Purification and Characterization of a Protein-Tyrosine Kinase from Bovine Thymus*

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A protein-tyrosine kinase has been isolated from a soluble extract of bovine thymus based on its ability to phosphorylate the tyrosine-containing peptide angiotensin I. The purification procedure employs sequential chromatography on columns of DEAE-cellulose, heparin-agarose, casein-agarose, butyl-agarose, and Sephadex G-75. The purified enzyme (p40) is a monomer of $M_r = 40,000$. The p40 kinase contains an ATP-binding site as determined by photoaffinity labeling experiments and catalyzes an intramolecular autophosphorylation reaction that leads to its modification on tyrosine. Of several proteins tested only the cytoplasmic domain of the erythrocyte band 3 protein serves as a good substrate for p40 ($K_M = 12 \mu M$). Increasing concentrations of NaCl stimulate the phosphorylation of angiotensin I, inhibit the phosphorylation of band 3, and have no effect on the autophosphorylation of p40. At low concentrations of NaCl, Mn$^{2+}$ is the preferred divalent cation. Peptide mapping experiments indicate that p40 is distinct from pp60$^{c-src}$ and from the major phosphorylating enzymes containing proteins of T and B lymphocyte membranes.

Despite the relatively low levels of phosphorylase found in nontransformed cells, the normal eukaryotic genome encodes multiple enzymes with protein-tyrosine kinase activity. Numbering among these gene products are the cell surface receptors for several mitogenic polypeptides and the cellular homologs of a number of viral oncogene products (for a review, see Ref. 1). The characterization of protein-tyrosine kinases from nontransformed cells has, in general, progressed at a slower rate than that of their viral counterparts in transformed cells. This has been partly due to the low levels at which these enzymes are typically expressed in normal cells.

The use of peptide substrates containing tyrosine residues has provided a convenient method for assaying the relatively low levels of this phosphotransferase activity (2-5). Using this assay, protein-tyrosine kinases have been detected in a number of cells and tissues including liver, brain, thymus, spleen, and platelets (4-10).

Among normal tissues, those populated by lymphoid cells express relatively high levels of protein-tyrosine kinase activity (4). Tyrosine kinase activity has been described in lymphocytes of both the T and B lineages (8, 11). A membrane-associated protein-tyrosine kinase of 56,000 daltons (p56) has been the most thoroughly characterized of the lymphocyte enzymes (2, 10, 12-15). This kinase bears a number of structural similarities to pp60$^{c-src}$ and is found at appreciable levels only in cells derived from T lymphocytes (14-16).

In an effort to further understand the role of tyrosine phosphorylation in lymphocyte function we have directed our research toward isolating and characterizing the major protein-tyrosine kinase activities in T lymphocytes. Our initial subcellular fractionation studies using calf thymus as a tissue source have indicated that much of the endogenous protein-tyrosine kinase activity can be attributed to soluble rather than particulate enzymes. This paper reports the purification and characterization of a novel 40,000-dalton protein-tyrosine kinase (p40) from bovine thymocytes.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Identification of Protein-Tyrosine Kinase Activity in Bovine Thymus—We have shown previously that protein-tyrosine kinase activity can be detected in detergent extracts of bovine thymus using angiotensin I as an exogenous substrate (5). The sensitivity of the assay could be greatly enhanced by the addition of high concentrations of NaCl. NaCl at a concentration of 2 M inhibited the activity of other protein-serine kinases endogenous to calf thymus, yet stimulated the phosphorylation of angiotensin I. Using these assay conditions we examined the distribution of protein-tyrosine kinase activity between the soluble and particulate fractions of bovine thymus. Angiotensin I kinase activity was distributed nearly equally between the soluble and postnuclear particulate fractions. The inclusion of NaCl at a concentration of 2 M in the reaction led to an approximate 12-fold increase in the activity of enzymes present in both fractions (data not shown).

