Tyrosine kinases in normal human blood cells

Platelet but not erythrocyte band 3 tyrosine kinase is $p60^{c\text{-}src}$

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Received 14 June 1986

The major tyrosine kinase from platelets was purified as a 51 kDa active enzyme which was shown to be a degradation product of the protooncogene product $p60^{c\text{-}src}$. Immuno-depletion experiments using a monoclonal antibody recognizing $p60^{c\text{-}src}$ failed to remove band 3 phosphorylating activity from red blood cell membranes. The erythrocyte tyrosine kinase was not at all immunoprecipitated by this antibody under conditions where the platelet enzyme was immunoprecipitated.

(Platelet, Red blood cell) Tyrosine kinase $p60^{src}$ Band 3 Differentiation

1. INTRODUCTION

Since tyrosine kinases were first reported as products of viral oncogenes [1–3], similar enzymatic activities have been found in different types of cells and tissues not infected by viruses [1,4–23]. Some of these tyrosine kinases have been shown to be associated with growth factor receptors [24–35]. The fundamental problem concerning these cellular tyrosine kinases is that phosphorylation at tyrosine of any endogenous substrate with a known physiological function has never been described. Several studies have dealt with tyrosine kinase activities of normal human hematopoietic cells [9,14–23], and our group has reported very high levels of phosphotyrosine in red blood cells and platelets [15]. In red blood cells the major substrate phosphorylated at tyrosine was found to be the membrane anion channel, band 3 [14,15]. A preliminary study has shown that the tyrosine kinase responsible for this band 3 phosphorylation was active in vivo in intact red blood cells, not associated with the insulin receptor of these cells, able to bind phosphocellulose at pH 8.5 and eluted from a HPLC-filtration column with an apparent molecular mass of 33 kDa [36]. We found that the major platelet tyrosine kinase behaved similarly on phosphocellulose and showed the same apparent molecular mass by HPLC-filtration (not shown). We therefore asked whether the major tyrosine kinase in human platelets and red blood cells was the same enzyme or not. We briefly report here purification of the major platelet tyrosine kinase and its identification as the $p60^{c\text{-}src}$ protein. We also show that endogenous phosphorylation at tyrosine of the anion channel band 3 in red blood cell membranes is not due to $p60^{c\text{-}src}$ activity.

2. MATERIAL AND METHODS

2.1. *Purification of platelet tyrosine kinase*

Platelets were obtained from blood freshly drawn from healthy volunteers or from outdated platelet units of the 'Centre National de Transfusion Sanguine'. After washing, they were solubilized in a 50 mM Tris-HCl buffer, pH 9, containing 1 mM EDTA, 1 mM β-mercaptoethanol, 10 mM ϵ-aminocaproic acid, 10% (v/v) glycerol, 1% (v/v) aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% (v/v) Nonidet P-40. In

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00145793/86/$3.50

some experiments, a 'cocktail' of antiproteolytic agents (20 mM benzamidine, 0.1 mM pepstatin, 0.1 mM antipain, 0.1 mM chymostatin, and 0.01 mM leupeptin) was added. Phosphocellulose binding was performed in the same buffer but at pH 8.5 and 0.1% (v/v) NP-40. NaCl (0.5 M) was added for elution. The active phosphocellulose eluate was subjected to a preparative flat-bed isoelectric focusing [37] using Ultrodex granulated gel and a 2117 Multiphor apparatus (LKB). Then two high-pressure liquid chromatography (HPLC) steps were performed on active fractions: firstly, an ion-exchange step on a TSK-DEAE-5PW column (7.5 × 75 mm) in a 5 mM Tris-HCl buffer, pH 8, containing 1 mM EDTA, 1 mM β-mercaptoethanol, 5% (v/v) glycerol, and 0.1% (v/v) NP-40; secondly, a filtration step on a TSK G 3000-SW column (7.5 × 600 mm) in a 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 10 mM $MgCl_2$ and 0.05% (v/v) NP-40. Fractions were subjected to one- [38] or two-dimensional [39] SDS-polyacrylamide gel electrophoresis, followed by Coomassie or silver [40] staining and autoradiography. Partial proteolysis was performed in tubes according to Cleveland et al. [41]. Phosphoamino acid analysis was performed as described [1,42].

