Purification and biochemical characterization of non-myristoylated recombinant pp60^{c-src} kinase

Nicholas B. LYDON,*† Brigitte GAY,* Helmut METT,* Brendan MURRAY,* Janis LIEBETANZ,* Alois GUTZWILLER,* Helen PIWNICA-WORMS,‡ Thomas M. ROBERTS§ and Elaine McGLYNN*

*Pharmaceuticals Division, Research Department, Ciba-Geigy Limited, CH-4002 Basel, Switzerland, ‡Department of Physiology, Tufts Medical School, Boston, MA 02111, U.S.A., and §Dana-Farber Cancer Institute, Boston, MA 02115, U.S.A.

To obtain sufficient material for the biochemical and biophysical study of pp60^{c-src}, we have utilized a recombinant pp60^{c-src} baculovirus lacking the myristoylation site at codon 2. On infection of Sf9 cells, this virus produced large amounts of soluble non-myristoylated pp60^{c-src}. The use of non-myristoylated pp60^{c-src} (1) increases production of pp60^{c-src} compared with the wild-type protein, (2) facilitates purification, (3) yields a stable product and (4) allows biochemical studies in the absence of detergents. Up to 20 mg of pp60^{c-src} of greater than 95% purity has been purified from 6 litres of Sf9 cells grown in a bioreactor. One major and multiple minor forms of pp60^{c-src} were separated by Mono Q f.p.l.c. Isoelectric focusing of purified pp60^{c-src} species revealed heterogeneity, some of which could be attributed to differences in the tyrosine phosphorylation state of the enzyme. Kinetic analysis of non-myristoylated pp60^{c-src} kinase in the presence of Mg²⁺ gave K_m values for angiotensin II and ATP of 2 mM and 30 μ M respectively and a V_{max} of 620 nmol/min per mg. The kinetic constants and metal ion preferences of a number of copolymers and peptide substrates have been compared. Polylysine and poly(GLAT), which was not phosphorylated by the pp60^{c-src} kinase, dramatically activated autophosphorylation of Tyr-416, suggesting a conformation modulation of pp60^{c-src} by charged polymers. This finding implies that Tyr-527 dephosphorylation is not sufficient for full activation of pp60^{c-src} *in vitro*.

INTRODUCTION

c-src, the normal cellular homologue of the Rous sarcoma virus oncogene (v-src), encodes the pp60^{c-src} tyrosine protein kinase. pp60^{c-src} is the prototype member of a family of tyrosine protein kinases which are tightly associated with cellular membranes via an N-terminal myristate group (Cross *et al.*, 1984; Schultz *et al.*, 1985; Kamps *et al.*, 1985). Although pp60^{c-src} is one of the most extensively studied tyrosine protein kinases, its physiological role still remains largely unknown. The phosphorylation state of pp60^{c-src}, as with many other kinases, is involved in the regulation of pp60^{c-src} kinase activity. A C-terminal phosphorylation site located at Tyr-527 negatively regulates pp60^{c-src} activity, whereas autophosphorylation at Tyr-416 is involved in enzyme activation (Kmiciek & Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Kmiciek *et al.*, 1988). The N-terminal region of pp60^{c-src} contains sites of tyrosine and serine/threonine phosphorylation which may be involved in the regulation of c-src (Cross & Hanafusa, 1983; Bollen *et al.*, 1985; Gould *et al.*, 1985; Ralston & Bishop, 1985; Gentry *et al.*, 1986; Gould & Hunter, 1988; Morgan *et al.*, 1989; Shenoy *et al.*, 1989). Ser-17, which is phosphorylated by the cyclic-AMP (cAMP)-dependent protein kinase *in vitro* and *in vivo*, has been suggested to affect the activity of pp60^{c-src} (Hunter & Cooper, 1985). The N-terminus of pp60^{c-src} also contains the SH2 homology domain (Pawson, 1988). This domain has been shown to bind to phosphotyrosine-containing peptides and polypeptides (Matsuda *et al.*, 1990; Moran *et al.*, 1990; Mayer *et al.*, 1991) and is thus likely to play an important role in regulation via interactions with cellular phosphoproteins. Attempts to purify either pp60^{v-src} (Levinson *et al.*, 1980; Donner *et al.*, 1981; Blithe *et al.*, 1982; Richert *et al.*, 1982; Fukani & Lipmann, 1985; Radziejewski *et al.*, 1989) or pp60^{c-src} (Varshney *et al.*, 1986; Presek *et al.*, 1988; Feder & Bishop, 1990) have met with limited success. Proteolytic cleavage of the N-terminus of pp60^{c-src} during purification has resulted in the isolation of proteins of varying size, purity and specific activity. Efforts to overcome this problem have utilized a number of recombinant expression systems, including *Escherichia coli* (Gilmer & Erikson, 1981; Gilmer *et al.*, 1982; McGrath & Levinson, 1982) and yeast (Kornbluth *et al.*, 1987; Cooper & Runge, 1987; Brugge *et al.*, 1987). Recently, expression of recombinant proteins in insect cells using vectors based on a baculovirus has emerged as the method of choice for high-level expression of protein kinases. This expression system, which exploits the baculovirus polyhedrin late promoter (Smith *et al.*, 1983; Summers & Smith, 1987), has been used to express a number of recombinant tyrosine protein kinases including pp60^{c-src} (Morgan *et al.*, 1989; Piwnica-Worms *et al.*, 1990; Zhang *et al.*, 1991) and v-src (Zhang *et al.*, 1991). Inactive pp60^{c-src} (containing a Lys-295 to methionine mutation at the ATP-binding site) has been purified to near-homogeneity from Sf9 insect cells (Morgan *et al.*, 1989) using immunoaffinity chromatography. Recombinant pp60^{v-src} and pp60^{c-src} have also been partially purified by substrate affinity chromatography from Sf9 insect cells (Zhang *et al.*, 1991).

