGGAGCAGCAAG-AGCAAGC-3′, SR-2: 5′-GGGGATCCAGGCCTATAGGTTCTC-TCCA-3′) and 2.5 units of recombinant *Taq* DNA polymerase (*AmpliTaq*; Perkin Elmer Cetus, Norwalk, CT). Cycling reactions were performed in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). Each cycle consisted of denaturation at 94°C for 90 s, annealing at 60°C for 60 s, and extension at 72°C for 90 s. A total of 30 cycles were performed. Since additional sequences containing restriction endonuclease recognition sites were attached to the 5′ end of the PCR extension primers, these sequences were incorporated in the PCR product after each subsequent PCR cycle: *Eco*RI and *Bam*HI sites in SR-1 and SR-2, respectively. The PCR product was purified and enzymatically digested with *Eco*RI and *Bam*HI. The purified DNA fragment was ligated into pRIT5 plasmid DNA, which was digested with *Eco*RI and *Bam*HI by using T4 ligase. Competent bacteria cells, DH5α, were transformed with the ligation mixture. The transformed bacteria colonies were screened by the colony hybridization method, and positive colonies were picked and grown. The DNA sequences of the *src*NP cDNA inserted into the plasmid were confirmed by the dideoxynucleotide chain termination method [22]

2.2. *Expression of protein A/truncated c-*src *fusion protein in* E. coli

Bacteria was grown in LB media containing ampicillin (50 μg/ml) overnight and harvested by centrifugation. To determine the preferable culture condition for the recombinant protein, two different temperatures (37°C and 25°C) were used for culturing the bacterial cells. Periplasmic proteins were collected by osmotic shock treatment as follows Bacterial pellets were resuspended in 1/4 volume of sucrose solution containing 20% sucrose, 0.3 M Tris-HCl (pH 8), 1 mM EDTA, and 0.5 mM $MgCl_2$ and incubated for 10 min at room temperature. The cells were centrifuged and resuspended in 1/4 volume of 0.5 mM ice-cold $MgCl_2$. After incubation on ice for 10 min, the cells were centrifuged at 10,000 × *g* for 10 min at 4°C. The periplasmic proteins were harvested from supernatant and utilized for purification, kinase assays and immunoblot analysis.

2.3. *Kinase assay*

In vitro phosphorylation assays were performed as previously described [23] with modifications. The periplasmic proteins (250 μg total protein) were incubated with IgG-Sepharose 6FF (Pharmacia, Piscataway, NJ) at 4°C for 1 h. After 3 washes with washing buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl and 0.1% Triton-X), the pellet was incubated in 50 μl of a kinase buffer containing 20 mM HEPES, pH 7.0, 6 mM $MgCl_2$, 20 mM sodium orthovanadate, and 10 μCi per reaction of [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear) to perform the kinase assay. The reactions were allowed to proceed for 10 min at 23°C. The samples were subjected to 8–16% SDS-PAGE, and radioactive bands were detected by autoradiography.

To study the effects of staurosporin on the protein A/*src*NP fusion protein kinase activity, the IgG-Sepharose 6FF precipitated periplasmic protein was incubated with increasing concentrations of staurosporin (0, 1, and 5 μg/ml) for 10 min at 23°C prior to initiation of the kinase assay as described above. The amount of the protein which bound to the IgG-Sepharose after the drug treatment was evaluated by immunoblot assay as described below.

To examine phosphorylation of an exogenous substrate, 10 μl of 6 mg/ml poly(Glu:Tyr) (Sigma) was added before addition of kinase buffer, after which the kinase assay was performed as described above.

2.4. *Purification of protein A/truncated c-*src *fusion proteins*

Bacterial periplasmic proteins from 2 l of culture were clarified by centrifugation at 45,000 × *g* for 30 min at 4°C. Clarified bacterial periplamic proteins were applied to IgG-Sepharose 6FF column (Pharmacia, Piscataway, NJ) and then washed with 10 bed vols. of washing buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl and 0.1% Triton-X) and 2 bed vols. of 5 mM ammonium acetate, pH 5.0. The fusion proteins were eluted from the column with 0.5 M ammonium acetate. The fractions with significant protein concentration were pooled, dialized against phosphate buffered saline (PBS), and concentrated by using Centricon-10 concentrators (Amicon, Beverly, MA) Protein concentration was determined according to Bradford [24]. The purified proteins were resolved on an acrylamide gel by SDS-PAGE. In vitro autophosphorylation activity of the purified protein was examined by the method described above.

