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Partial purification and characterization of cytosolic Tyr-protein kinase(s) from human erythrocytes

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Tyrosine-protein kinase, phosphorylating tyrosine residues of transmembrane band 3 protein, has been partially purified from human erythrocyte cytosol by DEAE-Sepharose chromatography followed by heparin-Sepharose chromatography.

Such a Tyr-protein kinase (36 kDa), as distinct from the Ser/Thre-protein kinases (casein kinase S and TS), appears to display a broader site specificity than does the previously described human erythrocyte *P*-Tyr-protein phosphatase, dephosphorylating band 3 protein. That is, it is able to phosphorylate not only the highly acidic copolymer poly(Glu-Tyr)$_{4:1}$ but also angiotensin II, lacking an acidic amino acid sequence around the target Tyr residue. Moreover, the phosphorylation of these two substrates exhibits a different pH dependence and a different response to NaCl and 2,3-bisphosphoglycerate.

These results suggest that in intact erythrocytes the cytosolic Tyr-protein kinase might phosphorylate band 3 not only on Tyr-8, surrounded by several acidic side-chains (as demonstrated preferentially to occur in isolated ghosts), but also on other Tyr residues surrounded by other amino acid sequences.

The multifunctional transmembrane band 3 protein (95 kDa) of human erythrocytes has been found to be endogenously phosphorylated not only on serine/threonine residues [1] but also on tyrosine residues [2].

Up to now attention has been focused on the characterization of human erythrocyte Tyr-protein kinase(s) associated with membrane structures [3–7].

The present research has been undertaken to characterize the endogenous Tyr-protein kinase(s) located in the cytosol and presumably involved in the Tyr phosphorylation of band 3, taking into account the fact that the endogenous phosphorylation of this transmembrane protein has been found [2] to occur preferentially on its NH_2-terminal region (23 kDa). Since this region protrudes from the membrane bilayer into the cytoplasm, it is expected to be accessible to the cytosolic protein kinases.

On the other hand, human erythrocyte *P*-Tyr-protein phosphatase(s), dephosphorylating band 3 *P*-tyrosine residues, has been found to be located predominantly in the cytosol [8, 9].

The results reported here indicate that cytosolic Tyr-protein kinase(s), and *P*-Tyr-protein phosphatase(s), involved in the Tyr phosphorylation state of band 3 protein, do not exhibit the same substrate specificity. That is, the Tyr-protein kinase(s) described here is able markedly to phosphorylate not only the very acidic random copolymer poly(Glu-Tyr)$_{4:1}$ but also angiotensin II, lacking acidic amino acid sequences around the target Tyr residue. By contrast, the *P*-Tyr-protein phosphatase, previously described [8, 9], actively dephosphorylates [^{32}P]*P*-Tyr-poly(Glu-Tyr) but very poorly, if at all, [^{32}P]*P*-Tyr-angiotensin II.

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Enzyme. Tyr-protein kinase (EC 2.7.1.112)

MATERIALS AND METHODS

Human erythrocytes (9 ml packed cells), free of leucocytes and platelets, were prepared from venous blood (45 ml) (freshly collected from healthy donors), according to Beutler et al. [10]. Thereafter they were washed once in isotonic phosphate buffer pH 8 and lysed in hypotonic (5 mM) phosphate buffer pH 8 according to Dodge et al. [11], except that the solutions contained 0.05 mM phenylmethylsulphonyl fluoride.

The membranes were recovered by centrifugation (at 20000 × *g* for 20 min) and the red hemolysate supernatant was made 39% (mass/vol.) in ammonium sulfate (60% saturation) with continuous stirring. After 60 min at 0 °C, the precipitate was collected by centrifugation and dissolved in 100 mM Hepes buffer pH 7.5 containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM mercaptoethanol and 0.1 mM phenylmethylsulphonyl fluoride and then dialyzed overnight against buffer A (25 mM Hepes pH 7.0, 10% glycerol, 0.1% Triton X-100, 10 mM mercaptoethanol, 1 mM EDTA, 0.05 mM phenylmethylsulphonyl fluoride and 0.02% NaN_3). The dialyzed ammonium sulfate fraction was applied to a DEAE-Sepharose CL-6B column (2 × 12 cm) equilibrated with buffer A. The column was first washed with equilibrium buffer until no more reddish substance emerged from the column and was subsequently eluted with 400 ml of a linear NaCl gradient (0–0.7 M) in buffer A. 3-ml fractions were collected and assayed for Tyr-protein kinase and casein kinase activity.