2.2. *Tyrosine kinase activity assays*

During purification, tyrosine kinase activity was monitored by phosphorylation of poly(Glu-Tyr) (glutamic acid:tyrosine, 4:1, Sigma) [36,43]. Autophosphorylation of the purified tyrosine kinase was performed as described [36]. The effect of TLCK [44] and phosphorylation of casein, histones, angiotensin II [6], phosphatidylinositol (Ptd Ins) and phosphatidylinositol 4-monophosphate (Ptd Ins-4-P) [45,46] were assayed as described. Platelet and red cell membrane tyrosine kinase activities were also assayed using band 3 as substrate. Briefly, the phosphocellulose-unbound fraction from red blood cell membranes (which has been shown to contain band 3 itself and not band 3 tyrosine kinase [36]) was incubated 10 min at 30°C in the presence of the tyrosine kinase preparation and of 2 mM $MnCl_2$, 30 μM vanadate and 7–8 μM [γ^{32}P]ATP (20–40 Ci/mmol, NEN). Band 3 phosphorylation was then investigated by Laemmli's method of electrophoresis [38].

2.3. *Immunoprecipitation experiments*

The purified autophosphorylated 51 kDa protein was immunoprecipitated [47] by an antiserum raised against the peptide Lys-Arg-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Val-Ala-Arg-Gln-Gly [48–51] coupled to anatoxin from tetanos virus (kind gift from S. Fischer) or by the antisera raised in two rabbits immunized with the 51 kDa band excised from polyacrylamide preparative gels and homogenized with Freund complete or incomplete adjuvant. Immunoprecipitation experiments on unlabeled whole platelet or ghost extracts were performed using the antiplatelet 51 kDa protein or a monoclonal antibody, GD11 (IgG_1 type), strongly reacting with $p60^{c\text{-}src}$ in several species [52] (kind gift from S.J. Parsons). Autophosphorylation was then assayed by adding to the immune-complex pellet 10 mM $MnCl_2$, 25 mM Tris-HCl, pH 7.4, (final concentrations) and 10 μCi [γ^{32}P]ATP (NEN, 3000 Ci/mmol).

2.4. *Immuno-depletion experiments*

Rabbit antimouse IgG antiserum was bound to protein A-Sepharose (Pharmacia) then incubated with either normal mouse IgG1 or GD11 antibodies [52]. Solubilized extracts (1%, v/v, NP-40) from red blood cell ghosts were preincubated (3 h at 4°C) with the immobilized immune or nonimmune IgGs. After removal of the resin, supernatants were endogenously phosphorylated [36] in the presence of 2 mM $MnCl_2$, 30 μM vanadate and 7–8 μM [γ^{32}P]ATP (20–40 Ci/mmol, NEN) and analyzed by Laemmli's method of electrophoresis [38]. Phosphocellulose eluates from ghost or platelet extracts and platelet purified tyrosine kinase were also incubated under the same conditions with the immobilized IgGs and the effect on poly(Glu-Tyr) and band 3 kinase activities were assayed.

3. RESULTS AND DISCUSSION

3.1. *Purification of the platelet tyrosine kinase*

As the platelet tyrosine kinase was 50–100-times more active on an exogenous substrate, poly(Glu-Tyr) [43], than was the red blood cell tyrosine kinase (not shown), we undertook purification from platelets. This tyrosine kinase was purified after solubilization of whole platelets at high detergent concentration as described in section 2.

