Isolation and partial characterization of distinct forms of tyrosine protein kinases from rat spleen

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Three peaks of tyrosine protein kinase activity (TK-I, TK-II and TK-III) can be resolved when the extract of rat spleen particulate fraction is subjected to DEAE-cellulose gradient chromatography. TK-I and TK-II, insensitive to both EGF and insulin, have been further purified by Sephacryl S200 gel filtration and characterized. TK-I has an apparent m_R of 65000, by far prefers Mn^{2+} over Mg^{2+} as activator, can use GTP besides ATP as phosphate donor and is stimulated 2-3-fold by polylysine. TK-II, whose m_R approximates 50000, is equally activated by Mg^{2+} and Mn^{2+}, does not use GTP and is insensitive to polylysine. TK-I and TK-II can phosphorylate the synthetic peptide Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly (as well as its derivative with Orn in place of Arg), angiotensin II and poly(Glu, Tyr) 4:1 which exhibits different k_m values with TK-I and TK-II, (100 and 10 μM, respectively). When TK-I was incubated with [γ-^{32}P]ATP and $MnCl_2$ a doublet of alkali-stable radiolabeled bands with molecular masses of 55 and 60 kDa were observed. Under identical conditions TK-II gives rise to a single alkali-stable radiolabeled band of 51 kDa, which may represent the autophosphorylation product of TK-II itself.

Protein kinase *Phosphotyrosine* *Protein phosphorylation* *(Spleen)*

1. INTRODUCTION

It was not until recently that the phosphorylation of tyrosyl, rather than seryl and threonyl, residues of proteins was detected. The first reports concerned the tyrosine specific protein kinase activities associated with the transforming products of oncogenes [1–3], but it soon became clear that the phosphorylation of tyrosyl residues also occurs in normal tissues [3–5] where it may be involved in the regulation of cell growth since several receptors of polypeptide mitogens have been shown to possess tyrosine protein kinase activity [6–10].

Spleen displays an especially high tyrosine kinase activity when compared with other tissues [5]. Here we show, using synthetic peptides as substrates, that the tyrosine protein kinase activity associated with the particulate fraction of rat spleen can be resolved by DEAE-cellulose chromatography and gel filtration into at least 3 distinct fractions. Two fractions that are fairly stable have been characterized and shown to exhibit quite different properties.

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Abbreviations: $D_{10}G_1$ and $D_{10}G_1$-Orn, peptides Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly and its derivative with Orn in place of Arg; EGF, epidermal growth factor; SDS-PAGE, 11% polyacrylamide gel electrophoresis in 0.1% SDS; PMSF, phenylmethylsulfonyl fluoride; poly(Glu,Tyr) 4:1, synthetic random polymer with a 4:1 ratio of glutamic acid residues and tyrosine residues

2. MATERIALS AND METHODS

The ornithyl derivative of the synthetic peptide $D_{10}G_1$ (peptide code as in [5]) was prepared by the procedure in solution (to be described elsewhere); by subsequent amidination [11] it was converted into $D_{10}G_1$. Angiotensin II and the random

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polymer poly(Glu,Tyr) 4:1 were purchased from Sigma.

Rat spleen soluble and particulate fractions were prepared as in [5]. No significant tyrosine protein kinase activity could be detected in the soluble fraction using $D_{10}G_1$ as phosphorylatable substrate. The tyrosine protein kinase activity of the particulate fraction was solubilized by extraction with buffer A (100 mM Tris-HCl, pH 9, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 50 μM PMSF) including 3% Triton X-100. The extracts were dialyzed overnight against 25 mM Hepes, pH 7, containing 10% glycerol, 0.1% Triton X-100 and 1 mM EDTA before being subjected to the purification procedure (see fig.1).

Tyrosine protein kinase activity was routinely determined by incubating 50 μg of the peptide $D_{10}G_1$ (or its ornithyl derivative) at 30°C for 15 min in 50 μl of a medium containing 50 mM Tris, pH 7.5, 10 mM $MnCl_2$, 10 μM vanadate, 0.05% Triton X-100, 20 μM [γ-^{32}P]ATP (500–1000 cpm/pmol). In some experiments Mn^{2+} was replaced by other divalent cations and $D_{10}G_1$ was replaced with either angiotensin II or poly(Glu,Tyr) 4:1. The reaction was stopped with 50 μl of 5% trichloroacetic acid and an aliquot was spotted on phosphocellulose paper (Whatman P-81). The phosphocellulose paper squares were washed 4 times in 0.5% phosphoric acid and once in acetone. The radioactivity adsorbed on dried papers was determined in a liquid scintillator. Whenever poly(Glu,Tyr) 4:1 was the substrate the reaction was stopped with trichloroacetic acid (10% final concentration) and 200 μg bovine serum albumin was added. The precipitate was washed 3 times with trichloroacetic acid and counted in a liquid scintillator.

