Generation of an Active Protein-Tyrosine Kinase from Lymphocytes by Proteolysis*

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The major NaCl-stimulated protein-tyrosine kinase activity found in soluble thymus extracts, as measured by the phosphorylation of angiotensin I, is a 40-kDa enzyme known as p40 (Zioncheck, T. F., Harrison, M. L., and Gehlen, R. L. (1986) J. Biol. Chem. 261, 15637–15643). Antibodies prepared against p40 cross-react with a 72-kDa protein-tyrosine kinase (p72) from spleen or thymus that is closely related to p40 by peptide-mapping experiments. The recovery of p40 from spleen homogenates is reduced while the recovery of p72 is enhanced by the addition of high concentrations of leupeptin or soybean trypsin inhibitor to the homogenization media. The generation of p40 in spleen homogenates occurs with a concomitant increase in protein-tyrosine kinase activity. Activated catalytic fragments of 38–43 kDa can be generated by the treatment of partially purified p72 with trypsin or papain. The p72 protein-tyrosine kinase is found at the highest levels in spleen, thymus, and lung, tissues that also have high protein-tyrosine kinase activity and generate high levels of p40 following homogenization. p72 is also found in certain T and B cell-derived cell lines and in NIH3T3 cells.

As important mediators of cellular and humoral immune responses, B and T lymphocytes interact with a variety of external factors that influence their growth and differentiation. Many of the biochemical mechanisms of signal transduction that govern these interactions remain to be fully characterized. The relatively high level of protein-tyrosine kinase activity found in lymphocytes (1) suggests that changes in the phosphorylation of proteins on tyrosine may be important in lymphocyte activation. In support of this hypothesis, several investigators have described changes in protein-tyrosine kinase activity in lymphocytes resulting from the actions of both mitogens and growth factors. For example, the activation of T lymphocytes with polyclonal mitogens (2, 3) or with specific antigens (4) leads to the rapid phosphorylation of specific cellular proteins on tyrosine residues. Also, the phosphorylation of proteins on tyrosine is an early event in the actions of the T cell growth factor, interleukin-2 (5).

The specific protein-tyrosine kinases that catalyze these phosphorylations have not been identified. It is likely that different aspects of lymphocyte activation will involve different protein-tyrosine kinases. It is important, therefore, to understand the nature of the protein-tyrosine kinases that are found in lymphocytes. Several investigators (6–8) have reported the isolation of protein-tyrosine kinases from tissues populated with lymphoid cells including a 40-kDa enzyme (p40) that we have isolated from bovine thymus (9). In this study we have conducted a number of experiments addressing the activation of the p40 tyrosine kinase. Initial experiments with polyclonal antibodies generated against p40 indicated the presence of a 72-kDa (p72) protein cross-reactive with p40. Experiments with proteases and protease inhibitors indicated that p40 was most likely an in vivo proteolytically derived fragment of p72. p72 appears to be a protein-tyrosine kinase capable of autophosphorylation. The protein is found at the highest levels in spleen, thymus, and lung and is distributed between the cytosolic and particulate fractions. The p72 kinase is also found in cultured lymphoid cell lines as well as NIH3T3 cells.

EXPERIMENTAL PROCEDURES

Protein Kinase Assays—Protein-tyrosine kinase activity was detected by the phosphorylation of angiotensin I. Reaction mixtures contained, in a volume of 25 μl, 1.2 mm angiotensin I (Bachem), 50 μM [γ-32P]ATP (2000–4000 cpm/pmol) (Du Pont-New England Nuclear), 50 mM MgCl2, 5 mM p-nitrophenylphosphate, 25 mm Hepes, pH 7.4, 2.0 mm NaCl, and varying amounts of enzyme. Reactions were carried out at 30 °C for 3.0 min. Phosphopeptides were isolated on Whatman P81 phosphocellulose paper as described previously (10).

