Partial purification and characterization of the *lck* protein-tyrosine kinase from bovine thymus

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Soluble extracts prepared from bovine thymus contain an angiotensin-I-phosphorylating activity that is activated several-fold by high concentrations of NaCl. Fractionation of this protein-tyrosine kinase activity by chromatography on DEAE-cellulose yields a major diffuse peak of activity. The enzymes responsible for this activity are found at much higher levels in extracts from bovine thymus than from bovine spleen. The peak of activity from the DEAE-cellulose column can be further separated into two major peaks by chromatography on heparin-agarose. The second peak to elute from the heparin-agarose column was previously purified through several chromatographic steps to yield a 40 kDa protein-tyrosine kinase (p40). We have now partially purified the early-eluting peak of kinase activity by chromatography on columns of butyl-agarose, protamine-agarose and Sephacryl S200. The enzyme was identified following covalent modification with 5'-fluorosulphonylbenzoyl-adenosine (FSBA) by reactivity with anti-FSBA antibodies. This procedure labelled a series of 52–56 kDa proteins. These proteins were also recognized by polyclonal anti-peptide antibodies raised against the C-terminal 33 amino acids of p56^{lck}, a major T lymphocyte protein-tyrosine kinase. Peptide maps of the partially purified enzyme were identical to maps generated from p56^{lck} obtained from LSTRA cells. These data suggest that bovine thymus p56^{lck} is responsible for the activity found in the early-eluting peak from heparin-agarose. Antibodies raised against a peptide corresponding to amino acids 39–64 of p56^{lck}, a sequence found near the N-terminus, recognized the slower-migrating, but not the faster-migrating, form of the enzyme, indicating that a fraction of the protein had been proteolysed near the N-terminus during purification. The partially purified bovine enzyme exhibited a restricted substrate specificity *in vitro* and did not readily phosphorylate human erythrocyte band 3, casein or histone, but was able to phosphorylate acid-treated enolase. The dilute enzyme present in fractions eluting from chromatography columns was unable to catalyse an autophosphorylation reaction. Autophosphorylation could be detected in more concentrated enzyme samples and was readily observed in immune-complex assays. The phosphorylation of angiotensin I by bovine thymus p56^{lck} was weakly activated by polyionic compounds such as heparin and polylysine, and was strongly activated by high concentrations of NaCl.

INTRODUCTION

The proliferation and differentiation of T lymphocytes is under the regulation of numerous extracellular factors including antigens, growth factors and cell surface molecules. The biochemical mechanisms that govern the response of T cells to these signals are now being elucidated, and are likely to include the activation of specific protein-tyrosine kinases. Increases in protein-tyrosine phosphorylation have been found to be early events in the activation of resting T cells by antigens interacting with the T cell receptor [1–3] and in the proliferation of activated T cells in response to interleukin 2 [4–6]. Each of these pathways appears to function independently and leads to the activation of distinct protein-tyrosine kinases [7].

Such observations have generated considerable interest in the nature of the protein-tyrosine kinases that are found in lymphocytes. T cells have been reported to express several distinct protein-tyrosine kinases, including p56^{lck}, p59^{c-lyn} and p62^{c-yes} [8–10]. In addition, protein-tyrosine kinases of 40 and 72 kDa have been reported from thymus [11,12], and several kinases ranging from 40 to 64 kDa have been described in spleen [13–16]. The isolation and characterization of lymphocyte protein-tyrosine kinases are important steps toward understanding how they are regulated in response to extracellular signals. Our laboratory has been interested in the identification and subsequent isolation of T cell protein-tyrosine kinases based on their ability to

phosphorylate synthetic tyrosine-containing peptide substrates. We previously demonstrated that soluble extracts prepared from bovine thymus contained an angiotensin-I-phosphorylating activity that was activated several-fold by high concentrations of NaCl [11,17]. In the present study we have partially purified a NaCl-activated kinase activity and have identified the enzyme as p56^{lck}, a major T lymphocyte protein-tyrosine kinase, on the basis of covalent modification with 5'-fluorosulphonylbenzoyl-adenosine (FSBA) and reactivity with anti-FSBA antibodies, reactivity with polyclonal anti-peptide antibodies and peptide mapping.

