Platelet membrane glycoproteins IIb and IIIa are substrates of purified pp60$^{c\text{-}src}$ protein tyrosine kinase

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Human platelet glycoproteins IIb and IIIa form the receptor for fibrinogen, von Willebrand factor and fibronectin. Isolated human glycoproteins IIb-IIIa are phosphorylated by purified pp60$^{c\text{-}src}$ protein tyrosine kinase. Analysis of the phosphorylated proteins on SDS-PAGE showed that under reducing conditions both phosphoproteins change their relative molecular masses from 135 to 120 kDa and from 97 to 105 kDa, which are characteristic properties of glycoproteins IIb-IIIa. Phosphorylated proteins could be immunoprecipitated with an antiserum against glycoproteins IIb-IIIa but not by control serum. Some kinetic properties of the glycoprotein phosphorylations are also investigated. How the glycoprotein IIb-IIIa complex acquires its receptor activity in stimulated platelets is unknown; however, phosphorylation could be an important mechanism.

pp60$^{c\text{-}src}$; Protein tyrosine kinase; Glycoprotein IIb; Glycoprotein IIIa; Integrin; (Platelet)

1. INTRODUCTION

Glycoproteins IIb-IIIa (GP IIb-IIIa) are two major integral membrane components in human blood platelets. They exist as a noncovalently associated asymmetrical complex requiring Ca^{2+} to maintain a heterodimeric structure [1,2]. The two glycoproteins belong to the integrin family [3] and the complex acts as a receptor for fibronectin, von Willebrand factor and fibrinogen [4]. In unstimulated platelets the GP IIb-IIIa complex does not bind to any of these ligands but the mechanism which controls the receptor activity is not understood.

Platelets express high levels of pp60$^{c\text{-}src}$, which is the cellular homologue of the transforming protein of Rous sarcoma virus [5-7]. pp60$^{c\text{-}src}$ represents the prototype of nontransmembrane normal protein tyrosine kinases (PTKs) of which the physiological function and regulation in platelets are still unknown.

We have recently reported that pp60$^{c\text{-}src}$ seems to be involved in tyrosine phosphorylation of various platelet membrane proteins [8]. As the localisation and relative molecular masses of two of these proteins (105 and 120 kDa) correspond under reducing conditions in SDS-PAGE analysis with the GP IIb and IIIa of human platelets we studied the role of platelet pp60$^{c\text{-}src}$ PTK upon phosphorylation of these glycoproteins.

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Abbreviations: PTK, protein tyrosine kinase; pp60$^{c\text{-}src}$, the 60 kDa phosphoprotein encoded by the cellular (c-) src-gene; GP IIb-IIIa, glycoproteins IIb and IIIa; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

Here, we show that the isolated glycoproteins IIb and IIIa serve as substrates of purified pp60$^{c\text{-}src}$ from human platelets.

2. MATERIALS AND METHODS

2.1. *Materials*

Concavalin A-Sepharose (10-15 mg concavalin A/ml), heparin-agarose (800 μg heparin/ml), Mops (4-morpholinepropanesulfonic acid), O-phosphoserine and O-phosphothreonine were purchased from Sigma, München, FRG. Sephacryl S-300 was from Pharmacia, Freiburg, FR O-phosphotyrosine was prepared as in [9]. [γ-^{32}P] ATP (3000 Ci/mmol, 1 Ci = 3.7 × 10^{10} Bq) was purchased from Amersham Buchler, Braunschweig, FRG.

2.2. *Isolation of glycoproteins IIb and IIIa and pp60$^{c\text{-}src}$ from human platelets*

Clinically outdated human platelet concentrates (10-15 days after blood collection) were obtained from the local blood bank. Platelets (10 g wet weight) were isolated as described [8]. Glycoproteins IIb-IIIa were purified according to Fitzgerald et al. [10] with the following modifications: (i) Concavalin A-Sepharose (25 ml) was equilibrated with buffer A (10 mM Tris/HCl, 100 mM NaCl, 1 mM $CaCl_2$, 0.05%, NaN_3, 0.1% (v/v) Triton X-100, pH 7.4) additionally containing 1 mM $MnCl_2$. Before loading onto the concavalin A-Sepharose, the extract (supernatant) was supplemented with 1 mM $MnCl_2$. The flow-through was again applied to the column. (ii) Instead of heparin-Sepharose, heparin-agarose (25 ml) was used. (iii) Sephacryl S-300 was packed into a column with a diameter of 2 cm. Bed volume was 257 ml.

pp60$^{c\text{-}src}$ PTK was purified according to [7] modified as described by Reuter et al. (submitted).