Protein Phosphorylation—The phosphorylation of endogenous proteins was carried out in a 50-μl reaction containing 10 mM MnCl2, 25 mm Hepes, pH 7.4, 5 mm p-nitrophenylphosphate, and 30 nm [γ-32P]ATP for 1 min at 30 °C. Reactions were terminated by the addition of a SDS-sample buffer. Phosphoproteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. The phosphoamino acid content of radiolabeled proteins separated by gel electrophoresis was determined as described (9). In one experiment, the polyacrylamide gel was treated with 1 m KOH at 55 °C for 2 h to aid in the identification of phosphotyrosine-containing proteins (11). Phosphoproteins were compared by one-dimensional phosphopeptide mapping using the procedure described by Cleveland et al. (12). Phosphoproteins were excised from SDS-polyacrylamide gels and re-electrophoresed on a second 17% polyacrylamide gel in the presence of 35% of papain (Sigma).

Preparation and Use of Antibodies—The protein-tyrosine kinase p40 was purified from bovine thymus using the procedure that we described previously (9). For the preparation of antibodies, New Zealand White rabbits were immunized by multiple subcutaneous injections of p40 (approximately 50 μg) in complete Freund's adjuvant. Rabbits were boosted at 2-week intervals by the subcutaneous injection of enzyme (25 μg) in incomplete Freund's adjuvant. The appearance of immunoreactivity against p40 was monitored by Western blot analysis. Immunoblotting was carried out by the method of Towbin et al. (13) except that 7% powdered nonfat milk was used in place of bovine serum albumin. The rabbit antisera was used at a

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride.
dilution of 1:1000. Immunoreactive proteins were detected by a second incubation with either goat anti-rabbit IgG conjugated to alkaline phosphatase or with 125I-protein A. Relative amounts of antigen were quantified by densitometry. For immunoprecipitations, protein phosphorylation reactions were terminated in radioimmune precipitation buffer (100 mM NaCl, 0.1 M Tris/Cl, pH 7.2, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS). Phosphoproteins were immunoprecipitated with 2 µl of antisera using the procedure described by Rohrschneider et al. (14).

Cells—L10A cells were the generous gift of Dr. A. DeFranco, University of California at San Francisco. K562 cells (15) were purchased from ATCC. L10A cells, K562 cells, and LSTRA cells were cultured as described previously for LSTRA cells (16). Hep G2 cells (17, 18) were obtained from Dr. Barbara Knowles, The Wistar Institute and NIH3T3 cells from Dr. Curtis Ashendel, Purdue University. NIH3T3 and Hep G2 cells were cultured in minimum Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin G, and 100 µg/ml streptomycin. Splenocytes were obtained by teasing cells from murine spleens through a wire screen followed by removal of red blood cells by hypotonic lysis.

Preparation of Cell and Tissue Extracts—Murine spleens and other organs were obtained from CF-1 mice (25-30 g), minced, and homogenized in a glass tissue grinder in 5 mM Hepes, pH 7.4, 1 mM MgCl2, and various protease inhibitors as indicated. Nuclei and unbroken cells were removed by centrifugation at 1000 × g for 10 min at 2 °C. These samples were used for tissue distribution studies. When needed, soluble extracts of these tissues were prepared by removal of particulate proteins by a second centrifugation at 12,000 × g for 10 min.

In order to clarify particulate fractions were prepared from splenocytes or cultured cells by Dounce homogenization as described previously (19). For the analysis of the cellular distribution of tyrosine kinases, cultured cells were collected, washed with phosphate-buffered saline, and suspended in 10 mM Hepes, pH 7.4, 10 mM NaCl, 5 mM MgCl2, 20 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and allowed to swell for 10 min on ice. Cells were lysed by the addition of 1.5% Triton X-100 and were disrupted with a Dounce homogenizer. A supernatant fraction was collected by centrifugation at 1000 × g for 5 min at 2 °C. For the rapid release of soluble proteins from cultured cells, cells washed in phosphate-buffered saline were resuspended in 250 mM sucrose and lysed by the addition of 1 mg/ml digitonin, 1 mM EDTA using the procedure described by Mackall et al. (20).