MATERIALS AND METHODS

Protein kinase assays

Routine assays for the measurement of angiotensin-I-phosphorylating activity were carried out for 3 min at 30 °C in 25 µl reactions containing 1.2 mM-angiotensin I (Purdue University Peptide Synthesis Facility), 50 µM-[γ-³²P]ATP (1500–3000 c.p.m./pmol) (DuPont–New England Nuclear), 50 mM-MgCl₂, 5 mM-*p*-nitrophenyl phosphate, 25 mM-Hepes, pH 7.4, 2.0 M-NaCl and an aliquot of enzyme. The [³²P]phosphopeptide products were recovered on Whatman P81 phosphocellulose paper as described previously [18]. Protein substrates were phosphorylated for 3 min at 30 °C in a total volume of 50 µl that contained 10 mM-MnCl₂, 10 mM-*p*-nitrophenyl phosphate,

Abbreviations used: FSBA, 5'-fluorosulphonylbenzoyl-adenosine; PMSF, phenylmethanesulphonyl fluoride.

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40 mM-Hepes, pH 7.4, 30 nM-[γ - 32 P]ATP and one of the following protein substrates: casein (50 μ g), acid-treated enolase [19] (40 μ g), histone types IIS, IIIS or VIIS (Sigma) (50 μ g), or the cytoplasmic domain of erythrocyte band 3 (kindly provided by Dr. P. S. Low, Purdue University) (20 μ g). Reactions were terminated by the addition of SDS sample buffer. Phosphoproteins were separated on SDS/10%-polyacrylamide gels and detected by autoradiography.

Protein purification

Soluble extracts of bovine thymus (200 g) were prepared and batch-adsorbed on to DEAE-cellulose (150 ml) as described previously for the purification of p40 [11]. Kinase activity was eluted with a 800 ml linear gradient of 50–500 mM-NaCl in 5 mM-Hepes (pH 7.4)/5% sucrose. Protein concentrations were determined by the method of Bradford [20] using BSA as a standard. Protein-tyrosine kinase activity was detected by the phosphorylation of angiotensin I. The major peak of kinase activity was pooled and precipitated with 25% poly(ethylene glycol) (molecular mass = 3350 Da; Sigma). The precipitate was collected by centrifugation at 16000 g for 25 min. The resulting pellets were resuspended in three-quarters of the initial volume of 10 mM-Hepes (pH 7.4)/10% sucrose (buffer A) containing 0.1 mM-phenylmethanesulphonyl fluoride (PMSF) and applied to a 50 ml column of heparin-agarose (Sigma) pre-equilibrated with buffer A. The proteins were eluted with a 400 ml linear gradient of 0–400 mM-NaCl in buffer A. Fractions that contained protein-tyrosine kinase activity were pooled and again precipitated in 25% poly(ethylene glycol). The collected protein pellets were resuspended in one-half of the initial volume of buffer A and transferred to a 25 ml column of butyl-agarose (Sigma), which was pre-equilibrated with buffer A. The column was then developed with a 120 ml linear gradient of 0–500 mM-NaCl in buffer A. The fractions containing kinase activity were then pooled and directly applied to an 8 ml column of protamine-agarose (Sigma). The kinase activity was then eluted with a 40 ml gradient of 0–1 M-NaCl in buffer A. The fractions that contained kinase activity were pooled, concentrated to 1 ml with a Centri-prep 10 (Amicon) and chromatographed on a 50 ml column of Sephacryl S200 pre-equilibrated with buffer A containing 100 mM-NaCl. The fractions containing kinase activity were pooled, dialysed against 10 mM-Hepes (pH 7.4)/100 mM-NaCl/50% glycerol and stored at -20°C .

Detection of proteins labelled with FSBA

Aliquots (30 μ l) of fractions eluted from the Sephacryl S200 column were incubated at 30°C for 20 min with 0.1 mM-FSBA (Sigma). FSBA-labelled proteins were separated by SDS/PAGE, transferred to nitrocellulose and detected by affinity-purified anti-FSBA antibodies as described [21].

Anti-peptide antibodies

Peptides having the following sequences: CKERPEDRP-TFDYLRVLEDDFTATEGQYQPQP (peptide A) (residues 478–509 of p56^{lck} plus an N-terminal cysteine) or CRNGSEVRDPLVTYEGSNPPASPLQDN (peptide B) (residues 39–64 of p56^{lck} plus an N-terminal cysteine) were prepared by the Purdue University Peptide Synthesis Facility. The peptides were coupled to BSA using 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester as a cross-linker as described previously [22,23]. New Zealand White rabbits were immunized by multiple subcutaneous injections of the BSA-coupled peptides. For the immunoblotting experiments, the proteins were separated on SDS/polyacrylamide gels and transferred to either Immobilon-P or nitrocellulose membranes as described by Towbin *et al.* [24], except that 5% powdered non-fat milk was used in place of BSA.

