Purification and characterization of a tyrosine-specific protein kinase of M_r 60 000 and comparison with a kinase of M_r 56 000 from rat spleen

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A tyrosine-specific protein kinase of M_r 60 000 (TK-I) was purified to near homogeneity from the particulate fraction of rat spleen. The purification procedure involved sequential chromatography of the detergent-solubilized enzyme on DEAE-Sephacel and hydroxyapatite columns. Polyacrylamide-gel electrophoresis under denaturing conditions showed one major polypeptide, of M_r 60 000. Gel filtration of the enzyme on Sephacryl S-200 column showed a single peak of kinase activity, of apparent M_r 60 000. On incubation with [γ - 32 P]ATP, it showed a phosphoprotein of M_r 60 000 as a result of autophosphorylation. The autophosphorylation of the kinase occurred only at tyrosine residues. Incubation of TK-I with ATP (but not with ADP) resulted in an increase in its tyrosine-specific protein kinase activity. The time course of autophosphorylation of TK-I was very similar to the time course of activation by ATP. These and other experiments suggest that autophosphorylation might be responsible for activation of TK-I observed on incubation with ATP. A second tyrosine-specific protein kinase (TK-II) was isolated from the particulate fraction of rat spleen. A highly purified preparation of TK-II on incubation with [γ - 32 P]ATP gave a major phosphoprotein, of M_r 56 000. TK-II was different from TK-I in several properties: (a) substrate specificity; (b) chromatographic behaviour; (c) phosphopeptide maps; and (d) inhibition by tosyl-lysylchloromethane. Antisera raised against TK-I did not cross-react with TK-II. These results suggest that TK-I and TK-II are distinct proteins, perhaps coded by two different genes.

INTRODUCTION

Protein phosphorylation at tyrosine residues has been studied in many transformed and some normal cells. Transforming proteins of some RNA tumour viruses and the receptors for some growth factors (e.g. epidermal growth factor, insulin, platelet-derived growth factor and colony-stimulating factor-1) are associated with tyrosine-specific protein kinase activity [1–6]. In addition to these two classes, there are some other tyrosine-specific protein kinases which are not known to be associated with either an oncogene product or a receptor for growth factors [6–8]. It has been suggested that protein phosphorylation at tyrosine is involved in the process of cell transformation and in the regulation of cell growth. The distribution of tyrosine-specific protein kinase activity in various tissues of rat, however, does not show any correlation between the amount of tyrosine kinase activity and the proliferative state of the tissues [7,8]. To obtain more definitive information about the function of tyrosine-specific protein phosphorylation in normal cells, it would be helpful to isolate and characterize the enzymes involved in the phosphorylation and dephosphorylation of tyrosine residues of proteins in normal cells. For this purpose we have chosen rat spleen as the source of tyrosine-specific protein kinase, since it shows very high activity of this enzyme [7]. So far only a few attempts have been made to purify cellular tyrosine kinases which are not associated with receptors for growth factors or with oncogene products. Partial

purification of the tyrosine kinase from rat liver, spleen, thymus and lung has been reported previously [9–17].

Here we report the isolation of two distinct tyrosine-specific protein kinases (TK-I and TK-II) from the particulate fraction of rat spleen. One of these enzymes (TK-I) was purified to near homogeneity, as judged by polyacrylamide-gel electrophoresis in the presence of SDS. This kinase (TK-I) was partially characterized. A comparison of the properties of TK-I and TK-II suggests that these kinases are distinct proteins, perhaps coded by different genes. These kinases can be distinguished easily by using Tos-Lys-CH₂Cl, which preferentially inhibits TK-II only.