Detection of Protein-Tyrosine Kinases in Crude Protein Fractions—To rapidly visualize autophosphorylating kinases, cells or tissues were disrupted in a variety of ways, as described above. Proteins (1-3 mg) were batch-adsorbed to heparin-agarose (0.1 ml) resin equilibrated in 10 mM Hepes, pH 7.4, 10% sucrose. The resin was washed extensively with the above buffer containing 200 mM NaCl. Protein-tyrosine kinase activity was eluted with the above buffer containing 650 mM NaCl. Heparin-agarose was washed extensively under "Experimental Procedures" and reacted with 2 µl of immune serum (lanes 1-3) or preimmune serum (lane 4). Immunoreactive proteins were identified by incubation with alkaline phosphatase-conjugated secondary antibody. Immunoprecipitation of autophosphorylated p40, p40 (0.25 µg) was phosphorylated in vitro as described under "Experimental Procedures" and reacted with 2 µl of immune serum (lane 1), preimmune serum (lane 2), or no serum (lane 3). Antigen-antibody complexes were recovered using protein A-Sepharose. Labeled proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. C. Detection of immunoreactive proteins in crude extracts by Western blotting. A soluble extract from murine spleen (250 µg) was fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted using anti-p40 antisera. Immunoreactive proteins were visualized using 125I-protein A followed by autoradiography.

**Fig. 1. Characterization of antibodies to p40.** A. detection of purified p40 on Western blots. 0.125 (lane 1), 0.25 (lane 2), or 0.5 µg of p40 (lanes 3 and 4) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with immune serum (lanes 1-3) or preimmune serum (lane 4). Immunoreactive proteins were identified by incubation with alkaline phosphatase-conjugated secondary antibody. B. Immunoprecipitation of autophosphorylated p40. p40 (0.25 µg) was phosphorylated in vitro as described under "Experimental Procedures" and reacted with 2 µl of immune serum (lane 1), preimmune serum (lane 2), or no serum (lane 3). Antigen-antibody complexes were recovered using protein A-Sepharose. Labeled proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. C. Detection of immunoreactive proteins in crude extracts by Western blotting. A soluble extract from murine spleen (250 µg) was fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted using anti-p40 antisera. Immunoreactive proteins were visualized using 125I-protein A followed by autoradiography.

**Series of 40-43-kDa Proteins could also be detected by Western blotting, but only following partial purification of the extract on heparin-agarose and application of a high concentrations of protein to the SDS-polyacrylamide gels (not shown). Similar blotting patterns were also observed with extracts from murine thymus or bovine thymus.**

**Characterization of the 72-kDa Protein.**—The Western blot data suggested the presence of spleen and thymus of proteins immunologically related to p40. We hypothesized that some of these proteins might also represent proteintyrosine kinases. To examine this possibility, we looked at the enzymes present in L10A cells, a mature B lymphoma cell line that we found to express readily detectable amounts of protein-tyrosine kinase activity and, as shown below, high levels of the immunoreactive 72-kDa protein.

To identify protein-tyrosine kinase in L10A cells, we took advantage of the fact that many of these enzymes catalyze autophosphorylation reactions. Such reactions are often useful for identifying potential protein-tyrosine kinases present in crude mixtures of proteins. We had previously shown that the purified thymocyte protein-tyrosine kinase p40 could catalyze an autophosphorylation reaction in vitro (9). The ability of p40 present in cellular homogenates to catalyze an autophosphorylation reaction was greatly enhanced if the enzyme was first adsorbed to beads of heparin-agarose and then eluted with a buffer of high ionic strength (9). We used a similar strategy to examine L10A cells for the presence of a protein-tyrosine kinase of molecular weight higher than 40,000. A soluble fraction was prepared from L10A cells, adsorbed to heparin-agarose, and eluted with buffer containing 650 mM NaCl as described under "Experimental Procedures." Endogenous proteins were phosphorylated and separated by SDS-polyacrylamide gel electrophoresis. The resulting autoradiogram is shown in Fig. 2A. Several phosphoproteins were
observed including a phosphoprotein with an apparent molecular weight of 72,000. This phosphoprotein could be immunoprecipitated with anti-p40 antisera (Fig. 2B), suggesting that the protein was related to p40 and to the protein observed on Western blots (a small amount of p40 was also present in the extract and was also precipitated by the antisera (Fig. 2B)). Phosphoamino acid analysis indicated that the 72-kDa protein was phosphorylated solely on tyrosine (Fig. 2C).

