Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 566-572

PARTIAL PURIFICATION AND CHARACTERIZATION OF PHOSPHOTYROSYL-PROTEIN PHOSPHATASE(S) FROM HUMAN ERYTHROCYTE CYTOSOL

Giulio Clari[+], Anna Maria Brunati and Vittorio Moret

[+]Istituto di Chimica Biologica dell'Università,
Via Menegone, 37100 Verona , Italy
Istituto di Chimica Biologica dell'Università,
Via F. Marzolo 3, 35131 Padova , Italy

Received April 11, 1986

Phosphotyrosyl-protein phosphatase activity of human erythrocyte cytosol can be resolved into two fractions by DEAE-cellulose chromatography followed by P-cellulose chromatography. Both ^{32}P-Tyr-phosphatases are able to dephosphorylate ^{32}P-Tyr of poly (Glu-Tyr) 4:1 but not angiotensin II and synthetic peptide Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Gly, previously phosphorylated on tyrosine residues by rat spleen tyrosine-protein kinase. Both ^{32}P-Tyr-phosphatase activities distinctly differ from either ^{32}P-Ser-casein phosphatase activity or "acid" and "alkaline" p-nitrophenylphosphatase activities with regard to catalytic and physico-chemical properties such as substrate specificity, chromatographic behaviour, response to various effectors.

A number of protein kinases phosphorylating the membrane proteins on ser and/or threo residues have been isolated from human erythrocytes and extensively characterized by several laboratories, including our own. Electrophoretic band 2 (β-subunit of spectrin) and band 3 (the predominant transmembrane protein) are the major phosphate-acceptor substrates for these protein kinases.

More recently (1-3) endogenous tyrosine-protein phosphorylation has been evidenced in the human erythrocyte membranes, although the identity of its major specific target substrate(s) (93 kDa anion transporter protein or comigrating 95 kDa insulin-receptor) is still a matter of controversy.

Tyrosine-protein kinase(s) have been partially purified from human erythrocytes (3, 4). By contrast, to our knowledge, no information is available on the isolation and characterization of the corrisponding P-Tyr-protein phosphatase(s).

The present paper shows that, in addition to the already described protein phosphatases dephosphorylating P-Ser/Threo residues of isolated ^{32}P-spectrin and ^{32}P-casein (12, 13), at least two forms of ^{32}P-Tyr-phosphatase activity,

0006-291X/86 $1.50

dephosphorylating ^{32}P-Tyr residues of acidic poly (Glu-Tyr) 4:1 can be isolated from human erythrocyte cytosol.

Methods

Human erythrocytes, free of leucocytes and platelets, were prepared from fresh venous blood (150 ml) of healthy volunteers, according to Beutler et al. (5). Thereafter they were washed and lysed according to Dodge et al. (6), except that all solutions contained 0.05 mM phenyl-methyl--sulphonyl-fluoride (PMSF).

The red hemolysate supernatant was dialysed overnight against buffer A (10 mM imidazole-HCl buffer pH 7.3, 30 mM mercaptoethanol, 1 mM EDTA, 0.02% NaN_3, 0.05 mM PMSF) and then mixed with DEAE-cellulose (150 g dry weight/100 ml) previously equilibrated with buffer A. The slurry was stirred slowly for 30 min at 4° and then packed into a column (3.5 cm diameter). The column was first washed with about 1000 ml of buffer A (to completely remove the unadsorbed hemoglobin) and then eluted with a 1000 ml linear gradient ranging from 0 to 1 M NaCl in buffer A. 5 ml fractions were collected and separately assayed for ^{32}P-Tyr-phosphatase, ^{32}P-casein-phosphatase, "acid" and "alkaline" p-nitrophenylphosphatase (pNPPase) activities. The eluted fractions displaying P-Tyr-phosphatase activity were pooled together, dialysed against buffer A to remove NaCl and then submitted to phosphocellulose chromatography on a column (2.4 cm x 12.5 cm) previously equilibrated with buffer A. The column was first washed with about 150 ml of buffer A and then eluted with 500 ml linear gradient ranging from 0 to 0.5 M NaCl in buffer A. 4 ml fractions were collected and assayed for the above phosphatase activities.

All above operations were carried out in a cold room at 0°C.