The rabbit antisera were used at either a dilution of 1:2000 for antibodies raised against peptide A or a dilution of 1:500 for antibodies raised against peptide B. The immunoreactive proteins were detected by a second incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:1000 dilution). Anti-p56^{lck} monoclonal antibodies were obtained from Oncogene Sciences, and anti-phosphotyrosine antibodies were from Calbiochem.

Immunoprecipitations and immune-complex tyrosine kinase assays

Aliquots of partially purified enzyme were incubated with rabbit anti-(peptide A) antiserum or with rabbit preimmune serum at 4°C for 3 h. The immune complexes were collected on Protein A-Sepharose (Sigma) and then washed 3 times with buffer containing 40 mM-Hepes, pH 7.4, 10 mM-MnCl₂, 100 mM-NaCl, 10 μ g of aprotinin/ml, 10 μ g of leupeptin/ml, 0.1 mM-PMSF and 0.1 mM-sodium orthovanadate. For protein phosphorylation, the immune complexes were incubated with 10 μ M-[γ - 32 P]ATP in the above buffer for 3 min at 30°C . The separation and detection of phosphoproteins were as described above. For peptide phosphorylation, the immune complexes were incubated with 1.2 mM-angiotensin I at 30°C for 3 min as described above.

LSTRA cells

The LSTRA cell line was cultured as described previously [25]. Particulate fractions containing p56^{lck} were prepared from LSTRA cells as described [12,25]. For the partial purification of LSTRA p56^{lck}, 2% Triton X-100 extracts of particulate fractions from 1×10^8 cells were chromatographed on a 10 ml column of DEAE-cellulose pre-equilibrated in buffer A. The column was then developed with a 75 ml linear gradient of 0–500 mM-NaCl in buffer A containing 2% Triton X-100. The protein-tyrosine kinase activity was detected by the angiotensin I phosphorylation assay as described above. The peak of kinase activity was pooled and stored at -20°C in 50% glycerol/10 mM-Hepes (pH 7.4)/100 mM-NaCl.

Peptide mapping experiments

Peptide mapping experiments were carried out essentially as described by Cleveland *et al.* [26] using partial proteolysis with *Staphylococcus aureus* V8 protease (17 ng). The resulting peptides were detected following electrophoretic transfer to Immobilon-P membranes by immunoblotting with anti-(peptide A) antibodies.

RESULTS

Partial purification of NaCl-activated protein-tyrosine kinase

Soluble extracts prepared from bovine thymus contain angiotensin-I-phosphorylating activity that is activated several-fold by high concentrations of NaCl [11,17]. Fractionation of this protein-tyrosine kinase activity by chromatography on DEAE-cellulose yielded a major diffuse peak of activity ([11] and Fig. 1). A comparison of soluble extracts from bovine thymus and spleen indicated that this activity was present to a much greater extent in thymus (Fig. 1). We have demonstrated previously that the major peak of activity from the DEAE-cellulose column chromatography of bovine thymus extracts could be further separated into two major peaks by chromatography on heparin-agarose [11]. Initial characterization of these activities indicated that they represented at least two distinct enzymes [11]. The second peak to elute from the heparin-agarose column was previously purified by several chromatographic steps to yield a 40 kDa protein-tyrosine kinase (p40) [11].

Fig. 1. Chromatography of bovine thymus or spleen extracts on DEAE-cellulose

Soluble extracts were prepared from bovine thymus (a) or bovine spleen (b) as described in the Materials and methods section. Extracts were applied to columns of DEAE-cellulose and proteins were eluted with a linear gradient of increasing NaCl concentration (□). Kinase activity was detected by the phosphorylation of angiotensin I (●).

To identify the enzyme responsible for the early-eluting peak of NaCl-activated kinase activity, the peak fractions of kinase activity were pooled and further fractionated by chromatography on columns of butyl-agarose (Fig. 2b), protamine-agarose (Fig. 2c) and Sephacryl S200 (Fig. 2d) as described in the Materials and methods section. A single major peak of kinase activity was observed at each stage of purification, indicating that the activity was most likely due to a single enzyme. The enzyme eluted from the final Sephacryl S200 column at a position intermediate between the elution positions of BSA (66 kDa) and ovalbumin (45 kDa). A summary of the partial purification of the enzyme is shown in Table 1. At this stage of purification the enzyme could be stored in 50% glycerol/10 mM-Hepes (pH 7.4)/100 mM-NaCl at -20 °C for several months without substantial loss of activity.