The 72-kDa phosphoprotein was compared to p40 from murine spleen by one-dimensional phosphopeptide mapping using partial proteolysis with papain. As shown in Fig. 2D, the peptide maps of the two proteins were nearly identical suggesting that the proteins were closely related. Furthermore, preincubation of the anti-p40 antisera with purified p40 blocked the binding of antibodies to p72 on Western blots (not shown).

Generation of p40 in Cellular Homogenates—To examine potential precursor-product relationships between the 72-kDa phosphoprotein and p40, we evaluated the effect of protease inhibitors on the appearance of p40 in tissue homogenates. As mentioned above, p40 can be rapidly visualized in crude cell extracts by its ability to catalyze an autophosphorylation reaction after chromatography of the extract on heparin-agarose (9). This technique is illustrated in Fig. 3 for the detection of p40 in spleen lysates.

We had previously shown that the inclusion of the protease inhibitors PMSF and leupeptin in extraction buffers for the fractionation of bovine thymocytes did not decrease the amount of p40 observed following chromatography on heparin-agarose (9), suggesting that p40 was not generated from a larger molecular weight precursor by proteolysis. We re-examined this question by using a number of different protease inhibitors at concentrations 10-fold higher than we had employed previously. The results of one of these studies are shown in Fig. 3. Murine spleens were homogenized in the presence or absence of different protease inhibitors. Supernatants of a 12,000 x g spin were adsorbed to heparin-agarose.
The resins were washed and then eluted with buffer of high ionic strength as described under “Experimental Procedures.” Endogenous proteins were then phosphorylated. As shown in Fig. 3A, the extent of recovery of p40 was altered by the presence of certain protease inhibitors. The inclusion of leupeptin at 100 μg/ml resulted in a partial reduction in the amount of p40 that was observed and an increase in the level of the 72-kDa phosphoprotein. PMSF appeared to stabilize a higher molecular weight form of p40 that migrated with an apparent molecular mass of 43 kDa but did not protect the 72-kDa protein from degradation. Soybean trypsin inhibitor was most effective at reducing the amount of the 40–43-kDa phosphoproteins and also increased the level of the 72-kDa phosphoprotein. The phosphoproteins observed in the 40–43-kDa range were all phosphorylated solely on tyrosine residues, were related to one another by peptide-mapping experiments, and could be immunoprecipitated with anti-p40 antisera (not shown). Protease inhibitors at these concentrations did not affect the ability of purified p40 to catalyze an autophosphorylation reaction (not shown). These results indicated that p40 was most likely generated by a proteolytic event.

We took advantage of the apparent PMSF stabilization of p40 to look at the effect of the appearance of p40 on the protein-tyrosine kinase activity of tissue homogenates. Mouse spleens were homogenized in the presence of PMSF and incubated at room temperature for varying periods of time prior to chromatography on heparin-agarose. As shown in Fig. 3B, both the protein-tyrosine kinase activity and the concentration of phosphorylated 40–43-kDa proteins increased with time of incubation. In contrast, the level of the 72-kDa phosphoprotein decreased with time of incubation.

**Protein-Tyrosine Kinase Activity of the 72-kDa Protein**—The data of Figs. 1–3 supported a precursor-product relationship between the 72-kDa protein and p40. These results suggested, therefore, the possibility that the 72-kDa protein was also a protein-tyrosine kinase. To examine this further, we compared the chromatographic behavior of the phosphorylatable 72-kDa protein and the endogenous protein-tyrosine kinase activity of L10A cells. To make this determination, we needed to minimize the generation of p40 in cell or tissue homogenates. We found that by lysing cultured cells rapidly with digitonin in the presence of soybean trypsin inhibitor we could avoid the proteolytic generation of p40.