The peak fraction of Tyr-protein kinase activity was pooled as indicated by the bar in Fig. 1, dialyzed overnight against buffer A and then applied to a heparin-Sepharose CL-6B column (1.5 × 3 cm) previously equilibrated with buffer A. The subsequent elution was carried out with 180 ml 0–0.8 M NaCl linear gradient in buffer A. 1.9-ml fractions were collected and assayed for Tyr-protein kinase and casein kinase activity. All the above operations were carried out in a cold room at 0 °C.

Assay of protein kinases

Tyr-protein kinase activity was assayed by incubating the enzyme sample (30 µl) at 30 °C for 10–30 min in a medium (100 µl) containing 100 mM Hepes buffer pH 7.5, 20 µM vanadate, 5 mM $MnCl_2$, 5 µM [γ-^{32}P]ATP (2.5×10^6 cpm/nmol) and 4 µg poly(Glu-Tyr)$_{4:1}$ or 250 µg angiotensin II.

Casein kinase activity was assayed under the same conditions, except that poly(Glu-Tyr)$_{4:1}$ was replaced by 100 µg whole casein as substrate, and 5 mM $MnCl_2$ by 10 mM $MgCl_2$.

When poly(Glu-Tyr) or casein was the substrate, the reaction was stopped by addition of 3.6 ml 10% trichloroacetic acid, 0.4 ml silicotungstic acid solution [12] and 0.1 mg phosvitin as carrier, followed by centrifugation. The precipitate was washed four times with the above stopping solution and counted by a liquid scintillation counter.

When angiotensin II was the substrate, the reaction was stopped by addition of 100 µl 5% trichloroacetic acid and an aliquot was spotted on phosphocellulose paper (Whatman P-81). The phosphocellulose paper squares were washed four times in 0.5% phosphoric acid and once in acetone. The radioactivity adsorbed on dried papers was counted by a liquid scintillation counter. 1 unit of Tyr-protein kinase was defined as the amount of enzyme which catalyzed the incorporation of 1 pmol ^{32}P into poly(Glu-Tyr)$_{4:1}$ (or 0.35 pmol into angiotensin II) in 1 min under the above standard conditions. 1 unit of casein kinase was defined as the amount of enzyme catalyzing the incorporation of 1 pmol ^{32}P into whole casein.

Phosphorylation of the membrane proteins

In order to remove hemoglobin, the pink native ghosts recovered by centrifugation of red hemolysate were washed once with lysis buffer and twice with 25 mM Hepes buffer pH 7.5 containing 0.1 mM EDTA, 0.1 mM mercaptoethanol and 0.1 mM phenylmethylsulphonyl fluoride.

The phosphorylation of membrane proteins was assayed by incubating the white ghosts (100–250 µg protein) at 30 °C for 15 min in 125 µl reaction mixture containing 75 mM Pipes buffer pH 6.5, 8 µM vanadate, 50 µM EDTA, 5 mM $MnCl_2$ (or 20 mM $MgCl_2$), 5 µM [γ-^{32}P]ATP (6×10^6 cpm/nmol) and the protein kinase sample (varying amounts). The incubation was stopped by adding 12 µl 20% sodium dodecylsulfate (SDS) (final concn 2%), as described in [13]. An aliquot of the solubilized membranes (approx. 40 µg) was subjected to electrophoresis on 0.1% SDS/10% polyacrylamide slab gels, essentially according to Laemmli [14]. After electrophoresis the slab gels were stained with Coomassie brilliant blue according to Laemmli [14]. Some of the gels were treated in a 2 M NaOH solution at 55 °C for 1 h and then fixed again. Dried gels were autoradiographed at −80 °C with intensifying screens. Alkali-treated and untreated gels were exposed for the same length of time.

