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# Comparative characterization of membrane-associated and cytosolic Tyr-protein kinases in human erythrocytes

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In recent years, two protein-tyrosine kinase activities, phosphorylating tyrosine residues on the transmembrane band-3 protein, have been isolated from human erythrocyte membranes and partially characterized by different laboratories, i.e. one extracted by non-ionic detergent (Triton X-100 or Nonidet P-40), the other solubilized by 0.25 M NaCl from the detergent-insoluble residue.

The present paper shows that these two membrane-associated Tyr-protein kinases purified, in the presence of bovine serum albumin, by phosphocellulose chromatography followed by heparin-Sepharose chromatography, have the same apparent molecular mass (36 kDa) determined by Ultrogel Ac44 filtration. Moreover, both Tyr-protein kinases exhibit several identical properties, including $K_m$ values for band 3, the random acidic copolymer poly(Glu,Tyr)$_{4:1}$ and angiotensin II, pH dependence, response to $Mn^{2+}$ and $Mg^{2+}$, response to NaCl and 2,3-bisphosphoglycerate.

All these properties are identical or very similar to those exhibited by the Tyr-protein kinase previously isolated by us from human erythrocyte cytosol.

These results suggest that the two membrane-associated and the cytosolic Tyr-protein kinase activities are mediated by the same enzyme, distributed between the cytosol and the membrane structures.

Most Tyr-protein kinases described to date have been found to be associated with the plasma membrane of mammalian tissues. In the past few years, two Tyr-protein kinases phosphorylating the transmembrane band-3 protein have been isolated and partially characterized from human erythrocyte membranes, i.e. one extracted from native ghosts by non-ionic detergent [1] and the other from the detergent-insoluble residue by 0.25 M NaCl [2–4]. More recently, a Tyr-protein kinase capable of phosphorylating band-3 protein has been isolated by our laboratory [5] from cytosol (hemolysate supernatant). The interrelationship between cytosolic and membranous Tyr-kinases remained to be established.

This prompted us to investigate whether, in human erythrocytes, the cytosolic and membranous Tyr-protein kinase activities are catalyzed by the same or by distinct enzymes, taking into account that in other blood cells (such as platelets [6] and bone marrow erythroid cells [7]), the cytosolic and membranous Tyr-protein kinase activities have been suggested to be catalyzed by unrelated enzymes.

The present paper shows that, in human erythrocytes, both membranous Tyr-kinase activities solubilized by non-ionic detergent or by 0.25 M NaCl share several physicochemical and catalytic properties with the cytosolic Tyr-protein kinase, thus suggesting that they are mediated by the same enzyme distributed between the cytosol and the membrane structures.

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*Abbreviation.* BSA, bovine serum albumin.

*Enzyme.* Tyr-protein kinase, protein-tyrosine kinase (EC 2.7.1.112).

## MATERIALS AND METHODS

Human erythrocytes, free of leucocytes and platelets, were prepared according to Beutler et al. [8] from human blood (about 320 ml), freshly collected from healthy donors. 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. [9], except that the buffered solutions contained 0.05 mM phenylmethylsulphonyl fluoride. The membranes (ghosts) were separated from the red hemolysate supernatant by centrifugation (at 20000 × *g* for 20 min).

### *Membrane-associated Tyr-protein kinases*

The pelleted membranes (about 300 mg protein, equivalent to 120 ml packed erythrocytes) were washed twice with lysis buffer to remove hemoglobin and twice more with buffer A (25 mM Hepes pH 7.6, 0.1 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% glycerol and 0.02% $NaN_3$) and then extracted with buffer A containing 0.1 mM EGTA, 0.5% Triton X-100, 0.5% Nonidet P-40 and 0.5 mg/ml BSA for 1 h at 0 °C, with gentle stirring.

The supernatant (referred to as detergent extract) was recovered by centrifugation at 105000 × *g* for 30 min. The pellet was then extracted again under the same above conditions and the supernatant recovered by centrifugation. The final detergent-insoluble residue was suspended in 100 ml

buffer A containing 0.05% Triton X-100, 0.05% Nonidet P-40, 0.5 mg/ml BSA and 0.25 M NaCl and allowed to stand at 0°C with continuous gentle stirring. After 60 min, the supernatant (referred to as NaCl-extract) was recovered by centrifugation. The two pooled detergent extracts, and the NaCl extract were separately dialyzed against buffer B (buffer A containing 0.1% Triton, 0.1% Nonidet and 0.5 mg/ml BSA) and then submitted to sequential chromatographic procedures on phosphocellulose, heparin-Sepharose and Ultrogel Ac44, under the conditions reported below.

*Sequential chromatography of detergent extract*

The two dialyzed detergent extracts, pooled together, were submitted to phosphocellulose chromatography on a column (3.2 × 7 cm) pre-equilibrated with buffer B. The column was first washed with about 150 ml buffer B and then eluted with a 500 ml linear gradient of 0–0.8 M NaCl in buffer B. 3.6-ml fractions were collected and assayed for Tyr-protein kinase and casein kinase activity.

The phosphocellulose peak of Tyr-protein kinase, pooled as indicated by the bar in Fig. 1, was applied to a heparin-Sepharose column (1.5 × 5 cm) previously pre-equilibrated with buffer B. The column was first washed with 50 ml buffer B and then eluted with 180 ml of a linear 0–0.8 M NaCl gradient in buffer B. 1.4-ml fractions were collected and assayed for Tyr-protein kinase activity.

