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CHARACTERIZATION OF HUMAN RED BLOOD CELL TYROSINE KINASE

Françoise Phan-Dinh-Tuy°, Joëlle Henry and Axel Kahn

INSERM U 129, Institut de Recherches en génétique et pathologie moléculaires
CHU COCHIN, 24 rue du Fg St Jacques, 75674 PARIS CEDEX 14

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A new tyrosine kinase in human red blood cells has been characterized and partially purified. The major substrate was a protein of molecular weight 93 K which could be phosphorylated both in whole red blood cells incubated with inorganic [^{32}P] orthophosphate and in ghost preparations incubated with [γ^{32}P] ATP. This tyrosine kinase displayed an alkaline isoelectric pH (around 8.5), a molecular weight of 32-33 K and does not seem to be autophosphorylable. Some kinetics of the enzyme are reported. This red blood cell tyrosine kinase is unrelated to EGF and insulin or insulin-like receptor subunits. This enzyme may represent a novel class of tyrosine kinases.

Since the original discovery of the tyrosine kinase activity of the Rous Sarcoma Virus transforming protein (1,2) the presence of phosphotyrosine has been under active investigation in normal (3-8) and malignant or growth-stimulated cells and tissues (6,9-12). Recent reports also point to the possible crucial role of phosphatidyl inositol and diacylglycerol phosphorylation by these "so-called" tyrosine kinases (13,14).

In human red blood cell membranes, Dekowski et al (15) and the authors (16) reported a tyrosine phosphorylation of the 93 K band 3. Recently, Grigorescu et al (17) described an insulin-dependent tyrosine phosphorylation in human red blood cell membranes, due to the autophosphorylation of the insulin receptor β subunit (95 K).

In the present paper, we report some biological and biochemical features of the tyrosine kinase phosphorylating band 3 in human red blood cell membranes. This protein kinase seems to correspond to a new class of enzymes.

MATERIAL AND METHODS

Endogenous phosphorylation of red cell membranes

Phosphorylation of red cell membrane endogenous substrates was carried out as described previously (at 30° C with 2 mM $MnCl_2$, 30 µM vanadate (Na_3VO_4), 7-8 µM [γ^{32}P] ATP 20-40 Ci/mmole, NEN) (16,7). The reaction was stopped (98° C, 3 min in a 2 % SDS, 5 % β-mercaptoethanol sample buffer). Part of the material was hydrolysed (6 N HCl, 110° C, 2 h) and

° To whom reprint requests should be sent.

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phosphoaminoacids analysed by one or two dimension high-voltage electrophoresis (1,18). SDS-polyacrylamide gradient gel electrophoresis was also performed (19) and protein alkaliresistance in gel was determined as described (16). In some experiments, radioactive bands were eluted from dried gels (20) and submitted to phosphoaminoacid analysis.

Phosphorylation of whole red blood cells

Two types of incubation mixtures were tested.

Purified red blood cells were washed three times and preincubated at 4°C overnight in a 35 mM Tris-HCl buffer, pH 7.4 (2 mM $MnCl_2$, 5 mM KCl, 0.1 mM sodium phosphate) made isotonic with either 280 mM glucose alone or 130 mM Nacl plus 3.5 mg/ml bovine serum albumin plus 20 mM glucose.

Pelleted red blood cells (200 µl) were washed again and incubated (4 h, 37° C) in the same buffer with 1 mM adenosine, 1 mM sodium phosphate and 1 mCi ^{32}Pi, carrier free (Amersham, 10 mCi/ml) (500 µl). In some experiments, 30 µM vanadate were added to the incubation mixture and 10,000 ng/ml insulin (Choay, France) to both preincubation and incubation mixtures. Red blood cells were then washed (60 mM sodium phosphate pH 7.4, 75 mM KF, 5 mM sodium pyrophosphate, 0.1 mM Zn acetate) and lysed (5 mM sodium phosphate, pH 7.4, 5 mM sodium pyrophosphate, 0.1 mM Zn acetate) in the presence of vanadate (30 µM) and paranitrophenylphosphate (4 mM). Ghosts were pelleted and washed once in lysis buffer. Phosphoaminoacid analysis and SDS-PAGE were performed as indicated above.

Tyrosine kinase activity assay

Phosphorylation of the random polyaminoacid glutamic acid-tyrosine (4:1) (Sigma ; poly glu-tyr, mol. wt 20 K to 50 K) (21) was assayed with 0.1 to 10 µg of material (22° C, 20 min in 20 µl Tris-HCl, pH 7.5, 30 µM vanadate, 20 mM $MgCl_2$, 2.5 mg/ml poly glu-tyr, 200 µM [$\gamma^{32}P$] ATP 1 Ci/mmole). The total reaction mixture was pipetted onto Whatman paper and the TCA-precipitable radioactivity was determined (22).