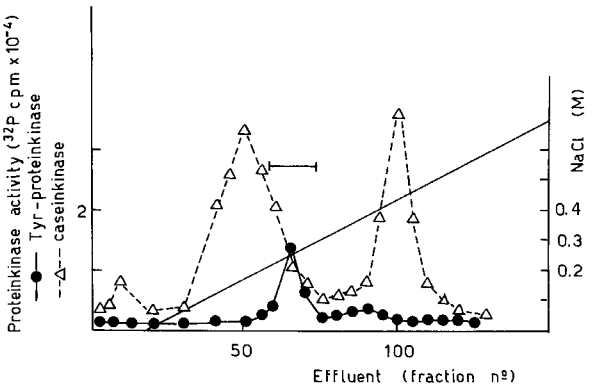

Fig. 1. *DEAE-Sepharose chromatographic profile displayed by protein kinase activity of human erythrocyte cytosol.* (●——●) Tyr-protein kinase activity on poly (Glu-Tyr)$_{4:1}$; (△– – –△) casein kinase activity on whole casein. Experimental conditions for the chromatography and the protein kinase assay as described in Materials and Methods

Phosphoamino acid analysis of ^{32}P-labelled membranes

A sample (about 300 µg protein) of ^{32}P-labelled membranes was hydrolysed in 6 M HCl for 2 h at 100 °C. An equal amount of ^{32}P-labelled membranes, prior to acid hydrolysis, was treated in a 2 M NaOH solution at 55 °C for 1 h and then dialyzed against H_2O to remove NaOH. The two acid-hydrolysed samples were separately lyophilized, redissolved in H_2O and lyophilized again to remove HCl and finally analyzed by two-dimensional electrophoresis on cellulose thin-layer plates (DC-Fertigplatten CE 20 × 20 cm) at pH 1.9 (H_2O/formic acid/acetic acid 897:25:78) for 120 min at 2000 V and then at pH 3.5 (H_2O/acetic acid/pyridine 89:10:1) for 90 min at 1000 V.

Miscellaneous

Angiotensin II, phosvitin, bovine serum albumin and poly(Glu-Tyr)$_{4:1}$ (ranging from 20 kDa to 50 kDa; reported average approx. 43 kDa) were purchased from Sigma; casein from Merck. [^{32}P]ATP from Amersham International. Cellulose thin-layer plates (DC-Fertigplatten CE 20 × 20) were from Riedel-deHaën AG (Seelze-Hannover).

Protein content was determined according to [15].

RESULTS

Purification of Tyr-protein kinase

When the dialyzed ammonium sulfate fraction from the red hemolysate supernatant was submitted to DEAE-Sepharose chromatography, as described in Materials and Methods, the elution profile of protein kinase activity reported in Fig. 1 is obtained.

As shown in Fig. 1, Under these conditions the previously described [16] cytosolic casein kinase activity (assayed on whole casein) is eluted from the column into two clearly distinct peaks: the less retarded peak (eluted at 0.17 M NaCl) was proven to be casein kinase S, being able to phosphorylate whole casein almost exclusively on serine residues, whereas the more retarded peak (eluted at 0.42 M NaCl) is due to casein kinase TS, being able to phosphorylate whole casein on both serine and threonine residues to about the same extent [16]. On the other hand, Tyr-protein kinase [assayed on poly(Glu-Tyr)$_{4:1}$] is eluted into a single peak at 0.25 M NaCl,

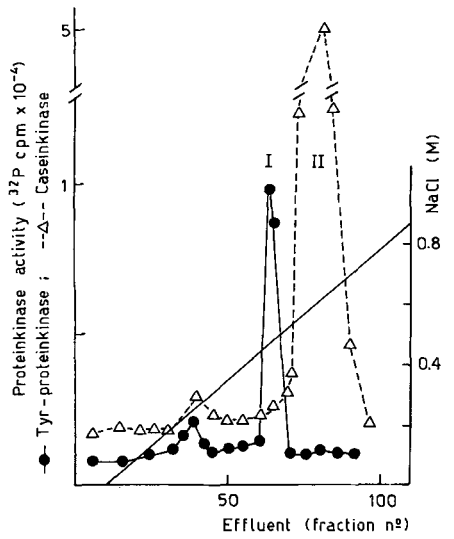

Fig. 2. *Heparin-Sepharose chromatographic profile of protein kinase activity displayed by DEAE-Sepharose peak of Tyr-protein kinase, pooled as indicated by the bar in Fig. 1.* (●——●)Tyr-protein kinase activity assayed on poly (Glu-Tyr)$_{4:1}$; (△---△) casein kinase activity assayed on whole casein

Table 1. *Purification of Tyr-protein kinase from cytosol of human erythrocytes (9 ml packed cells)*

Procedure	Activity	Protein	Specific activity	Yield	Purification
	units	mg	units/mg	%	fold
Crude hemolysate	944	1.487	0.635	100	–
Ammonium sulfate fraction	1.054	43.8	24.0	110	37.8
DEAE-Sepharose chromatography	918	12.45	73	97	114
Heparin-Sepharose chromatography	287.7	0.684	420	30	660

well separated from casein kinase TS, but heavily contaminated by the leading edge of the casein kinase S peak.