Assay of ^{32}P-Tyr-phosphatse activity Such an activity was assayed by incubating the enzyme sample (10 µl) at 37°C for 10 min in a medium (100 µl) containing: 10 µmole imidazole-HCl buffer pH 7.3, 30 pmol ^{32}P-poly (Glu-Tyr) 4:1 (Sigma) (corresponding to 3.10^4 cpm) previously phosphorylated by tyrosine-protein kinase isolated from rat spleen (7). The reaction was stopped as in (8) and inorganic [^{32}P]-Pi released was determined by the molybdate-extraction procedure as in (8). The reaction rate was linear with respect to time over a period of 10 min, under conditions in which no more than 30% of the substrate was dephosphorylated.

Assay of ^{32}P-Ser-casein phosphatase activity. Such an activity was assayed by incubating the enzyme sample (15 µl) under the same above conditions, except that imidazole-HCl buffer pH 7.3 was replaced by 10 µmole imidazole-HCl buffer pH 6.0 and synthetic ^{32}P-Tyr substrate was replaced by 20 µg of ^{32}P-casein (containing 30 pmole of [^{32}P] Pi, corresponding to 3.10^4 cpm) previously phosphorylated on serine residues by casein kinase purified from human erythrocyte membrane (9).

Assay of p-nitrophenylphosphatase (pNPPase) activity

The "alkaline" pNPPase activity was assayed by following spectrophotometrically at 418 nm the increase of p-nitrophenol released from 20 mM pNPP, in the presence of 20 mM Mg^{2+}, by enzyme sample (75 µl) at 37° in 15 min (10), by using a control lacking enzyme as a blank. The assay mixture (500 µl) contained Tris/HCl pH 8.6. The "acid" pNPPase activity was measured by the same procedure, except that 50 mM Tris.-HCl buffer pH 8.6 was replaced by 50 mM sodium acetate buffer pH 5.2.

Results

The phosphatase activity of the human erythrocyte hemolysate supernatant is resolved by DEAE-cellulose chromatography, as reported in Fig. 1.

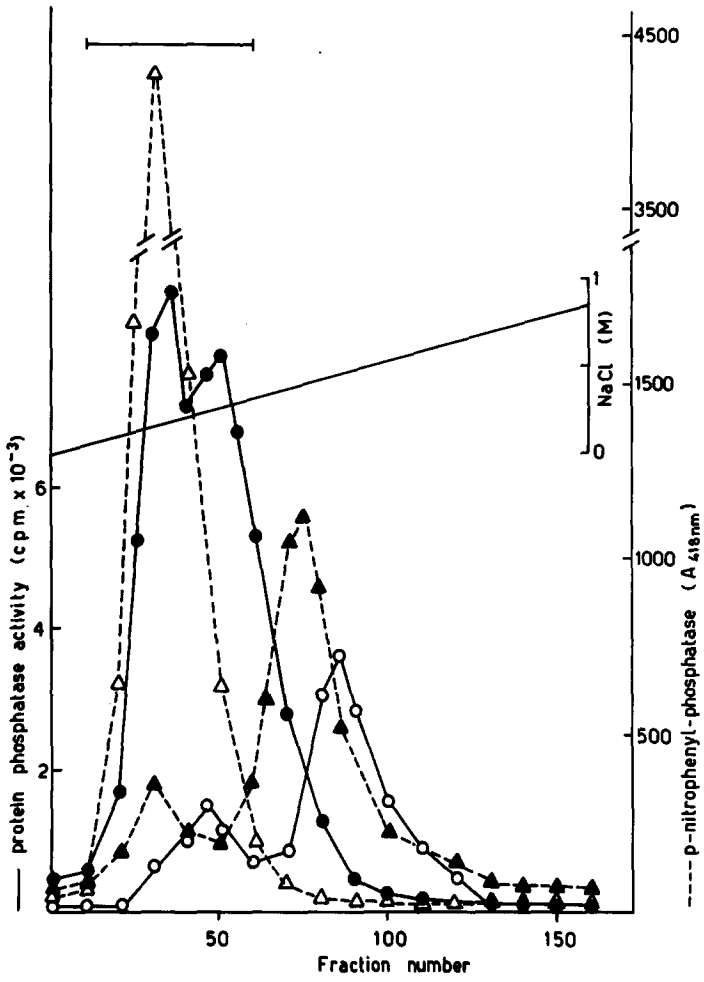

Fig. 1.
DEAE-cellulose chromatography of various phosphatase activities from human erythrocytes cytosol.
●—● ^{32}P-Tyr phosphatase activity, o—o ^{32}P-Ser-casein phosphatase activity, △--△"acid" pNPPase, ▲---▲"alkaline" pNPPase.