The heparin-Sepharose peak of Tyr-protein kinase, pooled as indicated by the bar in Fig. 3A, was submitted to gel filtration on an Ultrogel Ac44 column (2 × 87 cm), pre-equilibrated and eluted with buffer B containing 0.5 M NaCl (see Fig. 4A). 1.8-ml fractions were collected and assayed for Tyr-protein kinase activity. All chromatographic procedures were carried out in a cold room at 0–4°C.

*Chromatography of NaCl extract*

The NaCl extract was dialyzed against buffer B and then submitted to the sequential chromatographic procedures on phosphocellulose (see Fig. 2), heparin-Sepharose (see Fig. 3B) and Ultrogel Ac44 (see Fig. 4B), under the conditions described above for the detergent extract.

*Chromatography of cytosolic Tyr-protein kinase(s)*

The red hemolysate supernatant was brought to 60% saturation with ammonium sulfate with continuous stirring. After 60 min at 0°C, the precipitate was collected by centrifugation, dissolved in and dialyzed against buffer A containing 0.1% Triton X-100 and 0.1% Nonidet P-40, before being submitted to sequential chromatographic procedures (on phosphocellulose, heparin-Sepharose and Ultrogel Ac44) under the conditions described above for the membrane extracts, except that the buffer employed for equilibrating and eluting the phosphocellulose column lacked BSA.

*Tyr-protein kinase activity assay*

Tyr-protein kinase activity was assayed by incubating the enzyme sample (20 µl) in the presence of 0.7 µM poly-$(Glu,Tyr)_{4:1}$ or 2.5 mM angiotensin II as substrate, under the conditions previously described [5].

One unit of Tyr-protein kinase was defined as the amount of enzyme which catalyzed the incorporation of 1 pmol $^{32}P$ into angiotensin II [or 3 pmol into $poly(Glu,Tyr)_{4:1}$] in 1 min.

*Phosphorylation of human erythrocyte membrane proteins*

The white ghosts (100–250 µg protein) were phosphorylated by the protein kinase sample (varying amounts of the phosphocellulose peak) under the conditions described in a previous paper [5], except that the Pipes buffer pH 6.5 was replaced by 75 mM Hepes buffer, pH 7.5. After incubation, an aliquot (≈ 40 µg) of $^{32}P$-labelled membrane proteins was analyzed by SDS/PAGE as previously described in [5].

*Phosphoamino acid analysis of $^{32}P$-labelled membrane proteins*

Acid hydrolysis (6 M HCl at 110°C for 2 h) of $^{32}P$-labelled membrane proteins (300 µg protein) before and after alkali treatment (2 M NaOH at 55°C for 1 h) and two-dimensional electrophoresis of the acid hydrolysate on cellulose thin-layer plates were carried out under the conditions previously described [5].

*Miscellaneous*

Angiotensin II, $poly(Glu,Tyr)_{4:1}$ (molecular mass ranging over 20–50 kDa; average ≈ 43 kDa) were purchased from Sigma; casein from Merck; [$\gamma$-$^{32}P$]ATP from Amersham International plc, UK; cellulose thin-layer plates (DC-Fertigplatten CE 20 × 20 cm) were from Riedel de Haën, Seelze, FRG.

Phosphocellulose, heparin-Sepharose CL-6B and Ultrogel Ac44 were from Sigma, Pharmacia and LKB, respectively. Protein content was determined according to [10].

## RESULTS

*Detergent-extracted Tyr-protein kinase activity*

When detergent extract, prepared from human erythrocyte ghosts as described in Materials and Methods, was submitted to phosphocellulose chromatography in the presence of BSA, the elution pattern shown in Fig. 1A was obtained.

The Tyr-protein kinase activity was recovered as a single peak eluted at 0.38 M NaCl and was slightly contaminated by casein kinase activity, whereas band 3 was recovered in the excluded fraction (Fig. 1B), as it did not adsorb to the resin.

As shown by the SDS/PAGE patterns of the eluted fractions, no band-3 protein was detectable by Coomassie-blue staining in the eluted fractions displaying Tyr-protein kinase activity, thus suggesting that the enzyme was removed, by the ionic strength of chromatographic buffer, from band 3, to which it has been suggested [4] to be electrostatically bound in the native membranes.

It has to be pointed out that when the same volume of detergent extract was submitted to phosphocellulose chromatography in the absence of BSA in the eluting buffer, the Tyr-protein kinase peak eluted from the column was much smaller, suggesting that the enzyme, once removed from band 3, undergoes a drastic loss of activity unless it is stabilized by the addition of BSA [4]. For this reason, BSA was also added to the buffered solutions employed for the extraction of residual Tyr-protein kinase activity from the detergent-insoluble residue and for its characterization by the sequential chromatographic procedures described below.

*NaCl-extracted Tyr-protein kinase activity*

The Tyr-protein kinase extracted by 0.25 M NaCl from the detergent-insoluble residue, as described in the Materials