Further purification of Tyr-protein kinase is achieved when the DEAE-Sepharose peak, pooled as indicated by the bar in Fig. 1, is submitted to heparin-Sepharose chromatography (Fig. 2), which allows the separation of the Tyr-kinase from most of casein kinase S activity. This procedure yields 30% of the initial Tyr-protein kinase activity and an enzyme preparation (peak I) with specific activity of 420 pmol min^{-1} mg^{-1} (Table 1). This enzyme preparation (Fig. 2, peak I), stored at −5 °C, is stable for several weeks. However, when submitted to molecular filtration on an Ultrogel Ac44 column, equilibrated and eluted with buffer A containing 0.1% Nonidet and 0.5 M NaCl), it undergoes a dramatic loss of activity. The remaining activity was recovered from the column at an elution volume corresponding to an apparent molecular mass of 36 kDa.

Therefore the properties described below have been studied by using the heparin-Sepharose peak I.

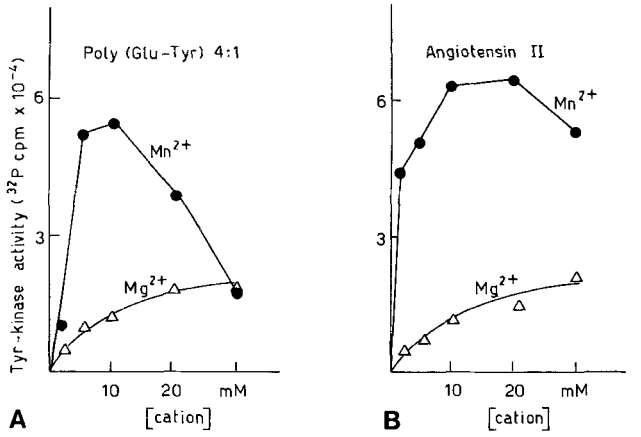

Fig. 3. *Effect of Mn^{2+} and Mg^{2+} on Tyr-protein kinse activity assayed on poly(Glu-Tyr)$_{4:1}$ (Fig. 3A) and on angiotensin II (Fig. 3B).* (●——●) Mn^{2+}; (△---△) Mg^{2+}

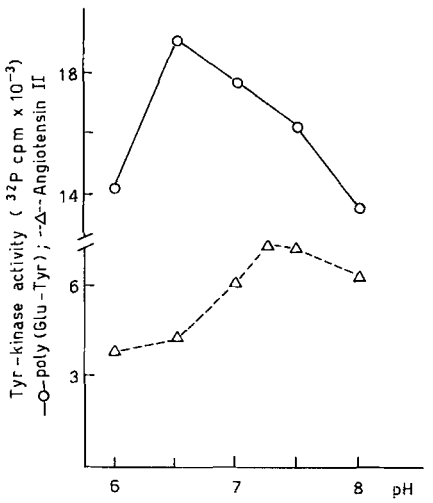

Fig. 4. *pH dependence profile of Tyr-protein kinase-catalyzed phosphorylation of poly(Glu-Tyr)$_{4:1}$ (○——○) and angiotensin II (△---△)*

Properties of Tyr-protein kinase

Cytosolic Tyr-protein kinase is able markedly to phosphorylate angiotensin II in addition to copolymer poly(Glu-Tyr)$_{4:1}$.

Fig. 3 shows the response of the phosphorylation of these two substrates to Mg^{2+} and Mn^{2+} as function of the cation concentration. Mn^{2+} appears to be more effective than Mg^{2+}. However, as the cation concentration increases, the activation by Mg^{2+} follows normal saturation kinetics, rising to a limiting level at higher cation concentrations (over approx. 20 mM), while the activation by Mn^{2+} rises to a maximum level at lower cation concentrations (up to 10–15 mM) and thereafter declines, thus suggesting that Mn^{2+} displays two opposite effects on phosphorylation of these two substrates, depending on cation concentration.

The enzyme is active between pH 6 and 8. In this range, however, the pH-dependence profile exhibited by the phosphorylation of the two substrates is different, the maximal activity occurring at pH 6.5–7.0 for poly(Glu-Tyr) and at pH 7.3–7.5 for angiotensin II (Fig. 4).