Identification of p56^{lck}

Many protein-tyrosine kinases are capable of catalysing intramolecular autophosphorylation reactions. These reactions are often useful for the identification of the partially purified enzyme. However, when aliquots of fractions eluting from the final Sephacryl S200 column were incubated with MnCl₂ and [γ -³²P]ATP and were analysed by SDS/PAGE, no substantial phosphorylation of any endogenous proteins on tyrosyl residues was observed.

As a second approach to identifying the NaCl-activated kinase, we sought to identify an ATP-binding protein that co-eluted from the column with the protein-tyrosine kinase activity.

Proteins present in aliquots of fractions from the S200 column were incubated with 0.1 mM-FSBA, separated by SDS/PAGE and transferred to nitrocellulose. The FSBA-modified proteins were then detected by immunoblotting using polyclonal, affinity-purified, anti-FSBA antibodies. These antibodies were raised in rabbits to FSBA-modified glutamate dehydrogenase and were affinity-purified on ATP-agarose. Their production and characterization has been described in detail [21]. The anti-FSBA antibodies detected a closely spaced series of 52–56 kDa proteins that co-eluted from the sizing column with the protein-tyrosine kinase activity (Fig. 3). This apparent size was consistent with that observed by gel filtration (Fig. 2d). Coomassie-Blue-stained protein bands on an SDS/polyacrylamide gel that corresponded to the FSBA-labelled proteins represented 7% of the total protein in the final preparation.

The apparent size of the NaCl-activated enzyme and its tissue distribution suggested the possibility that it was bovine p56^{lck}. This kinase is expressed predominantly in cells of the T lineage and is found at much higher levels in thymus than in spleen [8,9]. To verify the identity of the kinase, antibodies were raised against a peptide corresponding to residues 478–509 of p56^{lck} (peptide A). Antibodies prepared against this sequence had previously been shown by others to be reactive against p56^{lck} on Western blots [23]. To verify the specificity of the antiserum, particulate fractions prepared from the cell line LSTRA, a T cell lymphoma that overexpresses p56^{lck}, were analysed by immunoblotting. Antibodies reacted selectively with p56^{lck} (Fig. 4a). Immunoreactivity against p56^{lck} could be completely blocked by preincubation with peptide A (results not shown). These antibodies also reacted on Western blots with the partially purified thymus enzyme (Fig. 4a), and could immunoprecipitate angiotensin-I-phosphorylating activity (results not shown), suggesting that the NaCl-activated enzyme was, in fact, bovine p56^{lck}. These immunoreactive proteins co-eluted with protein-tyrosine kinase activity at each stage of purification (results not shown). The enzyme did not react on Western blots with a monoclonal anti-pp60^{src} antibody or anti-p40 antisera.

To further establish the identity of the partially purified enzyme, the kinase was compared with p56^{lck} from LSTRA cells by one-dimensional peptide mapping. Protein bands corresponding to the partially purified kinase and to LSTRA p56^{lck} were excised from SDS/polyacrylamide gels and re-electrophoresed on a second gel in the presence of 17 ng of *S. aureus* V8 protease. The resulting peptides were transferred to Immobilon membranes and detected by immunoblotting with the anti-(peptide A) antibodies described above. As shown in Fig. 4(b), the peptide maps obtained from the two proteins were nearly identical, confirming that the two proteins were highly related and probably represented the same enzyme.

Partial proteolysis of p56^{lck} during purification

Much of the partially purified bovine p56^{lck} migrated on SDS/polyacrylamide gels with a molecular mass of 52 kDa, which is lower than that of the native enzyme. Incubation of the partially purified enzyme with an excess of alkaline phosphatase or acid phosphatase failed to alter its migration, indicating that the altered mobility of the enzyme was not a result of its phosphorylation state. This suggested that the enzyme was most likely subject to partial proteolysis during purification. A similarly sized enzyme was also rapidly formed when detergent extracts of LSTRA cells were chromatographed on DEAE-cellulose (Fig. 4a). The retention of immunoreactivity of the partially purified bovine kinase with the anti-(peptide A) antibody, coupled with the one-dimensional peptide mapping data, indicated that the C-terminus of the protein was intact. The

Fig. 2. Partial purification of p56^{lck}

Details of the purification are given in the Materials and methods section. (a) Chromatography on heparin-agarose; (b) chromatography on butyl-agarose; (c) chromatography on protamine-agarose; (d) chromatography on Sephacryl S200. ●, Angiotensin I kinase activity; △, protein concentration; □, NaCl concentration determined by conductivity. The elution positions of BSA and ovalbumin (OV) are indicated in (d).