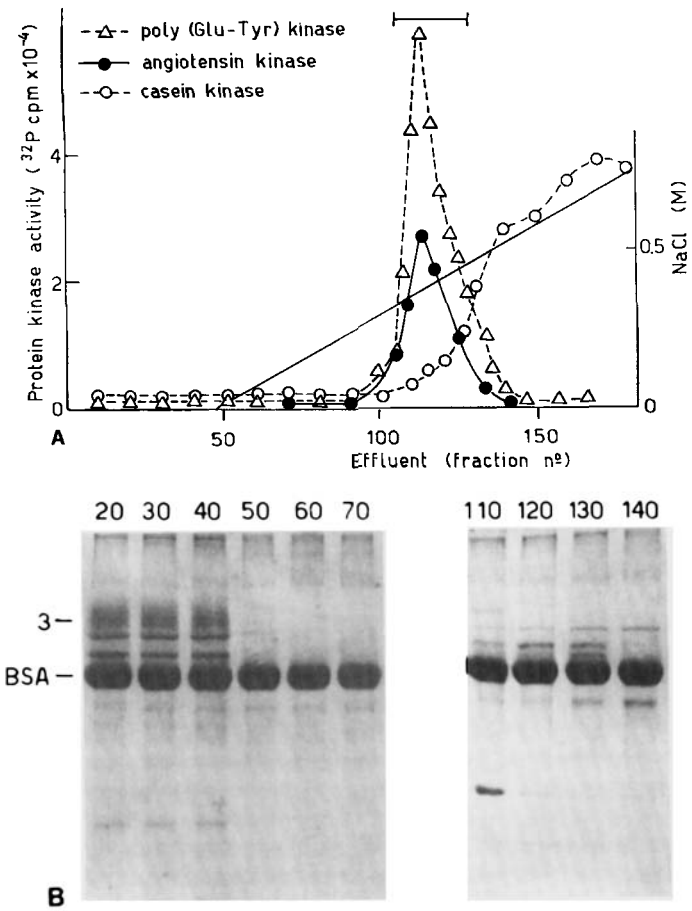


Fig. 1. *Phosphocellulose chromatography profile of Tyr-protein kinase activity of detergent extract from human erythrocyte membranes.* Experimental conditions for the chromatography and for the Tyr-protein kinase assay as described in Materials and Methods. (A) Phosphocellulose column profile; (B) Coomassie-blue-stained patterns of selected fractions from the column analyzed by SDS/PAGE as described in Materials and Methods. The numbers of the lanes correspond to those of the eluted fractions

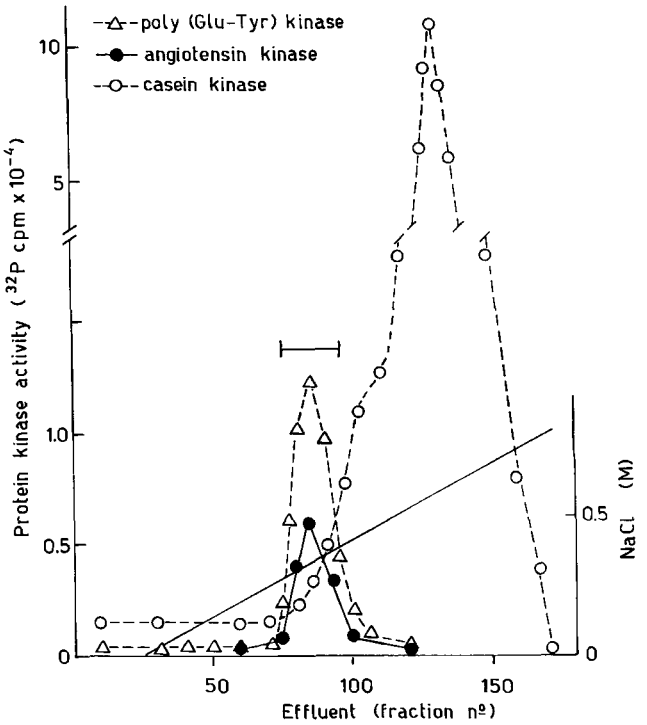


Fig. 2. *Phosphocellulose chromatography profile of Tyr-protein kinase activity of NaCl extract from the detergent-insoluble residue of human erythrocyte membranes.* The Tyr-protein kinase activity was assayed on poly(Glu,Tyr)$_{4:1}$ (△), angiotensin II (●) and whole casein (○). Experimental conditions for chromatography and for Tyr-protein kinase assay as in Materials and Methods

and Methods, exhibited the same chromatographic behaviour on phosphocellulose chromatography (Fig. 2) as the Tyr-protein kinase extracted by detergent, being eluted in a single peak at 0.38 M NaCl (contaminated by casein kinase activity).

Based on the angiotensin kinase activity of the two phosphocellulose peaks, we estimated, in agreement with [1], that approximately 90% of membrane-associated Tyr-protein kinase was recovered in the detergent extract. (The comparative assay in the crude extracts was difficult, because the Tyr-protein kinase activity of the detergent extract was masked by the high blank values due to the endogenous phosphorylation of band 3.)

The two phosphocellulose peak fractions of Figs 1 and 2 were employed for studying the properties reported below.

### *Chromatographic properties of membranous Tyr-protein kinases*

Both detergent- and NaCl-extracted Tyr-protein kinases, recovered in the phosphocellulose peaks of Figs 1 and 2, exhibited the same chromatographic behaviour on heparin-Sepharose chromatography (Fig. 3), both being eluted at 0.47 M NaCl. Both detergent- and NaCl-extracted Tyr-protein kinases (recovered in the heparin-Sepharose peaks) exhibited the same behaviour, when submitted to molecular filtration on an Ultrogel Ac44 column at high ionic strength (0.5 M NaCl) in the presence of BSA, both being recovered at the same elution volume, corresponding to an apparent molecular mass of 36 kDa (Fig. 4).

### *Kinetic properties of membranous Tyr-protein kinases*

Both detergent extracted and NaCl-extracted Tyr-protein kinase preparations were able to phosphorylate not only poly(Glu,Tyr)$_{4:1}$ but also angiotensin II although with much lower affinity. The apparent $K_m$ value of both enzymes for angiotensin II was 3.4 mM and for poly(Glu,Tyr) was 5–10 μM, depending on the NaCl concentration present in the medium (Table 1).

Both Tyr-protein kinases exhibited the same pH-dependence profile (not shown), the maximal activity occurring at pH 7.5–8.3 for poly(Glu,Tyr) phosphorylation and at pH 7–7.5 for angiotensin II phosphorylation (Table 1).