The phosphorylation of angiotensin II is practically unaffected or slightly inhibited by rising NaCl concentrations up

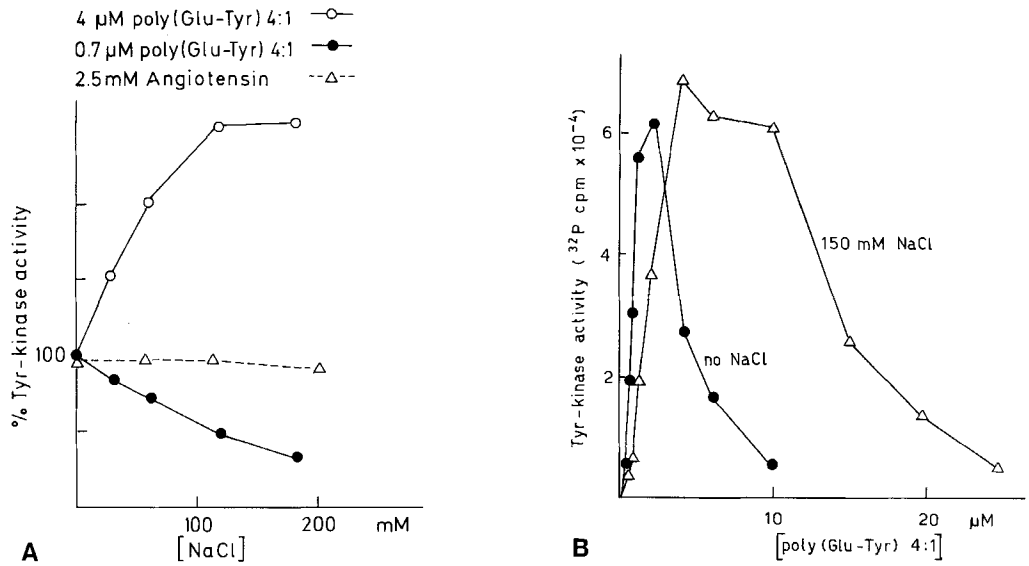

Fig. 5. *(A) Effect of NaCl on Tyr-protein-kinase-catalyzed phosphorylation of angiotensin II and of poly(Glu-Tyr)$_{4:1}$ at two different concentrations. (B) Substrate-dependence of Tyr-protein kinase activity on poly(Glu-Tyr)$_{4:1}$ in the absence or in the presence of 150 mM NaCl.* The molarities of poly(Glu-Tyr)$_{4:1}$ are related to the concentration of polymer