3. RESULTS

The profile of tyrosine protein kinase activity obtained by subjecting the Triton X-100 extract from rat spleen particulate fraction to DEAE-cellulose gradient chromatography is reported in fig.1A. It can be seen that 3 distinct peaks of activity, TK-I, TK-II and TK-III, are detectable whenever the peptide $D_{10}G_1$ or its derivative with Orn in place of Arg, is the substrate. By using the synthetic poly(Glu,Tyr) 4:1 random polymer as phosphate acceptor a superimposable pattern was

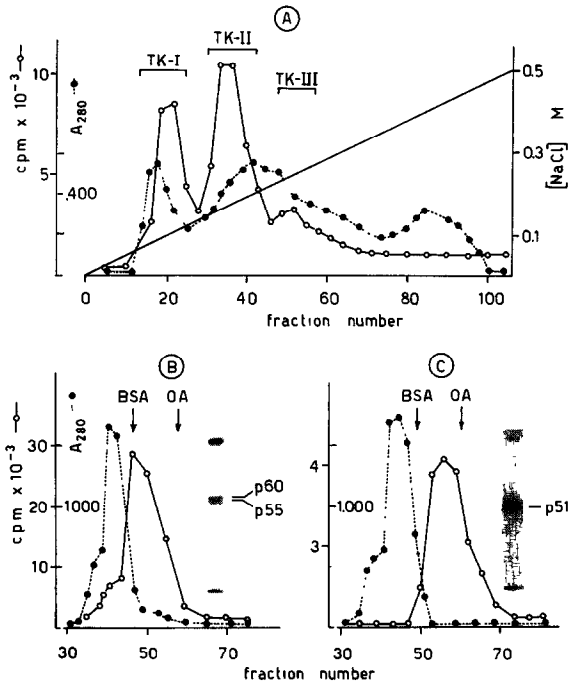

Fig.1. Isolation of distinct tyrosine protein kinase fractions by DEAE-cellulose and Sephacryl S200 chromatographies. (A) 180 ml of extract from rat spleen particulate fraction (see section 2) were applied to a DEAE-cellulose column (2.5 × 12 cm) equilibrated with 25 mM Hepes, pH 7, 10% glycerol, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol and 50 μM PMSF. The column was first washed with 150 ml of the equilibrium buffer which did not elute any tyrosine protein kinase activity. Then a linear NaCl gradient (0–0.5 M) in the same buffer was started. 3.6 ml fractions were collected and assayed for their absorbance at 280 nm and for tyrosine protein kinase activity toward the peptide $D_{10}G_1$-Orn (1.5 mM). (B,C) The first (TK-I) and second peak (TK-II) of tyrosine protein kinase activity were collected as indicated in (A), concentrated by ultrafiltration and separately submitted to gel filtration through a Sephacryl S200 column (2 × 80 cm) equilibrated and eluted with 125 mM Hepes, pH 7, 5% glycerol, 0.2 M KCl, 0.05 Triton X-100, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 μM PMSF. 3 ml fractions were collected. Proteins and protein kinase activity were evaluated as in (A). The arrows denote the elution volumes of bovine serum albumin (M_r 68000) and ovalbumin (M_r 45000). In the insets the autoradiographies are shown of TK-I and TK-II radiolabeled after the gel filtration step. Aliquots were incubated with [γ-^{32}P]ATP under conditions described in section 2, except for the absence of any phosphorylatable substrates, and subjected to SDS-PAGE. The slab was heated for 2 h in 1 N KOH at 56°C [12] and dried before autoradiography.

obtained although the relative sizes of the 3 peaks were somewhat different. With either the decapeptides or the random polymer, Tyr-^{32}P was the only radiolabeled phosphoamino acid detectable after acid hydrolysis (not shown).