In the present report we describe the expression and purification of recombinant non-myristoylated pp60^{c-src} using a recombinant baculovirus. The use of non-myristoylated pp60^{c-src} represents a significant advance in that it (1) increases enzyme production compared with the wild-type protein, (2) facilitates enzyme purification in the absence of detergents and (3) yields an enzymically stable product of high specific activity. The availability of large amounts of characterized enzyme can now form the basis for studies on the regulation of pp60^{c-src}.

Abbreviations used: Random polymers poly(GLAT), poly(Glu,Lys,Ala,Tyr, 2:5:6:1), poly(GT), poly(Glu,Tyr, 4:1), and poly(GAT), poly(Glu,Ala,Tyr, 6:3:1); Sf9, *Spodoptera frugiperda* cell line; AcNPV, *Autographa californica* nuclear polyhedrosis virus; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; mAb, monoclonal antibody; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; cAMP, cyclic AMP.

† To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Pepstatin, phenylmethanesulphonyl fluoride (PMSF), benzamidine, angiotensin II and random polymers poly(Glu,Lys,Ala,Tyr, 2:5:6:1) [poly(GLAT)], poly(Glu,Ala,Tyr, 6:3:1) [poly(GAT)] and poly(Glu,Tyr, 4:1) [poly(GT)] were from Sigma. Leupeptin and aprotinin were obtained from Boehringer. [γ - 32 P]ATP was from Amersham. The hybridoma-producing monoclonal antibody (mAb) 327 directed against pp60^{c-src} was kindly provided by Dr. J. Brugge (Lipsich *et al.*, 1983). Monoclonal anti-phosphotyrosine antibody 4G10 (Drucker *et al.*, 1989) was used for Western blotting. Affi-Gel Blue, goat anti-(mouse horseradish peroxidase) conjugate and horseradish peroxidase substrate were from Bio-Rad. Rabbit muscle cAMP-dependent protein kinase (catalytic subunit) was a gift from Dr. B. Hemmings (FMI, Basel, Switzerland). Sodium orthovanadate was from Fisher Chemical. Enzymes were from New England Biolabs or Boehringer-Mannheim. Ex-Cell 400 medium was from JRH Biosciences (Lenexa, KS, U.S.A.). Cell culture reagents and materials were from Gibco/BRL. Phenyl-Sepharose FF, Sephadex G-25, Mono Q HR 10/10, Superose 12 HR 10/30 and Pharmalyte were obtained from Pharmacia LKB. Casein/agarose was obtained from United States Biochemical Corporation (Cleveland, OH, U.S.A.).

Mutagenesis of pp60^{c-src} and vector construction

Virus construction was carried out as previously described for the baculovirus encoding wild-type pp60^{c-src} (Piwnica-Worms *et al.*, 1990). The c-src cDNA in SP68-4RBS (Piwnica-Worms *et al.*, 1986) in which codon 2 had been changed from GGA to GCA by oligonucleotide-directed mutagenesis was kindly provided by Dr. K. Balmer (FMI). DNA sequencing was used to confirm that the mutation was in place and that the remainder of the cDNA was unaltered. Since the cDNA in SP68-4RBS is derived from pHB5 (Iba *et al.*, 1985), it encodes a D residue at position 63 instead of the G encoded in wild-type pp60^{c-src}. This change has never been found to have any effect on the biochemical or biological activities of pp60^{c-src}. A recombinant baculovirus [pAC373(295)] carrying the c-src gene with a lysine to methionine mutation at position 295 was constructed as previously described (Piwnica-Worms *et al.*, 1986).

Cells and virus

Standard methods of gene expression using the baculovirus vector system were used as outlined by Summers & Smith (1987). The cell line *Spodoptera frugiperda* (Sf9) was propagated as a monolayer or in suspension culture in medium containing 10% fetal calf serum (Gibco/BRL). *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant virus stocks were propagated using spinner cultures of Sf9 cells. Baculovirus expression vectors, wild-type virus and Sf9 cells were obtained from Dr. M. Summers (Texas A & M University, College Station, TX, U.S.A.).

Large-scale expression of pp60^{c-src} kinase in Sf9 cells

Sf9 insect cells were first adapted to Ex-Cell 400 media. Large-scale suspension cultures were grown in a 6-litre stirred bioreactor with a single marine impeller (Biolifte SA, Paris, France). The bioreactor was equipped with a polarographic oxygen electrode for measurement of dissolved O₂. The O₂ concentration was controlled by addition of O₂ to the body of the culture as required. Air was continuously added to the head space to prevent foaming. The impeller speed was maintained at 125 rev./min throughout the run. The pH was monitored throughout the process. pH was regulated by the use of an adequately

buffered medium. Additional regulation during the run was not necessary. The temperature (28 °C) was controlled by a heat-exchange coil. Sf9 cells were seeded at a cell density of 2×10^5 cells/ml and were grown to a cell density of 3×10^6 cells/ml. The culture was then infected with the recombinant virus at a multiplicity of infection (m.o.i.) of 10. After 72 h, cells were harvested by centrifugation (1000 g) washed once in phosphate-buffered saline and frozen at -70 °C.

Purification of pp60^{c-src} from Sf9 cells

Cells were lysed by freeze/thawing in buffer A [25 mM-Tris/HCl, pH 8.0, 5 mM-EDTA, 1 mM-PMSF, 10 μ g of leupeptin/ml, 10 μ g of pepstatin/ml, 1 μ g of aprotinin/ml, 1 mM-benzamidine, 10% glycerol, 1 mM-dithiothreitol (DTT)]. The lysate was clarified by centrifugation at 30000 g for 30 min at 4 °C. The clarified lysate was loaded on to a 200 ml casein/agarose affinity column at a flow rate of 4 ml/min. The column was washed with buffer A until the absorbance of the effluent had returned to baseline. Bound protein was eluted with buffer B (buffer A containing 300 mM-NaCl). The eluted proteins were pooled. (NH₄)₂SO₄ was added to the pooled material with continuous stirring, to a final concentration of 0.5 M. After centrifugation, the 0.5 M-(NH₄)₂SO₄ supernatant was loaded on to a phenyl-Sepharose FF chromatography column (1.5 cm $\times$ 30 cm) at a flow rate of 5 ml/min. The column was washed with buffer C [buffer A containing 0.5 M-(NH₄)₂SO₄] at 5 ml/min. The column was then eluted with a linear gradient from buffer C to buffer A at a flow rate of 1 ml/min. The pooled pp60^{c-src}-containing fractions were rapidly desalted over a Sephadex G-25 column (2.6 cm $\times$ 30 cm) at a flow rate of 8 ml/min in buffer A. The desalted material was injected directly on to a Mono Q HR 10/10 column at a flow rate of 3 ml/min. The column was washed with buffer A. pp60^{c-src} was eluted with a linear NaCl gradient to 300 mM in buffer A. The column was then washed with buffer A containing 1 M-NaCl to remove the remaining bound proteins. All peaks eluted in the gradient consist predominantly of pp60^{c-src}. Fractions from all purification steps were analysed by SDS/PAGE and Western blotting.