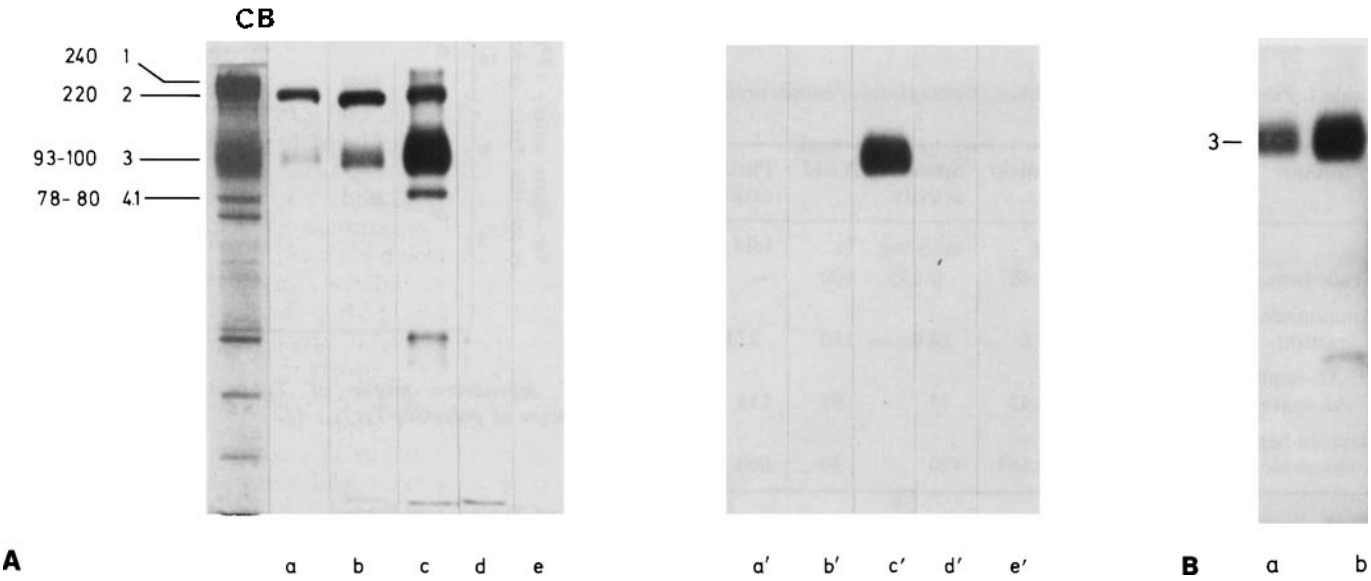

Fig. 6. *(A) Autoradiograms showing the phosphorylation patterns of erythrocyte ghost proteins by the two heparin-Sepharose peaks in the presence of 5 mM Mn^{2+}. (B) Autoradiograms (alkali-treated gels) showing the Tyr phosphorylation of band 3 protein(40 μg ghost protein) by the same amount of heparin-Sepharose peak I in the presence of 20 mM Mg^{2+} (lane a) or 5 mM Mn^{2+} (lane b).* (A) Lanes a′—e′ show the alkali-treated gels corresponding to the alkali-untreated gels a—e. Human erythrocyte ghosts (40 μg/protein) were incubated alone (lanes a and a′) or in the presence of (lane b,b′) a peak II sample containing 16 units of casein kinase and 0.1 unit Tyr-protein kinase; (lanes c,c′) a peak I sample containing 4 units Tyr-protein kinase and 2.1 units casein kinase. Lanes d,d′ and e,e′: phosphorylation controls of samples from peak II and peak I, respectively, incubated alone (enzyme blanks). The lane CB shows the Coomassie-blue-stained gel. On the left the protein bands are designated by Steck's nomenclature [17] and their corresponding molecular masses are reported (kDa). Autoradiograms were exposed for 24 h

to 200 mM (Fig. 5A). By contrast, the response of poly(Glu-Tyr) phosphorylation to NaCl depends on the concentration of the substrate, i.e. the phosphorylation is inhibited at lower concentrations of poly(Glu-Tyr), while it is enhanced at higher substrate concentrations, probably because of reversal of inhibition by the substrate (Fig. 5B).

The apparent K_m value for angiotensin II, evaluated from Lineweaver-Burk plots, is 3.8 mM. The apparent K_m value for poly(Glu-Tyr)$_{4:1}$ can not be accurately determined owing to the inhibitory effect of this substrate at concentrations above approximately 2 μM in the absence of added NaCl or 5 μM in the presence of 150 mM NaCl (Fig. 5B). However,the apparent K_m value, evaluated by extrapolating the linear part of Lineweaver-Burk plots and expressed as the molarity of the polymer (molecular mass approx. 43 kDa), is approximately 5 μM in the absence of added NaCl and approximately 20 μM in the presence of 150 mM NaCl.

The phosphorylation of both substrates, in the presence of Mg^{2+}, is inhibited by increasing the concentration of 2,3-bisphosphoglycerate (not shown). However, the phosphoryla-

