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Properties of a membrane-bound tyrosine kinase phosphorylating the cytosolic fragment of the red cell membrane band 3 protein

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Band 3 protein of human erythrocyte membrane is phosphorylated on a tyrosine residue located near the NH_2 terminal by an endogenous tyrosine kinase activity (Dekowski, S., Rybicki, A. and Drickamer, K. (1983) J. Biol. Chem. 258, 2750–2753). A tyrosine kinase phosphorylating the band 3 protein in situ has been extracted from ghosts by non-ionic detergent and partially characterized (Phan-Dinh-Tuy, F., Henry, J. and Kahn, A. (1985) Biochem. Biophys. Res. Commun. 126, 304–312). We have studied the properties of the tyrosine kinase activity which remains bound to the ghosts after detergent extraction using the 43 kDa fragment of protein 3 as substrate. This activity, solubilized from the detergent-resistant material at 0.25 M NaCl and concentrated by phosphocellulose and tyrosine-agarose chromatographies, remains linked to high molecular weight complexes. It is specific for tyrosine. Assayed with the purified 43 kDa fragment it requires the presence of Mn^{2+} which cannot be replaced by Mg^{2+}. Its affinity for 43 kDa fragment is very high with a K_m of 3.3 μM. ATP acts as a phosphoryl donor with a K_m of 0.55 μM. The tyrosine kinase activity was not modified by insulin, DMSO, phorbol ester and epidermal growth factor, vanadate and xanthine derivatives. Polyamines spermidine and the polylysine are inhibitors in the presence of Mn^{2+} but not in the presence of Mg^{2+}. Heparin is a competitive inhibitor of ATP. 2,3-Diphosphoglycerate is an inhibitor at physiological concentrations (K_i = 2 mM). Purified red cell actin is not phosphorylated by the tyrosine kinase. These properties distinguish the red cell membrane-bound tyrosine kinase from other tyrosine kinases extracted from normal cells.

Introduction

Since the identification of the gene product of the Rous sarcoma virus and some other retrovirus with tyrosine-specific protein kinases (see reviews, Refs. 1 and 2) there has been a considerable interest in the study of these enzymes. It was demonstrated that besides the proteins encoded by transforming genes, receptors of several mitogenic growth factors such as the epidermal growth factor [3,4] and platelet-derived growth factor [5,6] and of hormones such as insulin [7,8] also possessed an endogenous tyrosine kinase activity. These observations suggested that tyrosine-specific protein kinases might be involved in peptide hormone effects and in processes of cell prolifera-

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Abbreviations: DMSO, dimethyl sulfoxide; phorbol ester, 4β-phorbol 12β-myristate 13α-acetate; FB3, cytosolic fragment of red cell membrane protein 3 (43 kDa); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; Mes, 4-morpholineethanesulfonic acid; SDS, sodium dodecyl sulfate; EGF, epidermal growth factor; FGF, fibroblast growth factor.

tion. Other tyrosine kinases were recently extracted and partially purified from normal tissues such as rat lung [9] and liver [10,11], blood and bone marrow cells [12–15]. Concerning the red cells, Dekowski et al. [16] demonstrated that the cytosolic fragment of the membrane protein 3 (FB3) carried a tyrosine residue which could be phosphorylated by an endogenous tyrosine kinase activity, and recently Phan-Dinh-Tuy et al. [17] extracted a tyrosine kinase from red cell membrane with a non-ionic detergent.

The present work was undertaken in an attempt to complete the knowledge of the red cell membrane protein kinases and to look for the possible role of the red cell protein 3 tyrosine phosphorylation.

Materials and Methods

Materials

Poly(GluNa, Ala, Tyr) 6 : 3 : 1, poly(GluNa, Tyr) 4 : 1; poly(Lys · HBr, Ala, Glu, Tyr) 5 : 6 : 2 : 1, insulin, DMSO, phorbol ester, heparin, spermidine and polylysine, L-serine, L-threonine, L-tyrosine, phosphoserine, phosphothreonine and phosphotyrosine, and alkaline phosphatase from *Escherichia coli* were obtained from Sigma. Pentoxyfiline was a gift from Hoechst. 2,3-Diphosphoglycerate was obtained from Boehringer. HPLC cation exchanger was from Durrum (DC6A), phosphocellulose from Whatman, and wheat germ lectin-Sepharose from Pharmacia. Ultrogel AcA-44 was from IBF France. [γ-^{32}P]ATP (3 Ci/mM) and [γ-^{32}P]GTP (54 Ci/mM) were from Amersham. Vanadate (ortho-) and usual reagents were from Merck.