In addition to two peaks of ^{32}P-Ser-casein phosphatase activity, two peaks of ^{32}P-Tyr-phosphatase activity, partially overlapping with each other, are detected.

Moreover, Fig. 1 shows also the elution profile of "acid" (pH 5.2) and of "alkaline" (pH 8.6) pNPPase activities. The two DEAE-peaks of ^{32}P-Tyr-phosphatase activity are separated from a major peak of ^{32}P-Ser-casein-phosphatase activity eluted at 0.45 M NaCl and from a major peak of "alkaline pNPPase", eluted ay 0.35 M NaCl. However, they are contaminated by "acid pNPPase" and by minor peaks of ^{32}P-casein-phosphatase and "alkaline pNPPase" activity. This latter pNPPase peak is due likely to the residual activity displayed at pH 8.6 by "acid pNPPase", since it is detectable, like the major overlapping "acid pNPPase" peak, also in absence of added Mg^{2+}, whereas the "alkaline pNPPase"

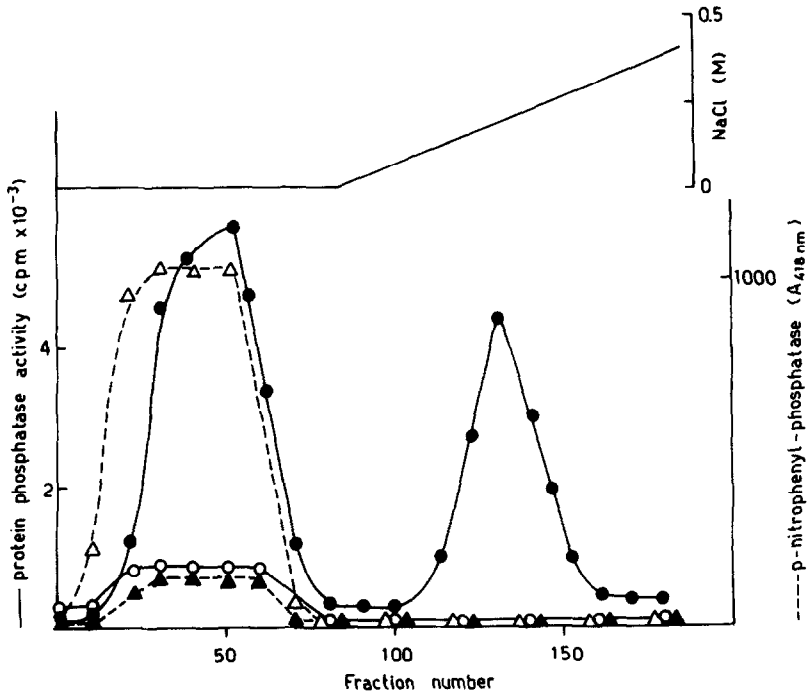

Fig. 2.
P-cellulose chromatography of the two DEAE-peaks of ^{32}P-Tyr-phosphatase activity reported in Fig. 1. ●—● ^{32}P -Tyr-phosphatase, o—o ^{32}P -Ser-casein phosphatase, △--△ "acid" pNPPase, ▲---▲"alkaline" pNPPase.

eluted at 0.35 M NaCl, is not detectable in the absence of Mg^{2+}, being strictly dependent on this cation.

Further purification is achieved when the two DEAE-fractions, pooled together as indicated by the bar in Fig. 1 and overnight dialyzed to remove NaCl, are submitted to P-cellulose chromatography as in the Methods (Fig. 2).

Under these conditions, a peak of ^{32}P-Tyr-phosphatase activity, completely devoid of ^{32}P-casein-phosphatase and of "acid" and "alkaline" pNPPase (in the presence and absence of Mg^{2+}) is eluted at 0.2 M NaCl, whereas another unadsorbed ^{32}P-Tyr-phosphatase activity, still contaminated by pNPPase and ^{32}P-casein-phosphatase activity is recovered into a large flow-through fraction.

The former peak of purified ^{32}P-Tyr-phosphatase has been employed for the study of its properties reported below.

It displays its activity in the pH range 6 to 8.5 the optimum pH being 7.0-7.3. While stimulated by physiological levels (1-3 mM) of ATP, ADP and 2,3-DPG, it is inhibited by increasing concentrations of NaCl and Mg^{2+} (Table I). Moreover it is 20% inhibited by 3 mM Ca^{2+}, 95% by 2 mM Zn^{2+}, 30% by 2 mM Mn^{2+}, 40% by 3 mM Molybdate, but unaffected by 3 mM pNPP, by 3 mM vanadate and by 200 mM NaF.