MATERIALS AND METHODS

Assay of tyrosine-specific protein kinase activity

TK activity was assayed by the phosphorylation of a synthetic peptide, E₁₁G₁, with [γ - 32 P]ATP as described previously [11]. In some experiments the tyrosine kinase activity was assayed by using poly(Glu₄Tyr₁), a synthetic random copolymer of glutamic acid and tyrosine (Sigma), as substrate. The assay mixture contained 25 mM-Hepes buffer (pH 7.5), 25 mM-MgCl₂, 0.1 mM-sodium orthovanadate, 0.2 mg of poly(Glu,Tyr)/ml, 0.2 mM-[γ - 32 P]-ATP (100–400 c.p.m./pmol), 10% (v/v) glycerol and a sample of the enzyme preparation, in a total volume of 0.05 ml. After incubation for 15 min at 24 °C, the reaction was stopped by spotting a 0.04 ml sample of the reaction mixture on Whatman 3MM paper (2 cm × 2 cm) and

Abbreviations used: TK, tyrosine-specific protein kinase; Tos-Lys-CH₂Cl, tosyl-L-lysylchloromethane ('TLCK'); E₁₁G₁, Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly.

dipping this paper in cold 10% (v/v) trichloroacetic acid. The papers were washed four times in 10% trichloroacetic acid containing 2 mM-sodium pyrophosphate.

Peptide mapping by V-8 protease

Peptide mapping was carried out by a modification of the procedure of Cleveland *et al.* [18]. Equal amounts of TK-I or TK-II were incubated with 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 25 mM-MgCl₂, 25 mM-Hepes buffer (pH 7.5), 10 μ M-vanadate and 10% glycerol, in a total volume of 50 μ l. After incubation for 1 h at 24 °C, 10 μ l of 2% (w/v) SDS was added, followed by addition of V-8 protease. The protease reaction was stopped by adding 10 μ l of 10% SDS containing 50% (v/v) mercaptoethanol. The sample was then heated for 2 min at 100 °C and analysed by electrophoresis on 15%-acrylamide gel, followed by autoradiography.

Purification of TK-I and TK-II

Particulate fraction from rat spleen was prepared as described previously [7]. TK activity was solubilized from the particulate fraction by extracting with 5% Triton X-100/20% glycerol in 50 mM-N-ethylmorpholine/acetate buffer (pH 8.5) containing 2 mM-EDTA, 0.5 mM-phenylmethanesulphonyl fluoride, 0.1 mM-vanadate and 7 mM-mercaptoethanol. After centrifuging for 1 h at 30000 g, the supernatant (200 ml) was mixed with 20 ml of DEAE-Sephacel. After 15 min at 4 °C, this suspension was poured into a DEAE-Sephacel column (2.6 cm $\times$ 15 cm) equilibrated with buffer A [25 mM-N-ethylmorpholine/acetate (pH 7.1) containing 10% glycerol, 0.5 mM-EDTA, 5 mM-2-mercaptoethanol, 0.1% Triton X-100 and 0.1 mM-vanadate]. After washing with 300 ml of buffer A, the kinase activity was eluted with 80 ml of 50 mM-NaCl in buffer A, followed by 100 mM-NaCl in buffer A (150 ml). Two peaks of TK activity were obtained (Fig. 1). Washing with 50 mM-NaCl was necessary to obtain good separation of the two kinases. These two peaks of kinase activity were pooled separately (fractions 15–16 for TK-I and fractions 28–31 for TK-II) and dialysed against buffer B [10 mM-sodium phosphate (pH 7.1), 50% glycerol, 0.5 mM-EDTA, 5 mM-2-mercaptoethanol, 0.1 mM-vanadate, 0.1% Triton X-100]. TK-I was further purified on a Bio-Gel HTP column (0.7 cm $\times$ 5 cm) equilibrated with buffer C [10 mM-phosphate (pH 7.1), 20% glycerol, 0.1% Triton X-100, 0.1 mM-vanadate, 0.5 mM-EDTA and 5 mM-2-mercaptoethanol]. After loading of the enzyme, the HTP column was washed with 8 ml of 40 mM-phosphate in buffer C. Kinase activity was eluted from this column with 100 mM-phosphate (pH 7.1) in buffer C. The active fractions were pooled and dialysed against buffer B and stored in this buffer at –10 °C.

TK-II was further purified by chromatography on a poly(A)-Sephacryl column (5 ml) equilibrated with buffer A. The kinase activity from this column was eluted with 0.15 M-NaCl in buffer A. After chromatography on poly(A)-Sephacryl, TK-II was subjected to gel filtration on a Sephacryl S-200 column (1.6 cm $\times$ 62 cm) equilibrated with buffer A containing 0.2 M-NaCl. The enzyme was then dialysed against buffer B and stored at –10 °C.