Preparation of ghosts

Blood samples from normal donors or patients treated by bleeding for hemochromatosis were collected on heparin and used within 24 h. Erythrocytes were washed three times in phosphate-buffered saline (pH 8), then hemolyzed according to Dodge et al. [18] in 5 mM phosphate buffer/0.1 mM PMSF (pH 8). White ghosts were washed once in 25 mM Hepes buffer/0.1 mM PMSF/0.1 mM mercaptoethanol/0.1 mM EDTA (pH 7.6) (buffer A).

Enzyme extraction

Ghosts were extracted with 1 vol. buffer A/0.2 mM EGTA/1% Triton X-100/1% Nonidet P-40 for 1 h at 0°C with gentle stirring. They were centrifuged for 30 min at 14000 rpm. The supernatant was discarded and the pellet was extracted again. The suspension of ghosts was centrifuged as above and the supernatant was discarded. The pellet was then extracted with 1/3 of the initial volume of ghosts with 0.25 M NaCl in buffer A for 1 h at 0°C with gentle stirring, then centrifugated at 20000 rpm for 30 min. The supernatant was used as the enzyme source (crude extract). The activity of the extract remained stable at −70°C for several weeks.

Partial enzyme purification

Phosphocellulose chromatography

A phosphocellulose column (10 × 1 cm) was equilibrated with buffer A/0.1% Nonidet/0.1% Triton/10% glycerol (buffer B). 25 ml of crude extract which had been dialyzed overnight against 1000 ml buffer B were loaded onto the column. Elution was performed with a NaCl gradient from 0.25 to 1 M at a flow rate of 15 ml/h. 3-ml fractions were collected and enzyme activity was assayed as described below.

Tyrosine-agarose interaction

Preparation of tyrosine-Sepharose. Activation of Sepharose 4B with BrCN was performed according to Ref. 19. Coupling was done in 0.1 M $NaHCO_3$ buffer (pH 10.7) allowing for complete tyrosine solubilization. 12.3 μmol tyrosine were bound per ml of gel. Tyrosine-Sepharose was kept in 1 M NaCl with 0.02% azide.

A column of tyrosine-Sepharose (1.5 × 15 cm) was equilibrated overnight with 25 mM Hepes buffer/0.1 mM mercaptoethanol/0.1 mM EDTA/0.1 mM PMSF/0.1% Triton/10% glycerol (pH 7.6) (buffer C), then loaded with about 30 ml of pooled active fractions from phosphocellulose chromatography, previously dialyzed overnight against buffer C. Elution was obtained by a $(NH_4)_2SO_4$ gradient from 0 to 0.5 M in buffer C over 4 h with a flow rate of 20 ml/h. 3-ml fractions were collected. Active fractions were pooled, concentrated up to 10% of initial volume

by vacuum dialysis against buffer C and kept frozen at −70°C.

Enzyme activity assay

During standard assays, enzyme activity was assayed in 25 mM Mes buffer (pH 6.5) in the presence of 5 mM $MnCl_2$, 0.1 mM PMSF, 0.1 mM EDTA and 5 μM [γ-^{32}P]ATP (final concentration) plus amounts of substrate and enzyme source as specified below. When the substrate used was the cytosolic fragment of band 3 (FB3) the reaction mixture, at a total volume of 320 μl, contained 10 μl enzyme source and 5 μl FB3 (0.5 mg/ml in final concentration).

Under standard assay conditions the reaction rate with respect to time was linear for at least 60 min.

When intact or detergent-extracted ghosts were used as enzyme sources, the reaction mixture was incubated at 0°C: at this temperature the activity of other protein kinases still present in enzyme sources was very limited as compared to that of the tyrosine kinase. With the partially purified enzyme, incubation temperature was either 0°C or 30°C according to the substrate used.

The reaction mixture was incubated for 30 min and then treated using the following methods.

(1) Assay of total radioactivity incorporated into the substrate, by trichloracetic precipitation as previously reported [20].