Effect of insulin on tyrosine kinase activity

Ultracentrifugation (100,000 x g x 1 h) supernatant of 1 % (v/v) Nonidet P-40-solubilized ghosts (10 µg for phosphorylation of endogenous substrates and 2.5 µg for phosphorylation of poly glu-tyr) was preincubated (1 h, 22°C, in 50 mM Hepes pH 7.6) with or without 10,000 ng/ml insulin. For phosphorylation of endogenous substrates, $MnCl_2$ (5 mM), vanadate (30 µM), and [$\gamma^{32}P$] ATP (9 µM ; 20-40 Ci/mmole) were added (final volume 50 µl) and incubation was prolonged for 20 min at 22° C. After dissociation (98° C, 3 min) in the presence of 2 % (w/v) SDS, with or without 5 % (v/v) β-mercaptoethanol, SDS-PAGE was performed under reducing or non-reducing conditions (19). For phosphorylation of poly glu-tyr, $MgCl_2$ (20 mM), vanadate (30 µM), glu-tyr (2.5 mg/ml) or H_2O, and [$\gamma^{32}P$] ATP (100 µM ; 1 Ci/mmole) were added to the preincubation mixture and the poly glu-tyr kinase activity was determined as described above.

Chromatographies

Red blood cell ghosts were solubilized in a 50 mM Tris-HCl buffer, pH 8.5 (1 mM EDTA, 10 mM ε-aminocaproic acid, 1 mM β-mercaptoethanol, 10 % (v/v) glycerol, 1 % (v/v) aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 % (v/v) Nonidet P-40) then ultracentrifuged (100,000 x g x 1 h). Affinity chromatography of the supernatant on Wheat Germ Agglutinin (WGA)-Sepharose 4B was carried out as described (23). Phosphocellulose chromatography of the same supernatant was performed at pH 8.5 ; the enzyme was fixed to the resin in the solubilization buffer and eluted by a linear ionic strength gradient (50-500 mM NaCl in the same buffer). High performance liquid chromatography (HPLC) was performed on a TSK G-3000 column (LKB) equilibrated with a 50 mM Tris-HCl buffer, 0.01 % (v/v) Nonidet P-40, 1 M NaCl and pH 8.5, or 0.4 M NaCl and pH 7.5 (22).

RESULTS

Phosphorylation of tyrosine residues in whole red blood cells

Two incubation mixtures were tested, made isotonic with either glucose alone or NaCl. Using the NaCl medium, no phosphotyrosine could be observed

TABLE 1 : Phosphotyrosine content of membrane phosphoproteins after phosphorylation of ghost preparations or whole red blood cells. Results of a typical experiment.

Experiment	Phosphorylation of ghosts					Phosphorylation of whole red blood cells					
						- Vanadate	+ Vanadate				
Sample/band	Ghosts	93 K	42 K	25 K	23 K	Ghosts	Ghosts	93 K	42 K	25 K	23K
Phosphotyrosine (in % of total phosphoaminoacids)	23 %	40 %	< 2 %	50 %	< 2 %	< 2 %	30 %	35 %	< 2 %	nd	nd

Phosphorylations were performed as described in material and methods. Whole red blood cells were incubated in the glucose medium ± 30 µM vanadate. Ghosts were submitted to SDS-PAGE and phosphoproteins eluted (20). Phosphoaminoacid analysis was performed as described on ghost samples and eluted bands (nd : not determined).

even in the presence of 30 µM vanadate (7) in the preincubation and incubation mixtures (figure 1). Using the glucose medium without vanadate, we could not detect reproducibly a significant amount of phosphotyrosine. In contrast, when 30 µM vanadate were present in the preincubation and incubation mixtures, the amount of membrane phosphotyrosine was about 30 % of the total phosphoaminoacids (table 1 and figure 1). The same major alkali-resistant band of mol. wt. 93 K was phosphorylated in these conditions as observed in ghost phosphorylation experiments (16) (figure 2A). Additional alkali-resistant bands were observed in the case of ghost phosphorylation (42 K, 25 K, 23 K). Under both types of phosphorylation conditions, the 93 K protein was highly phosphorylated at tyrosine at about the same level (40 % and 35 %). The 25 K band was also mainly phosphorylated at tyrosine while the 42 K and 23 K bands proved to correspond to serine-phosphoproteins (table 1). Such alkali-stability of some phospho-serine and -threonine ester bonds has already been reported (24).