Fig. 5 shows the response to $Mn^{2+}$ and $Mg^{2+}$ of poly(Glu,Tyr) and angiotensin II phosphorylation catalyzed by detergent-extracted Tyr-protein kinase.

The activation by $Mg^{2+}$, as a function of cation concentration, followed nearly normal kinetics, rising to a limiting level (saturation level), whereas the activation by $Mn^{2+}$ rose to a maximal level at 2–3 mM and thereafter declined more or less dramatically.

A similar response to $Mn^{2+}$ and $Mg^{2+}$, as a function of their increasing concentration, was exhibited by the poly(Glu,Tyr) (Fig. 6A) and angiotensin-II phosphorylation (Fig. 6B) catalyzed by the NaCl-extracted Tyr-protein kinase.

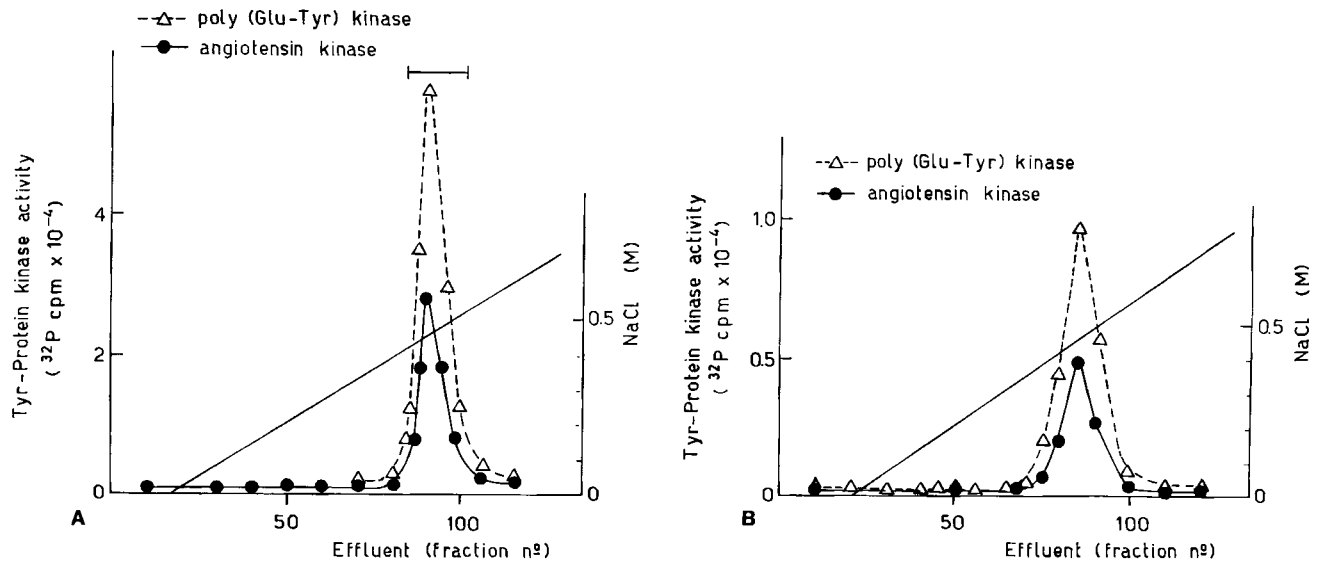


Fig. 3. *Heparin-Sepharose chromatography profiles of detergent-extracted and NaCl-extracted Tyr-protein kinases recovered in the phosphocellulose peaks of Figs 1 and 2, respectively.* (A) Detergent extract; (B) NaCl extract