Table 1. Summary of partial purification of p56^{lck}

Fraction	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (-fold)
Extract	6441	1265	0.20	100	1
DEAE-cellulose	180	269	1.58	21	7.5
Heparin-agarose	40	195	4.9	15.4	24.5
Butyl-agarose	15.6	160	10.3	12.7	52
Protamine-agarose	2.6	104	40	8.2	200
Sephacryl S200	0.6	74	122	5.8	610

FSBA-labelling experiments further indicated that the ATP-binding domain remained intact in the proteolysed enzyme.

To determine which end of the bovine p56^{lck} protein had been affected by proteolysis, a second antibody was prepared against a synthetic peptide (peptide B) corresponding to residues 39–64 in p56^{lck}, a site that is located near the N-terminus of the kinase. To facilitate coupling of the peptide to the carrier, an additional cysteine residue was added to the N-terminus. Antibodies to a similar peptide from this region had been prepared previously by

Fig. 3. Identification of the FSBA-modified kinase with anti-FSBA antibodies

Aliquots of fractions eluted from the Sephacryl S200 column were reacted with 0.125 mM-FSBA, separated by SDS/PAGE and transferred to nitrocellulose. FSBA-modified proteins were detected using affinity-purified anti-FSBA antibodies. The FSBA-labelled protein bands at 52–56 kDa (arrow, lanes 4 and 5) co-eluted with the peak of angiotensin-I-phosphorylating activity from the sizing column. Lanes 1–8 correspond to fractions 34, 36, 38, 40, 42, 44, 46 and 48 respectively from Fig. 2(d).

Fig. 4. Detection and mapping of p56^{lck} with anti-(peptide A) antibodies

(a) Western blot analysis of p56^{lck} partially purified from detergent extracts of LSTRA cells by chromatography on DEAE-cellulose (lane 1), p56^{lck} partially purified from bovine thymus (lane 2) or particulate fraction proteins prepared from LSTRA cells (lane 3). Proteins were separated by SDS/PAGE, transferred to nitrocellulose and detected by reactivity with anti-(peptide A) antibodies. (b) One-dimensional peptide map of p56^{lck} partially purified from bovine thymus (lane 1) or p56^{lck} from a LSTRA cell particulate fraction (lane 2). Peptides were generated by partial digestion with *S. aureus* V8 protease, transferred to Immobilon and detected by immunoblotting with anti-(peptide A) antibodies.

Fig. 5. Detection of p56^{lck} with anti-(peptide B) antibodies

Western blot analysis of p56^{lck} partially purified from bovine thymus (lanes 1 and 3) and particulate fraction proteins from LSTRA cells (lane 2). Proteins were separated by SDS/PAGE, transferred to nitrocellulose and detected by reactivity with either anti-(peptide A) antiserum (lanes 1 and 2) or anti-(peptide B) antiserum (lane 3). The migration position of p56^{lck} is indicated by the arrow.

others, and these had been shown to react specifically with p56^{lck} [10,27]. The polyclonal rabbit anti-(peptide B) antibodies specifically recognized p56^{lck} in particulate fractions from LSTRA cells, as determined by Western blotting analyses, and preincubation of the antiserum with peptide B completely inhibited immunoreactivity (results not shown). These antibodies also reacted with that portion of the purified bovine enzyme that migrated at 56 kDa (Fig. 5). However, the antibodies did not react with the portion of the enzyme that migrated at 52 kDa, indicating that this epitope was missing or interrupted in the proteolysed form of the kinase. This suggested that the faster-migrating form of the kinase was missing a portion of the *N*-terminus.