Influence of insulin and epidermal growth factor on tyrosine kinase activity

We phosphorylated purified membranes and whole red blood cells in the presence or absence of 10,000 ng/ml insulin. This hormone dose is sufficient to activate both true insulin receptors and IGF-type receptors (21). No significant increase in the amount of phosphotyrosine could be detected after phosphorylation in the presence of insulin (not shown). Reducing agents were omitted in all buffers used and phosphorylated products were electrophoresed under either reducing or non-reducing conditions. Whole cell (not shown) and

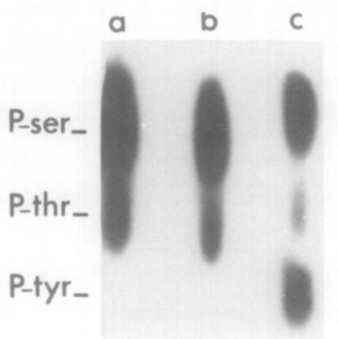

Fig. 1 : Electrophoretic phosphoaminoacid analysis of red blood cell membranes phosphorylated in whole cells.

Phosphorylation was performed (a) in the NaCl medium (cf Material and Methods) or (b,c) in the glucose medium and with (a,c) or without (b) 30 µM vanadate. Phosphoserine (P-ser), phosphothreonine (P-thr) and phosphotyrosine (P-tyr) were mixed with the samples and visualized by ninhydrin staining.

Autoradiographs were allowed to develop at -80° C on Kodak X-Omat AR films using intensifying screens.

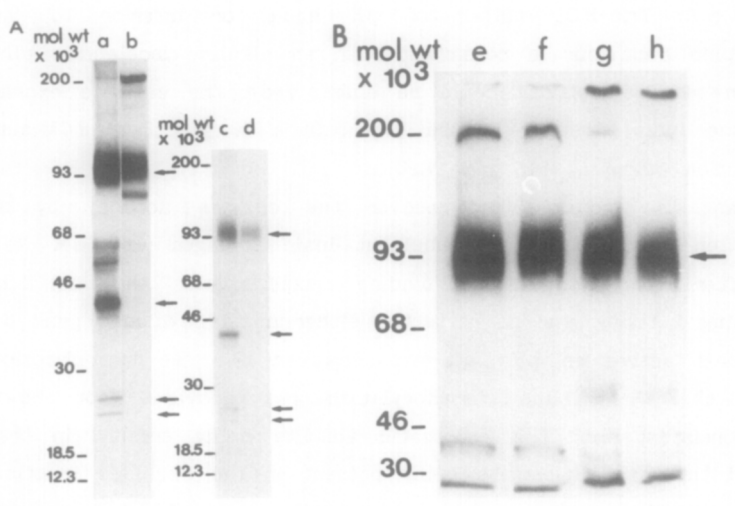

Fig. 2 : SDS-polyacrylamide gel electrophoresis analysis of red blood cell membrane phosphoproteins.

A. Phosphorylation was performed on ghosts with [γ^{32}P] ATP (a,c) or on whole cells with ^{32}P, carrier free (b,d). Samples were run in gradient gels (8 to 20 %, w/v, polyacrylamide). The gels were treated (c,d) or not (a,b) by 2 M NaOH (55° C, 1 h).

B. Ghosts were phosphorylated in the presence of [γ^{32}P] ATP with (f,h) or without (e,g) 10,000 ng/ml insulin. Samples were treated (e,f) or not (g,h) by 5 % (v/v) ß-mercaptoethanol and run in 7.5 % (w/v) polyacrylamide gels.

Autoradiographs were allowed to develop at -80° C on Kodak X-Omat AR films using intensifying screens. ^{14}C-labeled mol. wt. markers were used : myosin (200 K), phosphorylase b (93 K), bovine serum albumin (68 K), ovalbumin (46 K), carbonic anhydrase (30 K), lactoglobulin (18.5 K) and cytochrome c (12.3 K). The 93 K, 42 K, 25 K and 23 K red blood cell bands are indicated by arrows.

ghost (figure 2B) phosphorylation patterns were identical with or without insulin. Moreover the major phosphorylated alkali-resistant 93 K band was also observed under non-reducing conditions (fig. 2B). No higher mol. wt protein corresponding to the native phosphorylated insulin or IGF receptors (> 300 K) could be detected (21). Insulin was also without any activity on the polyaminoacid Glu-tyr kinase activity of solubilized red blood cell membranes (not shown).