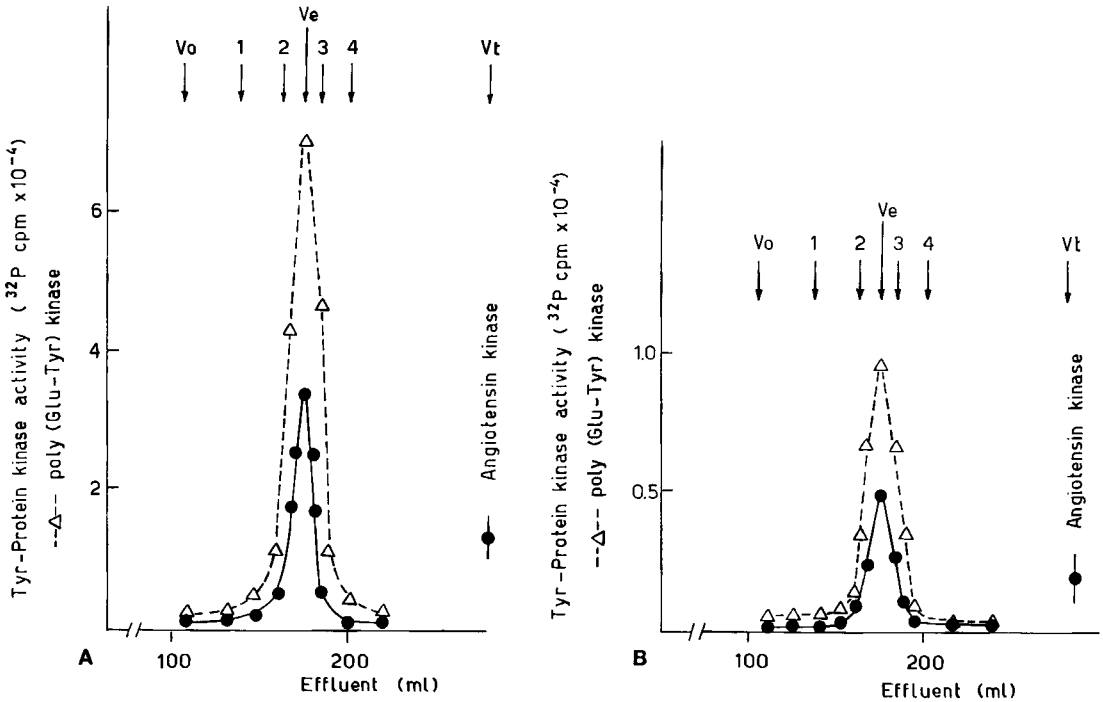


Fig. 4. *Ultrogel Ac44 filtration profiles of detergent-extracted and NaCl-extracted Tyr-protein kinases recovered in the heparin-Sepharose peaks of Fig. 3 A and B, respectively.* The arrows indicate the elution volume of Tyr-protein kinase ($V_e$) and of the standard proteins chromatographed under the same conditions: (1) bovine serum albumin (68 kDa); (2) ovalbumin (45 kDa); (3) carbonic anhydrase (29kDa); (4) chymotrypsinogen (25 kDa). $V_o$, void volume; $V_t$, total volume of the chromatographic bed; $V_e$, elution volume of the enzyme. (A) Detergent extract; (B) NaCl extract

Both Tyr-protein kinases exhibited an identical response to NaCl, i.e. phosphorylation of 0.7 μM poly(Glu,Tyr) by both enzymes was inhibited by increasing NaCl concentration (approximately 25% by 150 mM NaCl), whereas the phosphorylation of angiotensin II was unaffected or even stimulated (approximately 10% by 150 mM NaCl) (Table 1).

*Phosphorylation of band-3 protein in native membranes*

When the native membranes from human erythrocytes were separately incubated, in the presence of 5 mM $Mn^{2+}$, with the two membrane Tyr-protein kinase preparations (phosphocellulose peaks), calibrated on the basis of having the same angiotensin-II kinase activity, and subsequently

Table 1. *Properties of cytosolic and membrane-associated Tyr-protein kinases*

| Parameter | Tyr-protein kinase from | | |
|---|---|---|---|
| | cytosol | detergent extract | NaCl extract |
| $K_m$ for poly(Glu,Tyr)$_{4:1}$ in the presence of 150 mM NaCl | 5 μM | 8 μM | 7 μM |
| $K_m$ for angiotensin II | 3.8 mM | 3.4mM | 3.4 mM |
| $K_m$ for band-3 protein | 3.7 μM | 2 μM | 2.3 μM |
| Apparent molecular mass (kDa) | 36 | 36 | 36 |
| Optimal pH range for poly(Glu,Tyr) phosphorylation | 6.5–7.5 | 7.5–8.3 | 7.5–8.3 |
| Optimal pH range for angiotensin phosphorylation | 7.3–7.5 | 7.0–7.5 | 7.0–7.5 |
| Optimal pH range for band-3 phosphorylation | 7.0–8.5 | 7.0–8.5 | 7.0–8.5 |
| Elution from phosphocellulose at NaCl concentration | 0.26–0.45 M | 0.30–0.43 M | 0.28–0.42 M |
| Elution from heparin-Sepharose at NaCl concentration | 0.40–0.50 M | 0.40–0.52 M | 0.37–0.52 M |
| Inhibition of poly(Glu,Tyr) phosphorylation by 150 mM NaCl | 30% | 23% | 25% |
| Inhibition of band-3 phosphorylation by 150 mM NaCl | 45% | 48% | 46% |
| Response of angiotensin phosphorylation to 150 mM NaCl | none | none | none |

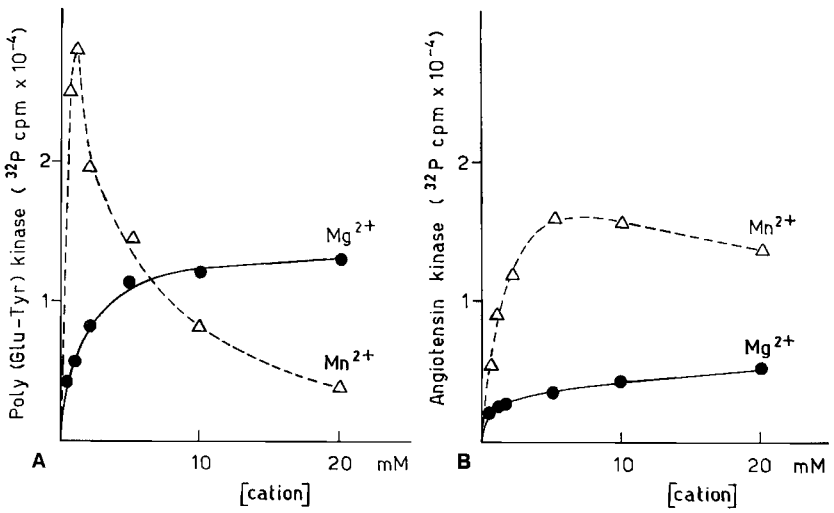


Fig. 5. *Effect of $Mn^{2+}$ and $Mg^{2+}$ on detergent-extracted Tyr-protein kinase activity.* (A) 1 μM Poly(Glu,Tyr)$_{4:1}$ as substrate; (B) 2 mM angiotensin II as substrate