2.5. *Immunoblot analysis*

The protein A fusion proteins produced in bacterial cells were detected by immunoblot analysis. The IgG-Sepharose 6FF precipitated periplasmic protein were resolved on an 8–16% gradient acrylamide gel by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) for 2 h at 100 V at 4°C. The membrane was incubated with PBS containing 5% non-fat dry-milk and 0.05% Tween 20 for 30 min. Since protein A could be detected by rabbit IgG, the membrane was incubated with horseradish peroxidase conjugated rabbit immunoglobulin (Amersham Corp., Arlington Heights, IL) for 1 h at room temperature. After rinsing for 30 min with PBS containing 0.3% Tween 20, the membrane was treated with the enhanced chemiluminescence (ECL) reagent (Amersham Corp., Arlington Heights, IL) and then exposed to Amersham Hyperfilm for 5 min.

3. RESULTS

3.1. *Bacterial expression of protein A/c-*src*NP fusion protein*

The bacterial expression vector was constructed using the protein A fusion vector pRIT5 (Fig. 1). The truncated c-*src* cDNA fragment, designated as c-*src*NP, was prepared by PCR using pRSVc-*src*NP plasmid as a PCR template and ligated into the multi-cloning site of the vector. c-*src*NP contains a deletion of amino acids

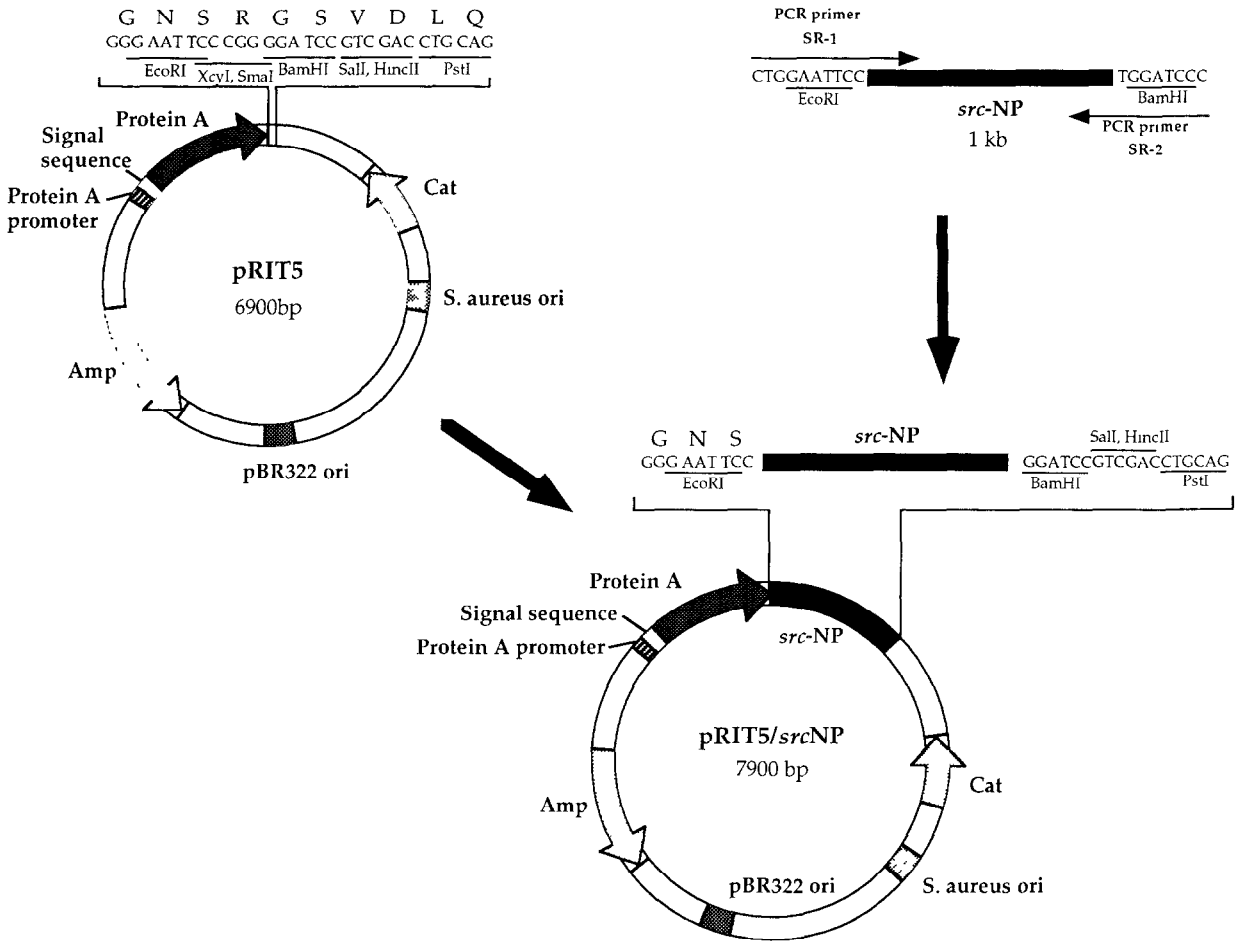

Fig. 1 Construction of the protein A/truncated c-*src* fusion protein expression plasmid The truncated c-*src* fragment, designated c-*src*NP, was amplified by PCR using a pair of primers, SR-1 and SR-2, and pRSVc-*src*NP plasmid as a PCR template. The purified PCR-amplified DNA was digested with *Eco*RI and *Bam*HI and ligated into pRIT5 plasmid. The truncated c-*src* gene was linked at the 3′ end of the protein A gene in the same reading frame.

15 to 225 in the c-*src* molecule and reportedly has been the smallest form of truncated c-*src* protein that has tyrosine kinase activity in the eukaryotic expression system [20]. The *E. coli* strain DH5α was then transformed by this expression vector containing the c-*src*NP fragment. Since the inserted c-*src*NP fragment was prepared by the PCR method, which is potentially capable of incorporating a wrong base during the amplification, the DNA sequence of the insert was confirmed. The plasmid clone containing the authentic c-*src*NP cDNA insert, designated pRIT5/*src*NP, was used for further experiments.