(2) Electrophoretic analysis and autoradiography, by adding SDS and mercaptoethanol to a final concentration of 2% SDS and 5 mM mercaptoethanol. Samples were heated and kept at 100°C for 3 min. Electrophoresis was performed in polyacrylamide SDS gel, either in a 5–15% acrylamide gradient or in 10% acrylamide gel, following Laemmli's [21] method. Each sample was electrophoresed in duplicate: one half of the gel was stained by Coomassie blue as described by Fairbanks et al. [22] and the other half was submitted to hydrolysis by 2 M NaOH at 55°C for 1 h. The dried gels were autoradiographed on Kodak X-Omat film with Dupont-kronex intensifying screens.

(3) HPLC phosphoamino acid analysis using 10% trichloroacetic acid precipitation; the precipitate was washed three times to eliminate free [γ-^{32}P]ATP, dried, and hydrolyzed with 6 M HCl at 110°C under vacuum for 2 h. The hydrolysate was evaporated and dissolved in 10 mM citric acid. It was passed through a HPLC column (25 × 0.4 cm) filled with DC6A Durrum cation exchanger using an adaptation of the Capony and Demaille method [23] in which citric acid replaces trifluoroacetic acid.

Preparation of the FB3 substrate

The cytosolic fragment of band 3 was obtained from normal erythrocyte membranes by partial proteolysis by the method of Bennett and Stenbuck [24]. In SDS-polyacrylamide gel electrophoresis this purified fragment was resolved into two major peptides, 43 and 41 kDa, and minor bands, 30 and 20–25 kDa.

Results

Preparation of concentrated tyrosine kinase

The extraction of ghosts with non-ionic detergent Triton X-100 and/or Nonidet P-40 eliminates most of but not all the protein band 3 and about two-thirds of the overall membrane tyrosine kinase activity involving the insulin receptor beta-subunit. Following this process, the pellet retains significant tyrosine protein kinase activity which can be extracted by increasing the ionic strength. We selected an NaCl concentration of 0.25 M because it gave a satisfactory extraction and did not cause the enzyme inhibition which occurs at higher ionic strength.

When the crude extract was passed through a phosphocellulose column under the conditions described in Material and Methods, tyrosine kinase activity eluted in a single peak at 0.47–0.53 M NaCl with a yield of about 80%. Tyrosine kinase activity was demonstrated with FB3 as a substrate, by both the autoradiography of phosphorylated FB3 after gel exposure to NaOH and phosphoaminoacid analysis using HPLC.

Part of the tyrosine kinase activity obtained from the phosphocellulose chromatography did not bind to tyrosine-agarose (about 15%). Two peaks were eluted during the development of an $SO_4(NH_4)_2$ gradient (Fig. 1); one minor peak, corresponding to about 15% of the total activity loaded onto the column, eluted at 0.05 M $SO_4(NH_4)_2$; a major peak containing about 30%

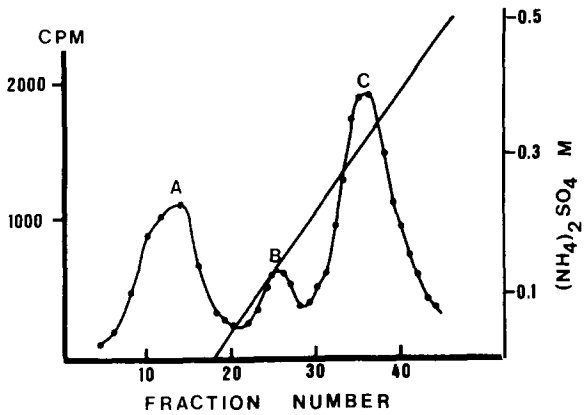

Fig. 1. Tyrosine-agarose chromatography. 30 ml of active fractions eluted from the phosphocellulose column were poured on a 1.5×15 cm tyrosine-agarose column and eluted with an $(NH_4)_2SO_4$ concentration gradient from 0 to 0.5 M as described in Material and Methods. Peak A corresponds to the unbound activity. Active fractions of peak C were collected and concentrated.

of the initial activity eluted at 0.2 M $SO_4(NH_4)_2$. Fractions of the main peak were collected. After desalting and a 10-fold concentration by vacuum dialysis, these fractions were considered as partially purified enzyme, but the level of protein was too low to be reliably measurable.

In an attempt to determine the molecular weight of the enzyme, a gel filtration column of AcA-44 (100 × 1.5 cm) was loaded with a sample of the active fractions. Less than 10% of activity eluted in the void volume, and the rest beyond the total volume of the column, indicating the presence of some interaction between the enzyme and the matrix of the gel filtration medium.