Assay of tyrosine protein kinase activity

Tyrosine protein kinase activity was assayed with either angiotensin II (2 mM) or the random polymeric substrate poly(GT) (100 μ g/ml). The reaction mixtures (40 or 50 μ l) contained 25 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂ (or 1 mM-MnCl₂ as described in the Figure legends), 10 μ M-[γ - 32 P]ATP (0.1 μ Ci/assay) and 10 μ l of enzyme (50 ng of protein). Reactions were terminated with 10 μ l of 0.5 M-EDTA, pH 8.0. Portions of the reaction mixture were spotted on to P81 paper (Whatman, Maidstone, Kent, U.K.) and processed as described previously (Geissler *et al.*, 1990). Autophosphorylation assays were performed essentially as described above in the absence of exogenous peptide substrate, with either 2 μ M- or 10 μ M-ATP. Reactions were terminated with 40 μ l of 2 $\times$ Laemmli sample buffer and boiled for 5 min. Samples were analysed by SDS/PAGE [10% (w/v) polyacrylamide] (Laemmli, 1970), dried and exposed to X-OMAT-AR film with screen. cAMP-dependent protein kinase phosphorylation of pp60^{c-src} was carried out using 0.02 unit of enzyme and 100 ng of pp60^{c-src}.

Kinetics

For kinetic determinations, pp60^{c-src} from peak 1 (see Fig. 2) was preincubated for 10 min with 50 μ M-ATP in order to autophosphorylate the pp60^{c-src} enzyme. The enzyme/ATP mixture was then diluted and assayed at 20 °C for 10 min in the presence of either a constant amount of peptide substrate and various concentrations of ATP or a constant amount of ATP

and various peptide substrate concentrations. The non-variable substrate was used at approximately K_m and the variable substrate at $0.2\text{--}5 \times K_m$.

Western blotting

After SDS/PAGE (10 % gels), proteins were transferred on to nitrocellulose membranes (Bio-Rad) by semi-dry blotting. Membranes were blocked with PBS/Tween (0.2 % Tween 20), and incubated with primary antibody (0.5 $\mu\text{g}/\text{ml}$ in PBS containing 0.05 % Tween 20) for 1 h. After being washed in TBS/Tween, membranes were incubated with peroxidase-conjugated secondary antibody (goat anti-mouse IgG) and developed using the Bio-Rad peroxidase substrate kit.

Peptide mapping

Staphylococcus aureus V8 proteinase mapping of pp60^{c-src} was carried out as described by Collet *et al.* (1979) using 300 ng of V8 proteinase per digest. CNBr mapping was performed as described by Jove *et al.* (1987). Fragments were separated on a 16.5 % (w/v) polyacrylamide gel as described by Schagger & Von Jagow (1991). Tyrosine phosphorylation of peptides was detected by direct autoradiography or by Western blotting using an anti-phosphotyrosine mAb.

Isoelectric focusing analysis of pp60^{c-src}

Samples (100–200 ng of protein) were loaded on to a pH 5–8 isoelectric focusing polyacrylamide gel (5 % polyacrylamide containing 2 % Pharmalyte pH 5–8 and 0.4 % Pharmalyte pH 3–10). Isoelectric focusing was carried out in a Bio-Rad SDS/PAGE Minigel apparatus for 30 min at 150 V constant voltage, then at 200 V for 2 h. The anode and cathode solutions were 0.01 M-phosphoric acid and 0.02 M-NaOH respectively. After focusing, proteins were transferred to nitrocellulose and processed as described above.

Protein determination

Protein concentrations were determined by the method of Bradford (1976) with BSA as standard.

RESULTS

Expression of pp60^{c-src}

A recombinant c-src baculovirus was constructed using a cDNA in which amino acid 2 had been mutated from glycine to alanine by site-directed mutagenesis (Fig. 1a). This construct was designed to produce a non-myristoylated pp60^{c-src} protein which could be purified in the absence of detergents. When used to infect Sf9 cells, the recombinant c-src baculovirus produced a protein of the predicted size (Fig. 1b, lanes 1–4) which was found in the soluble fraction from virus-infected Sf9 cells (results not shown). Expression of pp60^{c-src} in Sf9 cells resulted in extensive tyrosine phosphorylation of cellular proteins (Fig. 1b, lane 5). This tyrosine phosphorylation was not seen in control cells (results not shown) or in cells infected with a c-src baculovirus which contains a kinase-inactivating mutation (Piwnicka-Worms *et al.*, 1986) within the ATP-binding pocket (Fig. 1b, lane 6). Initial small-scale purifications of pp60^{c-src} utilized Sf9 cells grown in 1-litre spinner cultures. However, owing to the difficulty of producing sufficient quantities of cells, we subsequently utilized a 6-litre submarine impeller bioreactor for scaling up growth of Sf9 cells [the optimization of the bioreactor for growth of Sf9 cells is described in McGlynn *et al.* (1992)]. Reproducible cell-culture and viral-infection conditions were found to be the major variables in expression of pp60^{c-src} in Sf9 cells. Using a bioreactor we have been able to grow cells to high density in a reproducible manner. Sf9 cells were infected at a cell density of 3×10^6 at an

m.o.i. of 10 using a high-titre viral stock. The time course of expression of pp60^{c-src} after viral infection was determined. Harvesting of cells at 72 h after infection was found to give the maximum expression levels (Fig. 1b, lane 4). For the routine purification of pp60^{c-src}, cells were harvested 72 h after infection, washed once in PBS and frozen at -70°C . Freezing and storage of Sf9 cells was found to have no noticeable effect on degradation of pp60^{c-src} or on the recovery of enzyme activity.

