Activation of a cellular tyrosine-specific protein kinase by phosphorylation

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Received 6 June 1985

A tyrosine-specific protein kinase of M_r 56000 was purified over 200-fold from rat spleen. Incubation of this kinase preparation with ATP and Mg^{2+} results in about 10-fold increase in the protein kinase activity. The activation of the kinase was unaffected in the presence of soyabean trypsin inhibitor. Polyacrylamide gel electrophoresis of the enzyme preparation after phosphorylation with ATP showed one phosphoprotein band of M_r 56000. During purification of this kinase a large decrease in enzyme activity was observed which could be prevented by adding 10 μM vanadate, as inhibitor of tyrosine-specific protein phosphatases. These results suggest that the activation of the protein kinase by ATP is due to phosphorylation of the enzyme.

Tyrosine kinase *Enzyme activation* *Rat spleen* *Autophosphorylation*

1. INTRODUCTION

Tyrosine-specific protein kinases were first reported in transformed cells [1]. Since then a variety of transformed and normal cells have been shown to have appreciable amounts of tyrosine-specific protein kinase activity. A survey of tyrosine-specific protein kinase activity in normal tissues by Swarup et al. [2,3] showed that rat spleen and human T lymphocytes had a very high level of this enzyme as compared to other tissues. Only a few attempts have been made to purify cellular tyrosine kinases which are not associated with receptors for growth factors. Partial purification of a tyrosine kinase of M_r 75000 has been reported from rat liver cytosol [4]. The particulate form of tyrosine-specific protein kinase from rat spleen has been solubilised and partially purified earlier [2]. We have now purified this enzyme over 200-fold. We have also found that incubation of this kinase preparation with ATP results in a large increase in its tyrosine-specific protein kinase activity. The activation of the kinase by ATP appears to be due to its phosphorylation.

2. MATERIALS AND METHODS

The tyrosine-specific protein kinase activity was measured by the phosphorylation of a synthetic peptide $E_{11}G_1$ (Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly) with [γ-^{32}P]ATP essentially as described by Swarup et al. [2]. The assay mixture contained 25 mM Hepes buffer (pH 7.5), 25 mM $MgCl_2$, 0.2 mM $E_{11}G_1$, 10 μM sodium orthovanadate, 0.2 mM [γ-^{32}P]ATP (200–400 cpm/pmol), 10% glycerol, and an aliquot of enzyme preparation (0.3 to 0.8 μg protein), in a total volume of 0.05 ml. After incubation for 15 min at 24°C the reaction was stopped by adding 0.05 ml of 10% trichloroacetic acid. The reaction mixture was then centrifuged for 2 min in an Eppendorf centrifuge and the assay continued as described previously [2].

The tyrosine-specific protein kinase from rat spleen was solubilized and purified on DEAE-Sephacel and Sephacryl S-200 columns as described earlier [2] but on a larger scale. During extraction of the kinase 0.25 mM phenylmethanesulfonyl fluoride was added. The kinase preparation after

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00145793/85/$3.30

the Sephacryl S-200 column step was further purified by chromatography on a 2′,5′-ADP-Sepharose column (5 ml) equilibrated with buffer A [25 mM *N*-ethylmorpholine acetate (pH 7.1), 0.5 mM EDTA, 10% glycerol, 0.1% Triton X-100, 7 mM 2-mercaptoethanol]. The column was washed with 10 ml buffer A and the kinase activity was eluted with 0.25 M NaCl in buffer A. Kinase active fractions (2 ml each) were pooled and dialysed to remove salt. This enzyme preparation showed one major Coomassie blue staining band of M_r 56000 and a few minor bands, after polyacrylamide gel electrophoresis in the presence of SDS. Overall purification achieved was at least 200-fold.

Protein concentration was estimated by the method of Bradford [5].