L10A cells were lysed with digitonin, and the soluble fraction was chromatographed on a column of heparin-agarose. The 72-kDa protein eluted from the column at a NaCl concentration just below that required to elute p40 (9). As shown in Fig. 4A, the 72-kDa phosphoprotein co-eluted from heparin-agarose column. The fractions eluted from the heparin-agarose column were phosphorylated in vitro, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Immunoreactive bands were detected by blotting with anti-p40 antisera, followed by alkaline phosphatase-conjugated antibody (lanes 1–3). Radiolabeled proteins were detected by autoradiography of the nitrocellulose (lanes 4–6). The migration position of p72 is indicated by the arrow.

**Activation of a Protein-Tyrosine Kinase by Proteolysis**—We wanted to determine if we could mimic the generation of p40 by the addition of exogenous proteases to a partially purified preparation of p72. p72 was obtained from a digitonin extract of L10A cells chromatographed on heparin-agarose. Protein samples containing p72 were reacted with varying concentrations of two different proteases and then incubated with [γ-32P]ATP to allow the active kinase fragments to catalyze autophosphorylation reactions. Samples were also examined by immunoblotting for angiotensin I phosphorylating activity. As shown in Fig. 4A, treatment of the sample with trypsin or papain at the concentrations indicated generated fragments of 43 and 38 kDa, respectively. The protein-tyrosine kinase activity of the sample was also increased in the presence of either protease (Fig. 5B). Treatment of the sample with lower concentrations of papain first generated fragments of 42–43 kDa, which were further cleaved to generate the 38-kDa fragment (not shown). The fragments that were generated were phosphorylated solely on tyrosine. The identity of the fragments was verified by phosphopeptide mapping. The generation of similar size fragments using different proteases suggested the existence of a sensitive exposed “hinge” region in p72. When p72 was first phosphorylated and then subjected to proteolytic digestion, we found that the major in vitro phosphorylation sites remained with the 38–43-kDa fragments (not shown). The phosphoprotein present in the p72 preparation that migrated at an apparent molecular weight of 60,000 was phosphorylated on threonine and did not appear to be related to p72 or p40 (not shown).

These results were consistent with the possibility that p72 served as the precursor to p40. The amount of p72 that we could detect by Western blotting experiments was not decreased if intact L10A cells were rapidly lysed by treatment with boiling SDS-sample buffer (not shown). Therefore, we think it is unlikely that p72 is also generated from a larger precursor unless the conversion occurs extremely rapidly or prior to the homogenization of the cell.

**Tissue Distribution of p72**—Prior to examining the distribution of p72 in tissues and cells, we first examined spleen...
tracts of LLOA cells by chromatography on heparin-agarose as shown in Fig. 4A. The peak fractions were pooled (0.25 mg/ml protein) and incubated at 0°C (lane 1) or at room temperature (lanes 2-6) for 30 min in the absence of added exogenous proteases (lanes 1 and 2) or in the presence of 2 µg/ml trypsin (lane 3), 4 µg/ml trypsin (lane 4), 2 µg/ml papain (lane 5), or 4 µg/ml papain (lane 6). Endogenous proteins were then phosphorylated as described under "Experimental Procedures," separated by SDS-polyacrylamide gel electrophoresis, and examined by autoradiography. Procedures," separated by SDS-polyacrylamide gel electrophoresis, and examined by autoradiography. Lane 7 contains in vitro auto-

phosphorylated p40 from bovine thymus. The migration positions of 72- and 40-kDa proteins are indicated. B, effect of proteases on tyrosine kinase activity. The samples generated in experiments outlined for lanes 1-6 of panel A were assayed for angiotensin I-phosphorylating activity.