The protein A/*src*NP fusion protein was precipitated with IgG-Sepharose 6FF from the periplasmic proteins of pRIT5/*src*NP transformed bacterical cells and subjected to a kinase assay as described in section 2. As shown in Fig. 2, several phosphorylated proteins were detected in the pRIT5/*src*NP transformed bacterial cells, whereas no phosphoprotein was detected in the pRIT5 transformed bacterial cells. A prominent band was observed at the predicted molecular weight of the fusion protein (68 kDa), as well as at the site of four phosphoproteins of lower molecular weights (65, 50, 47, 25 kDa) when the transformed bacterial cells were cultured at 37°C. However, in the low-temperature culture condition (25°C), the 68-kDa protein was predominantly expressed, and the intensities of the lower-molecular-weight bands were reduced. Additionally, Northern blot analysis revealed that the transformed bacterial cells cultured in 37°C had only one transcript detected by c-*src* cDNA probe (data not shown). These results indicated that the four lower-molecular-weight proteins are most likely proteolytic products of the primary translated 68-kDa protein. Further analyses were performed using the recombinant fusion protein from the cells cultured at 25°C.

In addition to autophosphorylation, the protein A/*src*NP fusion protein phosphorylated exogenously added poly Glu:Tyr in vitro (Fig. 3). This result confirms the tyrosine kinase activity of the fusion protein.

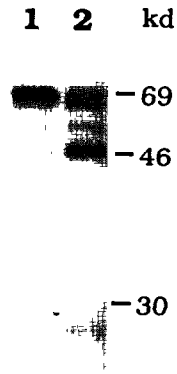

Fig. 2. Autophosphorylation of the bacterially expressed protein A/truncated c-*src* fusion protein. The fusion protein was precipitated by IgG-Sepharose and applied to the in vitro kinase assay as described in section 2. Bacteria were grown at 25°C (lane 1) or 37°C (lane 2).

The soluble protein A/*src*NP fusion proteins were purified by using IgG affinity chromatography. As shown in Fig. 4, the 68-kDa full-length fusion protein was purified with the 47-kDa and the 25-kDa proteins. We confirmed that these two co-purified proteins are proteolytic products of the full-length fusion protein by peptide mapping procedure using cyanogen bromide (data not shown). Approximately 100–500 μg of the 68-kDa full-length fusion protein was obtained from a 2 liters bacterial culture. Moreover, the affinity purified soluble protein reserved an autophosphorylation activity (Fig. 4, lane 3).

Suppression of the kinase activity by missense point mutation in the ATP-binding domain of the truncated c-*src*.