Purification of pp60^{c-src}

A purification starting from 844 mg of cell lysate, derived from 25 % of a bioreactor, is summarized in Table 1. As expected, non-myristoylated pp60^{c-src} from lysed cell was found only in the soluble fraction as detected by immunoblotting using mAb 327

Fig. 1. Construction of recombinant baculovirus vectors and expression of pp60^{c-src} in Sf9 cell

(a) Structure of c-src proteins including sites of phosphorylation (Tyr-416 and Tyr-527) and myristoylation (Gly-2): (1) Structure of wild-type pp60^{c-src}; (2) non-myristoylated c-src. This cDNA was derived from pHB5 (Iba *et al.*, 1985) which contains a Gly $\rightarrow$ Asp mutation at codon 63 in addition to the Gly $\rightarrow$ Ala transversion at codon 2; (3) enzymically inactive c-src generated by mutation of codon 295 (Lys $\rightarrow$ Met) within the ATP-binding consensus sequence (Piwnicka-Worms *et al.*, 1986). (b) A 6-litre bioreactor was infected with the c-src baculovirus as described in the Materials and methods section. Cells were harvested 48 h (lanes 1 and 2) and 72 h (lanes 3 and 4) after the infection and analysed for pp60^{c-src} expression by Western blotting using mAb 327. Analysis was performed on 2 μg of protein (lanes 1 and 3) and 20 μg of protein (lanes 2 and 4). Lanes 5 and 6 show a Western-blot analysis, using an anti-phosphotyrosine mAb, of cell extracts from Sf9 cells infected with either a non-myristoylated c-src baculovirus (lane 5) or with a mutated virus which produces an inactive pp60^{c-src} (lane 6). Lane M contains molecular-mass markers (kDa).

Table 1. pp60^{c-src} purification

Purification scheme starting from cells from 25 % of a bioreactor. Kinase assays were performed as described in the Materials and methods section with 10 μ M-ATP, 10 mM-MgCl₂ and poly(GT) (100 μ g/ml) as substrate.

Step	Volume (ml)	Protein (mg)	Activity (nmol·min ⁻¹)	Specific activity (nmol·min ⁻¹ ·mg ⁻¹)	Yield (%)	Purification (fold)
1. Cell extract	175	844	5429	6.4†	100	1
2. Casein/agarose	144	44	1133	25.7	20.8	4.01
3. Phenyl-Sepharose	48	11	1214	110.0‡	22.3	17.2
4. Sephadex G-25	65	7	731	104.4	13.4	16.2
5. Mono Q						
Peak 1*	20	5	660	130	12.1	20.6
Peak 2*	10	0.4	49.6	124	0.91	19.4

* Peaks 1 and 2 refer to Fig. 2.

† This value may overestimate the activity of pp60^{c-src} since the cell extract may contain endogenous Sf9 tyrosine protein kinases.

‡ Assay contains 0.1 mM-(NH₄)₂SO₄.

Fig. 2. Analysis of the pp60^{c-src} purification

(a) pp60^{c-src}-containing fractions were loaded on to a Mono Q HR 10/10 f.p.l.c. column and eluted with a linear NaCl gradient. The A_{280} elution profile is shown. Peaks 1 and 2 are indicated. (b) Fractions were analysed by SDS/PAGE: Peak 1 (lane 1) and peak 2 (lane 2). Lanes 3 and 4 show the leading and trailing edge of peak 1. (c) Coomassie Blue-stained SDS/polyacrylamide gel and (d) Western blot using anti-pp60^{c-src} mAb 327 of pooled fractions from each stage of the pp60^{c-src} purification. Crude cell extract (lane 1), casein/agarose pool (lane 2), phenyl-Sepharose FF pool (lane 3), Sephadex G-25 pool (lane 4), Mono Q peak 1 (lane 5) and Mono Q peak 2 (lane 6). All lanes contain 2.4 μ g of protein except for lanes 1 and 6 which contain 5 μ g and 1.2 μ g of protein respectively. Lane m contains molecular masses (kDa).

(results not shown). In addition to full-length pp60^{c-src}, degradation products were detected in Sf9 cells before lysis (Fig. 1b, lane 4). Since mAb 327 recognizes the N-terminal domain of pp60^{c-src} (peptides V3 and V4 generated by proteinase V8, Fig. 7), these degradation products are unlikely to have enzyme

Fig. 3. Substrate phosphorylation by the pp60^{c-src} kinase

(a) Polymeric substrates poly(GAT) (lanes 1–4), poly(GLAT) (lanes 5–8) and poly(GT) (lanes 9–12) were phosphorylated as described in the Materials and methods section in the presence of either 1 mM-Mn²⁺ or 10 mM-Mg²⁺. (b) Autophosphorylation of pp60^{c-src}. 50 ng of purified pp60^{c-src} was phosphorylated in assay buffer containing either Mg²⁺ (lane 1) or Mn²⁺ (lane 2). (c) Stimulation of pp60^{c-src} autophosphorylation by poly(GLAT): 50 ng of purified pp60^{c-src} was phosphorylated in assay buffer containing 1 mM-MnCl₂ in the absence (lane 1) or presence (lane 3) of poly(GLAT). Phosphorylation of poly(GT) under the same reaction conditions is shown in lane 2. Reaction products were separated by SDS/PAGE (10% acrylamide) and exposed to X-ray film.

activity, since their size would predict deletions within the kinase domain of pp60^{c-src}. No tyrosine phosphorylation of these degradation products could be detected by using the anti-phosphotyrosine antibody. The clarified cell lysate was loaded on to a casein/agarose column. After the column had been washed, pp60^{c-src} was eluted with buffer containing 0.3 M-NaCl. (NH₄)₂SO₄ (final concentration 0.5 M) was added to the pooled pp60^{c-src} peak (fractions 21–29). After clarification by centri-

Fig. 4. Bivalent metal ion preference of pp60^{c-src} for substrate phosphorylation

Kinase assays were performed as described in the Materials and methods section with various concentrations of either MgCl₂ (■, ●) or MnCl₂ (□, ○) and poly(GT) (■, □) or angiotensin II (●, ○) as substrates. Poly(GT) and angiotensin II were used at 100 μg/ml and 250 μg/ml respectively.