The poly Glu-tyr kinase activity was not significantly retained by WGA column chromatography which is a method for purifying insulin receptors from different cells (25). In addition, ghosts and whole cells were phosphorylated in the presence of absence of 10^{-7} M Epidermal Growth Factor (EGF, Collaborative Research, USA). There was no detectable effect of EGF on tyrosine phosphorylation (not shown).

Chromatographic properties of the red blood cell tyrosine kinase

The red blood cell tyrosine kinase activity could be solubilized in 1 % (v/v) Nonidet P-40 as described in "Material and Methods". About 90 % of the poly glu-tyr kinase activity was recovered in the ultracentrifugation supernatant. The enzyme was almost totally retained on the cationic phosphocellulose resin in a 50 mM Tris-HCl buffer of pH equal or inferior to 8.5. This chromatographic behaviour is consistent with an alkaline isoelectric pH (around 8.5). Tyrosine kinase activity could be eluted from the cationic exchanger by increasing the ionic strength (figure 3A) : in a 50 mM Tris HCl buffer (pH 8.5), the elution began at 115 mM NaCl.

It was also possible to recover the enzyme activity by substrate affinity elution with ATP at a subeluent ionic strength (100 mM NaCl) (not shown). After phosphocellulose binding experiment, the 93 K band phosphorylating activity was not detected either in the excluded peak or in the eluted fraction active on poly glu-tyr. However if these two fractions were recombined, the 93 K band phosphorylation was recovered (not shown). This result is consistent with the enzymatic activity being present in the eluted fraction and the 93 K substrate being present in the excluded fraction. When the eluted partially purified enzyme preparation was subjected to endogenous phosphorylation in the presence of 10 mM $MgCl_2$ or 2 mM $MnCl_2$, several alkali-labile discrete phosphoprotein bands appeared ; this phosphorylation proved to occur almost exclusively on serine and threonine residues, even after a previous treatment with alkaline phosphatase to dephosphorylate hypothetical phosphorylable tyrosines of the enzyme (not shown). Then ion exchange chromatographic step alone was not sufficient to give a homogeneous preparation. The partially purified enzyme was however used to perform qualitative characterizations. The phosphocellulose-eluted active preparation was concentrated, then chromatographed on a high performance liquid chromatography TSK G 3000 column (figure 3 B). The activity was eluted with

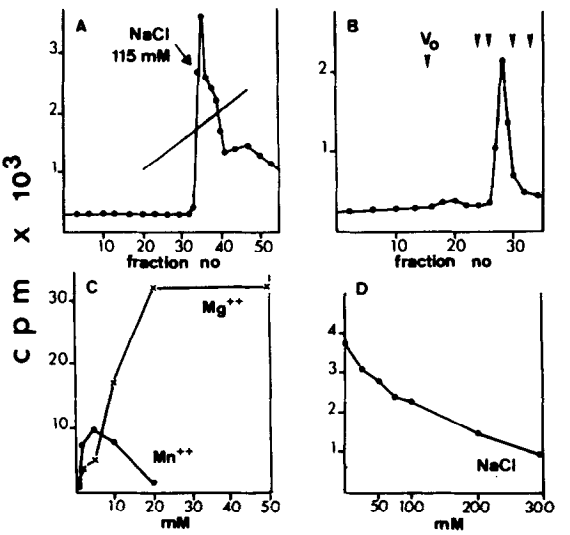

Fig. 3 : A) Phosphocellulose chromatography of the red blood cell tyrosine kinase activity. B) Chromatography on HPLC column of the active fractions eluted from phosphocellulose. The HPLC chromatography was performed in 1 M NaCl. The positions of dextran blue (Vo), bovine serum albumine (68 K), ovalbumin (46 K), chymotrypsin (25 K) and ribonuclease A (13.7 K) (▼) are indicated.
C and D) Effect of Mg^{++} (C, X—X), Mn^{++} (C, ●—●) and NaCl (D) on the tyrosine kinase activity eluted from phosphocellulose.
All activity assays were performed by phosphorylation of poly glu-tyr as described in Material and Methods.

an apparent mol. wt of 32-33 K, both when elution was performed in 0.4 M NaCl at pH 7.5 and in 1 M NaCl at pH 8.5.

In one experiment, the sample applied to the TSK column was previously subjected to endogenous phosphorylation and the active poly glu-tyr kinase peak was concentrated, then analysed by SDS-PAGE : no alkali-resistant phosphorylated band was observed (not shown). This result is in agreement with the absence of an alkali-resistant phosphorylated band in the range 32-33 K after endogenous phosphorylation of the phosphocellulose-eluted preparation as noted above.