SDS-polyacrylamide gel electrophoresis of partially purified enzyme from a tyrosine-agarose column according to Laemmli [21] in a 10% acrylamide concentration only displayed high molecular weight complexes which did not penetrate the gel.

Tyrosine specificity of the enzyme (Table I)

The ability of the purified enzyme to phosphorylate tyrosine was proven by using synthetic substrates devoid of serine and threonine, as proposed by Braun et al. [25]. Three different peptides were used: poly(GluNa, Ala, Tyr) (6 : 3 : 1), poly (GluNa, Tyr) (4 : 1) and poly(Lys · HBr, Ala, Glu, Tyr) (5 : 6 : 2 : 1). The three peptides were phosphorylated but to a differing degree, decreasing from poly(GluNa, Ala, Tyr) to poly(GluNa, Tyr) and poly(Lys · HBr, Ala, Glu, Tyr). Such phosphorylation differences between synthetic substrates have been reported previously [25].

TABLE I

PHOSPHORYLATION OF FB3 AND SYNTHETIC SUBSTRATES BY PARTIALLY PURIFIED TYROSINE KINASE

Enzyme assays were performed as described in Material and Methods at 30 °C with substrates at the concentration of 1 mg/ml (final concentration). Tyrosine phosphorylation of FB3 was confirmed by HPLC analysis of phosphoamino acids and autoradiography of polyacrylamide gel electrophoresis after NaOH exposure.

Substrate	pmol/mg
Poly(GluNa, Ala, Tyr) 6 : 3 : 1	54
Poly(GluNa, Tyr) 4 : 1	30
Poly(Lys · HBr, Ala, Glu, Tyr) 5 : 6 : 2 : 1	34
FB3	137

As with many other proteins phosphorylated on their tyrosine by tyrosine-specific protein kinases (such as $pp60^{v\text{-}src}$ or insulin receptor), FB3 also possesses serine and threonine residues which can be phosphorylated by corresponding protein kinases.

Using FB3 as substrate, tyrosine specificity of the partially purified enzyme was demonstrated in two ways: (1) hydrolysis of ^{32}P-phosphorylated FB3 and subsequent analysis of labelled phosphoaminoacids by high performance liquid chromatography; (2) SDS-polyacrylamide gel electrophoresis of ^{32}P-phosphorylated FB3 followed by autoradiography of gels with and without previous exposure of gel to NaOH. The autoradiographic method showed that FB3 phosphorylation was alkali-resistant and therefore supported by phosphotyrosine; by HPLC analysis, almost all of the radioactivity corresponded to phosphotyrosine, with traces of labelled phosphoserine and phosphothreonine. Because of the great sensitivity of phosphotyrosine to acidic hydrolysis in comparison to the relative resistance of phosphoserine and phosphothreonine, the method is not really quantitative and entails an underestimation of the phosphotyrosine level; in experimental conditions

traces of phosphoserine and phosphothreonine are in fact negligible.

Functional properties of the enzyme

Effect of ions (Fig. 2). The tyrosine kinase activity requires the presence of Mn^{2+} with an optimal concentration of 5 mM. Mg^{2+} cannot substitute for Mn^{2+}: in the presence of 5–20 mM Mg^{2+} and 5–500 μM ATP the enzyme activity was only 10–13% of that obtained at Mn^{2+} and ATP concentrations giving maximal activity.

Enzyme activity was not influenced by Na^+ and K^+ ions at low concentrations. However, concentrations higher than 0.3 M decreased enzyme activity; at 0.5 M NaCl there was a 30% inhibition, increasing with ionic strength.

Affinity for ATP and FB3 (Figs. 3 and 4). Affinity for FB3 was studied at four FB3 concentrations form 0.465 to 4.65 μM and four ATP concentrations from 0.125 to 0.8 μM in the presence of 10 mM $MnCl_2$. The Michaelis constant calculated from Lineweaver-Burk plots was 3.3 μM (0.143 mg/ml).

Affinity for ATP was studied at four ATP concentrations from 0.125 to 0.8 μM and four FB3 concentrations from 0.465 to 4.65 μM in the presence of 10 mM $MgCl_2$. The Michaelis constant calculated from the inverse plots was 0.55 μM.

GTP was very poorly used as compared to ATP; with [γ-^{32}P]GTP enzyme activity was slower than 5% of that obtained under conditions giving the maximal velocity with ATP. $K_{0.5}$ GTP was about 500 μM.