TK-I and TK-II were further purified by Sephacryl S200 gel filtration (fig.1B, C) giving rise to single peaks of apparent M_r approximating 65000 and 50000, respectively. TK-III on the contrary was very unstable and once subjected to gel filtration its activity could no longer be recovered. Two peaks, corresponding to the elution volumes of TK-I and TK-II, could be resolved also by applying the crude Triton X-100 extract directly on the Sephacryl S200 column (not shown). If partially purified preparations of TK-I and TK-II are incubated with [γ-^{32}P]ATP in the presence of 10 mM Mn^{2+} and subjected to SDS-PAGE followed by treatment with NaOH and autoradiography, the patterns shown in the insets of fig.1B,C are obtained. TK-I gives rise to a doublet (or sometimes a triplet) of radiolabeled bands (p55 and p60) somewhat less mobile than the single band (p51) which is radiolabeled in TK-II. The intensity of these bands is not decreased by addition of large excess of peptide substrates and thermally inactivated TK-I and TK-II could not undergo phosphorylation at their specific bands by addition of TK-II and TK-I, respectively (not shown).

Both TK-I and TK-II require divalent cations for their activity, however, as shown in fig.2 the activation curves by Mn^{2+}, Mg^{2+} and Co^{2+} with the 2 tyrosine protein kinases are very different. In particular Mg^{2+}, which is almost as effective as Mn^{2+} with TK-II, is a relatively poor activator of TK-I. Co^{2+} on the other hand is less effective than the other 2 cations with TK-II while its effect on TK-I is quite peculiar; exhibiting maximal stimulation at about 10 mM followed by a dramatic fall of activity if its concentration is further increased. Neither Ca^{2+} nor Zn^{2+} can activate the tyrosine protein kinases; moreover Zn^{2+} acts as a powerful inhibitor displaying half-maximal inhibition at about 60 μM with both TK-I and TK-II (table 1). Under our conditions both enzymes are insensitive to EGF, insulin, and to 20 μM quercetin, which is a powerful inhibitor of the tyrosine protein kinase activity associated with the Rous sarcoma virus transforming gene product [13]. TK-I, unlike TK-II, is 2–3-fold stimulated by polylysine

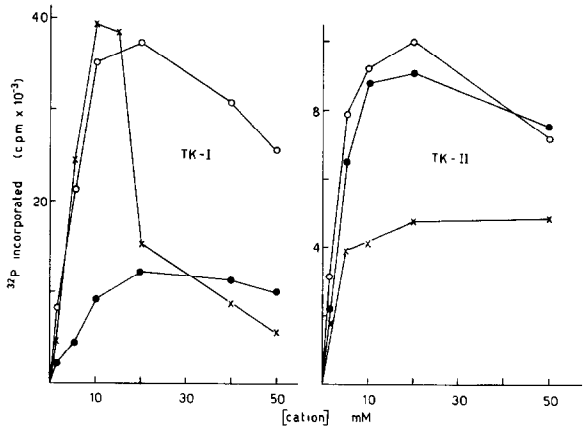

Fig.2. Activation of TK-I and TK-II by different divalent cations. The activities of partially purified TK-I (left) and TK-II (right) were tested on the peptide $D_{10}G_1$-Orn under standard conditions (see section 2) in the presence of increasing concentrations of either $MnCl_2$ (○) or $MgCl_2$ (●) or $CoCl_2$ (×).

Table 1

Response of TK-I and TK-II to various effectors

Effectors	Tyrosine protein kinase activity[a]	
	TK-I	TK-II
None	100	100
$ZnCl_2$ (50 μM)	68	74
$ZnCl_2$ (100 μM)	35	26
EGF (0.2 μM)	98	101
Insulin (0.1 μM)	101	98
Quercetin (20 μM)	103	100
Polylysine (20 μM)	257	99
Polyarginine (16 μM)	96	98
Spermine (2 mM)	101	103
GTP (25 μM)	69	75
GTP (150 μM)	12	76

[a] Expressed as percent of controls and determined on $D_{10}G_1$-Orn in the presence of 10 mM $MnCl_2$

(200 μg/ml) but not by polyarginine nor by spermine (table 1).