The different steps of the purification procedure were: phosphocellulose exchange at pH 8.5, preparative isoelectric focusing, HPLC DEAE exchange and HPLC filtration (not shown). Starting from the solubilization supernatant, a final purification of 1004-fold and an activity yield of 2.2% were obtained. Both are probably greatly underestimated due to a large decrease of activity during the purification procedure. The final purified fraction was autophosphorylated in the presence of 7–8 μM [γ^{32}P]ATP, 2 mM $MnCl_2$ and 30 μM vanadate, then analyzed by SDS-polyacrylamide gel electrophoresis (fig.1A, lanes a and b). Two bands were detected after Coomassie blue or silver staining: a major band of 51 kDa which was the only phosphorylated protein detected on the autoradiogram and a minor non-phosphorylated band of 44 kDa. Two-dimensional electrophoresis [39] showed a pH_i of 6.4 for the 51 kDa protein (not shown). The only phosphoamino acid present in the autophosphorylated purified fraction was phosphotyrosine (fig.1B). Two rabbits were immunized with the 51 kDa band excised from the acrylamide gel. The antisera obtained specifically immunoprecipitated the 51 kDa protein which was able to autophosphorylate in the immunecomplex (fig.1A, lanes c–f) and was therefore throught to be the tyrosine kinase itself. Phosphorylation of the IgG heavy chain was not observed.

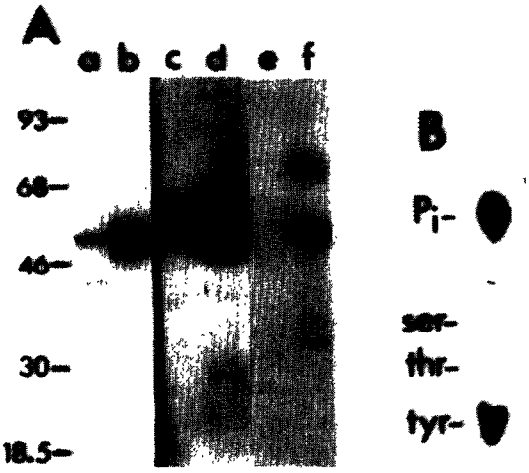

Fig.1. (A) SDS-polyacrylamide gel electrophoresis. Purified platelet tyrosine kinase was radiolabeled in the presence of [γ^{32}P]ATP: (a) Coomassie staining, (b) autoradiogram. Platelet tyrosine kinase was immunoprecipitated by normal rabbit serum (c,e) or antiplatelet 51 kDa band (d,f) from final purified tyrosine kinase fraction (c,d) or phosphocellulose eluate from platelet extract (e,f). Autophosphorylation was then assayed in the immune complex itself. Positions of M_r markers are indicated. (B) Phosphoamino acid analysis of the autophosphorylated purified tyrosine kinase from platelets. Positions of free phosphate (P_i), phosphoserine (ser), phosphothreonine (thr) and phosphotyrosine (tyr) are indicated.

3.2. *Characterization of the platelet tyrosine kinase*

The autophosphorylated 51 kDa tyrosine kinase was also specifically immunoprecipiated by an antiserum raised against the tyrosine autophosphorylation peptide of $p60^{src}$ [48–51] (not shown). In addition to poly(Glu-Tyr) the purified platelet tyrosine kinase was able to phosphorylate casein, whole histones, histone 2B, erythrocyte band 3 and angiotensin II, exclusively at tyrosine, but was unable to phosphorylate either Ptd Ins or Ptd Ins-4-P (not shown) [53]. 2 mM TLCK induced a 80% inhibition of the autophosphorylation of the 51 kDa band (not shown) [44].

No cellular tyrosine kinase of 51 kDa has so far been clearly characterized, but it is well known that

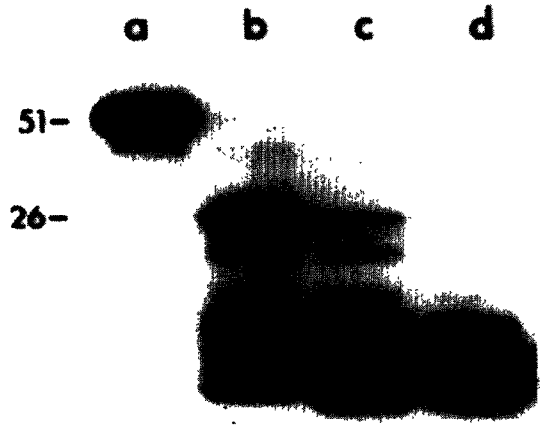

Fig.2. Partial proteolytic digestion of purified platelet tyrosine kinase. Final purified fraction was autophosphorylated in the presence of [γ^{32}P]ATP. Proteolytic cleavage was then omitted (a) or performed in the presence of (b) 1, (c) 5, (d) 10 μg/ml of *Staphylococcus aureus* V8 protease [41].