Table 2. Apparent kinetic constants for substrate phosphorylation by pp60^{c-src}

Apparent kinetic constants were determined at 20 °C in the presence of 10 μM-ATP and 10 mM-MgCl₂ as described in the Materials and methods section. Values are averages of at least two independent experiments.

Substrate	Apparent <i>K_m</i>	Apparent <i>V_{max}</i> (nmol · min ⁻¹ · mg ⁻¹)
Angiotensin II	2 mM	620
Angiotensin I	0.8 mM	60
Poly(GT)	15 μg/ml	350
Poly(GAT)	10 μg/ml	640
Casein	0.54 μg/ml	250

fugation, this material was loaded on to a phenyl-Sepharose FF column, washed and eluted using a linear gradient from 0.5 to 0 M-(NH₄)₂SO₄. The peak of pp60^{c-src} eluted from the phenyl-Sepharose FF column (fractions 27–29) was pooled, desalted over a Sephadex G-25 column, and loaded on to a Mono Q HR 10/10 f.p.l.c. column. A Coomassie-Blue-stained SDS/polyacrylamide gel (Fig. 2c) and Western blot (Fig. 2d) of the peak pp60^{c-src}-containing fractions from each purification step are shown in Fig. 2. The u.v.-absorbance profile (Fig. 2a) and SDS/polyacrylamide gel of pp60^{c-src}-containing fractions from the Mono Q column (Fig. 2b) are shown. pp60^{c-src} was eluted from the Mono Q column at two well-separated positions in the gradient. Both peaks were recognized by mAb 327, had identical apparent molecular masses (Figs. 2c and 2d, lanes 5 and 6) and contained essentially pure pp60^{c-src} (> 95 % purity). The major peak of pp60^{c-src} (peak 1) contained 5 mg of pp60^{c-src} kinase of greater than 95 % purity. In replicate purifications using cells from a different bioreactor, the major pp60^{c-src}-containing fraction was eluted at an identical position in the Mono Q gradient (peak 1). However, the profile and number of peaks were variable (results not shown).

The purification shown in Fig. 2 is summarized in Table 1. The specific activity of the purified kinase measured at 20 °C, with 10 μM-ATP, 10 mM-Mg²⁺ and poly(GT) as substrate, was 130 nmol/min per mg (peak 1) and 124 nmol/min per mg (peak

2). When assayed in the presence of Mn²⁺, the specific activity of pp60^{c-src} was 395 nmol/min per mg. With angiotensin II as substrate and Mg²⁺ as the bivalent cation, the specific activity was 1.2-fold higher than that determined with poly(GT) as substrate. The yield of pp60^{c-src} protein from a quarter of the contents of a bioreactor was 5.4 mg. The yield of pp60^{c-src} in a replicate purification which used cells from an entire bioreactor (4200 mg of cell extract derived from 10¹⁰ Sf9 cells) was 19.2 mg of pp60^{c-src} (> 95 % purity). Purified pp60^{c-src} was stored at -70 °C in buffer D without apparent loss of activity for over 6 months.

Substrate specificity and autophosphorylation of pp60^{c-src}

We have examined a number of substrates for phosphorylation by the purified pp60^{c-src} kinase from peak 1. These include angiotensins and the random polymers poly(GT), poly(GAT) and poly(GLAT) (Table 2). Random-polymer phosphorylation was assayed with either Mn²⁺ or Mg²⁺ as the bivalent cation and purified pp60^{c-src} from Mono Q peak 1 (Table 1). Phosphorylation of poly(GAT) and poly(GT) showed a slight preference for Mn²⁺, when assessed by SDS/PAGE (Fig. 3a, lanes 1–4 and 9–12 respectively). Poly(GLAT), which is a good substrate for v-abl kinase (results not shown), was not phosphorylated by pp60^{c-src} (Fig. 3a, lanes 5–8). Autophosphorylation of the pp60^{c-src} kinase had a preference for Mn²⁺ (Fig. 3b, lanes 1 and 2). However, addition of the polymer poly(GLAT) resulted in a dramatic stimulation of autophosphorylation (Fig. 3c, lanes 1 and 3). This activation of autophosphorylation could also be seen using polylysine (results not shown). The optimal metal ion concentrations for poly(GT) and angiotensin II phosphorylation was investigated (Fig. 4). The optimal bivalent cation concentration for poly(GT) phosphorylation was 10 mM. The activity of pp60^{c-src} for poly(GT) phosphorylation was 2.3-fold higher with Mn²⁺ than with Mg²⁺. With angiotensin II as substrate, the optimal Mn²⁺ and Mg²⁺ ion concentrations were 1 mM and 10 mM respectively. Under optimal ion conditions, the rate of poly(GT) and angiotensin II phosphorylation was 2-fold higher in the presence of Mn²⁺.

Kinetic properties of purified pp60^{c-src}

For kinetic determinations, pp60^{c-src} from peak 1 (Fig. 2) was preincubated for 10 min with 50 μM-ATP in order to autophosphorylate the pp60^{c-src} enzyme. Using optimized assay conditions, the apparent kinetic constants for pp60^{c-src} were determined for ATP and angiotensin II in the presence of Mg²⁺ (Table 2). *K_m* values of 37 μM and 2 mM were obtained for ATP and angiotensin II respectively. The apparent *V_{max}* value was 620 nmol/min per mg when assayed at 20 °C with angiotensin II as substrate. This corresponds to a turnover number of 39 mol/min per mol. The kinetic constants determined for polymeric substrates are also shown in Table 2. The *K_m* values for poly(GT) and poly(GAT) determined in the presence of Mg²⁺ were 15 μg/ml and 10 μg/ml respectively. The corresponding *V_{max}* values for poly(GT) and poly(GAT) phosphorylation were 350 nmol/min per mg and 640 nmol/min per mg. The kinetic constants for phosphorylation of casein were *K_m* = 0.54 μg/ml and *V_{max}* = 240 nmol/min per mg.