Preparation of antiserum against TK-I

Purified TK-I was dialysed against phosphate-buffered saline [19], pH 7.4, mixed with an equal volume of

complete Freund's adjuvant and emulsified. This emulsion was injected subcutaneously on the back of a New Zealand White rabbit at 10–12 sites. Booster injections were given in incomplete adjuvant on days 29, 36 and 51. For each immunization 0.2–0.3 mg of TK-I was used. At 1 week after the last boost, blood was taken from the eye (from the retro-orbital plexus) and allowed to clot for 2 h at room temperature. Serum was removed carefully and centrifuged for 2 min in a Microfuge. Clear supernatant was used for testing the presence of antibodies against TK-I by double diffusion in agar plates, prepared on microscope slides as described in [19].

Immunoprecipitation

Protein A-Sepharose (0.5 ml) was incubated with 0.1 ml of rabbit anti-TK-I serum in phosphate-buffered saline for 2 h at 0 °C and washed several times with phosphate-buffered saline. TK-I and TK-II were phosphorylated with 10 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 25 mM-MgCl₂ for 30 min at 24 °C. Unlabelled ATP was added to give a final concentration of 2 mM. Then 0.5 ml of phosphate-buffered saline was added, followed by 0.05 ml of Protein A-Sepharose coated with anti-TK-I antibodies. After 60 min at 0 °C it was centrifuged for 15 s in an Eppendorf centrifuge. The pellet was washed three times with phosphate-buffered saline. Then 0.1 ml of sample buffer (3% SDS, 0.1 M dithiothreitol, 10% glycerol, 25 mM-Tris, pH 7.1) was added, and the mixture heated at 100 °C for 5 min. After centrifugation the supernatant was analysed by electrophoresis in the presence of SDS in a 12%-polyacrylamide gel [20], followed by autoradiography. For reaction with control serum, Protein A-Sepharose was coated with control serum.

Chemicals

DEAE-Sephacel, Sephacryl S-200 and poly(A)-Sepharose were obtained from Pharmacia; Bio-Gel HTP was from Bio-Rad; poly(Glu,Tyr), adenosine 5'- $[\beta\gamma\text{-imidol}]\text{triphosphate}$ and Tos-Lys-CH₂Cl were from Sigma; and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from Isotope Division, Bhabha Atomic Research Centre, Trombay, India.

RESULTS

Purification of a tyrosine-specific protein kinase of M_r 60000 from the particulate fraction of rat spleen

The particulate fraction from rat spleen contains two major tyrosine-specific protein kinases, which after solubilization could be separated by chromatography on a DEAE-Sephacel column (Fig. 1). These two kinases, designated TK-I and TK-II, showed different substrate specificity with synthetic substrates. The kinase TK-I, which prefers E₁₁G₁ as substrate, was purified to near homogeneity by a procedure involving two chromatographic steps. The purification procedure is summarized in Table 1. Starting with 15 g of rat spleen, 0.5 mg of purified kinase could be obtained within 4–5 days. This kinase preparation was stable for several months when stored in buffer B (which contains 50% glycerol) at –10 °C. TK-I represents about 40–50% of total E₁₁G₁ kinase activity recovered from the DEAE-Sephacel chromatography step. Taking into account the fact that

Fig. 1. Separation of two tyrosine-specific protein kinases by chromatography on DEAE-Sephacel

The kinase activity of column fractions (5 ml) was measured by using 0.2 mg of poly(Glu, Tyr)/ml or 0.05 mM- $E_{11}G_1$. The details are described in the Materials and methods section. The arrow indicates change in salt concentration from 50 to 100 mM. The concentration of salt was changed from 0 to 50 mM from fraction 1.

8–10-fold enrichment of tyrosine kinase activity is obtained in the preparation of particulate fraction, the overall purification obtained was about 1000-fold for $E_{11}G_1$ kinase activity of TK-I. This does not take into account the fact that TK-I represents only 40–50 % of the total $E_{11}G_1$ kinase activity. The TK-II preparation, although purified several hundred-fold, showed two major contaminants (results not shown). Calculation of purification factor is further complicated by the finding that these kinases are activated on incubation with ATP (see below).