Isolation of a Soluble Protein-Tyrosine Kinase—Soluble proteins extracted from bovine thymus were chromatographed on a column of DEAE-cellulose (Fig. 1A). NaCl-stimulated protein-tyrosine kinase activity eluted as a major, irregular peak along with at least two smaller peaks of activity, indicating that multiple enzymes or multiple forms of an enzyme were present in the soluble fraction. A significant amount of activity, representing nearly 40% of that found in the initial

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* Portions of this paper (including "Experimental Procedures," Figs. 1-3, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-298, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Protein-tyrosine kinase activity was quite labile during this stage of purification. This precluded the use of lengthy dialysis steps needed for reducing the ionic strength of the pooled sample prior to chromatography on subsequent columns. Consequently, precipitation of the enzyme with polyethylene glycol was introduced as a mechanism of stabilizing the enzyme and changing the buffer composition for subsequent chromatographic steps. We could consistently recover greater than 90% of the protein-tyrosine kinase activity present in the sample pooled from the DEAE-cellulose column using this procedure.

Chromatography of the resolubilized enzyme on a column of heparin-agarose yielded three major peaks of activity (Fig. 1B). The angiotensin I phosphorylating activity of each of these enzymes was stimulated by the addition of 2 M NaCl to the reaction mixture. To examine potential relationships between these peaks of activity, each was pooled separately, allowed to incubate overnight at 4 °C, and rechromatographed on a column of heparin-agarose. Rechromatography of peak 3, the enzyme form eluting at the highest ionic strength, consistently yielded a mixture of peaks 2 and 3, indicating that peak 2 was generated from peak 3 during the overnight incubation. There was no apparent precursor-product relationship between the first peak and the second two. Other preliminary studies have indicated that peak 1 exhibits a substrate specificity different from that of peak 3, suggesting that peak 1 represents a distinct enzyme. We have concentrated our efforts on the further purification of the major peak of activity (peak 3) that eluted from the column at the highest ionic strength.

The pooled peak from the heparin-agarose column was further purified by chromatography on columns of casein-agarose (Fig. 1C), butyl-agarose (Fig. 1D), and Sephadex G-75 (Fig. 2). The enzyme eluted from the sizing column as a symmetrical peak, just following the elution position of ovalbumin. A summary of the overall purification is shown in Table I. At this stage of purification the enzyme was very labile during the overnight incubation. The loss of enzyme activity that was membrane-associated and was not solubilized by the extraction procedure.

Identification of p40—Most protein-tyrosine kinases that have been described to date are capable of catalyzing auto-phosphorylation reactions that lead to their covalent modification on a tyrosine residue. Incubation of fractions with kinase activity that eluted from the Sephadex G-75 column with [γ-32P]ATP and Mn2+ resulted only in the phosphorylation of a protein that often appeared as a closely spaced doublet of M, = 40,000 on SDS-polyacrylamide gels (Fig. 2A, inset). The extent of phosphorylation of the 40-kDa proteins that was observed correlated with the amount of angiotensin I phosphorylating activity of each fraction. Analysis of the phosphoamino acid composition of the modified proteins indicated that only tyrosine had been phosphorylated (Fig. 2B). A protein of identical molecular weight constituted the major Coomassie Blue-staining protein in the enzyme preparation (Fig. 3).

To further confirm that the 40-kDa protein (p40) corresponded to the protein-tyrosine kinase, the presence of an ATP-binding site was verified using photoactivatable analogs of ATP. An example of such an experiment is shown in Fig. 4. In the absence of activating light, 8-N3-[γ-32P]ATP was able to serve as a substrate for the phosphorylation of p40, indicating that the photoprobe was capable of interacting with the kinase at the ATP-binding site. Activation of the probe by photolysis led to a substantial increase in the labeling of p40. The addition of unlabeled ATP to the mixture of enzyme and photoprobe prior to photolysis was able to block both the phosphorylation and the photolabeling of p40 by 8-N3-[γ-32P]ATP. When 8-N3-[α-32P]ATP was used as the photoprobe, we observed labeling of p40 only in the presence of activating light. Once again, photoincorporation of the probe could be blocked by the addition of unlabeled ATP (data not shown). These results indicated that p40 contained an ATP-binding site.

The photolabeling experiments suggested that p40 was most likely the enzyme responsible for catalyzing its own phosphorylation. To further examine this question, the effect of dilution on the rate of autophosphorylation of p40 was determined. As shown in Fig. 5, the total amount of phosphate transferred from ATP to p40 in each reaction was directly proportional to the amount of enzyme added to the assay. The rate of phosphorylation of p40 was independent of the enzyme concentration, indicating that the phosphorylation of p40 was proceeding via an intramolecular reaction. These reactions were carried out at 0 °C under conditions where the rate of autophosphorylation was linear with respect to time. Preparations of p40 exhibited no tendency to aggregate under conditions of low or high ionic strength as determined by gel filtration (data not shown), so it was unlikely that the lack of effect of dilution on the rate of p40 autophosphorylation was due to the presence of protein aggregates.