Phosphorylation of pp60^{c-src} in vitro

Autophosphorylation of pp60^{c-src} in vitro (Fig. 5a), lane 1) was found to occur exclusively in peptide V2 as assessed by V8 proteinase mapping (Iba *et al.*, 1985) (Fig. 5b, lane 1). Autophosphorylation was stimulated by polylysine or poly(GLAT). No tyrosine phosphorylation occurred on Tyr-527 in vitro as assessed by CNBr peptide mapping (Fig. 5c, lane 1). CNBr cleavage revealed label in the 10 kDa (Tyr-416) fragment, but

Fig. 5. *pp60^{c-src}* phosphorylation sites *in vitro*

pp60^{c-src} (50 ng) was phosphorylated *in vitro* in the absence (a, lane 1) or presence (a, lane 2) of 0.02 unit of cAMP-dependent protein kinase. Labeled *pp60^{c-src}* was excised from the polyacrylamide gel (a) and subjected to either proteinase V8 (b) or CNBr (c) peptide mapping. The location of phosphorylation sites and the expected size of peptides (Jove *et al.*, 1987) are shown in (d). The X-ray shown in (c) was overexposed to enable detection of low-level phosphorylation in the 4 kDa peptide.

not in the 4 kDa fragment that corresponds to Tyr-527 (Jove *et al.*, 1987).

Ser-17, which is the major site of serine phosphorylation *in vivo*, is the target for the cAMP-dependent protein kinase (Iba *et al.*, 1985; Patschinsky *et al.*, 1986). We have studied the effect of phosphorylation of Ser-17 in *pp60^{c-src}* on the kinetics of substrate phosphorylation. Addition of 0.02 unit of cAMP-dependent protein kinase to the kinase reaction *in vitro* resulted in a 3-fold increase in the incorporation of radioactivity into *pp60^{c-src}* relative to *pp60^{c-src}* autophosphorylation (Fig. 5a, lane 2). Peptide mapping of *pp60^{c-src}* using V8 proteinase and CNBr (Figs. 5b and 5c, lane 2) revealed that cAMP-dependent protein kinase treatment resulted in phosphorylation of the N-terminus of *pp60^{c-src}* (peptides V1, V3 and V4). Phosphorylation of Ser-17 was found to have no detectable effect on the K_m or V_{max} for poly(GT) phosphorylation by *pp60^{c-src}* (results not shown).

Characterization of purified *pp60^{c-src}*

To characterize the two peaks of *pp60^{c-src}* eluted from the Mono Q column, we have carried out analytical isoelectric focusing followed by Western blotting. Under native conditions, major heterogeneity was seen in both *pp60^{c-src}*-containing fractions (results not shown). The isoelectric-focusing pattern of *pp60^{c-src}* from peaks 1 and 2 was greatly simplified by performing

Fig. 6. Isoelectric focusing analysis of *pp60^{c-src}* heterogeneity

Vertical slab gel isoelectric focusing followed by Western blotting was used to separate the different isoelectric forms of *pp60^{c-src}*. Mono Q peaks 1 and 2 (200 ng each) were separated and transferred to nitrocellulose as described in the Materials and methods section. Blots were developed with either an anti-*pp60^{c-src}* mAb (a) or an anti-phosphotyrosine mAb (b). *pp60^{c-src}* from peak 1 (lane 2); *pp60^{c-src}* from peak 2 (lane 1). Arrowheads indicate the positions of the four *pp60^{c-src}* species.

Fig. 7. V8 proteinase mapping of tyrosine sites in *pp60^{c-src}* phosphorylated *in vivo* from Mono Q peaks 1 and 2

Proteinase V8 digests of *pp60^{c-src}* (0.2 μ g) from Mono Q peaks 1 and 2 were separated by SDS/PAGE (13% acrylamide gel), transferred to nitrocellulose and developed with anti-*pp60^{c-src}* mAb 327 (a) or an anti-phosphotyrosine mAb (b) as described in the Materials and methods section. *pp60^{c-src}* from peak 1 (lanes 1–3): non-digested (lane 1); V8 proteinase-digested (lane 2); *pp60^{c-src}* from peak 1 which had been phosphorylated *in vitro* in the presence of 1 mM-MnCl₂ and 10 μ M-ATP was used as a control to identify peptide V2 (lane 3). *pp60^{c-src}* from Mono Q peak 2 (lanes 4 and 5): partially digested (lane 4); V8 proteinase-digested (lane 5).

isoelectric focusing under denaturing conditions. Peak 1 was found to contain two forms of *pp60^{c-src}* (Fig. 6a, lane 2), one of which was phosphorylated on tyrosine (Fig. 6b, lane 2). V8 proteinase peptide mapping of peak 1 (Fig. 7, lanes 2) revealed that tyrosine phosphate was located in peptide V2. Additional tyrosine phosphorylation, which resulted in an isoelectric shift, occurred in *pp60^{c-src}* from peak 1 after incubation with ATP and MnCl₂. This tyrosine phosphorylation was mapped to proteinase V8 fragment V2 (Fig. 7, lanes 3). *pp60^{c-src}* from peak 2 contained two major and one minor isoelectric species, all of which were phosphorylated on tyrosine (Figs. 6a and 6b, lane 2). Addition of ATP to peak 2 had no apparent effect on its isoelectric profile. Peptide mapping of peak 2 using proteinase V8 revealed tyrosine phosphate in peptide V2 (Fig. 7, lanes 5).