Kinetics of the partially purified red blood cell tyrosine kinase

The pH dependence was investigated at a constant ionic strength as indicated for erythrocyte pyruvate kinase variant characterization by the International Committee for Standardization in Haematology (26). The enzyme was found active between pHs 6 and 9 but the curve obtained showed no narrow peak of activity in this range (not shown).

Monovalent and divalent cation dependence was also tested for phosphorylation of poly glu-tyr (figures 3 C and D). Mn^{++} was a poor effector of the enzyme for this substrate (about 30 % of the activity obtained with 20 mM Mg^{++}) although it was the only efficient divalent cation for phosphorylation of endogenous red blood cell substrate (16). With Ca^{++} (0.5 to 5 mM) only

3 % and with Zn^{++} (0.1 to 2 mM) only 13 % of the activity measured with 20 mM Mg^{++} were obtained (not shown). Increasing the ionic strength was rather inhibitory but residual activity was still observed up to 500 mM NaCl. The Michaelis constant values of the red blood cell tyrosine kinase were about 110 µM for ATP and 0.66 mg/ml for the artificial substrate poly glu-tyr.

DISCUSSION

The human red blood cell tyrosine kinase reported here represents a major enzymatic activity, as established by phosphorylation of whole red blood cells and ghosts. Is this tyrosine kinase activity related to the described (17) red blood cell insulin-dependent tyrosine kinase activity of the insulin receptor β subunit ? Three types of results argue against this possibility : (i) Tyrosine kinase activity is not at all insulin-dependent, when tested on either endogenous substrates of whole red blood cells or ghosts, or on poly glu-tyr substrate for solubilized ghosts ; (ii) The tyrosine-phosphorylated 93 K protein is also observed when analysed under non-reducing conditions and there is no shift to a higher mol. wt band which could represent the native insulin receptor complex (> 300 K) ; (iii) Most of the red blood cell tyrosine kinase activity is not retained on the lectin WGA which is known to bind the insulin receptor (25). Consequently, it seems that, even if the insulin receptor β subunit is indeed autophosphorylable on a tyrosine residue in red blood cells as in other cells (20, 21, 27), this activity does not represent the major tyrosine kinase activity of the red blood cells. The major tyrosine phosphorylated substrate, that is the 93 K band, is not the insulin receptor β subunit. This 93 K protein probably represents the red blood cell membrane anion channel, as already investigated in details (15).

Several features allow us to consider that the tyrosine kinase described here is distinct from tyrosine kinases previously described in normal, growth-stimulated, or malignant cells and tissues ; (i) The chromatographic behaviour of the enzyme indicates that its pHi is more basic than pHis so far reported for tyrosine kinases (4, 28) ; (ii) A molecular weight of 32-33 K is lower than those of all the other known tyrosine kinases (2, 4, 11, 21, 29-34). This apparent molecular weight was not modified by adding various antiproteolytic agents (benzamidine, phenylmethylsulfonylfluoride...) to the extracts and no tyrosine kinase of higher molecular weight was detected. These two points argue against the possibility that the 32-33 K enzyme could result from proteolysis of a higher molecular weight protein ; (iii) We identified for this tyrosine kinase a single substrate protein of 93 K, which is not the kinase itself. The 32-33 K red blood cell tyrosine kinase does not seem to be autophosphorylable, in contrast with all so far described receptor and/or oncogene product tyrosine kinase. The aminoacid sequence close to the phosphorylable tyrosine residue of these autophosphorylable tyrosine kinases is

highly conserved (35) and the lack of autophosphorylation activity of the 33 K red blood cell tyrosine kinase could indicate that it belongs to a different class of enzymes.

Tyrosine phosphorylation of the 93 K protein has been shown to occur in whole red blood cells, but in very unphysiological experimental conditions (low NaCl, high glucose medium). So the actual role of this tyrosine kinase in such non-proliferative cells as red blood cells remains to be elucidated. It is possible that the tyrosine phosphorylation process is of physiological importance and the 93 K protein is the physiological substrate. In this case, the enzyme could be implicated in regulation of ion exchange processes. Alternatively this red blood cell tyrosine kinase could be more concerned with a totally different metabolic pathways such as the inositol phospholipid pathways (13, 14, 36), as it is known that the inositol lipid cycle is present in red blood cells (37). In any case, the discovery of a new protein kinase enzyme active in phosphorylating tyrosine residues of natural and artificial substrates, abundant in non-proliferating cells and belonging to a different class from all so far described tyrosine kinase, is probably a finding of significance which could shed light on the actual physiological role of the "so-called" tyrosine kinases in the different tissues.

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