Characterization of the enzyme

During isolation, the dilute enzyme present in fractions eluting from various chromatography columns did not appear to catalyse

Fig. 6. Autophosphorylation of p56^{lck}

A partially purified preparation of p56^{lck} was incubated with preimmune serum (lanes 1 and 3) or anti-(peptide A) immune serum (lanes 2 and 4) as indicated in the Materials and methods section. Antibody-antigen complexes were recovered on beads of Protein A-Sepharose. The supernatants (lanes 1 and 2) or the resulting immune complexes resuspended in the original volume (lanes 3 and 4) were phosphorylated with [γ -³²P]ATP as described. Phosphoproteins were separated by SDS/PAGE and detected by autoradiography.

an autophosphorylation reaction. Incubation of aliquots of the enzyme with [γ -³²P]ATP in the presence of either Mn²⁺ or Mg²⁺ as the bivalent cation did not lead to the extensive phosphorylation of any endogenous proteins on tyrosyl residues. This was surprising, since the enzyme readily catalyses an autophosphorylation reaction when present in crude membrane fractions [18,25]. The inclusion of the phosphatase inhibitors *p*-nitrophenyl phosphate and sodium orthovanadate in the reaction mixture did not increase phosphorylation. Since the autophosphorylation of p56^{lck} is often observed in immune-complex assays [28,29], we repeated the autophosphorylation experiment using enzyme that had been reacted with anti-(peptide A) antibodies and collected on beads of protein A-Sepharose. Under these conditions, autophosphorylation of p56^{lck} was readily observed (Fig. 6). Phosphoamino acid analysis indicated that this phosphorylation occurred selectively on tyrosine (results not shown). Autophosphorylation was also observed when peak fractions containing the highest activity from the protamine-agarose column were pooled selectively and concentrated by dialysis against 50% glycerol prior to addition of [γ -³²P]ATP. One-dimensional phosphopeptide maps of the autophosphorylated enzyme were essentially identical to those generated from autophosphorylated LSTRA-cell p56^{lck} (results not shown).

The partially purified kinase showed a limited ability to phosphorylate exogenous substrates *in vitro*. Several proteins that have previously been shown to serve as substrates for other protein-tyrosine kinases were not phosphorylated efficiently by this enzyme under the reaction conditions employed. These included histone, casein and the cytoplasmic domain of erythrocyte band 3. This latter protein was previously shown to be an excellent substrate for the p40 protein-tyrosine kinase from bovine thymus [11]. The enzyme was, however, able to catalyse the phosphorylation of enolase on tyrosine, providing that the substrate was first acid-treated (Fig. 7). Prior autophosphorylation of the kinase was not required for substrate phosphorylation. As shown in Fig. 7, the phosphorylation of enolase was linear with respect to time with no discernible lag as would be expected for a kinase activated by autophosphorylation (e.g. the insulin receptor [30]). In addition, prephosphorylation of p56^{lck} with ATP before addition of the substrate did not significantly alter the rate of enolase phosphorylation (results not shown).

Fig. 7. Time course of enolase phosphorylation by $p56^{lck}$

Acid-treated enolase was phosphorylated by partially purified $p56^{lck}$ as described, except that reactions contained $100 \mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Aliquots were removed at the following timed intervals (min): 0.5 (lane 1), 1 (lane 2), 2 (lane 3), 4 (lane 4), 6 (lane 5), 10 (lane 6), 15 (lane 7) and 20 (lane 8). Phosphoproteins were separated by SDS/PAGE and detected by autoradiography (a), or ^{32}P enolase was excised from the gel and quantified by liquid scintillation spectrometry (b). The migration positions of enolase (arrow) and $p56^{lck}$ (p56) are indicated.

Fig. 8. Effect of monovalent and bivalent cations on the activity of $p56^{lck}$

(a) Effect of increasing concentrations of MgCl_2 on the phosphorylation of angiotensin I by partially purified $p56^{lck}$. (b) Effect of increasing concentrations of MnCl_2 on the phosphorylation of angiotensin I. Assays for (a) and (b) were carried out in the presence of 2 M-NaCl. (c) Effect of increasing concentrations of NaCl on the phosphorylation of angiotensin I in the presence of 50 mM- MgCl_2 (■) or 2.5 mM- MnCl_2 (□).

The phosphorylation of the peptide angiotensin I *in vitro* was strongly affected by changes in the ionic strength of the reaction medium. When Mg^{2+} was used as the bivalent cation, the increase in angiotensin-I-phosphorylating activity as a function of salt concentration was nearly linear up to 1.5 M-NaCl (Fig. 8c). The phosphorylation of angiotensin I was weakly stimulated by high

Table 2. Effects of various agents on the angiotensin-I-phosphorylating activity of $p56^{lck}$

The angiotensin-I-phosphorylating activity of partially purified $p56^{lck}$ was determined in assays containing 1.2 mM-angiotensin I and 50 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the Materials and methods section. Reactions containing heparin or polylysine were carried out using 50 mM- MgCl_2 in the absence of added NaCl. Activity is expressed as % of control activity observed in reactions carried out in the absence of added heparin or polylysine. Reactions containing CaCl_2 or CoCl_2 were carried out using 2 M-NaCl and no additional bivalent cation. Activity is expressed as % of control activity measured in reactions containing 50 mM- MgCl_2 and 2 M-NaCl.