2.3. *In vitro phosphorylation assay of glycoproteins IIb and IIIa*

The phosphotransferase assay was performed for 20 min at 30°C in a total volume of 50 μl containing 2 mM $MgCl_2$, 2 mM $MnCl_2$, various amounts of glycoproteins IIb and IIIa, purified PTK and ATP concentrations as indicated and 25 mM Mops, pH 6.5. The reaction was terminated by addition of 20 μl SDS-sample buffer containing either 10% 2-mercaptoethanol or none. The samples were analysed by SDS-

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PAGE according to Laemmli [11]. The dried gels were exposed to Kodak X-Omat films for autoradiography. Glycoproteins IIb-IIIa were cut out from the dried gels and radioactivity determined by Cerenkov counting.

2.4. *Immunoprecipitation of the phosphorylated glycoproteins IIb and IIIa*

For immunoprecipitation the phosphotransferase reaction was stopped by addition of neutralised EDTA (pH 7.0) and $Na_3 Vo_4$ at final concentrations of 10 mM and 200 μM, respectively. Phosphorylated GP IIb-IIIa (65 μg) was incubated with 10 μl GP IIb-IIIa antiserum or 10 μl control serum for 1 h at 4°C. The immunocomplex was adsorbed onto swollen Protein A-Sepharose (4 mg dry weight per sample) for an additional 1.5 h at 4°C. The following steps were performed as described recently [12].

2.5. *Two-dimensional polyacrylamide gel electrophoresis*

Two-dimensional analysis of the phosphorylated proteins (isoelectric focusing and SDS-PAGE with 8% polyacrylamide gels in the second dimension) was performed according to O'Farrel [13] with the BioRad mini gel system (Bio Rad, München, FRG). Ampholine reagents (Serva, Heidelberg, FRG) with pH 4–5 and 5–7 were used for isoelectric focusing. Reducing conditions were applied throughout.

2.6. *Analysis of phosphorylated amino acids*

Two-dimensional separation and identification of phosphoamino acids were performed according Hunter and Sefton [14].

2.7. *Protein determination*

Protein was determined by the Coomassie dye-binding assay [15].

3. RESULTS

Intact $pp60^{c\text{-}src}$ purified from human blood platelets [Reuter et al, submitted) phosphorylates the human GP IIb-IIIa. Two-dimensional separation of the phosphorylated proteins under reducing conditions yields only two phosphoproteins of 120 and 105 kDa (fig.1A). This result excludes the possibility that the glycoprotein preparation was contaminated with other proteins of the same relative molecular masses which could also serve as substrates of the purified PTK. Both proteins are exclusively phosphorylated on tyrosine residues (fig.1B). Densitometric analysis on the Coomassie stained gels show that GP IIb-IIIa isolated according to Fitzgerald et al. [10] form about 60% of total protein of the preparation. The ratio between glycoprotein IIb and IIIa was 1:1. The glycoprotein preparation did not show any endogenous kinase activity.

Glycoprotein IIb consists of a large α-chain and a small β-chain linked by disulfide bonds. Glycoprotein IIIa, however, is a single polypeptide chain containing one or more intramolecular disulfide bond(s) [4]. Therefore, under reducing conditions both glycoproteins typically change their electrophoretic mobility on SDS-PAGE. Analysis on SDS-PAGE revealed that both phosphorylated proteins apparently change their relative molecular masses in the presence of 2-mercaptoethanol from 135 to 120 kDa and from 97 to 105 kDa (fig.2A). The phosphoproteins on the resulting autoradiograms correspond with the Coomassie-stained bands. Furthermore, the phosphorylated proteins could be immunoprecipitated by an antiserum against GP IIb-IIIa, but not by control serum (fig.2B). The titre of the antiserum was positive up to 1:12 800 on the ELISA test system. These data clearly show that the two proteins phosphorylated by purified $pp60^{c\text{-}src}$ PTK are the human platelet glycoproteins IIb-IIIa. The 25 kDa β-chain of GP IIb is not phosphorylated.