Another remarkable difference between TK-I and TK-II concerns their ability to use GTP instead of ATP as phosphate donor. While the radiolabeling of the tyrosyl peptide by TK-II is only slightly decreased, in a dose-independent man-

ner by the addition of up to 15-fold excess unlabeled GTP over [γ-^{32}P]ATP (table 1), the radiolabeling by TK-I is drastically and dose-dependently prevented by unlabeled GTP which behaves as a typically competitive inhibitor with respect to [γ-^{32}P]ATP (fig.3A). Conversely, the slight inhibition of TK-II by GTP is non-competitive in nature (fig.3B). The apparent K_i of GTP for TK-I was calculated to be 10 μM which is also the value of the apparent K_m of ATP with either TK-II or TK-I.

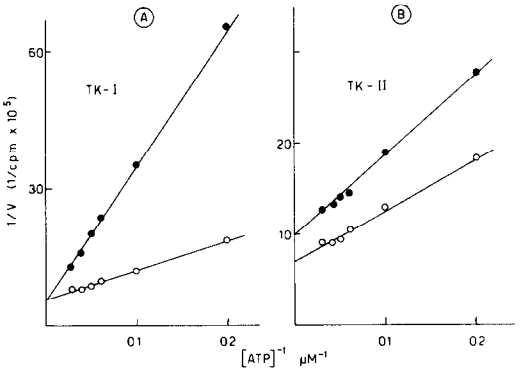

Fig.3. Effect of unlabeled GTP on the tyrosine protein kinase activities of either TK-I (left) or TK-II (right), as a function of increasing concentrations of [γ-^{32}P]ATP. The double-reciprocal plots are presented of kinetic experiments where the initial phosphorylation rates of $D_{10}G_1$-Orn (4 mg/ml) by increasing concentrations of [γ-^{32}P]ATP in the absence (○) and presence (●) of 50 μM unlabeled GTP were determined.

Table 2

Kinetic constants of tyrosyl peptide substrates with TK-I and TK-II

	Apparent K_m (mM)		Relative V_{max}	
	TK-I	TK-II	TK-I	TK-II
$D_{10}G_1$	2.0; 5.2	1.3; 6.0	100	100
Angiotensin II	2.0	0.77	34	53
Poly(Glu,Tyr) 4:1	0.10	0.009	88	70

The kinetic constants were calculated by the double-reciprocal plot method from initial velocities as a function of increasing concentration of peptide substrates. In the case of $D_{10}G_1$ with both enzymes a biphasic curve was obtained from which 2 limiting K_m values could be extrapolated. The K_m values of poly(Glu,Tyr) are expressed as molarity of tyrosine residues. The V_{max} are expressed relative to that of $D_{10}G_1$ (100%)

In table 2 the kinetic constants of tyrosyl peptide substrates with TK-I and TK-II are reported. While the V_{max} values vary in a similar way, significant differences in the K_m values were observed; in particular the K_m of poly(Glu,Tyr) 4:1 with TK-II is one order of magnitude lower than that with TK-I.

4. DISCUSSION

This report shows that the tyrosine protein kinase activity detectable with the aid of peptide substrates in rat spleen particulate fraction is heterogeneous, being resolved by DEAE-cellulose chromatography and Sephacryl S200 gel filtration into at least 3 different fractions (TK-I, TK-II and TK-III). No attempt has been made to establish whether these enzymes are differently distributed between T and B lymphocyte populations which have been recently shown to express distinct tyrosine protein kinases [14,15].

While the remarkable instability of TK-III hindered its characterization, the properties of the other 2 fractions have been studied and compared. TK-I by far prefers Mn^{2+} over Mg^{2+} as activator, is stimulated by polylysine, it can also use GTP besides ATP as phosphate donor and it exhibits an M_r of 65000 by gel filtration. Once partially purified TK-I still exhibits a doublet of alkali-stable phosphorylatable bands whose molecular mass values determined by SDS-PAGE (55 and 60 kDa) are reminiscent of 2 bands which are phosphorylated at tyrosine in B lymphocytes [14,15] and which were previously detected in a crude tyrosine protein kinase preparation from spleen [5]. This coincidence as well as their similar behaviour on DEAE-cellulose and their ability to use GTP may suggest the identification of TK-I with that enzyme. On the other hand the properties of TK-II are quite distinctive: its M_r by gel filtration approximates 50000, it is maximally stimulated by Mg^{2+} as well as by Mn^{2+}, insensitive to polylysine and does not exhibit competitive kinetics with unlabeled GTP. Likely TK-II as well as TK-III were not detected in a previous study on spleen tyrosine protein kinases [5] since they bind

rather tightly to DEAE-cellulose, being eluted by NaCl concentrations higher than those employed in [5].