Table I: Effect of various effectors on the different phosphatase activities

Addition	^{32}P-Tyr-phosphatase	^{32}P-casein phosphatase	pNPPase
none	100	100	100
3 mM Mg^{2+}	45	---	125
5 mM "	25	---	130
7 mM "	15	---	130
75 mM NaCl	65	60	98
150mM "	30	40	100
0.5 mM ADP	165	35	97
1 mM "	205	25	100
0.5 mM ATP	155	25	100
1 mM "	145	16	100
1.5mM 2,3-DPG	195	70	100
3 mM "	167	50	100

All enzyme preparations, prior to assay, were overnight dialyzed against buffer A to remove NaCl. Phosphatase activity is expressed as percent relative to control containing no addition.

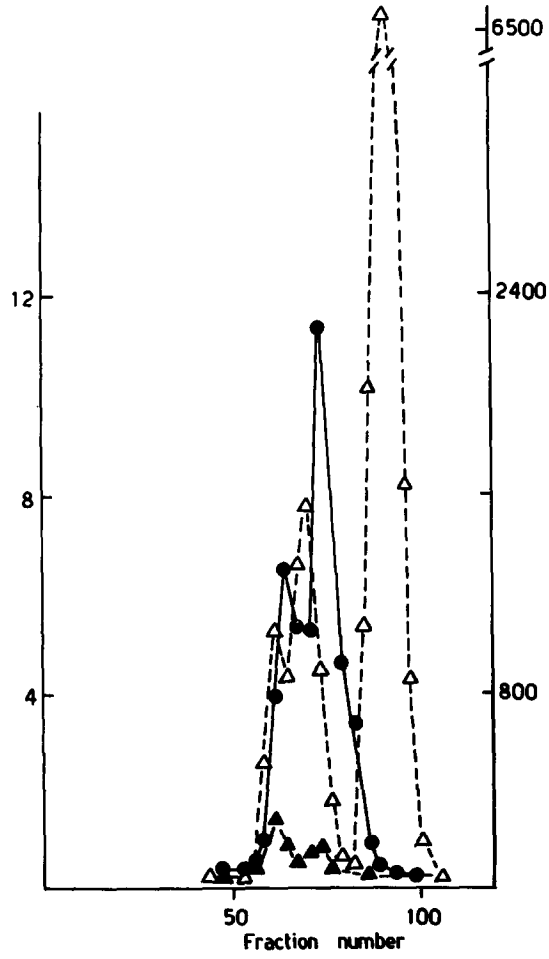

Fig. 3.
Ultrogel AcA44 elution profile of ^{32}P-Tyr-phosphatase and pNPPase activities. The two DEAE peaks of Fig. 1, pooled together, were applied to a Ultrogel AcA44 column (2 cm x 90 cm) previously equilibrated with buffer A containing 0.5 M NaCl and then eluted with the same equilibration buffer. 2.5 ml fractions were collected and assayed for: ●—● ^{32}P-Tyr-phosphatase, △-△"acid" pNPPase, ▲--▲ "alkaline" pNPPase.

Very similar properties are exhibited by ^{32}P-Tyr-phosphatase activity recovered into P-cellulose unadsorbed fraction, whereas the response to some ions by co-eluted "acid" pNPPase and ^{32}P-casein-phosphatase is different (Tab. I), i.e., in contrast to ^{32}P-Tyr-phosphatase, co-eluted pNPPase activity is unaffected by NaCl and slightly stimulated by Mg^{2+}, while ^{32}P-casein-phosphatase is inhibited by above anionic phosphate compounds (Table I).

The above differential response suggests that ^{32}P-Tyr-phosphatase and "acid" pNPPase activities may be mediated by distinct enzymes. Such a view is strengthened: 1) by their different elution profile (Fig. 3) observed when the two DEAE-cellulose peaks of Fig. 1 or even the crude cytosol, (freed from hemoglobin) are submitted to molecular sieve chromatography on Ultrogel AcA 44 at high ionic strength (0.5 M NaCl). Under these conditions, most of "acid" pNPPase activity is separated from ^{32}P-Tyr-phosphatase activity which is recovered into two peaks eluted at position corresponding to Mr 65 and 35 kDa, respectively. Moreover, the remaining pNPPase activity is eluted into two minor peaks which are not stricktly overlapping with those of ^{32}P-Tyr-phosphatase; 2) by differential behaviour (not shown) of the two activities observed when the P-cellulose unadsorbed peak of Fig. 2 is submitted to Ultrogel AcA44 or Sephadex G-100 gel filtration under these conditions, ^{32}P-Tyr-phosphatase, unlike pNPPase, undergoes an unexpected dramatic loss of activity.