Characterization of TK-I

Polyacrylamide-gel electrophoresis of the purified TK-I preparation in the presence of SDS showed only one major Coomassie-Blue-staining band, of M_r 60000 (Fig. 2). This enzyme showed one peak of kinase activity on gel filtration on Sephacryl S-200, with an apparent M_r of about 60000 (results not shown). This suggested that TK-I is monomeric under the conditions of the experiments. On incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and Mg^{2+} , this kinase preparation showed one phosphoprotein band, of M_r 60000, on polyacrylamide-gel electrophoresis (Fig.

3), presumably owing to self-phosphorylation. Auto-phosphorylation of the kinase occurred only at tyrosine residues, as determined by high-voltage paper electrophoresis of the partial acid hydrolysate of the phosphorylated enzyme (results not shown).

Incubation of the TK-I preparation with ATP and Mg^{2+} for 30 min resulted in a several-fold increase in its tyrosine-specific protein kinase activity with $E_{11}G_1$ as substrate. The increase in kinase activity was time-dependent; maximal activation of the enzyme was obtained after about 30 min of incubation of the enzyme with ATP. The activity of the enzyme did not increase on incubation without ATP. Mg^{2+} was also required for the activation of the enzyme. Incubation with Mg^{2+} alone in the absence of ATP did not activate the kinase. The time course of peptide phosphorylation before and after preincubation with ATP for 30 min is shown in Fig. 4. The time course of peptide phosphorylation was non-linear before incubation with ATP. After preincubation of the enzyme for 30 min with ATP the time course was linear, suggesting that activation was complete in about 30 min.

This activation of the enzyme was not observed when

Table 1. Purification of TK-I from 15 g of rat spleen

The recoveries of protein and tyrosine-specific protein kinase activity for each step of purification are presented. Tyrosine-specific protein kinase activity was measured by using 0.05 mM- $E_{11}G_1$ and 0.2 mM-ATP as described in the Materials and methods section. Protein concentration was measured by the method of Bradford [35].

	Total protein (mg)	Total activity (nmol)	Recovery (%)	Specific activity (nmol/min per mg)	Purification (fold)
1. Particulate fraction	360	13.6	100	0.038	1
2. Triton extract		9.7	71		
3. DEAE pool	4.2	6.9	50	1.65	43
4. HTP pool	0.55	2.2	16	4.15	109

Fig. 2. Polyacrylamide-gel electrophoresis of purified TK-I preparation in the presence of 0.1% SDS

The electrophoresis was carried out as described previously [11] in the buffer system of Laemmli [20]. The gel was stained with Coomassie Blue. Lane 1, M_r markers; lane 2, TK-I preparation (5 μ g of protein).

TK-I was preincubated with ADP or adenosine 5'-[β -imido]triphosphate (Fig. 4). This suggested that either hydrolysis of ATP or the transfer of the terminal phosphate of ATP was required for the activation of the kinase. These observations raised the possibility that autophosphorylation of the kinase may be responsible for the activation of the enzyme by ATP. This possibility was examined by comparing the time course of autophosphorylation and activation by ATP. As shown in Fig. 5, these time courses were similar. Increased kinase activity on preincubation of TK-I with ATP was observed with three different substrates, namely $E_{11}G_1$, poly(Glu,Tyr) (0.025 mg/ml) and [Val⁵]angiotensin II (1 mg/ml). The degree of activation with these three substrates was nearly the same. When TK-I was purified in the absence of vanadate, which is an inhibitor of phosphotyrosine-specific protein phosphatase [21], the kinase preparation showed much more activation on incubation with ATP (up to 20-fold). All these observations taken together suggest that autophosphorylation may be responsible for the ATP-dependent activation of TK-I.