Substrates for p40—A number of proteins that have been reported to be phosphorylated in vitro by other protein-tyrosine kinases were examined as potential substrates for p40. Of the protein substrates examined to date, only the cytoplasmic domain of band 3, which is the anion transport channel in red blood cells, was a good substrate for p40 (Fig. 6). Band 3 was phosphorylated by p40 with an apparent K, of 12 μM (determined at an ATP concentration of 50 μM).

The autoradiogram of p40 photolabeled with 8-N3-[γ32P]ATP as described under “Experimental Procedures.” The autoradiogram is shown in the inset. Lane 1, labeling of p40 with 8-N3-[γ32P]ATP in the presence of activating light; lane 2, labeling of p40 in the absence of activating light; lane 3, labeling of p40 in the presence of activating light and an excess of ATP.
strates for p40 under the reaction conditions employed.

Modifiers of Kinase Activity—Angiotensin I was phosphorylated by p40 with an apparent $K_a$ of 2.5 mM (at an ATP concentration of 50 μM). The apparent $K_a$ for ATP (at 2.5 mM angiotensin I) was 10 μM. The rate of phosphorylation of angiotensin I was enhanced under conditions of high ionic strength. The extent of activation was dependent on the nature of the divalent cation used in the reaction (Table II). At low salt concentrations, optimal activity was obtained using Mn$^{2+}$ in the reaction, however, a greater degree of NaCl dependency was seen with Mg$^{2+}$ as the divalent cation. Little activity was seen in assays using Zn$^{2+}$ and no activity was observed with Ca$^{2+}$ as the sole divalent cation.

In contrast to the results observed with angiotensin I, the rate of phosphorylation of the band 3 protein decreased as NaCl concentrations were increased. Therefore, the effect of NaCl on the activity of p40 was dependent, in part, on the nature of the substrate. The autophosphorylation of p40 was relatively insensitive to the concentration of NaCl present in the reaction. Mn$^{2+}$ was the preferred divalent cation for the phosphorylation of band 3 and for the autophosphorylation of p40 (data not shown).

Comparison of p40 to Other Protein-Tyrosine Kinases—p40 was compared to other protein-tyrosine kinases and phosphoproteins known to be present in lymphocytes by one-dimensional phosphopeptide mapping. The phosphoproteins examined were p56, a T cell-specific protein-tyrosine kinase that is elevated in the lymphoma cell line LSTRA and which catalyzes an autophosphorylation reaction (12, 13); p60, a predominant phosphotyrosine-containing protein formed when membranes from B lymphocytes are phosphorylated in vitro (8); and pp60$^{src}$, which was immunoprecipitated from membranes phosphorylated in vitro prepared from Rous Sarcoma virus-transformed rat-1 fibroblasts by using a commercially available monoclonal antibody. pp60$^{src}$ and pp60$^{src}$ are modified on analogous tyrosine residues when phosphorylated in vitro (22). As shown in Fig. 7, p40 did not appear to be identical to any of the phosphoproteins. We have repeated these experiments many times using a wide range of protease concentrations and have not been able to demonstrate a direct relationship between p40 and these other phosphoproteins. p40 also could not be immunoprecipitated with the monoclonal antibody to pp60$^{src}$ (data not shown).

Detection of p40 in Thymocyte Extracts—We found that the ability of p40, when present in a crude mixture of cytosolic proteins, to catalyze an autophosphorylation reaction was greatly enhanced following chromatography on heparin-agarose due to the removal of materials in the cytosol inhibitory to autophosphorylation. We have used this observation to devise a more rapid method for the detection of p40 in thymocyte cytosols. As shown in Fig. 8, p40 could be readily detected among cytosolic proteins that were batch eluted from heparin-agarose with 650 mM NaCl (lane 8). Peptide mapping experiments indicated that this phosphoprotein was p40. The two predominant but slower migrating phosphoproteins that were present in the fraction eluting at 650 mM NaCl were not related to p40 or p56 as determined by peptide mapping experiments (data not shown). The majority of the protein-tyrosine kinase activity that adhered to the resin also eluted in the high salt fraction. This technique provides a convenient method for the detection of p40 in cellular extracts. These results also indicate that p40 originated from bovine thymocytes and not from red blood cells or connective tissue present in the intact thymus.