Fig. 6. Cellular distribution of p72. A, subcellular distribution of p72 in murine splenocytes. Proteins from spleen cell lysates prepared as described under "Experimental Procedures" were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted with anti-p40 antisera followed by alkaline phosphatase-conjugated second antibody. Lane 1, 50 µg of cytosolic protein released from splenocytes by treatment with digitonin; lane 2, 100 µg of protein released from splenocytes by lysis with 1.5% Triton X-100; lane 3, 50 µg of postnuclear particulate fraction protein prepared from Dounced cells by differential centrifugation. B, cellular distribution of p72. Cultured cells were lysed with buffer containing 1.5% Triton X-100. Solubilized proteins (250 µg) were separated by SDS-polyacrylamide gel electrophoresis and detected by Western blotting. The cells lines used were L10A (lane 1), K562 (lane 2), 3T3 (lane 3), LSTRA (lane 4), and Hep G2 (lane 5). The migration position of the p72 doublet is indicated by the arrow.

Protein-Tyrosine Kinases from Lymphocytes

To examine the distribution of p72 in various mouse organs, tissues were homogenized as described under "Experimental Procedures." Nuclei and unbroken cells were removed by centrifugation, and the remaining sample consisting of both cytosolic and particulate fraction proteins was fractionated by electrophoresis on SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and probed with anti-p40 antisera. The relative levels of p72 were determined by densitometric scanning of the Western blots. The results are shown in Table I. The highest levels of p72 were found in spleen, followed by thymus and lung. Tissues with the highest levels of p72 also expressed the highest levels of protein-tyrosine kinase activity (measured by the phosphorylation of angiotensin I) (Table I). The tissue distribution studies indicated that the highest levels of p72 were found in organs populated by hematopoietic cells.

In a separate experiment, soluble fractions from these same tissues were chromatographed on heparin-agarose, phosphorylated in vitro, and separated by SDS-polyacrylamide gel electrophoresis to examine the relative levels of p40 that had been generated during homogenization. p40 could be readily detected in extracts from spleen, thymus, and lung. The amount of p40 from bovine thymus (as the sum of 40-43-kDa phosphoproteins) also correlated well with the level of protein-tyrosine kinase activity found in each tissue (ratio of kinase activity for spleen/thymus/lung: 100:22:11; ratio of p40, 100:23:14).

Cell Distribution of p72—We also examined a limited number of cultured cell lines by immunoblotting. L10A cells exhibited relatively high levels of the p72 protein doublet compared to the other cells tested (Fig. 6B). LSTRA, a T lymphoma cell line that overexpresses the protein-tyrosine kinase p56Lck (10, 23, 24) also expressed p72, although at levels lower (3-fold) than those seen in L10A cells. The antisera did not cross-react with p56Lck. We also observed in NIH3T3 cells levels of immunoreactive protein comparable to those seen in LSTRA cells. The 72 kDa protein was not readily detected in either K562 erythroleukemia cells or Hep G2 hepatocellular carcinoma cells.

Table I

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<thead>
<tr>
<th>Tissue</th>
<th>Relative kinase activity</th>
<th>Relative level of p72</th>
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<td>Spleen</td>
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<td>Lung</td>
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<td>Heart</td>
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* Indicates that p72 was not detected.
The cellular distribution of p72 with highest levels in lymphoid cells was consistent with its tissue distribution with the exception of its presence in 3T3 cells, a mouse embryo fibroblast-derivived cell line. To verify the presence of a protein-tyrosine kinase of this size in these fibroblasts, a detergent extract of 3T3 cells was made and adsorbed to heparin-agarose. The gel was then rehydrated in 1 M KOH, heated to 55 °C for 2 h, and redried. The autoradiograms obtained by exposure of the untreated and treated (KOH) gels are shown. The migration position of p72 is indicated by the arrow.

**DISCUSSION**

We had shown previously that the principle protein-tyrosine kinase activity of soluble bovine thymus extracts could be purified through several column steps to yield a 40-kDa enzyme known as p40 (9). Due to the relatively low molecular weight of the isolated kinase, we were suspicious that the enzyme might represent an active catalytic fragment of a larger kinase. The observations described in this report are consistent with the hypothesis that p40 results from the proteolysis of a larger molecular weight precursor. This proteolytic event gives rise to an enzyme fragment that readily catalyzes both an autophosphorylation reaction and the phosphorylation of an exogenous peptide substrate. These conclusions are based on the observations that (i) the exclusion of protease inhibitors from the homogenization media alters the extent of recovery of p40 and of a related larger molecular weight 43-kDa phosphoprotein (Fig. 1A), (ii) the formation of p40 from spleen homogenates increases with time of incubation (Fig. 1B), (iii) the rapid release of cytosolic enzymes from cultured cells with digitonin inhibits the formation of the 40-43-kDa phosphoproteins (Fig. 5), (iv) antibodies prepared against p40 recognize a larger 72-kDa kinase that is closely related to p40 by phosphopeptide mapping (Fig. 3), and (v) the addition of exogenous proteases to preparations containing the 72-kDa protein generates active 38–43-kDa kinase fragments (Fig. 5).