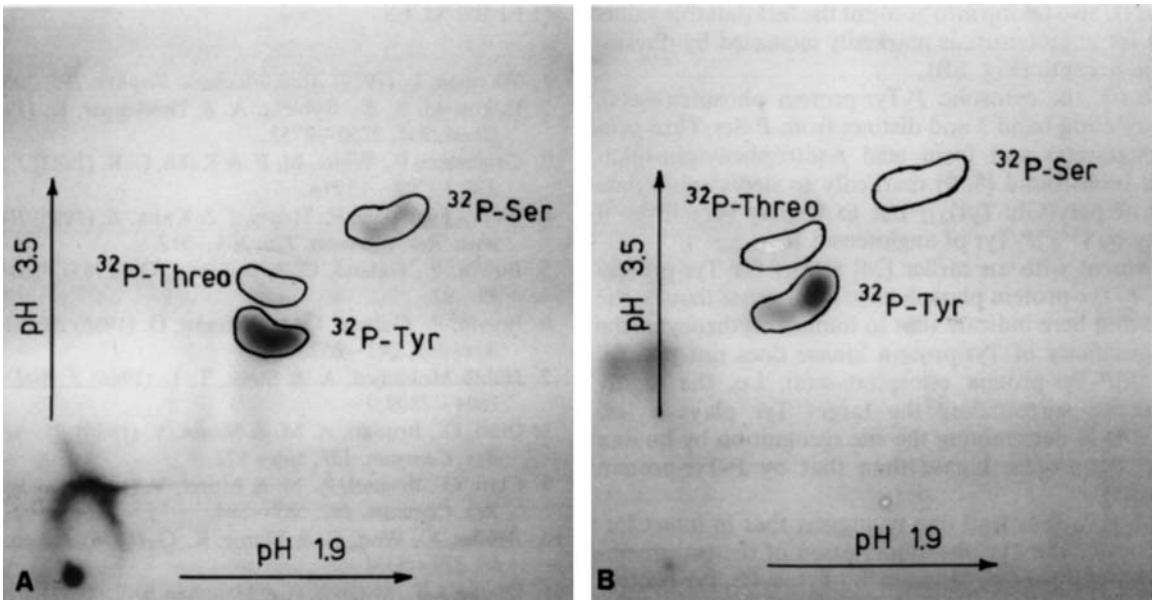

Fig. 7. *Autoradiograms showing the electrophoretic analysis of ^{32}P-phosphoamino acids in acid hydrolysate of ^{32}P-labelled membranes phosphorylated by heparin-Sepharose peak I.* ^{32}P-labelled membranes prior to acid hydrolysis (6 M HCl at 110 °C for 2 h) were submitted (B) or not (A) to alkali treatment (2 M NaOH at 55 °C for 1 h). Experimental conditions for the two-dimensional thin-layer electrophoresis were as in Materials and Methods. Standards of phosphotyrosine, phosphoserine and phosphothreonine were added to the hydrolysed sample and visualized by ninhydrin staining. Location of these three standards is indicated by circles. Autoradiograms were exposed for 4 days

tion of angiotensin II is less sensitive (about 15% inhibition by 3 mM 2,3-bisphosphoglycerate) than that of poly(Glu-Tyr) (about 45% inhibition). The effect of 2,3-bisphosphoglycerate could not be assayed in the presence of Mn^{2+}, since this cation has been found to interact with 2,3-bisphosphoglycerate giving rise to an insoluble complex (gel).

Phosphorylation of band 3

Fig. 6A shows the SDS-PAGE patterns of P^{32}-labelled membrane proteins obtained when the isolated native ghosts from human erythrocytes are separately incubated, in the presence of 5 mM Mn^{2+}, with the two protein kinase peaks obtained by heparin-Sepharose chromatography.

The endogenous phosphorylation (lane a) of spectrin β subunit (band 2) and of band 3 is differentially increased by the two heparin-Sepharose fractions.

A sample of peak II [containing 16 units of casein kinase S and negligible Tyr-poly(Glu-Tyr) kinase activity] enhances the ^{32}P labelling of both band 2 and band 3 (lane b) although to a much lesser extent than in the presence of Mg^{2+} (not shown). By contrast, the peak I sample (4 units of Tyr kinase ans 2.1 units of casein kinase S) greatly increases predominantly, if not exclusively, the ^{32}P labelling of band 3 (lane c).

Moreover, the peak-I-catalyzed phosphorylation of band 3 involves most predominantly, if not exclusively, Tyr residues as suggested by its alkali-stable ^{32}P labelling in alkali-treated gel (lane c′). This is confirmed by the [^{32}P]phosphoamino acid analysis (Fig. 7) of membrane proteins before and after alkali treatment. The two-dimensional thin-layer electrophoretic analysis reported in Fig. 7 shows that (a) the membrane proteins, in the presence of 5 mM Mn^{2+}, are phosphorylated by heparin-Sepharose peak I on Tyr residues and, to a lesser extent, on Ser residues, but negligibly on Thre residues (Fig. 7A); (b) the alkali-resistant [^{32}P]*P*-Tyr is the sole [^{32}P]phosphoamino acid detectable in the alkali-treated membranes (Fig. 7B). [^{32}P]*P*-Ser being hydrolysed by such alkali treatment [18, 19]. In contrast, the peak-II-catalyzed phosphorylation of bands 2 and 3 involves [^{32}P]*P*-Ser residues as indicated by the disappearance of ^{32}P labelling in alkali-treated gels (Fig. 6A, lane b′) [18, 19].