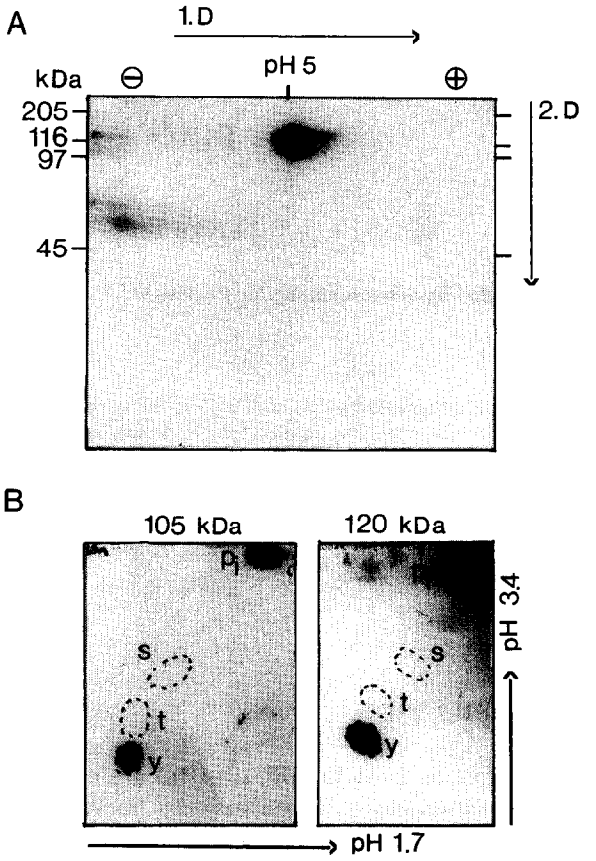

Fig.1. (A) Tyrosine phosphorylation of 105 kDa and 120 kDa platelet proteins by purified $pp60^{c\text{-}src}$. GP IIb-IIIa (28 μg) was phosphorylated in the presence of 180 ng purified PTK and 6 nM ATP. The phosphorylated proteins were analysed by two-dimensional gel electrophoresis. The autoradiogram of the phosphoproteins analysed by SDS-PAGE in the second dimension is shown (for details see section 2). (B) Two dimensional phosphoamino acid analysis of the 105 kDa and 120 kDa phosphoproteins. Separation and identification of the phosphoamino acids were performed according to Hunter and Sefton [14]. The dashed circles show the positions of the standard phosphoamino acids identified by ninhydrin. y, phosphotyrosine; t, phosphothreonine; s, phosphoserine; Pi, orthophosphate.

Phosphorylation of GP IIb-IIIa at 30°C was a linear time-dependent process completed within 30 min of incubation time (not shown). GP IIb and IIIa were phosphorylated by purified $pp60^{c\text{-}src}$ in a concentration-dependent manner (fig.3). The K_m values for GP IIb and GP IIIa were 1 and 0.75 μM, respectively. The K_m value of ATP for phosphorylation of both glycoproteins was 4 μM (fig.4) and coincides with that of $pp60^{c\text{-}src}$ for casein phosphorylation [7; Reuter et al., submitted].

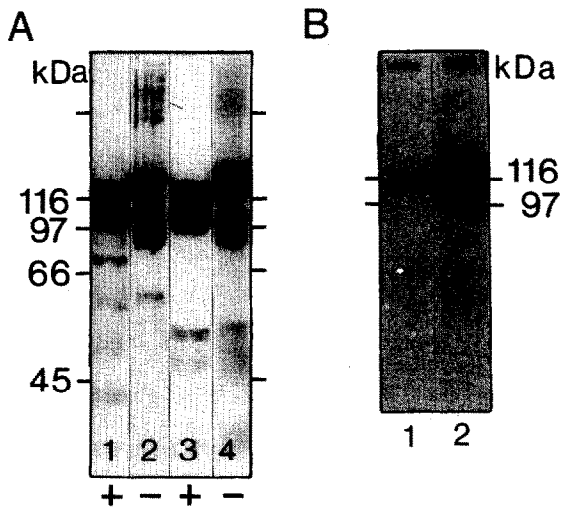

Fig.2. Identity of the tyrosine phosphorylated 105 and 120 kDa protein substrates of purified pp60$^{c\text{-}src}$ from human platelets. (A) Isolated GP IIb-IIIa from human platelets (11 μg) were phosphorylated by 70 ng purified PTK in the presence of 1 μM ATP as described in section 2. The proteins were analysed by SDS-PAGE in the absence (-) (2,4) or presence (+) of 2-mercaptoethanol (1,3). Coomassie stained proteins (1,2) and the autoradiograms of the corresponding ^{32}P-labeled proteins (3,4) are demonstrated. (B) The autoradiogram of the immunoprecipitated and phosphorylated proteins is shown. Isolated GP IIb-IIIa (384 μg) was phosphorylated by 200 ng purified PTK in the presence of 17 nM γ-[^{32}P]ATP. Immunoprecipitation of phosphorylated proteins was performed as described in section 2. Lane 1, control serum; 2, GP IIb-IIIa antiserum.

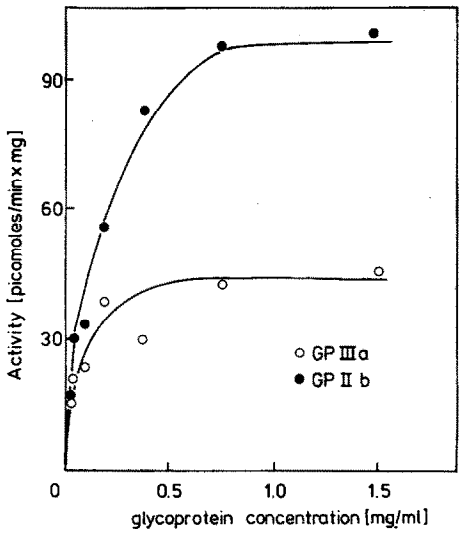

Fig.3. Substrate dependence of the glycoproteins IIb and IIIa phosphorylation by purified pp60$^{c\text{-}src}$. Samples containing 145 ng purified PTK, 1 μM ATP and various concentrations of GP IIb-IIIa were used in the assay performed as described in section 2. Enzyme activity is expressed as picomoles phosphate incorporated into the glycoproteins IIb and IIIa/min per mg pp60$^{c\text{-}src}$.