Although all the tyrosine peptide substrates used in this study are affected by both enzymes, the substrate specificities of TK-I and TK-II are not identical considering that the K_m of poly(Glu,Tyr) with TK-I is one order of magnitude greater than that determined with TK-II. The very low K_m values of this acidic polymer relative to other peptide substrates would indicate that acidic residues play a crucial positive role in determining the site recognition by both TK-II and TK-I. Consequently the biphasic saturation curves obtained with $D_{10}G_1$ (Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly) could be interpreted assuming 2 binding sites for this peptide; a high-affinity site (low K_m) recognizing the N-terminal acidic residues (and also responsible for the binding of poly(Glu,Tyr)) and a low-affinity site (high K_m) which seemingly may recognize the C-terminal cluster of arginyl residues. It should also be noted that the substrate specificities of TK-I and TK-II are quite different from those recently reported for EGF and insulin receptor kinases [16].

Although the endogenous substrates of the tyrosine kinase described here remain unknown, a doublet of bands of 54 and 55 kDa and a single 51 kDa band become radiolabeled at tyrosine(s) whenever the partially purified preparations of TK-I and TK-II, respectively, are incubated with [γ-^{32}P]ATP and Mn^{2+}. It has not been unambiguously established whether these radiolabeled bands are protein substrates or represent the autophosphorylation products of the tyrosine protein kinases. The latter hypothesis, however, seems more likely considering that such a phosphorylation, similar to other intramolecular autophosphorylations [17,18], is neither prevented by large excess of peptide substrate, nor can be restored by adding active TK-II to thermally inactivated TK-I and vice versa. Such a conclusion would also be consistent with the M_r values of the radiolabeled bands determined by SDS-PAGE which correspond fairly well with the M_r values of TK-I and TK-II evaluated by gel filtration under non-denaturing conditions.

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REFERENCES

[1] Erikson, R.L., Collet, S.M., Erikson, E.L. and Purchio, A.F. (1979) Proc. Natl. Acad. Sci. USA 76, 6260–6264.
[2] Witte, O.N., Dasgupta, A. and Baltimore, D. (1980) Nature 283, 826–831.
[3] Hunter, T. and Sefton, B.M. (1980) Proc. Natl. Acad. Sci. USA 77, 1311–1315.
[4] Wong, T.W. and Goldberg, A.R. (1983) Proc. Natl. Acad. Sci. USA 80, 2529–2533.
[5] Swarup, G., Dasgupta, J.D. and Garbers, D.L. (1983) J. Biol. Chem. 258, 10341–10347.
[6] Ushiro, H. and Cohen, S. (1980) J. Biol. Chem. 255, 8363–8365.
[7] Ek, B., Westermark, B., Wasteson, A. and Heldin, C.-H. (1982) Nature 295, 419–420.
[8] Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M. and Kahn, C.R. (1982) Nature 298, 667–669.
[9] Jacobs, S., Kull, F.C., Eurp, H.S., Svoboda, M.E., Van-Wyck, J.J. and Cuatracasas, P. (1983) J. Biol. Chem. 258, 9581–9584.
[10] Rubin, J.B., Shia, M.A. and Pilch, P.F. (1983) Nature 305, 438–440.
[11] Marchiori, F., Borin, G., Filippi, B., Moretto, V., Bonora, G.M. and Toniolo, C. (1979) Int. J. Peptide Protein Res. 14, 143–152.
[12] Cooper, J.A. and Hunter, T. (1981) Mol. Cell. Biol. 1, 165–168.
[13] Graziani, Y., Erikson, E. and Erikson, R.L. (1983) Eur. J. Biochem. 135, 583–589.
[14] Harrison, M.L., Low, P.S. and Geahlen, R.L. (1984) J. Biol. Chem. 259, 9348–9350.
[15] Earp, H.S., Austin, K.S., Gillespie, G.Y., Buessow, S.C., Davies, A.A. and Parker, P.J. (1985) J. Biol. Chem. 260, 4351–4357.
[16] Klein, H.H., Freidenberg, G.R., Cordera, R. and Olefsky, J.M. (1985) Biochem. Biophys. Res. Commun. 127, 254–263.
[17] Meggio, F. and Pinna, L.A. (1984) Eur. J. Biochem. 145, 593–599.
[18] Rees-Jones, R.W. and Taylor, S.I. (1985) J. Biol. Chem. 260, 4461–4467.