Both Mg^{2+} and Mn^{2+} could serve as bivalent cations for the kinase activity of TK-I with $E_{11}G_1$ or poly(Glu,Tyr) as substrate. However, Mg^{2+} always gave higher kinase activity (Fig. 6). No kinase activity was observed in the absence of a bivalent cation. Most of the other known tyrosine-specific protein kinases prefer Mn^{2+} to Mg^{2+} as the required bivalent cation.

Fig. 3. Autoradiograph showing phosphorylation of TK-I and TK-II by [γ - ^{32}P]ATP

Samples of TK-I or TK-II containing equal amount of $E_{11}G_1$ kinase activity were incubated with 10 μ M-[γ - ^{32}P]-ATP in 25 mM-Hepes buffer (pH 7.5) containing 25 mM- $MgCl_2$, 0.1 mM-sodium orthovanadate and 10% glycerol. After 60 min at 25 $^{\circ}C$, the reaction was stopped and the sample analysed by polyacrylamide-gel electrophoresis in 10% polyacrylamide gel in the presence of SDS, followed by autoradiography as described previously [11]. Lane 1, TK-I; lane 2, TK-II.

The apparent K_m for $E_{11}G_1$ was 2 mM, and apparent V_{max} after activation with ATP was 480 nmol/min per mg at 0.2 mM-ATP. With poly(Glu,Tyr) as substrate, the apparent K_m and V_{max} were 0.05 mg/ml and 120 nmol/min per mg. The apparent K_m for ATP was 0.012 mM at 0.1 mg of poly(Glu,Tyr)/ml.

Comparison of TK-I and TK-II

Besides differences in chromatographic behaviour on DEAE-Sephacel, these two tyrosine-specific protein kinases differ in their chromatographic behaviour on other types of resins (results not shown). The TK-II preparation on autophosphorylation gave a phosphoprotein of M_r 56000, whereas the TK-I preparation gave one of M_r 60000 (Fig. 3). TK-I showed higher activity with $E_{11}G_1$ as substrate as compared with TK-II (Fig. 1).

TK-II was preferentially inhibited by Tos-Lys- CH_2Cl , which is known to inhibit many protein kinases, including the kinase activity of viral *src* gene product [22-24]. At 10 mM, Tos-Lys- CH_2Cl gave over 90% inhibition of TK-II, whereas TK-I was inhibited by only 10-20%.

In spite of several differences in the properties of the two kinases, it still seemed possible that TK-II was

Fig. 4. Time course of phosphorylation of $E_{11}G_1$ by TK-I before (○) and after (▲) incubation with 0.2 mM-ATP for 30 min

The kinase assay was carried out with 0.2 mM- $E_{11}G_1$ as substrate and 1 μ g of enzyme for the indicated period of time as described in the Materials and methods section. In the third experiment (●), the kinase was incubated with 0.1 mM-adenosine 5'-[β - γ -imido]triphosphate for 30 min before adding ATP (0.2 mM) and $E_{11}G_1$.

derived from TK-I by proteolysis during purification. Therefore these two kinases were compared by peptide mapping with V-8 protease [18]. As shown in Fig. 7, the phosphopeptide maps of the two kinases are clearly distinct. This suggests that TK-II is not derived from TK-I by proteolysis and that TK-I and TK-II are distinct proteins, perhaps coded by two different genes.

The two kinases were further compared for their antigenic properties. For this purpose antiserum against TK-I was prepared by immunizing rabbits as described in the Materials and methods section. This antiserum showed one sharp precipitin line with TK-I when subjected to double diffusion in agar plates (Fig. 8); control serum did not show any precipitin line with TK-I (results not shown). TK-II did not show any cross-reaction with antiserum raised against TK-I (Fig. 8). When TK-I and TK-II were phosphorylated and then immunoprecipitated by using anti-TK-I serum and Protein A-Sepharose, only TK-I was precipitated by the antibodies, as shown in Fig. 9. Control serum did not precipitate TK-I or TK-II (results not shown). Some proteolysis of TK-I occurs during immunoprecipitation, as can be seen in Fig. 9. These results suggest that TK-I and TK-II are antigenically distinct proteins. These experiments, however, do not rule out the possibility of some weak interaction between TK-II and anti-TK-I antibody, which is not detectable by the methods used here.