It is known that some other protein-tyrosine kinases are also susceptible to proteolytic degradation during the fractionation of cells and tissues. For example, Wells and Collett (25) have made a study of the effect of protease inhibitors and homogenization conditions on the generation of the 52-kDa proteolytic fragment of pp60 on is (25). The conditions that lead to the generation of p52 are in many instances similar to those that lead to the generation of p40. In each case, lysis of cells with detergents allows a greater recovery of the intact enzyme. Also, the inclusion of leupeptin or soybean trypsin inhibitor, but not PMSF, in homogenization buffers helps to inhibit the formation of both pp52 and p40 (40-43-kDa phosphoproteins) (Fig. 1). In both cases, generation of the active proteolytic fragment appears to occur more readily when attempts are made to fractionate tissues as opposed to cultured cells (25, 26).

From our observations, the generation of p40 clearly occurs with a concomitant increase in protein-tyrosine kinase activity as measured both by the endogenous phosphorylation of proteins and by the phosphorylation of an exogenously added peptide substrate. It is difficult to obtain a direct comparison of the specific activities of p40 and of the native enzyme due to the fact that p40 itself is quite susceptible to proteolysis both by endogenous proteases and by exogenously added enzymes. The amount of p40 that is generated in the types of experiments illustrated by Figs. 1 and 5 is small and is generally below the detection limits of our Western blotting experiments unless the samples are concentrated prior to SDS-polyacrylamide gel electrophoresis. In comparison to the more abundant p72, p40 shows a greatly enhanced ability to catalyze an autophosphorylation reaction. Despite the low levels of p40 that are generated in tissue homogenates such as those shown in Fig. 1A, samples containing p40 exhibit an elevated protein-tyrosine kinase activity (Figs. 1B and 5). Using our antibodies, we have re-examined our purification protocol for the isolation of p40 from bovine thymus (9) and found that from the very first chromatographic step p40 accounts for the majority of the tyrosine kinase activity that is observed in thymus homogenates. Thus, the amount of protein-tyrosine kinase activity measured in cellular homogenates depends in large part on the extent to which p40 is generated. Much of the tyrosine kinase activity measured in tissue homogenates such as those reported in Table I is actually representative of the amount of p40 generated during homogenization.

These are many examples known where the partial proteolysis of a protein kinase releases the inhibitory effects of a regulatory domain on a catalytic domain. For example, protein kinase C was first described as a proteolytically activated catalytic fragment (27). Other regulated kinases such as phosphorylase kinase (28), the insulin receptor (29), and the epidermal growth factor receptor (30) are also stimulated by...
proteolysis. The elevation in protein-tyrosine kinase activity that accompanies the generation of p40 suggests the possibility that the precursor to p40 is a regulated enzyme. If so, it is possible that additional mechanisms other than proteolysis will also operate to increase the activity of the enzyme. It will be of interest to determine the nature of the physiological factors that are involved in its regulation.

A number of observations point to p72 as the most likely precursor to p40: (i) antisera raised against p40 also recognize the 72-kDa protein (Figs. 2 and 3); (ii) p72 is phosphorylated on tyrosine residues under conditions that also allow the phosphorylation of p40, the phosphorylation of both enzymes being enhanced following adsorption to and elution from heparin-agarose (Figs. 1 and 3); (iii) phosphopeptide maps of phosphorylated p72 and p40 are nearly identical (Fig. 3D); (iv) both p40 and p72 (Fig. 4) co-migrate during column chromatography with protein-tyrosine kinase activity, although the inherent activity of p40 appears to be much higher than that of p72; (v) both enzymes exhibit an unusual activation by high concentrations of NaCl (up to 2 M) when angiotensin I is used as a substrate; (vi) the tissues that express the highest levels of p72 are also those that generate the highest levels of p40 during homogenization (Table I).