Agent	Concentration	Activity (% of control)
Heparin	1 $\mu\text{g}/\text{ml}$	102
Heparin	1000 $\mu\text{g}/\text{ml}$	165
Polylysine	1 $\mu\text{g}/\text{ml}$	105
Polylysine	300 $\mu\text{g}/\text{ml}$	195
Ca^{2+}	5 mM	1.2
Ca^{2+}	50 mM	1.7
Co^{2+}	5 mM	4.4
Co^{2+}	50 mM	6.7

ionic strength with Mn^{2+} as the bivalent cation (Fig. 8c). These effects are similar to those reported previously for the p40 enzyme from thymus [11] and the p75 enzyme from liver [31]. Unlike the p40 enzyme, however, $p56^{lck}$ exhibited little angiotensin-I-phosphorylating activity in the absence of NaCl, regardless of the bivalent cation employed. The enzyme exhibited a preference for Mg^{2+} as the bivalent cation under conditions of high NaCl concentrations (Figs. 8a and 8b). Little activity was seen in assays using Co^{2+} or Ca^{2+} as the sole bivalent cation (Table 2).

Polyionic compounds such as heparin and polylysine were found to have modest stimulatory effects (approx. 2-fold) on the phosphorylation of angiotensin I (Table 2). Other effectors of kinase activity such as cyclic AMP, cyclic GMP or Ca^{2+} /calmodulin had no observable effects on activity (results not shown).

DISCUSSION

The protein-tyrosine kinase activity of bovine thymic extracts, when measured by the phosphorylation of angiotensin I, is strongly activated by high concentrations of NaCl [11,17]. We speculated previously that this NaCl-activated kinase might be $p56^{lck}$, since the angiotensin-I-phosphorylating activity of particulate fractions prepared from LSTRA cells, which overexpress this kinase, is also strongly activated by NaCl [17]. Attempts to isolate this activity from thymus, however, indicated that at least two distinct enzymes were responsible for the NaCl-activated kinase activity. One of these enzymes was previously isolated as a 40 kDa protein [11], which appears to be generated from a larger-molecular-mass precursor by proteolysis [12]. The other kinase, based on the studies reported here, is indeed $p56^{lck}$. This conclusion is based on several lines of evidence: (i) the kinase activity is found at much higher levels in extracts from bovine thymus than from bovine spleen; (ii) the NaCl-activated enzyme co-purifies with a set of ATP-binding proteins, as detected by labelling with FSBA, of 52–56 kDa; (iii) the partially purified protein reacts with polyclonal anti-peptide antibodies that recognize the C-terminus of $p56^{lck}$; (iv) the intact form of the enzyme reacts with polyclonal anti-peptide antibodies that recognize a region of $p56^{lck}$ that is unique to this member of the

src family of tyrosine kinases [10,27]; and (v) the one-dimensional peptide map generated from the partially purified enzyme is essentially identical to that generated from p56^{lck} from LSTRA cells.

The first clue to the identification of the partially purified kinase came from experiments using FSBA to identify ATP-binding proteins present in the enzyme preparation. FSBA is an affinity analogue of ATP that has been shown to bind to numerous adenine-nucleotide-binding sites, where it is capable of alkylating nucleophilic amino acid residues (for a review see [32]). Since many protein-serine/threonine and protein-tyrosine kinases have been shown to be covalently modified by FSBA at a conserved lysine residue present within the ATP-binding site [33,34], it was reasonable to assume that our enzyme would also be subject to modification with FSBA. To identify proteins that had been modified by FSBA, specific antibodies were raised that recognized the covalently attached sulphonylbenzoyladenine moiety [21]. By using this reagent it was possible to determine the relative sizes of the nucleotide-binding proteins that co-eluted from columns with kinase activity. This example illustrates the usefulness of this approach. p56^{lck} present in particulate fractions from LSTRA cells also reacts with FSBA and can be detected using these anti-FSBA antibodies [21]. The antibodies also identified some additional nucleotide-binding proteins contaminating the final preparation of p56^{lck} (Fig. 3). The identity of these proteins is unknown; however, the final preparation does contain protein-serine/threonine kinase activity, suggesting that at least one of these bands corresponds to a contaminating kinase.