In the course of subcloning the *src*NP cDNA into the pRIT5 plasmid, we obtained a plasmid that had a PCR-induced point mutation in the *src*NP cDNA. This plasmid was designated pRIT5/*src*NPmu. DNA sequence analysis revealed that the pRIT5/*src*NPmu had a missense mutation, resulting in a change of Ile (ATA) to Thr (ACA) at codon 294 (terminology refers to that the full-length c-*src* cDNA described by Takeya and Hanafusa [25]) (Fig. 5C). Codon 294 is located in the ATP-binding domain of the c-*src* [26]. Previous experiments revealed that mutations of full-length $pp60^{v\text{-}src}$ at codon 295 (Lys → Glu, Lys → His, or Lys → Arg) which is closed to this site resulted in a protein without functional kinase activity [27]. Therefore, the expression and activity of this protein derived from pRIT5/*src*NPmu transformed DH5α bacterical cells was determined. Immunoblot analysis revealed that this mutant fusion protein was expressed in the periplasmic space at the same level as the wild-type clone (Fig. 5A). However, the mutant fusion protein had very low in vitro autophosphorylation activity compared with that of the normal fusion protein (Fig. 5B). These results demonstrated that Ile-294 as well as Lys-295 is important for regulating the kinase activity of $pp60^{c\text{-}src}$. The results also revealed that the tyrosine kinase activity is attributable to the *src*NP fusion protein but not to contaminated bacterial kinases.

3.2. *Effects of staurosporin on kinase activity of the fusion protein*

To determine whether staurosporin, a well-known kinase inhibitor, could directly affect autophosphorylation of the truncated form of the c-*src* protein, the IgG-Sepharose-precipitated fusion protein was preincubated with increasing concentrations of the drug and processed for kinase assay. As shown in Fig. 6, staurosporin reduced autophosphorylation of the fusion protein kinase. Immunoblot analysis of the staurosporin treated fusion protein revealed that the fusion protein was not degraded by the drug treatment. These data suggest that staurosporin exerts its inhibitory effect on the carboxyl-terminal half of the c-*src* protein.

4. DISCUSSION

In the present study, we demonstrate that the bacterially expressed carboxy-terminal half of the c-*src* protein fused to the protein A moiety has tyrosine kinase activity in vitro. This recombinant truncated c-*src* protein is secreted in the periplasmic space of bacteria and can be purified in a simple one-step procedure using IgG-binding matrix. The protein demonstrates high efficiency in autophosphorylation and substrate phosphorylation.

Overexpression of certain eukaryotic proteins in bac-

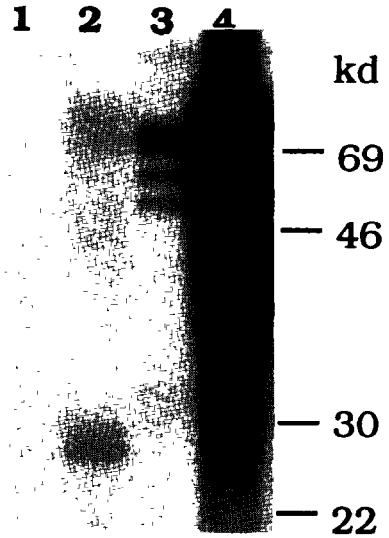

Fig. 3. Autophosphorylation and exogeneous substrate phosphorylation of the protein A/truncated c-*src* fusion protein The protein A expressed in pRIT5 plasmid transformed DH5α bacterial cells (lanes 1 and 2), and the protein A/truncated c-*src* fusion protein expressed in pRIT5/srcNP plasmid transformed DH5α bacterial cells (lanes 3 and 4) were precipitated by IgG-Sepharose and applied to in vitro kinase assay without (lane 1 and 3) and with poly(Glu:Tyr) substrate (lane 2 and 4). Remarkable autophosphorylation (lane 3) and substrate phosphorylation (lane 4) by the fusion protein were observed.