These observations indicate that band 3 is strongly phosphorylated on Tyr residues by cytosolic Tyr-protein kinase(s), (Fig. 6A, lane c′), but very poorly, if at all, by casein kinase S (Fig. 6A, lane b′), closely resembling casein kinase I [20] and casein kinase A [21]. The apparent K_m value, evaluated from Lineweaver-Burk plots, is approximately 3.7 μM, assuming that band 3 (95 kDa) constitutes approximately 30% of total ghost protein [22]. The phosphorylation of band 3 in the presence of 5 mM Mn^{2+}, is 50% inhibited by physiological ionic strength (150 – 160 mM NaCl).

The Tyr phosphorylation of band 3 occurs also in the presence of Mg^{2+}, although to a much lesser extent (Fig. 6B). However, in this context, it is worth noting that in red cells Tyr phosphorylation of band 3 might be activated by Mg^{2+} rather than by Mn^{2+}, this latter being by far less abundant (approx. 5 μM) than Mg^{2+} (4 – 5 mM) (reported in [23]). The Mg^{2+}-activated phosphorylation of band 3 is inhibited (about 20%) by physiological levels (3 mM) of 2,3-bisphosphoglycerate.

DISCUSSION

The above results show that cytosolic Tyr-protein kinase(s), phosphorylating band 3 protein, is able to phosphorylate angiotensin II besides the acidic copolymer poly(Glu-Tyr)$_{4:1}$, although the former synthetic peptide lacks the polyacidic amino acid sequence around the target Tyr residue. In this context it is of interest to underline the fact that the apparent K_m value for poly(Glu-Tyr)$_{4:1}$, expressed as tyrosine molarity, is about 1 mM, comparable to that for

angiotensin II, also taking into account the fact that this value, unlike that for angiotensin, is markedly increased by physiological ionic strength (Fig. 5B).

In contrast, the cytosolic *P*-Tyr-protein phosphatase(s), dephosphorylating band 3 and distinct from *P*-Ser/Thre-protein phosphatase(s) and from acid *p*-nitrophenylphosphatase(s), has been found [8, 9] markedly to dephosphorylate [^{32}P]*P*-Tyr of poly(Glu-Tyr)$_{4:1}$ but to display very little, if any, activity on [^{32}P]*P*-Tyr of angiotensin II.

In agreement with an earlier [24] report for Tyr-protein kinase and *P*-Tyr-protein phosphatase from other tissues, the results reported here indicate that in human erythrocytes the substrate specificity of Tyr-protein kinase does not parallel that of [^{32}P]*P*-Tyr-protein phosphatase(s), i.e. the highly acidic sequence surrounding the target Tyr plays a less important role in determining the site recognition by human erythrocyte Tyr-protein kinase than that by *P*-Tyr-protein phosphatase(s).

These observations lead one to suggest that in intact human erythrocytes the Tyr phosphorylation of the transmembrane band 3 cytoplasmic domain by cytosolic Tyr-protein kinase(s) might involve not only the Tyr-8-residue (at position 8 near the NH_2 terminus), surrounded by several acidic sidechains (as shown to occur preferentially by the endogenous phosphorylation in isolated ghosts [2]), but also other Tyr residues surrounded by other peptide sequences [25].

The our knowledge the present research is the first report on Tyr-protein kinase(s) isolated from hemolysate supernatant (cytosol) of human erythrocytes.

In recent years two membrane-associated Tyr-protein kinases have been described: one solubilized from human erythrocyte ghosts by non-ionic detergents, such as Triton X-100 [3] or Nonidet P40 [4], while the other is extracted (solubilized) from the detergent-insoluble pellet (membrane skeleton) by 0.25 M NaCl [5–7]. Both these membrane-associated Tyr kinases have been found to be capable of phosphorylating *in vitro* the band 3, although the physiological substrate (band 3 [4] or insulin receptor [3]) of detergent-extracted enzyme is still a matter for controversy.

The interrelationship between these membranous Tyr-protein kinases and the cytosolic enzyme described here remains to be established.

It is likely that the membranous NaCl-extracted and cytosolic Tyr-kinase activities are mediated by the same enzyme distributed between the membrane and the cytosol, partitioning equilibrium being regulated by ionic strength and/or by metabolic control. However, despite the presence of phenylmethylsulphonyl fluoride (proteolysis inhibitor) in the phosphate buffers, pH 8, used for washing of the erythrocytes and for preparation of hemolysate supernatant, the possibility that cytosolic and membranous enzymes may be generated from each other by limited proteolysis cannot be completely ruled out.

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