The p56^{lck} kinase was highly susceptible to proteolysis during purification, which resulted in the formation of smaller fragments, the most prominent of which was a 52 kDa fragment. Upon storage of the enzyme, we occasionally observed additional proteolysis, which generated even lower-molecular-mass, active, forms of the enzyme (see, for example, Fig. 6). The 52 kDa fragment retained the ATP-binding site, as measured by reactivity with FSBA, and retained immunoreactivity with the antibodies raised against a peptide corresponding to the C-terminal portion of the intact enzyme. Only the portion of the purified kinase that remained intact reacted with an antibody raised against an epitope found near the N-terminus. These results indicated that the proteolytic events that occurred during purification resulted in the removal of a portion of the N-terminus of the kinase. This type of proteolysis is similar to that seen previously with the pp60^{c-src} tyrosine kinase, which is also sensitive to proteolysis and is frequently recovered as a 52 kDa fragment due to the removal of an 8 kDa fragment from the N-terminus [35,36].

It was at first surprising to us that the partially purified enzyme did not catalyse an autophosphorylation reaction *in vitro* when present at dilute concentrations. The primary site of 'autophosphorylation' of p56^{lck} *in vitro* is Tyr-394, which lies in the catalytic domain in a site that is highly similar in amino acid sequence to the autophosphorylation site of pp60^{c-src} (Tyr-416). Studies with mutants of pp60^{c-src} indicate that the predominant mode of autophosphorylation is actually intermolecular [37]. If this is the case with p56^{lck}, then it is possible that the concentration of enzyme in dilute column fractions was too low for efficient catalysis of the autophosphorylation reaction. This lack of proximity is overcome in more concentrated samples or in immune complexes, conditions where autophosphorylation has been observed. This idea is consistent with the observation that Tyr-394 is not a prominent site of phosphorylation on cellular p56^{lck} [38] unless the CD4 or CD8 cell surface molecules with which the kinase is associated are cross-linked [39]. This cross-linking might bring p56^{lck} molecules into close enough proximity to allow them to phosphorylate one another at this site.

Of several proteins examined as potential substrates for p56^{lck}, only acid-treated enolase was efficiently phosphorylated *in vitro*. Although erythrocyte band 3 is an excellent substrate for several protein-tyrosine kinases, including p40 [11], it was not phosphorylated by the partially purified p56^{lck}. This observation, however, is consistent with previous observations indicating that this protein was also very poorly phosphorylated by particulate fractions from LSTRA cells [40]. Band 3 may prove to be a useful substrate for distinguishing between various kinases on the basis of substrate specificity. *In vivo*, the phosphorylation of p56^{lck} occurs primarily on Tyr-505, which is located near the C-terminus. Mutation of this residue to phenylalanine results in an activated form of p56^{lck} that is tumorigenic, suggesting that this site might normally have a negative regulatory effect on p56^{lck} activity [41,42]. The partially purified enzyme, however, did not react on Western blots with anti-phosphotyrosine antibodies, indicating that the activity of the kinase was not repressed due to phosphorylation at this site.

The ability of the isolated kinase to efficiently catalyse the phosphorylation of angiotensin I under conditions of high ionic strength is a property that the enzyme shares with a number of other kinases that have been described, including p40 [11] and p72 [12] from lymphoid cells and p75 from liver [31]. The phosphorylation of angiotensin I by the isolated p56^{lck} is even more dependent on the presence of NaCl for activity than is the activity of p40 [11], and there is little activity in the absence of salt, regardless of the nature of the bivalent cation present. p56^{lck} is further differentiated from p40 by its inability to bind to casein-agarose (results not shown), by its differential binding to heparin-agarose, by its inability to phosphorylate erythrocyte band 3 and by its lack of reactivity with anti-p40 antisera.

The selective expression of p56^{lck} in T lymphocytes [8,9] and its activation in response to the cross-linking of CD4 or CD8 receptors [39,43,44] strongly suggest a critical role for the enzyme in T cell signal transduction. The availability of the isolated enzyme should greatly aid in its characterization with respect to its interactions with various modulators of activity. For example, we have recently used the partially purified enzyme for the screening and evaluation of protein-tyrosine kinase inhibitors [45]. The isolated enzyme should also prove useful for studies on the kinetics of substrate phosphorylation, enzyme regulation and substrate specificity.

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