We have also used this procedure to investigate the possibility that p40 was generated by proteolysis from a larger
Our earliest attempts to isolate p40 were hampered by the lability of the enzyme. This problem was overcome, in part, by using polyethylene glycol to precipitate the enzyme from solution following chromatography on columns of both DEAE-cellulose and heparin-agarose. The overall yield and fold purification for the chromatographic steps described in Table I are difficult to accurately evaluate due to the presence of multiple activities in the initial extract and in the peak of enzyme activity that was eluted from DEAE-cellulose. The p40 kinase represents the major protein present in the final preparation, but low levels of other contaminating proteins can still be detected on polyacrylamide gels. We feel that the 40-kDa protein represents the protein-tyrosine kinase based on (i) its co-purification with angiotensin I phosphorylating activity on all columns tested, (ii) the presence of an ATP-binding site on the protein, and (iii) its ability to catalyze an intramolecular autophosphorylation reaction on tyrosine residues.

Studies using isolated bovine thymocytes indicate that p40 originates from this cell type. The enzyme does not, however, appear to be equivalent to other protein-tyrosine kinases that have been described in T lymphocytes. We can detect the T-cell-specific, membrane-associated protein-tyrosine kinase, p56, in membrane preparations from bovine thymocytes, but our peptide mapping experiments using papain or Staphylococcus aureus V8 protease do not indicate that p40 is derived from p56. Furthermore, in preliminary experiments, we have been unable to identify appreciable levels of p40 in LSTRA cells, which overexpress p56. Detergent-solubilized LSTRA cell membranes also serve as a relatively poor source of enzyme for the phosphorylation of band 3 (8), which is an excellent substrate for p40. p40 also appears to be distinct from pp60^c^-based on peptide mapping experiments, immunoprecipitation studies, and differences in the substrate specificity of the two activities (23–25).

We cannot completely rule out the possibility that p40 is generated within thymocytes by the proteolytic digestion of a larger protein-tyrosine kinase, but we have not been able to identify a larger molecular weight precursor that appears to be structurally related to p40. We also cannot generate increased amounts of p40 from larger protein precursors by fractionating thymocytes in the absence of protease inhibitors.

Many of the enzymatic properties of p40 are similar to those observed for other protein-tyrosine kinases. The phos-
phorylation of angiotensin analogs by p75, a soluble protein-tyrosine kinase from liver, is also enhanced at elevated concentrations of NaCl (6). The two enzymes differ, however, in their response to divalent cations, with p75 showing a much stricter dependency on Mn^{2+} for catalytic activity. The range of protein substrates effectively phosphorylated by p40 appears to be narrower than that exhibited by most protein-tyrosine kinases, almost all of which, for example, can catalyze the phosphorylation of casein.

The relatively high K_m for the phosphorylation of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) by p40 (2.5 mM) is similar to values reported for pp60^c-src (3), the protein-tyrosine kinase of the Abelson murine leukemia virus (26), the insulin receptor (27), and the epidermal growth factor tyrosine kinase of the Abelson murine leukemia virus (261. The phosphorylation of casein.

The relationships between the two forms of p40 is currently under investigation, but preliminary peptide mapping experiments suggest that these enzymes may also be involved in the response of cells to growth factors (32). One could speculate that other intracellular protein-tyrosine kinases such as p40 might also serve a role in the growth response of cells to external signals. Soluble protein-tyrosine kinases such as p40 might have access to a different subset of cellular substrates than do membrane-associated enzymes. It is known that in fibroblasts soluble proteins of 40–42 kDa are phosphorylated on tyrosine residues in response to various mitogens (33–37), but it is not yet known if these are related to p40.