4. DISCUSSION

The c-src gene product, pp60$^{c\text{-}src}$ is expressed in high levels in human blood platelets [5]. Platelets also possess high levels of total cell phosphotyrosine, which may in part be ascribed to pp60$^{c\text{-}src}$ protein tyrosine kinase activity [16]. However, the physiological function of pp60$^{c\text{-}src}$ in mature cells like platelets is still unknown.

We have recently reported that Zn^{2+} enhances tyrosine phosphorylation of various proteins in human blood platelets membranes including proteins of 105 and 120 kDa. These phosphorylation reactions were supressed after preincubation of the membranes with antibodies (TBR-IgG) which bind to pp60$^{c\text{-}src}$ [8]. We now show that isolated glycoproteins IIb and IIIa of human platelets are substrates of purified intact pp60$^{c\text{-}src}$ PTK (figs 1 and 2).

In intact platelets stimulation with the physiological agonist thrombin induces tyrosine phosphorylation of various proteins [16,17]. Among these are two proteins with relative molecular masses of 105 kDa and 126 kDa. [17]. In a recent report Ferrell and Martin [18] demonstrated that the glycoproteins IIb-IIIa are involved in regulation of platelet PTK activities. Inhibition of

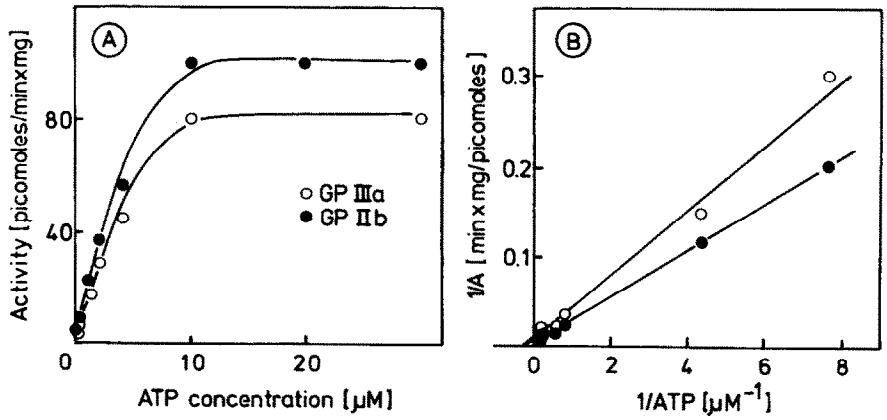

Fig.4. Dependence of glycoproteins IIb and IIIa phosphorylation on ATP. The experiment was performed in the presence of 0.45 μg isolated GP IIb and 0.4 μg GP IIIa, 145 ng purified PTK and various concentrations of ATP as described in section 2. The total volume was 50 μl. The saturation curve is shown in (A). The data of (A) are presented as a double reciprocal plot in (B).

ligand binding to GP IIb-IIIa inhibits the thrombin-induced tyrosine phosphorylation of 126, 130, 108 and 100 kDa proteins. However, it has not yet been studied whether any of these substrates could be GP IIb-IIIa. The cytoplasmic domain of GP IIIa has a tyrosine residue, which shows sequence homology with phosphorylation sites of insulin and epidermal growth factor receptors associated with transmembrane PTKs [4]. In chicken embryonic fibroblasts transformed by oncogenes coding for PTKs (v-src, v-yes, v-fps, v-erbB) bands 2 and 3 of the fibronectin receptor complex become phosphorylated at tyrosine residues [19]. The avian fibronectin receptor complex (CSAT antigen) and the platelet GP IIb-IIIa complex belong to the cell surface proteins of the integrin family. The members of the integrin family are transmembrane glycoproteins which link extracellular matrix proteins to the cytoskeleton [20]. How the stimulation-dependent receptor function of GP IIb-IIIa is regulated is unknown, but phosphorylation could be an important mechanism. The thrombin-induced phosphorylation of $pp60^{c\text{-}src}$ at Ser-12 and Tyr-527 seems not to influence its kinase activity [17]. But it is not excluded that these phosphorylations alter the substrate accessability of $pp60^{c\text{-}src}$ or its localisation. Therefore, the possible role of $pp60^{c\text{-}src}$ in regulation of the receptor activity of GP IIb-IIIa remains an interesting field for further investigations.

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