Effect of insulin, phorbol ester, epidermal growth factor and DMSO. Most of the functional properties of the enzyme were determined using partially purified enzyme and FB3 as the substrate. However, we used intact and/or Triton-extracted ghosts when the studied property could depend on a membrane receptor. In such cases, membranes or detergent-extracted ghosts were incubated with the studied substance prior to performing autophosphorylation. Controls were done for each sample. Detergent-extracted ghosts previously incubated with the substance were used as enzyme

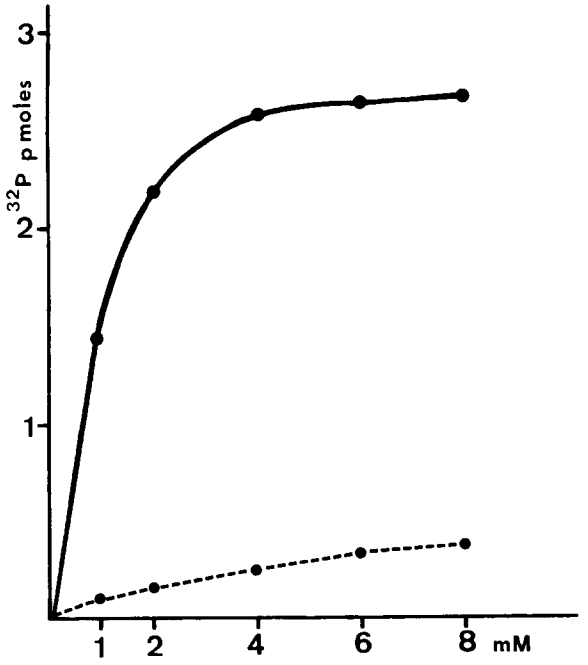

Fig. 2. Mn^{2+} dependence of tyrosine kinase activity. ————, enzyme activity in the presence of various Mn^{2+} concentrations; — — —, enzyme activity in the presence of the same Mg^{2+} concentrations.

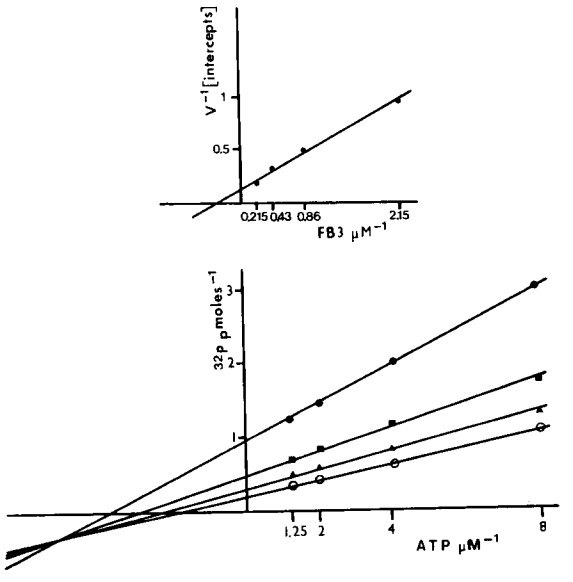

Fig. 3. Lineweaver-Burk plot of initial velocity as a function of ATP concentration with FB3. ●, 0.465 μM; ■, 1.163 μM; ▲, 2.325 μM; ○, 4.650 μM. The inset (top) shows a replot of the ordinate intercepts versus the reciprocal of the FB3 concentration.