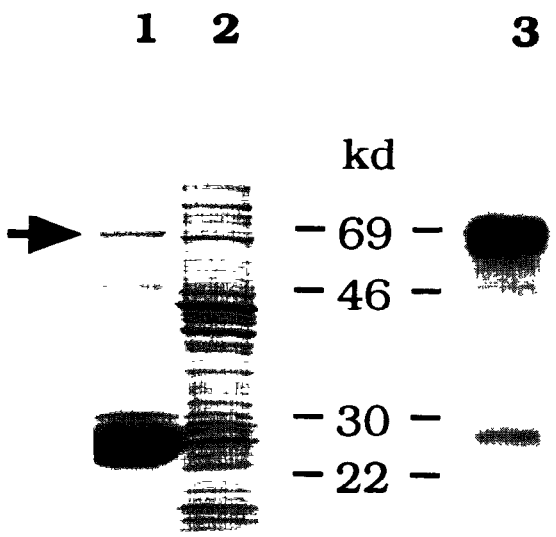

Fig. 4. Purification of the protein A/truncated c-*src* fusion protein using IgG-Sepharose affinity chromatography. Total cell extracts (lane 2) and the eluent from IgG-Sepharose column (lane 1) were analyzed by SDS-PAGE (8–16% gradient acrylamide gel) and Coomassie blue staining. The position of the full-length fusion protein (68 kDa) is indicated by an arrow. The purified fusion proteins were applied to the in vitro autophosphorylation assay and analyzed by SDS-PAGE and autoradiography (lane 3). The 68 kDa protein was predominantly phosphorylated. The amount of sample applied on each lane corresponded to 10 ml (lane 1), 100 μl (lane 2) and 1 ml (lane 3) of original bacterial culture.

teria present significant technical and biologic problems. Especially difficult has been the bacterial expression of PTK in an enzymatically active form. To date only several viral and proto-oncogene PTKs, including v-*src* [10], v-*abl* [11], v-*fps* [13], and c-*abl* [14], have been expressed in bacterial cells and purified in active form. Therefore, mammalian or insect cell expression systems, which are more laborious and expensive than bacterial expression systems, have been generally applied for production of enzymatically active PTK proteins, and these have also not always met with great success.

A variety of problems may be responsible for the difficulties in producing active PTKs in bacterial cells. Since there is no direct evidence supporting the possibility that bacterial cells contain endogenous PTKs, the exogenous recombinant PTKs may seriously affect the function of bacterial cellular proteins. In fact, although we attempted to transform 5 different *E. coli* strains (DH5α, HB101, SURE, MC1061 and NM522) with the pRIT5/*src*NP plasmid, only DH5α could grow with the expression of active truncated c-*src* whereas the other 4 strains did not make any transformed colonies (data not shown).

In addition to the toxicity, bacterially expressed PTKs are frequently aggregated in insoluble precipitates and an enzymatically inactive form during the high salt and detergent extraction of *E. coli* [9,28]. We attempted to express the c-*src*NP protein using the glutathione-*S*-transferase fusion protein expression system [29]. The expressed fusion protein, however, generated inclusion bodies, and the small amount of the soluble form of the protein did not have PTK activity (data not shown). The expression system used in the present study allows secretion of the fusion protein in the periplasmic space of *E. coli* [19]. Since the loss of cloned protein by aggregation into inclusion bodies can be prevented, the protein released slowly into the periplasmic space may constitute a biologically active form of the enzyme. Furthermore, the fusion of the c-*src* gene to the protein A moiety makes it possible to purify the protein by IgG-sepharose in a one-step procedure.

Nemeth et al. previously tested the function of the c-*src*NP protein using chicken embryo fibroblasts infected by retroviral expression plasmid pRSVc-*src*NP. The amino-terminal half-deletion of amino acids 15 to 225 restricted the ability of c-*src* to phosphorylate cellular substrates in vivo. Since c-*src*NP does not contain SH2 and SH3 domains, it may not be able to interact with critical intracellular molecules and may thus result in low levels of in vivo tyrosine phosphorylation and non-transformed phenotype of the transfected cells. However, the c-*src*NP protein displayed an increased in vitro PTK activity compared with the full-length c-*src* protein, suggesting that the amino-terminal half may