$p60^{src}$ tyrosine kinase is highly sensitive to proteolysis and is often recovered, after purification, as a 52–54 kDa active enzyme [54–57]. The question, therefore, was whether the 51 kDa tyrosine kinase we purified from platelets was the proteolyzed $p60^{c\text{-}src}$ enzyme. Four results argue in favour of this possibility. First, limited V8 protease digestion [41] of the purified autophosphorylated 51 kDa protein resulted in a pattern compatible with those reported previously for $p60^{src}$ [56–59], with a major peptide of 26–28 kDa phosphorylated at tyrosine (fig.2). Second, platelet extracts prepared from either freshly drawn blood, in the presence of a cocktail of antiproteolytic agents (see section 2.1), or outdated platelet units were immunoprecipitated with a strong monoclonal anti-$p60^{src}$ antibody, GD 11 [52]. Assay of autophosphorylation in the immune complex showed a highly phosphorylated 60 kDa band in fresh platelets while a highly phosphorylated 51 kDa band was observed in the outdated platelets, which were those generally used for the purification procedure (fig.3). Third, the two rabbit antisera raised against the 51 kDa protein were able to immunoprecipitate the active 60 kDa tyrosine kinase from fresh platelet extracts although much less efficiently that GD 11 (not shown). Fourth, the platelet tyrosine kinase activity assayed with poly(Glu-Tyr) or band 3 as substrate could be efficiently removed from a platelet phosphocellulose eluate or purified fraction by the GD 11 antibodies (fig.4, lane c). These findings confirm the data of Golden et al. [60] who reported a high level of $p60^{c\text{-}src}$ in human platelets.

3.3. *Band 3 phosphorylation in red blood cells*

Some common chromatographic properties suggested that the platelet and red blood cell tyrosine kinases could be the same enzyme. However immunoprecipitation experiments using either antiplatelet 51 kDa band antiserum or GD 11 antibody, followed by autophosphorylation assay in the immune complex, failed to detect a radiolabeled 60 or 51 kDa protein in red blood cell solubilized membranes (not shown). This could be due to a very low amount of $p60^{c\text{-}src}$ protein in red blood cells [60]. To establish or rule out the possibility that $p60^{c\text{-}src}$ is responsible for band 3 phosphorylation in red blood cell membranes, we examined whether the GD 11 anti-$p60^{src}$ antibody was able to

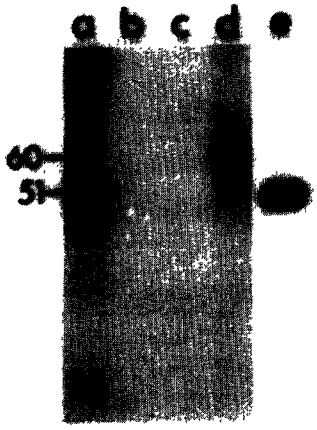

Fig. 3. Immunoprecipitation by anti $p60^{src}$ antibody. Soluble extracts were prepared from fresh drawn platelets (c,d) or outdated platelets (a,b) and immunoprecipitation was performed using GD 11 anti-$p60^{src}$ monoclonal antibody (a,d) or normal mouse IgG1 (c,d). Autophosphorylation was then assayed in the immune complex itself. Autophosphorylated purified tyrosine kinase (e). Positions of M_r 60000 and 51000 are indicated.