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Protein-Tyrosine Kinase from Bovine Thymus

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EXPERIMENTAL PROCEDURES

Materially: 9-(2-O-Acetamido-2-deoxy-β-D-glucopyranosyl)fluorescein (2-AMG) was purchased from BioMedical Sciences, Inc. 2-AMG-activated Sepharose (2-AMG-Sepharose) is a product of Bio-Rad Laboratories, Inc. All other reagents were of the highest grade commercially available.

Cell cultures were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS). The cells were harvested by trypsinization and centrifugation at 1000 x g for 5 min. The cell pellets were resuspended in 1N NaOH to solubilize DNA and then neutralized with 1N HCl. The cell lysates were sonicated and passed through a 0.45 µm filter to remove debris. The clarified lysates were then subjected to 90% ethanol precipitation followed by resuspension in 0.5M Tris-HCl, pH 8.0. The samples were then applied to a 2-AMG-Sepharose column and washed with 50 mM Tris-HCl, pH 8.0. The kinase activity was eluted with 200 mM NaCl in 50 mM Tris-HCl, pH 8.0. The eluates were then analyzed by SDS-PAGE and Western blotting.

The catalytic subunit of protein-tyrosine kinase was purified from bovine heart muscle by a modification of the method of Kao et al. [1]. The protein was then subjected to a second purification step on a second column of 2-AMG-Sepharose. The kinase was then dialyzed against 50 mM Tris-HCl, pH 8.0, containing 20% glycerol, and stored at -80°C.

The kinase activity of the purified protein-tyrosine kinase was assayed by incubating 20 µg of the protein with 200 µM [γ-32P]ATP and 100 µM of a peptide substrate in 50 mM Tris-HCl, pH 8.0, for 30 min at 30°C. The reaction was terminated by the addition of 5% TCA and the precipitated kinase was then subjected to SDS-PAGE and autoradiography.
Protein-Tyrosine Kinase from Bovine Thymus

Table I. Purification of p80

| Fraction | Protein | Activity | Specific Activity | Purification |
|----------|---------|----------|------------------|--------------|
|          | mg      | mol min⁻¹ | mol min⁻¹ mg⁻¹   |              |
| Extract  | 25.76   | 2196     | 0.08            | 1            |
| CM-Sepharose  | 6600   | 4134     | 0.64            | 2.3          |
| Hypervaporsor | 164   | 1100     | 6.44            | 27           |
| Cation-agarose | 9.6   | 439      | 45.1            | 103          |
| Butyl-agarose  | 3      | 280      | 95.2            | 289          |
| Sephacryl S-100 | 0.8   | 200      | 252.5           | 1259         |

Table II. Effect of cations on the phosphorylation of p80

| Cation | Protein-tyrosine kinase activity |
|--------|---------------------------------|
| 50 mM NaCl | pmol min⁻¹                           |
| Mg²⁺   | 0.3                             |
| Ca²⁺   | 0.5                             |
| Mn²⁺   | 59.1                            |
| Zn²⁺   | 10.3                            |
| Co²⁺   | 2.6                             |
| Cu²⁺   | 0.9                             |

*Levels of angiotensin I phosphorylation were not detectable above background.

Fig. 1. Phosphorylation of p80 chromatographed on Sephacryl S-100. A, the points at which the highest kinase activity eluted from butyl-agarose (Fig. 1) was chromatographed on Sephacryl S-100. The elution position of ovalbumin (66,000) is also indicated. The inset shows an autoradiogram of the gel of proteins from the sample column; proteins were phosphorylated in vitro using [β-³²P]ATP and electrophoresed on an SDS-polyacrylamide gel. The migration positions of proteins of known molecular weight are indicated. B, the phosphorylated protein bands migrating at an apparent molecular weight of 80,000 were excised and eluted with buffers containing 0.1 M 2-mercaptoethanol and 5% SDS. The samples were then subjected to SDS-polyacrylamide gel electrophoresis. The relative mobility of authentic standards and phosphorylated (P-ser), phosphorylated (P-tyr) and phosphatase (P-tyr) are indicated.

Fig. 2. Molecular weight determination by SDS-polyacrylamide gel electrophoresis. A, Coomassie blue-stained polyacrylamide gel of proteins of the peak pooled from the Sephacryl S-100 column. B, relative mobility of p80 (indicated by arrow) on a 12% SDS-polyacrylamide gel when compared to proteins of known molecular weight (bovine serum albumin, 66,000; ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,400).