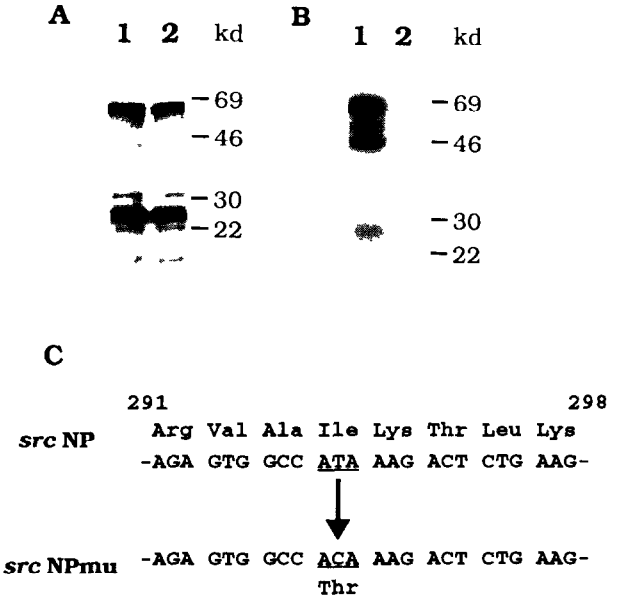

Fig. 5. Reduction of autophosphorylation activity of the protein A/truncated c-*src* fusion protein as a result of a missense mutation at the ATP-binding domain of the c-*src* gene. (A) Protein expression levels for pRIT5/*src*NP transformed bacteria (lane 1) and pRIT5/*src*NPmu transformed bacteria (lane 2) detected by immunoblot assay. (B) Autophosphorylation of the fusion proteins expressed in pRIT5/*src*NP transformed bacteria (lane 1) and pRIT5/*src*NPmu transformed bacteria (lane 2). (C) Comparison of DNA sequences of the ATP-binding domain of c-*src* cDNA between pRIT5/*src*NP plasmid and pRIT5/*src*NPmu plasmid

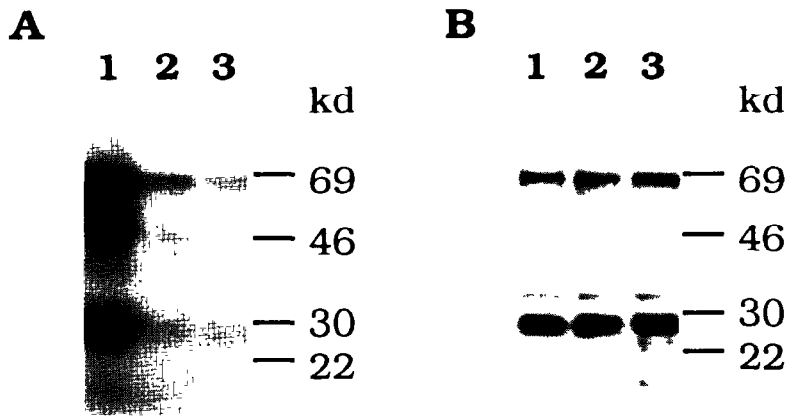

Fig 6. Suppression of autophosphorylation activity of the protein A/truncated c-*src* fusion protein by staurosporin treatment. The fusion protein precipited with IgG-sepharose was treated with staurosporin 0 μg/ml (control; lane 1), 1 μg/ml (lane 2), and 5 μg/ml prior to in vitro kinase assay (A) and immunoblot assay (B). Staurosporin reduced autophosphorylation of the fusion protein kinase, whereas the amount of the protein which bound to the IgG-Sepharose did not change by the drug treatment.

negatively effect the catalytic activity of the c-*src* protein [20]. Therefore, the carboxyl-terminal half of the c-*src* protein is useful for analyzing the catalytic action of the PTK in vitro as well as for understanding the effects of SH2 and SH3 domains on phosphorylation of substrates in vivo.

Since the fusion protein can be precipitated by IgG-sepharose, different mutated forms of c-*src* protein are easily analyzed. As we have shown in this study, one amino acid substitution (Ile to Thr) in the ATP-binding domain, which was caused by PCR misincorporation, significantly reduced the kinase activity of the c-*src*NP. Further analyses of the effects of other mutations on PTK function are in progress.

Several strategies are being employed in an attempt to lower levels of cellular oncogene products vital for transformation and thus inhibit malignant transformation in oncogene-activated cells. Drugs that inhibit the activity of oncogene-encoded PTKs may therefore provide a new strategy for cancer treatment. A major problem in this area of research has been the lack of a high-yield source of pure PTKs, especially cellular PTKs such as c-*src*. Moreover, to screen large numbers of putative oncogene-inhibiting compounds, it is necessary to create new methodologies that are fast, sensitive, quantitative, and cost effective. The protein A/c-*src*NP fusion protein is very useful for the evaluation of compounds that interact with the catalytic domain of c-*src* kinase as well as for crystallizing the catalytic domain of the enzyme. Our studies demonstrated that one known inhibitor, staurosporin, was effective in inhibiting the PTK activity of the fusion protein.

In summary, the strategy of expressing truncated c-*src* kinase fused to protein A is useful for structural analysis of the catalytic domain and diverse enzymological studies to elucidate the interactions of the catalytic domain with other molecules.

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