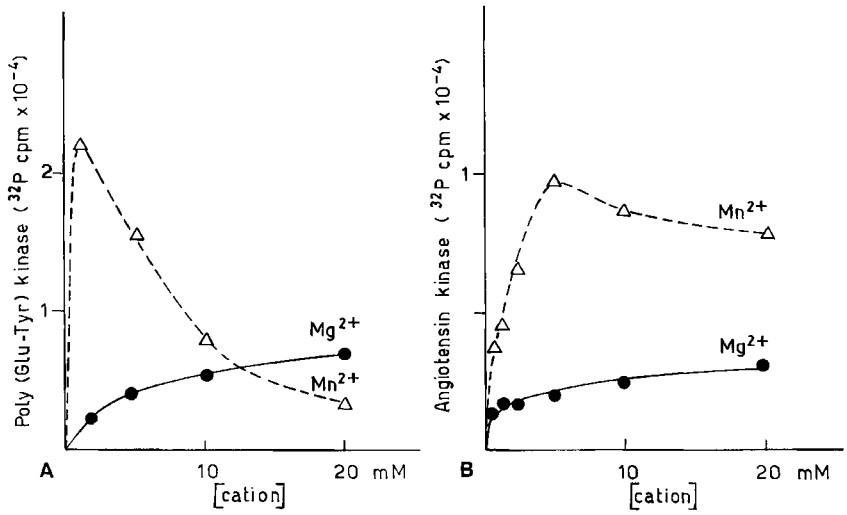


Fig. 6. *Effect of $Mn^{2+}$ and $Mg^{2+}$ on NaCl-extracted Tyr-protein kinase activity.* (A) 1 μM poly(Glu,Tyr)$_{4:1}$ as substrate; (B) 2 mM angiotensin II as substrate

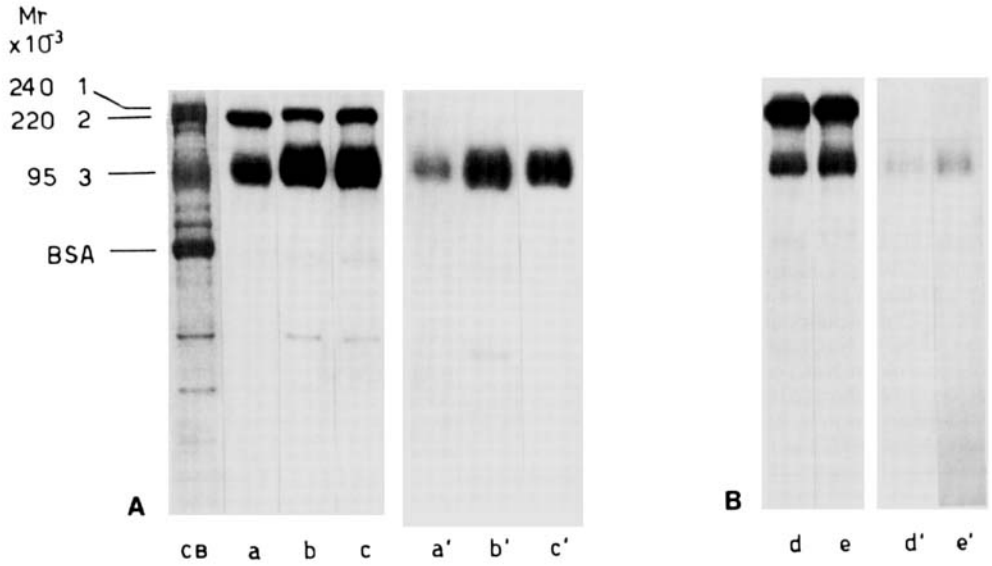


Fig. 7. *Autoradiograms showing the phosphorylation patterns of erythrocyte membrane proteins by detergent-extracted and NaCl-extracted Tyr-protein kinase preparations in the presence of 5 mM $Mn^{2+}$ or 20 mM $Mg^{2+}$.* Lanes a′–e′ show the alkali-treated gels corresponding to the alkali-untreated gels a–e. (A) 5 mM $Mn^{2+}$; (B) 20 mM $Mg^{2+}$. Human erythrocyte ghosts (≈ 100 μg protein) were incubated alone (lanes a, a′ and d, d′) or in the presence of: lanes b, b′ and e, e′, detergent-extracted Tyr-protein kinase (phosphocellulose peak of Fig. 1); (lanes c, c′) NaCl-extracted Tyr-protein kinase (phosphocellulose peak of Fig. 2). The phosphocellulose peak samples of detergent-extracted and NaCl-extracted Tyr-protein kinases employed in the assay were calibrated on the basis of having the same kinase activity (1.5 units) with angiotensin II. After incubation, an aliquot (≈ 40 μg) of $^{32}$P-labelled membrane proteins was analyzed by SDS/PAGE as described in [5]. Lane CB shows the Coomassie-blue-stained pattern. On the left the protein bands are designated by Steck's nomenclatue [17] and the corresponding molecular mass is given. Autoradiograms were exposed for 10 h

Table 2. *Phosphorylation of band-3 protein in the human erythrocyte ghosts in the absence and presence of the added membranous Tyr-protein kinase preparations*
Experimental conditions for incubation of the ghosts and subsequent SDS/PAGE analysis as described in the legend to Fig. 7. The $^{32}$P-labelled areas of band 3 were excised from lanes a–c and a′–c′ shown in Fig. 7A and counted for radioactivity in a liquid scintillation counter. The $^{32}$P-labelling is expressed as counts/min incorporated into the band 3. Letters in parentheses indicate lane in gel shown in Fig. 7A

| Sample | Untreated gels | Alkali-treated gels |
|---|---|---|
| | cpm/min | |
| Ghosts alone | 3400 (a) | 1470 (a′) |
| Ghosts + detergent-extracted Tyr-protein kinase | 9720 (b) | 4500 (b′) |
| Ghosts + NaCl-extracted Tyr-protein kinase | 9300 (c) | 4350 (c′) |

analyzed by SDS/PAGE, the protein phosphorylation patterns shown in Fig. 7 were obtained.