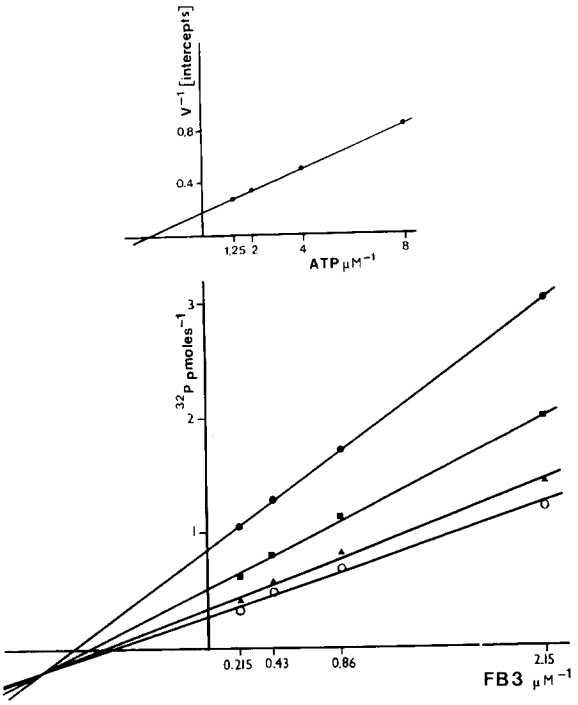

Fig. 4. Lineweaver-Burk plot of initial velocity as a function of the FB3 concentration with ATP. ●, 0.125 μM; ■, 0.25 μM; ▲, 0.5 μM; ○, 0.8 μM. The inset (top) shows a replot of the ordinate intercepts versus the reciprocal of the ATP concentration.

source when FB3 was used as a substrate.

Overall phosphorylation was assayed as in Material and Methods and labeled phosphotyrosine was determined by HPLC.

As shown in Table II, we did not find a significant effect of insulin, phorbol ester and epidermal growth factor on tyrosine phosphorylation of intact membranes, triton-extracted ghosts and FB3. DMSO at a concentration of 15% inhibited ^{32}P incorporation into membranes and FB3.

Effect of vanadate (Table III). In order to study the effect of vanadate on the red cell tyrosine kinase, we performed experiments of whole membrane phosphorylation with orthovanadate (30 μM in final concentration) added to the reaction mixture and in the presence of either Mn^{2+} or Mg^{2+}. The reaction time was 5 and 30 min, respectively; overall results were taken and tyrosine phosphorylation was determined as described above. In the presence of Mn^{2+} vanadate did not increase ^{32}P incorporation in membrane proteins, and labelled

TABLE II

EFFECT OF INSULIN, PHORBOL ESTER, DMSO AND EGF ON THE OVERALL PHOSPHORYLATION OF RED CELL MEMBRANES

Red cell ghosts were incubated with either insulin (30 μg/ml at 0°C for 30 min) or phorbol ester (200 ng/ml in 0.2% DMSO), EGF (1 ng/ml), DMSO (15% over 15 min), then autophosphorylated for 30 min in the presence of 5 mM $MnCl_2$. The incubation was performed at 0°C to limit the serine and threonine kinases activity. ^{32}P incorporated in phosphotyrosine was measured by HPLC as indicated in Material and Methods.

	Overall phosphorylation (pmol/mg)	HPLC [^{32}P]phosphotyrosine (cpm)
Control	85	828
+ Insulin	85,5	836
+ Phorbol	82,7	907
+ DMSO	49,3	232
+ EGF	87,5	824

phosphotyrosine was not modified. On the contrary, vanadate increased overall phosphorylation 1.5-fold in the presence of Mg^{2+}; autoradiographies of gels showed this increase to be predominant at the band 3 level; labelled phosphotyrosine counted by HPLC was increased at least 4-fold in comparison with the control without vanadate. However the activity of the purified enzyme was not modified by vanadate concentrations from 30 to 100 μM in the presence of either Mn^{2+} (10 mM) or Mg^{2+} (20 mM).

Effect of polyamines. The effect of two polyamines, spermidine and polylysine, on the whole membrane autophosphorylation was tested at 0°C in the presence of either Mn^{2+} or Mg^{2+}; phosphorylated substrates were identified by SDS-polyacrylamide gel electrophoresis and autoradiography and tyrosine phosphorylation was detected on gels using the alkali resistance of phosphotyrosine. Overall phosphorylation and phosphorylated substrates repartition were unchanged in the presence of polyamines and Mg^{2+}. In the presence of Mn^{2+}, both polyamines inhibited tyrosine phosphorylation of band 3 protein. Inhibition was stronger with spermidine than with polylysine.

Effect of heparin. As we have briefly reported

TABLE III

EFFECT OF VANADATE ON THE WHOLE MEMBRANE AND TYROSINE PHOSPHORYLATION IN THE PRESENCE OF Mg^{2+} AND Mn^{2+}

Vanadate increases tyrosine phosphorylation in the presence of Mg^{2+}.

	+ $MgCl_2$ (10 mM)	+ $MnCl_2$ (5 mM)
Membranes control, − orthovanadate	634 pmol/mg	104 pmol/mg
Membranes + orthovanadate	798 pmol/mg	97.7 pmol/mg
Phosphotyrosine peak (HPLC)		
− orthovanadate	160 cpm	
+ orthovanadate	805 cpm	

elsewhere [38], heparin inhibits the kinase activity; it was a non-competitive inhibitor with respect to the FB3 peptide and acted as a competitive inhibitor with respect to ATP.