DISCUSSION

The results presented here show that there are two different tyrosine-specific protein kinases present in the particulate fraction of rat spleen, which can be isolated

Fig. 5. Time course of autophosphorylation and activation of TK-I by ATP

Increase in kinase activity is expressed as a percentage of the maximal increase. Autophosphorylation is expressed as percentage of maximal autophosphorylation. The concentration of ATP was 0.1 mM for both the reactions. Kinase activity was measured with 0.2 mM- $E_{11}G_1$ as substrate as described in the Materials and methods section. The amount of enzyme used was 2 μ g for each assay. Autophosphorylation was measured by counting radioactivity in the kinase band after autoradiography of the dried 10%-polyacrylamide gel.

Fig. 6. Effect of Mg^{2+} and Mn^{2+} concentrations on the kinase activity of TK-I

The kinase activity was determined by using poly(Glu,Tyr) as substrate at various concentrations of Mg^{2+} or Mn^{2+} .

by simple purification procedures. One of the kinases, TK-I, has been purified to near homogeneity by a convenient procedure involving only two chromatographic steps. These two kinases were eluted together in the study reported previously [7]. This could have been due to the use of a small column of DEAE-Sepharose [7]. A tyrosine-specific protein kinase of M_r 56000 was described from rat spleen [11] by us, which was the same as TK-II described here. Since purification was not described in detail, no mention was made of the presence of TK-I obtained during purification [11]. Using a salt gradient for elution from a DEAE-cellulose column, Brunati *et al.* [10] have isolated three chromatographically distinct tyrosine-specific protein kinases

Fig. 7. Phosphopeptide maps of TK-II (lanes 1–4) and TK-I (lanes 5–8)

Peptide mapping was carried out as described in the Materials and methods section, by using an equal amount of $E_{11}G_1$ kinase activity of the two kinases. The amount of the V-8 protease used was $0.16 \mu\text{g}$ (lanes 2 and 6), $0.8 \mu\text{g}$ (lanes 3 and 7) and $4 \mu\text{g}$ (lanes 4 and 8). Lanes 1 and 5 are controls without any protease.

from rat spleen. Two of these kinases migrated with M_r values of 65000 (TK-I) and 50000 (TK-II), and may be the same as TK-I and TK-II described in the present work. However, we found that TK-I (and TK-II) prefers Mg^{2+} to Mn^{2+} as bivalent cation for its kinase activity, whereas TK-I of Brunati *et al.* [10] prefers Mn^{2+} to Mg^{2+} . The reason for this discrepancy is not clear. The TK-I preparation of Brunati *et al.* [10] may have contained proteolysed kinase, since it showed two phosphoproteins, of M_r 60000 and 55000. The proteolysed form of TK-I may have a somewhat different metal-ion requirement. Recently a tyrosine-specific protein kinase of M_r 50000 has been purified 3000-fold from bovine spleen particulate extract, which was 40% pure [25]. It prefers Mg^{2+} over Mn^{2+} as bivalent cation. This enzyme could be similar to TK-II described here. The purification factor for TK-I found by us is 109-fold from particulate fraction (Table 1). Since 8–10-fold purification is obtained in the preparation of particulate fraction, the overall purification is 1000-fold for $E_{11}G_1$ kinase activity of TK-I. The purification factor in [25] seems to be an over-estimate, since it would mean that purification to homogeneity from spleen homogenate would require 60000-fold purification. The specific activities (apparent V_{max}) of TK-I and TK-II are 480 and 300 nmol/min per mg. respectively, with $E_{11}G_1$ as substrate (0.2 mM-ATP). The specific activity reported for M_r -50000 kinase [25] is 202 nmol/min per mg with [Val⁵]antiotensin as substrate.

These kinases, TK-I and TK-II, differ from each other

Fig. 8. Reactivity of rabbit antiserum raised against TK-I with TK-I and TK-II

The immunodiffusion in agar plates was carried out by the method of Ouchterlony as described in [19]. The central well contained 0.01 ml of undiluted rabbit antiserum raised against TK-I. Wells 1, 2 and 3 contained TK-I, TK-II and phosphate-buffered saline respectively. After diffusion for 24 h at room temperature the slides were washed, stained with Coomassie Blue, destained, dried and photographed as described in [19].