As shown in Fig. 7, in the presence of 5 mM $Mn^{2+}$, the major phosphate acceptor proteins in the endogenous phosphorylation (lane a) were band 2 (spectrin, β-subunit) and band 3.

The $^{32}$P-labelling of band-3 protein was markedly enhanced by both Tyr-protein kinase preparations (Fig. 7, lanes b, c; Table 2). This labelling was due predominantly to the phosphorylation of tyrosine residues as indicated by its alkali-stability [11, 12] (Fig. 7, lanes b′, c′). This was confirmed by phosphoamino acid analysis showing that $^{32}$P-Tyr was by far the predominant, if not sole, phosphoamino acid recovered in the acid hydrolysate of alkali-treated $^{32}$P-labelled membranes (Fig. 8B).

By contrast, the $^{32}$P-labelling of band 2 was poorly, if at all, affected by added Tyr-protein kinases (Fig. 7, lanes a–c), suggesting that it was predominantly, if not exclusively, catalyzed by the endogenous protein kinases. Such a $^{32}$P-labelling was due almost exclusively to the phosphorylation of serine residues, as indicated by its alkali-lability [11, 12] (Fig. 7, lanes a′–c′). This was confirmed by the phosphoamino acid analysis of untreated membranes (Fig. 8A), showing that only $^{32}$P-Ser, besides $^{32}$P-Tyr, was recovered in the acid hydrolysate of these membrane proteins. Only trace amounts of $^{32}$P-Thr were detectable under these conditions. It is noteworthy that both detergent-extracted and NaCl-extracted Tyr-protein kinases phosphorylated Tyr residues of band 3 to the same extent, as indicated by the comparison of the alkali-treated gels (Fig. 7, lanes b′ and c′).

The apparent $K_m$ values of the two membranous Tyr-protein kinases for band 3, evaluated from Lineweaver-Burk plots, were virtually identical, i.e. 2.0 μM for detergent-extracted enzyme and 2.3 μM for NaCl-extracted enzyme preparation, assuming that band-3 protein (95 kDa) constitutes approximately 30% of total ghost protein [13]. These $K_m$ values were comparable to that for cytosolic Tyr-protein kinase [5].

The Tyr phosphorylation of band 3, in the presence of 5 mM $Mn^{2+}$, by both membranous Tyr-protein kinases was

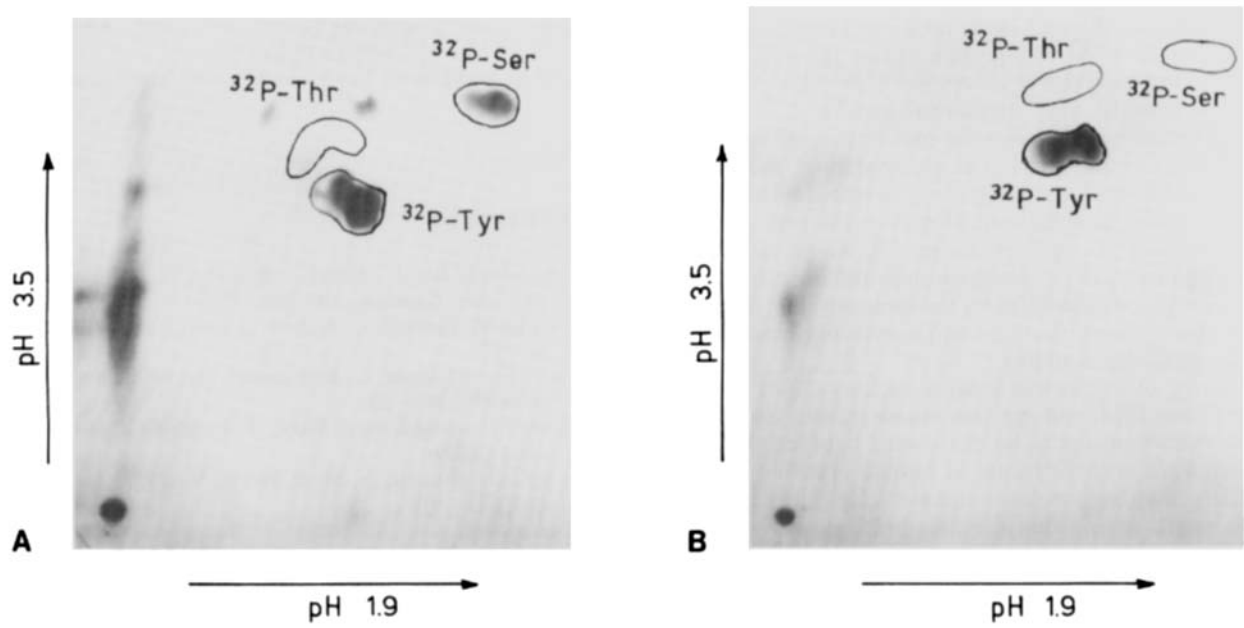


Fig. 8. *Autoradiograms showing the electrophoretic analysis of [$^{32}P$]phosphoamino acids in the acid hydrolysate of $^{32}P$-labelled membrane proteins (300 µg) phosphorylated in the presence of detergent-extracted Tyr-protein kinase preparation (7 units).* $^{32}P$-labelled membranes prior to acid hydrolysis (6 M HCl at 110°C for 2 h), were (B) or were not (A) submitted to alkali treatment (2 M NaOH at 55°C for 1 h). Experimental conditions for the two-dimensional thin-layer electrophoresis were as in [5]. 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 20 h

inhibited to the same degree by increasing concentrations of NaCl (about 50% inhibition by 150 mM NaCl) and of $Mn^{2+}$ above 5 mM (35% inhibition by 10 mM $Mn^{2+}$).