Discussion

The present paper shows that, in addition to the already described P-Ser/P-Threo-protein phosphatases (11-12), dephosphorylating isolated ^{32}P-spectrin as well as exogenous ^{32}P-casein, at least two forms of ^{32}P-Tyr-phosphatase activity, dephosphorylating ^{32}P-Tyr of acidic poly (Glu-Tyr) 4:1, can be isolated from human erythrocyte cytosol. Such ^{32}P-Tyr-phosphatase forms appear to be different not only from P-Ser/P-Threo-protein phosphatases but, unlike those of other tissues (13-15), also from "acid" and "alkaline" pNPPases.

Both ^{32}P-Tyr-phosphatases are unable to dephosphorylate angiotensin II and synthetic peptide Asp-Ala-Glu-Tyr(P)-Ala-Ala-Arg-Arg-Gly (not shown) previously phosphorylated by rat spleen tyrosine protein kinase (7).

This latter observation supports the idea (16) that apparently tyrosine-protein kinase and ^{32}P-Tyr-protein phosphatases do not exhibit the same specificity.

The above results raised the question of the identity of physiological substrate(s) for P-Tyr-phosphatases. A probable candidate might be the membrane-spanning band 3 protein (93 kDa anion transporter protein) (1, 3), whose NH_2-terminal region of cytoplasmic domain has been found (1) to contain a tyrosine residue (at 8th position close to N-terminus) which is surrounded by

sequences of several acidic aminoacids, like the tyrosine residue in poly (Glu-Tyr) 4:1, used in this study.

Addendum - A report of these findings has been given at the 1st Intern. Symposium on proteinphosphatases held in Leuven (Belgium) August 1985 (17). During preparation of this manuscript, a human erythrocyte pNPPase, dephosphorylating P-Tyr of chymotryptic fragment of band 3, has been reported (18).

References

1. Dekowski, S.A., Rybicki, A., and Drickamer, K. (1983) J. Biol. Chem. 258, 2750-2753.
2. Grigorescu, F., White, M.F., and Kahn, C.R. (1983) J. Bio. Chem. 258, 13708-13716.
3. Phan-Dihn-Tuy, F., Henry, J., and Kahn, A. (1985) Biochem. Biophys. Res. Comm. 126, 304-312.
4. Boivin, P., Galand, C., and Bertrand, O. (1985) FEBS Letters 186, 89-92.
5. Beutler, E., West, C., and Blume, K.G. (1976) J. Lab. Clin. Med. 88, 329-333.
6. Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119-130.
7. Brunati, A.M., Marchiori, F., and Pinna, L.A. (1985) FEBS Letters 188, 321-325.
8. Pinna, L.A., Donella, A., Clari, G., and Moret, V. (1976) Biochem. Biophys. Res. Comm. 70, 1308-1315.
9. Clari, G., and Ferrari, S. (1983) It. J. Biochem. 32, 174-188.
10. Ellory, J.C., and Lew, V.L. (1974) Biochim. Biophys. Acta 332, 215-220,
11. Clari, G., and Moret, V. (1981) Biochem. Intern. 2, 509-515.
12. Usui, H., Kinohara, N., Yoshikawa, K., Imazu, M., Imaoka, T., and Takeda, M. (1983) J. Biol. Chem. 258, 10455-10463.
13. Swarup, G., Cohen, S., and Garbers, D.L. (1981) J. Biol. Chem. 256, 8197-8201.
14. Chernoff, J., Li, H.-C., Cheng, Y.-S.E., and Chen, L,B, (1983) J. Biol. Chem. 258, 7852-7857.
15. Lau, K.-H.W., Farley, J.R., and Baylink, D.J. (1985) J. Biol. Chem. 260, 4653-4660.
16. Sparks, J.W., and Brautigan, D.L. (1985) J. Biol. Chem. 260, 2042-2045.
17. Clari, G.,and Moret, V. (1985) in Adv. in Protein phosphatases (Merlevede W. and Di Salvo J. eds.) vol. 2, p. 420 Leuven University Press.
18. Boivin, P., and Galand, C. (1986) Biochem. Biophys. Res. Comm. 134, 557-564.