Fig. 9. Immunoprecipitation of TK-I (lane 1) and TK-II (lane 2) by antiserum raised against TK-I

The details are described in the Materials and methods section.

in many properties. The strikingly different pattern of fragments obtained by V-8 protease digestion (Fig. 7) suggests that TK-I and TK-II are distinct proteins, perhaps coded by two different genes. This is supported by the observation that antiserum raised against TK-I does not show any cross-reactivity with TK-II. By using monoclonal antibodies which recognize the protein product of *c-src* gene, no cross-reacting protein was detected in spleen [26]. From this observation it was concluded that the *c-src* gene is expressed in spleen at a very low level [26]. Therefore it seems unlikely that any one of these kinases is the product of the *src* gene. However, these kinases could be the products of an as yet unidentified cellular oncogene. The protein products of *c-fgr* and some other cellular oncogenes, which are likely to be tyrosine kinases, have not been identified so far [27]. A lymphoma cell line, LSTRA, expresses a tyrosine-specific protein kinase of M_r 56000 [28]. The gene coding for this kinase, *lck*, is expressed at a relatively low level in spleen [29]. Whether TK-II described here is coded by the *lck* gene is a possibility that needs to be explored.

B and T lymphocytes isolated from rat spleen have been shown to have distinct tyrosine-specific protein kinases in the plasma-membrane preparations [30,31]. These kinases have not been isolated so far. The kinase activity from T-cell (but not from B-cell) membranes is inhibited by Tos-Lys-CH₂Cl [31]. In this respect TK-II described here is similar to the tyrosine-specific protein kinase activity from T lymphocytes.

Many normal tissues show appreciable tyrosine-specific protein kinase activity [7,8]. Some of these tyrosine-specific protein kinases have been partially purified. The properties of such kinases from liver, brain, spleen, lung, thymus and platelets differ from each other [8,17]. Although all of these kinases may not be different proteins, it is clear that there are several different tyrosine-specific protein kinases present in normal differentiated cells. Why should there be so many different tyrosine-specific protein kinases in normal cells? It seems unlikely that all or most of these tyrosine-specific protein kinases present in normal cells are involved in the regulation of cell growth only. One possible explanation for the presence of so many different kinases is that these kinases may be involved in the process of cell differentiation or in the maintenance of the differentiated state [32].

Incubation of TK-I with ATP results in an increase in its tyrosine-specific protein kinase activity. The following observations suggest that this ATP-dependent increase in kinase activity of TK-I may be due to the phosphorylation of the enzyme: (a) preincubation of TK-I with ADP or adenosine 5'-[β -imido]triphosphate does not activate the kinase; (b) the time courses of activation and autophosphorylation by ATP are very similar; (c) when the enzyme was purified in the absence of vanadate (which is an inhibitor of phosphotyrosine-specific protein phosphatase) it showed much more activation on incubation with ATP. Theoretical analysis by Lisman [33] has shown that a protein kinase which is activated by autophosphorylation (such as TK-I described here) can function as a molecular switch with only two stable states.

The kinase isolated from bovine spleen [25] showed a linear time course of peptide phosphorylation. From this observation, the authors concluded that autophosphorylation was not related to activation by ATP.

This conclusion is somewhat premature, since the kinase activity before and after preincubation with ATP was not measured. The linear time course implies that there is no activation during peptide phosphorylation. The high concentration of peptide (3 mg/ml) used during the assay [25] could perhaps inhibit autophosphorylation.

TK-II on incubation with ATP showed a major phosphoprotein of M_r 56000; some of the M_r -60000 component is also seen (Fig. 3). This is perhaps not due to contamination by TK-I, since this would have shown up in the immunoprecipitation experiments (Fig. 9). Since TK-II was not purified to homogeneity, this could be a contaminating protein. The other possibility is that it represents a different phosphorylated form of TK-II. It is relevant to point out here that a tyrosine-specific protein kinase from T lymphocytes can under certain conditions show phosphoproteins of M_r 56000, 59000, 64000 and 70000 [34].

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