In the presence of $Mg^{2+}$, the Tyr phosphorylation of band 3 by added Tyr-protein kinase (Fig. 7B, lanes d′, e′) was much lower than in the presence of $Mn^{2+}$ (Fig. 7A, lanes a′–c′).

By contrast, the alkali-labile phosphorylation of band-2 Ser, practically unaffected by added Tyr-protein kinase (Fig. 7, lanes d, e), was much higher with $Mg^{2+}$ than with $Mn^{2+}$ (lanes a, b), suggesting that it was catalyzed by endogenous casein kinase [14, 15], which, unlike the added Tyr kinase, is activated much more by $Mg^{2+}$ than by $Mn^{2+}$.

The Tyr phosphorylation of band 3 in the presence of $Mg^{2+}$ was inhibited by increasing concentration of 2,3-bisphosphoglycerate (approximately 15–20% by 3 mM 2,3-bisphosphoglycerate (data not shown).

### *Cytosolic Tyr-protein kinase*

The above properties of both membranous Tyr-protein kinases were very similar to those previously reported for cytosolic Tyr-protein kinase partially purified from human erythrocytes by DEAE-Sepharose followed by heparin-Sepharose chromatography [5] (Table 1).

Moreover, cytosolic Tyr-protein kinase exhibited the same chromatographic behaviour on phosphocellulose chromatography (where it was eluted at 0.38 M NaCl), followed by heparin-Sepharose chromatography (eluted at 0.45 M NaCl) and on Ultrogel Ac44 molecular filtration (eluted at a volume corresponding to 36 kDa).

However, it is noteworthy that the cytosolic Tyr-protein kinase, unlike the membranous Tyr-protein kinases, appeared to be stable once eluted from phosphocellulose column, even in the absence of BSA, probably because at this purification stage the enzyme was still electrostatically bound to some unidentified cytosolic component(s).

The addition of BSA was required during the subsequent filtration on Ultrogel Ac44 column, where the enzyme became labile, probably because it was dissociated from the stable complex by the higher ionic strength (0.5 M NaCl) of the eluting buffer.

## DISCUSSION

In addition to insulin-receptor kinase [16], two Tyr-protein kinases phosphorylating transmembrane band-3 protein have been described and partially characterized in human erythrocyte membranes, i.e. one extracted from native ghosts by non-ionic detergent [1] and the other from the detergent-insoluble residue by 0.25 M NaCl [2, 3]. The two Tyr-protein kinase preparations have been reported to differ from each other in their chromatographic behaviour on phosphocellulose and kinetic properties [3]. Moreover, while the molecular mass of the detergent-extracted enzyme has been estimated to be 32–33 kDa [1], the apparent molecular mass of the NaCl-extracted enzyme could not be determined [3] because its activity once subjected to gel filtration could no longer be recovered.

These observations raise the question [3] whether the two membrane-associated Tyr-protein kinase activities are catalyzed by distinct enzymes or by different forms of the same enzyme. The results reported here indicate that both the Tyr-protein kinase extracted from membranes by non-ionic detergent and that extracted from the detergent-insoluble residue by 0.25 M NaCl have the same apparent molecular mass (36 kDa) and exhibit many identical chromatographic and kinetic properties, including their $K_m$ for poly(Glu,Tyr)$_{4:1}$, angiotensin II and band-3 protein, pH dependence, response to $Mn^{2+}$, $Mg^{2+}$, NaCl and 2,3-bisphosphoglycerate (Table 1).

It is worth noting that these properties appear to be very similar to those of the Tyr-protein kinase previously [5] isolated from human erythrocyte cytosol (Table 1).

The possibility that membrane-associated Tyr-protein kinases differ from their cytosolic counterpart by subtle post-translational modifications (i.e. glycosylation, methylation, etc.), undetectable by the analytical systems employed in this research, cannot be ruled out. However, the present results, together with those reported earlier [5], would suggest that the two membranous Tyr-protein kinase and the cytosolic Tyr-kinase activities are mediated by the same enzyme, distributed between membrane structures and a cytosolic pool ($\approx 80\%$ of the total cellular activity).

Similarly, other protein kinases such as cAMP-depedent protein kinase I [18] and spectrin kinase (casein kinase I) [19, 20] have been reported to be distributed between the cytosol and membrane compartments of human erythrocytes, from which they have been isolated and partially characterized.

It is worth noting that the membrane casein kinase has been found [21] to interact electrostatically with spectrin (its major membrane phosphorylatable substrate) giving rise to a (soluble) complex which can be partially dissociated at physiological ionic strength (140–160 mM NaCl) and by physiological levels (3–6 mM) of 2,3-bisphosphoglycerate, the prominent erythrocyte metabolite.

This supports the view [22] that the subcellular distribution of the enzyme, probably depending on the affinity of binding sites of specific anchor proteins, might be regulated by the ionic strength and the metabolic state of the red cell.

Also, Tyr-protein kinase has been reported [4] to be reversibly bound to the membrane through electrostatic interactions with band-3 protein, its major, if not sole, membrane substrate. It has to be underlined that, under our conditions, the more purified the Tyr-protein kinase preparations are, the greater is the loss of the enzyme activity, unless BSA is added. This suggests that the enzyme, which is stabilized in the membrane by electrostatically binding to band 3, becomes stabilized in the cytosol by binding to unidentified anchor proteins.

It will be of interest to investigate whether the subcellular distribution of Tyr-protein kinase is regulated by metabolic control and/or by extracellular physiological agents, as has been shown for protein kinase C in other cells; for review see [23].

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