Effect of methylxanthines. 0.5–10 mM pentoxyfiline (3,7-dimethyl-1-(oxohexyl)xanthine) was added to the assay mixture in the presence of partially purified enzyme and 1 mg/ml (final concentration) FB3: the xanthine derivative did not modify the FB3 tyrosine phosphorylation, at least in the concentration range used.

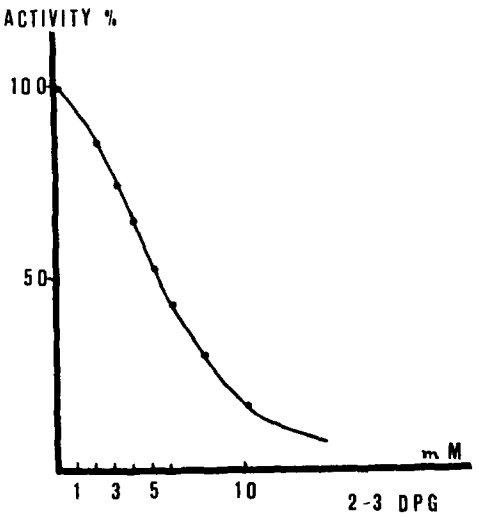

Fig. 5. Inhibition of tyrosine kinase activity by increased 2,3-diphosphoglycerate concentrations.

Effect of 2,3-diphosphoglycerate (Fig. 5). 2,3-diphosphoglycerate is a potent inhibitor of casein kinase II in erythroid cells [27–30]. We observed that it was also an inhibitor of the red cell tyrosine kinase: in the presence of saturating concentrations of ATP (5 μM) and FB3 (0.45 mg/ml), 50% inhibition was obtained with 5 mM 2,3-diphosphoglycerate and 90% with a 12 mM concentration.

A possible interaction between 2,3-diphosphoglycerate and ATP was studied by plotting the inverse of the reaction rate versus 2,3-diphosphoglycerate concentrations, from 0 to 7.5 mM, at four different ATP concentrations from 0.125 to 1 μM. It appears that inhibition was non-competitive with respect to ATP, with an inhibition constant of 1.9 mM. A possible interaction between 2,3-diphosphoglycerate and FB3 was studied in the same manner by plotting the inverse of the reaction rate versus 2,3-diphosphoglycerate concentrations from 0 to 9 mM at four different FB3 concentrations from 0.02 to 0.2 mg/ml: inhibition was also non-competitive with respect to FB3, with a K_i of 2 mM (not shown).

However it is worth noting that FB3 is in direct contact with the cytosol and that the 2,3-diphosphoglycerate inhibiting concentrations are very close to physiological values.

Absence of red cell actin phosphorylation. Actin, spectrin and protein 4.1 constitute the essential protein complex of the red cell membrane skeleton. Muscle actin is a substrate for $pp60^{v\text{-}src}$ [31–33] and insulin receptor [34]. We attempted to phosphorylate purified polymerized red cell membrane actin with red cell tyrosine kinase. 70 μg purified actin were added to the assay mixture with detergent-extracted ghosts as an enzyme source. After a 30 min incubation at 0°C, ghosts were removed by centrifugation: the supernatant was counted for radioactivity and submitted to polyacrylamide gel electrophoresis and autoradiography. Under these experimental conditions purified red cell actin was not phosphorylated.

Discussion

At least two tyrosine-specific protein kinase activities were present in the red cell membrane: one in the insulin receptor beta-subunit and the

other, phosphorylating the internal or cytosolic fragment of band 3 protein. One part of these activities can be extracted from ghosts by non-ionic detergents (Triton X-100, Nonidet P-40) and the other remains bound to the membrane pellet after detergent extraction. We have solubilized the latter after discarding the detergent extract to avoid contamination by the insulin receptor subunit and phosphatidylinositol kinases.

In order to perform our studies in conditions as close as possible to physiological ones we chose the isolated cytosolic fragment of protein 3 as a substrate rather than synthetic substrates. This fragment can be prepared in large quantities. It is a very convenient and inexpensive substrate for tyrosine kinase activity determination.

The properties of the tyrosine-specific protein kinase phosphorylating the cytosolic fragment of membrane band 3 protein distinguish it clearly from other membrane tyrosine kinases, especially with regards to the activity linked to the insulin and hormone receptors.