DISCUSSION

Previous attempts to purify either *pp60^{v-src}* (Levinson *et al.*, 1980; Donner *et al.*, 1981; Blythe *et al.*, 1982; Richert *et al.*, 1982; Fukani & Lipmann, 1985; Radziejewski *et al.*, 1989) or *pp60^{c-src}*

(Varshney *et al.*, 1986; Presek *et al.*, 1988) have been hampered by proteolysis, poor yields and a low degree of purity. These problems have not been overcome by utilizing a number of recombinant expression systems, including *E. coli* (McGrath & Levinson, 1982; Gilmer & Erikson, 1981; Gilmer *et al.*, 1982) and yeast (Kornbluth *et al.*, 1987; Cooper & Runge, 1987; Brugge *et al.*, 1987). Expression of recombinant proteins in insect cells has been used to express a number of recombinant tyrosine protein kinases including pp60^{c-src} (Morgan *et al.*, 1989; Piwnica-Worms *et al.*, 1990; Zhang *et al.*, 1991) and pp60^{v-src} (Zhang *et al.*, 1991). Enzymically active pp60^{c-src} has been partially purified from Sf9 insect cells using substrate affinity chromatography (Zhang *et al.*, 1991). This purification required the use of non-ionic detergents for the solubilization of pp60^{c-src} from the membrane fraction. In mammalian and avian cells, pp60^{c-src} is associated with the plasma membrane as a result of an N-terminal myristoylation. Previous studies with wild-type pp60^{c-src} expressed in Sf9 cells have shown that a fraction of pp60^{c-src} is myristoylated on glycine (Gly-2) (Piwnica-Worms *et al.*, 1990). However, the extent of myristoylation was not determined. Recent studies using the closely related pp56^{lck} kinase have shown that a significant fraction of pp56^{lck} is non-myristoylated when expressed in Sf9 cells (T. Roberts, unpublished work). To avoid heterogeneity due to variable myristoylation, and to circumvent the use of detergents during purification, we have utilized a recombinant pp60^{c-src} baculovirus in which codon 2 of c-src had been mutated from glycine to alanine by site-directed mutagenesis. This virus produced pp60^{c-src}, which was found in the soluble fraction from Sf9 cells.

Recombinant proteins have been produced as either fusion or non-fusion proteins with various levels of expression (Luckow & Summers, 1988). In general, protein tyrosine kinases are expressed in insect cells in the range 0.5–4 mg of protein/l of culture (Greenfield *et al.*, 1988; Herrera *et al.*, 1988; Wedegaertner & Gill, 1989). Under the conditions reported in the present paper, purification of 1–4 mg of pure pp60^{c-src} kinase per litre of Sf9 cells was achieved, implying expression levels above this value. This corresponds to approx. 1% of total soluble Sf9 cell protein. Western blotting suggests that the non-myristoylated form of pp60^{c-src} was produced at approx. 3–5-fold higher levels than wild-type pp60^{c-src} when expressed using the same vector constructions (T. Roberts, unpublished work). An objective of this work was to enable large-scale purification of the pp60^{c-src} kinase by classical purification methods which would avoid the need to use detergents or mAb affinity chromatography. This method has the advantage over using mAb affinity chromatography that it does not require harsh elution conditions and that scale up is not limited by the capacity of the antibody column. The purification scheme is rapid and simple to perform and results in a pp60^{c-src} kinase with the highest specific activity yet reported. From 6 litres of Sf9 cells we have purified up to 20 mg of pp60^{c-src} to greater than 95% purity.

The specific activity of pp60^{c-src} from peak 1 was not significantly different from that of peak 2. This finding implied that modifications that have occurred in pp60^{c-src} from peak 2 do not significantly affect the specific activity of the enzyme. Kinetic analysis of the purified pp60^{c-src} kinase from peak 1 (Table 1) gave a K_m value for angiotensin II which was similar to that reported for pp60^{v-src} (Wong & Goldberg, 1983). The K_m for ATP was 30 μ M. This is similar to the value reported for purified pp60^{v-src} with casein as substrate (Richert *et al.*, 1982), but 10-fold higher than that reported for pp60^{c-src} with enolase as substrate (Feder & Bishop, 1990). The V_{max} for pp60^{c-src}, when assayed in the presence of angiotensin II and Mg^{2+} , was 620 nmol/min per mg. This corresponds to a turnover number of 39 mol/min per mol. To our knowledge this represents the

highest turnover number reported for a tyrosine protein kinase. This compares with a turnover number for the intracellular domain of the epidermal-growth-factor receptor which was 10 mol/min per mol when assayed in the presence of 1 M-(NH₄)₂SO₄ (McGlynn *et al.*, 1992). This high activity probably reflects the lack of tyrosine phosphorylation on the pp60^{c-src} C-terminal regulatory site (Tyr-527) when expressed in Sf9 insect cells. It has previously been shown that only approx. 5% of baculovirus-expressed pp60^{c-src} from Sf9 cells was phosphorylated on Tyr-527, and that this phosphorylation was carried out by pp60^{c-src} *in vivo* (Piwnica-Worms *et al.*, 1990).

pp60^{c-src} has also been purified to near-homogeneity from human platelets (Feder & Bishop, 1990) by using immunoaffinity chromatography. The specific activity of this enzyme was 28 nmol/min per mg when assayed with enolase as substrate and Mn^{2+} as the bivalent cation. Since this enzyme was purified from human platelets, the lower specific activity reported may reflect phosphorylation of Tyr-527 before purification. This modification would inhibit the activity of pp60^{c-src}. However, the lack of characterization of the platelet enzyme for tyrosine phosphorylation, and the use of different substrates and bivalent cations, make direct comparison of specific-activity differences difficult.