3. RESULTS AND DISCUSSION

When the tyrosine-specific protein kinase preparation was incubated with 0.1 or 0.2 mM ATP and Mg^{2+}, there was a several-fold increase in the tyrosine kinase activity as measured by using $E_{11}G_1$ as substrate. This increase in kinase activity was time dependent; maximal activation of the enzyme was obtained after 30–60 min of incubation of the enzyme with ATP. The activity of the enzyme did not increase on incubation without ATP. Similarly, Mg^{2+} was also required for the activation of the enzyme. However, incubation with Mg^{2+} alone (in the absence of ATP) did not activate the enzyme. Fig.1 shows the time course of peptide phosphorylation by the kinase preparation before and after incubation with ATP for 1 h. The phosphorylation proceeded linearly with time after incubation of the enzyme with ATP for 1 h, suggesting that the enzyme was fully activated within 1 h. The activation of the kinase was observed at all the concentrations of the peptide substrate tested (0.2–2.0 mM) as shown in fig.2. The activation of the kinase was largely due to an increase in V_{max}, although some decrease in K_m for the peptide substrate was also observed. The apparent V_{max} of this kinase before and after incubation with ATP was 33 and 270 nmol · min^{-1} · mg^{-1} (calculated from double-reciprocal plots), respectively. The apparent K_m for the peptide substrate decreased from 1.4 to 0.5 mM after incubation with ATP.

The kinase preparation was incubated with

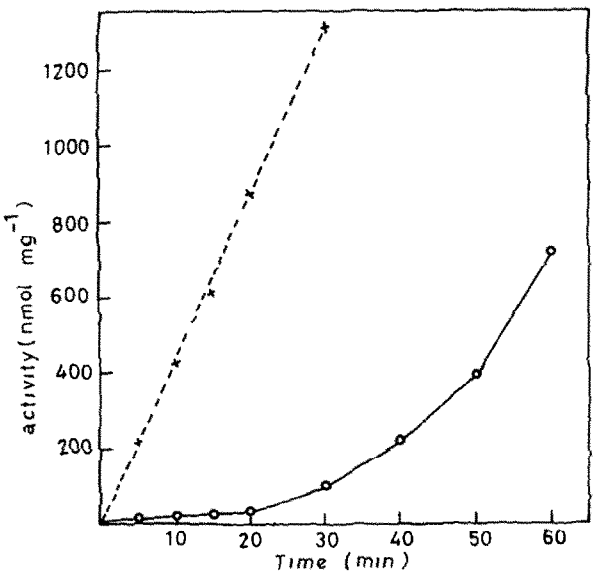

Fig.1. Time course of phosphorylation of $E_{11}G_1$ by the kinase preparation before (——) and after (– – –) incubation with 0.2 mM ATP for 1 h. The kinase assay was carried out using 0.2 mM $E_{11}G_1$ as substrate for the indicated period of time, as described in section 2.

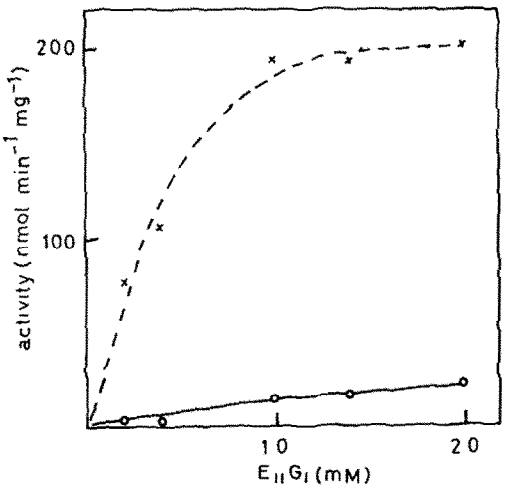

Fig.2. Phosphorylation of $E_{11}G_1$ by the tyrosine-specific protein kinase from rat spleen after incubation with and without ATP. The kinase preparation was incubated with 0.2 mM ATP (– – –) or without ATP (——) for 1 h at 24°C in the presence of 25 mM $MgCl_2$. The kinase assay was then started by adding various concentrations of $E_{11}G_1$ or $E_{11}G_1$ plus ATP. The assay was continued as described in section 2.

[γ-^{32}P]ATP and Mg^{2+}, and analysed by polyacrylamide gel electrophoresis in the presence of SDS followed by autoradiography. A major phosphoprotein band of M_r 56000 was observed. The phosphorylation of this band increased with time as shown in fig.3. This phosphoprotein of M_r