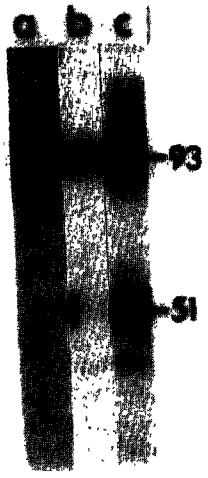

Fig.4. Phosphorylation of band 3 by the red blood cell enzyme in a soluble ghost extract (a) and by the platelet purified tyrosine kinase (b,c) (as described in section 2 after immuno-depletion by anti-$p60^{src}$ GD 11 antibody (b) or normal mouse IgG1 (c). Positions of M_r 93000 and 51000 are indicated.

abolish the band 3 phosphorylating activity in a red blood cell extract. Band 3 has already been shown to be a substrate for tyrosine kinase(s) from human B lymphocytes [17] and more recently for purified $p60^{src}$ protein and EGF receptor [61]. The role of these kinases in the endogenous phosphorylation of erythrocyte band 3, nevertheless, has never been proved. We confirm in this paper that platelet tyrosine kinase is also active in phosphorylating band 3 in vitro. However, in fractions partially purified by phosphocellulose chromatography, the ratio of band 3 kinase activity over poly(Glu-Tyr) kinase activity was at least 20-fold higher for the erythrocyte enzyme than for the platelet enzyme (not shown). Ghosts were prepared, solubilized and incubated with immobilized normal mouse IgG1 or GD 11 antibodies. Band 3 phosphorylating activity was then assayed on supernatants. Preincubation of the extracts with anti-$p60^{src}$ antibodies had no effect on band 3 phosphorylation (not shown). Immunodepletion experiments were also performed on purified platelet tyrosine kinase and on phosphocellulose eluates from either platelets or red blood cell membranes. Poly(Glu-Tyr) and band 3 kinase activities were then assayed on supernatants. Strong inhibition was observed in the case of the platelet tyrosine kinase towards both substrates either in purified fraction (more than 80% inhibition after 3 h incubation with immune IgG over the control with normal IgG7 or in phosphocellulose eluate (60–70% inhibition after 3 h incubation) (fig.4). In contrast, no decrease at all of the poly(Glu-Tyr) or band 3 tyrosine kinase activities was observed in the case of red blood cells after incubation with the $p60^{src}$ antibodies (not shown).

The results reported in this paper establish therefore that the major red blood cell tyrosine kinase is different from the major platelet tyrosine kinase and is not $p60^{c\text{-}src}$. While the anion channel band 3 is a good in vitro substrate for various tyrosine kinases and especially for $p60^{src}$ ([17,61]; present paper), the tyrosine kinase responsible for endogenous phosphorylation of band 3 in red blood cell membranes is not $p60^{c\text{-}src}$. The definitive characterization of the major red blood cell tyrosine kinase would require the purification of this enzyme, which has been so far difficult to perform due to its low activity on an exogenous substrate such as poly(Glu-Tyr) as compared to the high activity of $p60^{c\text{-}src}$ towards this substrate in platelets. This could be due not only to a low amount of enzyme but also to a low level of activity of the red blood cell band 3 kinase towards this substrate poly(Glu-Tyr). Indeed a very efficient phosphorylation of band 3 at tyrosine is observed in either membranes or intact red blood cells [36]. A close association between the enzyme and band 3 in the red cell membranes, suggested by Mohamed and Steck [62], could also account for the strong in vivo band 3 kinase activity [36].

In platelets as well as in erythrocytes, which represent terminal differentiation stages in hematopoietic development, the functional role of these tyrosine kinase activities remains to be elucidated. A relationship between tyrosine kinase activity and differentiation has been strongly suggested for $p60^{src}$ [63], namely in neural tissues [64] and in a transformed rat cell line where the v-src oncogene product is able to stop proliferation and to induce differentiation [65].

ACKNOWLEDGEMENTS

This work was supported by a grant from CNRS (ATP 95 5352). We thank S. Fischer, R. Fagard and G. Gacon for helpful discussion, S.J. Parsons for kind gift of GD 11 antibodies, A. Strickland for his help in preparing this paper, C. Brunner and T. Wetzel for typing the manuscript.

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