In the absence of clear physiological target substrates, a number of exogenous substrates have been used for tyrosine protein kinase assays *in vitro*. These substrates include proteins such as casein (Richert *et al.*, 1982) and enolase (Cooper *et al.*, 1984) and synthetic peptides (Geahlen & Harrison, 1990). Most of these substrates are phosphorylated only at very high substrate concentrations. In an attempt to define the primary sequence requirements for tyrosine phosphorylation, much effort has been devoted to finding peptide substrates with low K_m values. However, in contrast with the situation with the cAMP-dependent protein kinase (Kemp *et al.*, 1977), no clear phosphorylation consensus sequences for tyrosine protein kinases have been determined, suggesting that such sites represent three-dimensional conformations. Synthetic random polymers containing tyrosine and some other amino acids have been found to be good substrates for a number of tyrosine protein kinases (Braun *et al.*, 1984). We have compared a number of substrates for phosphorylation by pp60^{c-src}. The polymers poly(GT) and poly(GAT) were found to have low K_m values of 15 μ g/ml and 10 μ g/ml respectively. The V_{max} values were similar to those found with angiotensin II or casein. Both polymers were phosphorylated in the presence of either Mg^{2+} or Mn^{2+} . In contrast, poly(GLAT), which is a good substrate for the v-abl kinase (N. B. Lydon & J. Geissler, unpublished work) was not phosphorylated by pp60^{c-src}. Additionally, clear differences were seen between pp60^{c-src} and the epidermal-growth-factor-receptor kinase for phosphorylation of random polymers (McGlynn *et al.*, 1992). Thus tyrosine protein kinases differ in their preferences for polymeric substrates and bivalent cations.

The role of pp60^{c-src} autophosphorylation is not yet clear, but for several protein tyrosine kinases there is evidence that phosphorylation of Tyr-416 results in increased catalytic activity, possibly resulting from a conformational change that allows better access of substrate to the active site (Piwnica-Worms *et al.*, 1987). Poly(GLAT), which is not phosphorylated by the pp60^{c-src} kinase, dramatically activated autophosphorylation of pp60^{c-src}. This phosphorylation was mapped to proteinase V8 peptide V2, implying modification of Tyr-416. Activation of autophosphorylation may be due to the basic nature of poly(GLAT). Polylysine and other basic proteins have previously been found to activate the insulin receptor kinase (Morrison *et al.*, 1989). Hyperphosphorylation induced by polylysine also occurred with pp60^{c-src}. These findings suggest that basic charged polymers can

interact with pp60^{c-src} and induce a conformation which results in high enzyme activity. This finding would suggest that in addition to dephosphorylation of Tyr-527, additional interactions with charged molecules are required for the full activation of pp60^{c-src} *in vitro*. Thus the specific activity of the maximally activated form of pp60^{c-src} may be significantly higher than previously thought and may require interaction with additional cellular modulators. Recent findings with the related lyn kinase have shown that polylysine dramatically alters the selectivity of the lyn for peptide substrates (Donella-Deana *et al.*, 1992).

Ser-17, which is the major site of phosphorylation of pp60^{v-src} *in vivo* (Hunter & Cooper, 1985; Patschinsky *et al.*, 1986), is the target for the cAMP-dependent protein kinase. As yet, no role has been found for phosphorylation of Ser-17. Additionally, sites for other protein kinases are also located in the *N*-terminus of pp60^{c-src}, suggesting a regulatory role for the *N*-terminus. There is some evidence that phosphorylation of pp60^{v-src} by the cAMP-dependent protein kinase *in vitro* increases its activity (Hunter & Cooper, 1985). However, no determination of the effect of phosphorylation of Ser-17 has been carried out with purified pp60^{c-src}. We have investigated the effect of *N*-terminal phosphorylation of pp60^{c-src} by the cAMP-dependent protein kinase on the kinetics of substrate phosphorylation. Serine/threonine phosphorylation, which mapped to proteinase V8-generated peptides V3 and V4, was without apparent effect on the V_{max} or K_m of pp60^{c-src} for poly(GT) phosphorylation. This suggests that Ser-17 phosphorylation does not regulate enzyme activity directly, but may be involved in the interaction of pp60^{c-src} with other cellular elements.

Purified pp60^{c-src} from Sf9 cells showed isoelectric heterogeneity, which is at least partially due to tyrosine phosphorylation. Analysis of pp60^{c-src} revealed that peak 1 (from the Mono Q column) was a mixed population, consisting of two species of pp60^{c-src} which differed in their tyrosine-phosphorylation state. A non-phosphorylated and phosphorylated species of pp60^{c-src} were detected by isoelectric focusing. The tyrosine phosphate has been mapped to proteinase V8 peptide V2, which contains the Tyr-416 autophosphorylation site. Addition of ATP to peak 1 pp60^{c-src} resulted in additional tyrosine phosphorylation in V2. Peak 2 pp60^{c-src} was also found to contain phosphotyrosine which mapped to proteinase V8 peptide V2. However, addition of ATP to this fraction did not affect the isoelectric-focusing profile, implying that additional autophosphorylation did not occur. The specific activity given for pp60^{c-src} from peak 2 may therefore represent the activity of a mixed population of molecules with different kinetic properties. An exact analysis of the activity of such species will require their isolation and characterization. The heterogeneity found in peak 2 and the additional peaks found in replicated purifications could be due to a number of additional modifications, including serine/threonine phosphorylation by Sf9 cell kinases *in vivo*. A number of serine/threonine protein kinases have been reported to phosphorylate in the *N*-terminal region of pp60^{c-src} (Cross & Hanafusa, 1983; Bollen *et al.*, 1985; Gould *et al.*, 1985; Gould & Hunter, 1988; Morgan *et al.*, 1989; Ralston & Bishop, 1985). Alternatively, such heterogeneity could be due to other post-translational modifications, or to chemical modifications (such as deamidation) which occurred during purification.

In summary, we have described the purification and characterization of recombinant non-myristoylated pp60^{c-src} kinase. Conditions have been established for the large-scale growth of Sf9 cells using a laboratory-scale bioreactor which has enabled the purification of soluble pp60^{c-src} kinase by classic purification methods. The yield, purity and turnover number of the purified enzyme were significantly higher than previously reported for pp60^{c-src}. The purification of soluble non-myristoylated pp60^{c-src}

has enabled us to carry out a detailed biochemical study on the purified enzyme.

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