The p72 kinase that we have described most resembles the p75 protein-tyrosine kinase isolated by Wong and Goldberg (31) from rat liver. The enzyme from liver purifies as a 72–75-kDa doublet as observed on silver stained SDS-polyacrylamide gels (31). We also consistently observe a protein doublet of this approximate size by Western blotting experiments (Fig. 2). Both p72 from spleen and p75 from liver are activated by high concentrations of NaCl when angiotensin analogs are used as substrates (31). The liver p75 kinase, like p72, is also found both as a soluble and a microsome-associated enzyme (21 and Fig. 6). We have also found that, like the p75 kinase from liver (21), p72 can be quantitatively precipitated from soluble cell homogenates by decreasing the pH to 5.0.

Our tissue distribution studies, however, indicate that the levels of p72 are low in liver as compared to spleen, thymus, or lung (Table I). This does not rule out the possibility that p72 and p75 represent the same enzyme. It might be of interest, however, to determine the cell types present in intact liver tissue that actually give rise to the p72 (or p75) that is observed. The level of p72 present in the hepatocellular carcinoma cell line Hep G2 was below our level of detection by immunoblotting (Fig. 6). The limited distribution studies that we have conducted to date suggest that p72 is expressed at high levels in lymphocytes and in particular in lymphocytes of the B lineage (Table I and Fig. 6). It is clear from the studies using 3T3 cells, however, that the expression of p72 is not limited to hematopoietic cells (Figs. 6 and 7).

It is not known if the generation of p40 occurs in vivo as well as in vitro or, if so, whether this plays any significant physiological role. It is interesting to note that several investigators have reported the appearance of 38–45-kDa phosphorylase-containing proteins following the activation of a number of cell types with a diverse group of mitogens. It has been hypothesized that the phosphoproteins represent substrates for endogenous protein-tyrosine kinases, perhaps the growth factor receptors. These proteins have been reported in T lymphocytes activated by antigens (4), mitogens (3), or interleukin-2 (5) and in mitogen-activated fibroblasts (32–35), including 3T3 cells (35). An alternative explanation for the appearance of these phosphoproteins is that they represent proteolytic fragments of a larger molecular weight protein and that these fragments have now become activated and are catalyzing autophosphorylation reactions. While it is not known if these phosphoproteins are indeed related to p40, it is clear that in cells such as T lymphocytes and 3T3 cells where the appearance of the 38–45-kDa substrates has been described, there is a protein-tyrosine kinase (p72) that is capable of generating active fragments of this size (Ref. 9 and Fig. 5).

The role, if any, of p72 or p40 in the activation of lymphocytes remains to be determined. It is possible that the generation of p40 provides a mechanism for the physiologically relevant activation of a protein-tyrosine kinase. It is also possible that the generation of p40 reflects changes in the conformation of p72 that renders it more susceptible to proteolysis. The phosphorylation of proteins similar in size to p72 on tyrosine has been reported in a number of systems. The phosphorylation of a 66-kDa protein on tyrosine is an early event following the treatment of T lymphocytes with polyclonal mitogens (2). In BALB/c 3T3 cells, the phosphorylation of a 74-kDa protein is stimulated by platelet-derived growth factor (36). Finally, in certain hematopoietic progenitor cell lines, interleukin-3 and granulocyte-macrophage colony-stimulating factor stimulate the phosphorylation of proteins of 70–72 kDa (37). It is not yet known if any of these phosphoproteins are related to p72. It has also been reported that the activities of two protein-tyrosine kinases with properties similar to those of p72 and p40 are greatly decreased 24–48 h after mitogen activation of T cells (38). It will be of interest to determine how the enzymes that we have characterized are modified in activated cells.

REFERENCES