Recently Phan-Dinh-Tuy et al. [17] reported on the partial purification of a tyrosine-specific protein kinase contained in the detergent extract of human red blood cell membrane [26]. While this detergent extract includes the insulin receptor, the properties of the extracted enzyme were very different from those of this receptor tyrosine kinase activity. The authors noticed the absence of the stimulating effect of insulin and epidermal growth factor as we did ourselves. However the phosphocellulose chromatography pattern and kinetic properties are different from those of the enzyme bound to the pellet of detergent-extracted ghosts. A comparison of this tyrosine kinase with the one that we studied is difficult because the substrates used in vitro and the experimental conditions were different. It is possible that both enzymes are different; more likely, the red cell tyrosine kinase exists in two forms: one freely extractable by detergents and the other firmly attached to membrane proteins and only extracted at relatively high ionic strength. The constant presence of high molecular weight complexes in our preparations seems to argue in favor of such a hypothesis. These high molecular weight complexes, which are present in crude extract and phosphocellulose chromatography fractions, contained glycoproteins stained by the periodic acid-Schiff reaction. Dissociation of the high molecular weight complexes by dialysis against a low ionic strength buffer was accompanied by a parallel loss of enzyme activity and the appearance of a protein migrating as band 3 on polyacrylamide gel electrophoresis. It seems that we obtained the enzyme linked to protein 3 and that this linkage is necessary for the activity to remain. It could be suggested that the protein-linked enzyme activity is the physiological for of the enzyme.

When applied externally to cultured cells, vanadate mimics the effects of growth factors such as EGF, FGF [35] or insulin, with which it seems to act synergetically [36]. In the same way, the specific activity of the tyrosine kinase of $pp60^{v\text{-}src}$ is enhanced in cells cultured in the presence of vanadium [37].

Our results confirmed that membrane tyrosine phosphorylation is increased in the presence of vanadate and Mg^{2+} but that the mechanism is not direct stimulation of the tyrosine kinase suggested in some cases [38]. A phosphatase inhibition by vanadate and Mg^{2+} seems to explain our results, as it explains the modification of phosphorylation of $pp60^{v\text{-}src}$ observed in cells cultured in the presence of vanadate [37].

Recent reports from several laboratories have shown that polyamines are involved in controlling various types of protein kinase activities [39,40] and that the polyamine effect is counteracted by Mg^{2+} [41]. We observed that polyamines inhibited FB3 phosphorylation by tyrosine kinase in the presence of Mn^{2+}. The mechanism of this inhibition could be an interaction of polyamines either with the polyanionic structure of the band 3 NH_2-terminal peptide or with the enzyme itself. A phosphatase activation could also be suggested [42].

Methylxanthines interact with phosphorylation of proteins: they are potent inhibitors of cyclic nucleotide phosphodiesterase and of some kinases such as phosphatidylinositol kinase [43], thymidine kinase [44] and casein kinase II [45]. They also interfere with the insulin receptor [46]. It has been suggested that some tyrosine-specific protein kinases act as phosphatidylinositol kinases [47]; it was therefore of interest to test the effect of methylxanthines on the red blood tyrosine kinase

activity. We did not find any interaction between pentoxyfiline and tyrosine kinase. This result suggests that the tyrosine kinase differs from the red cell phosphatidylinositol kinase which is also extracted from ghosts at relatively high ionic strength [48].

If the tyrosine phosphorylation of protein 3 plays a physiological role it is very likely that this phosphorylation is under the control of a regulatory mechanism. This may involve the direct effect of inhibitors and activators upon the enzyme and a dephosphorylation process under the action of phosphotyrosine protein phosphatases. While little is known about the phosphorylation-dephosphorylation equilibrium, the phosphorylation inhibitory role of 2,3-diphosphoglycerate may be suspected, as it is for the cAMP-independent protein kinase of red cell cytosol. The inhibitor effect of polyamines in the presence of Mn^{2+} could also be considered.

Dephosphorylation of FB3 previously phosphorylated by our tyrosine kinase with [γ-^{32}P]ATP was easily obtained with alkaline phosphatase from *E. coli* at neutral pH (not shown); furthermore, we recently demonstrated that the red cell acid phosphatase is a phosphotyrosine-specific protein phosphatase [49], but other works are still necessary to define the role of band 3 tyrosine phosphorylation.

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