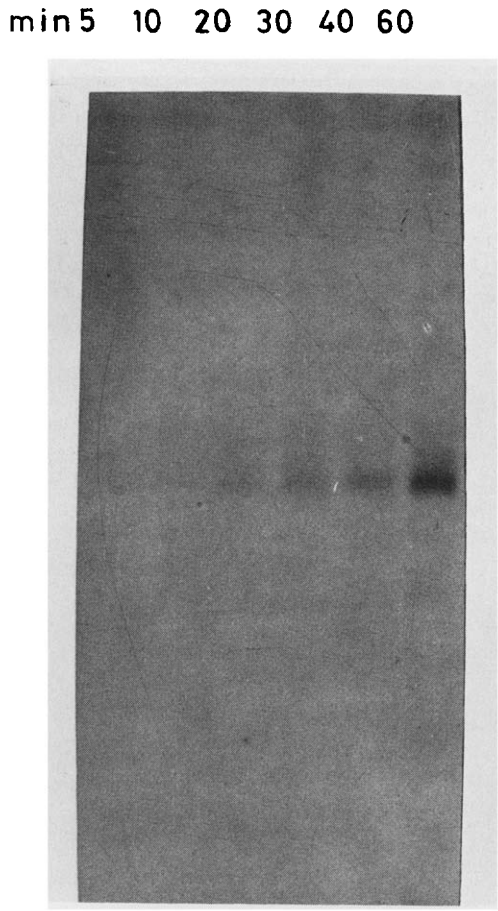

Fig.3. An autoradiograph showing phosphorylation of the tyrosine-specific protein kinase by ATP. An aliquot of the enzyme preparation (0.7 μg protein) was incubated with 10 μm [γ-^{32}P]ATP, 25 mM $MgCl_2$, and 10 μm orthovanadate in 25 mM Hepes buffer (pH 7.5), in a total volume of 0.05 ml. After incubating for the indicated time at room temperature, the reaction was stopped by adding 0.05 ml of a solution containing 6% SDS, 0.2 M dithiothreitol and 20% glycerol, in 50 mM Tris buffer (pH 7.1). The samples were then heated for 3 min at 100°C and electrophoresis was carried out using 10% polyacrylamide gel [8]. The gel was stained with Coomassie blue and dried for autoradiography.

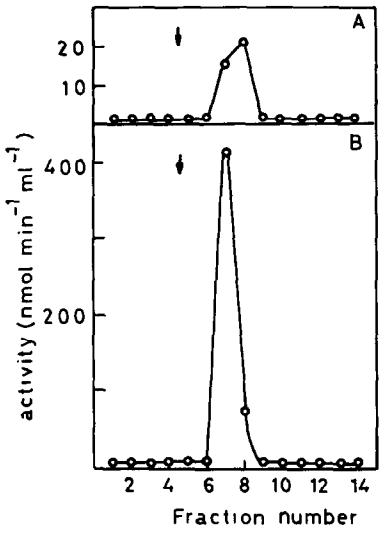

Fig.4. ADP-Sepharose chromatography of the tyrosine-specific protein kinase from rat spleen. Details are given in section 2. The purification was carried out in the absence (A) or presence (B) of 10 μM vanadate.

56000 is perhaps the tyrosine-specific protein kinase itself since this phosphoprotein comigrates with the kinase activity on gel filtration on the Sephacryl S-200 column [2] and also on ADP-Sepharose column chromatography (not shown).

During purification of this kinase a large decrease in enzyme activity was observed. This loss of kinase activity during purification could be prevented by including 10 μM sodium orthovanadate in all the buffers. Vanadate is known to be a potent inhibitor of tyrosine-specific protein phosphatases [6,7]. Fig.4 shows a comparison of the enzyme activities obtained after chromatography on an ADP-Sepharose column when the purification was carried out in the presence or the absence of vanadate. The enzyme purified in the presence of vanadate showed much less activation on incubation with ATP. These results are consistent with the suggestion that the active, phosphorylated form of this kinase is converted (during purification) into a less active (or inactive) form by the action of an endogenous tyrosine-specific protein phosphatase which is inhibited by vanadate.

The activation of the protein kinase does not appear to be due to proteolysis since the M_r of the phosphorylated enzyme on SDS-gel did not de-

crease after incubation with ATP for 60 min. In addition, the activation of the enzyme was observed even in the presence of soyabean trypsin inhibitor (0.2 mg/ml). It has been shown previously that this kinase phosphorylates itself at tyrosine residues [2]. Therefore, it is reasonable to suggest that the activation of the enzyme on incubation with ATP is due to its phosphorylation.

It has been shown recently that the insulin receptor kinase and $pp60^{src}$ kinase (the product of the *src* gene of Rous sarcoma virus) are activated on incubation with ATP [9,10]. It will be interesting to see whether other tyrosine-specific protein kinases are also activated by phosphorylation.

ACKNOWLEDGEMENTS

We are grateful to Drs P.M. Bhargava, Gopal Pande and K. Kannan for helpful comments on this manuscript.

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