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Tyrosine-specific phosphorylation of gpIIIa in platelet membranes

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In vitro phosphorylation of platelet subcellular fractions revealed that most of the alkali-resistant phosphoproteins and the majority of $pp60^{c\text{-}src}$ were in the surface membrane fraction. An alkali-resistant phosphoprotein of about 100 kDa was also immune precipitated by an anti-phosphotyrosine antibody and comigrated with gpIIIa. The phosphorylation of gpIIIa, but not gpIIb, was confirmed by the comparison of reduced and non-reduced gels, and this protein was phosphorylated exclusively on tyrosine. In contrast, both gpIIb and gpIIIa were phosphorylated when the purified complex was added to immunopurified, immobilised $pp60^{c\text{-}src}$. A synthetic peptide with partial homology to a putative tyrosine phosphorylation site in the cytoplasmic domain of gpIIIa was phosphorylated by antibody-purified $pp60^{c\text{-}src}$. Our results indicate that tyrosine-specific phosphorylation of gpIIIa by $pp60^{c\text{-}src}$ may play a role in the regulation of platelet function.

Platelet; Integrin; Tyrosine phosphorylation; GpIIb-IIIa; Membrane

1. INTRODUCTION

Platelets are small discoid cells which exhibit highly regulated adhesive properties and whose main physiological role is the prevention of blood loss by the formation of haemostatic plugs at the site of vascular injury. Activation by a wide variety of agonists which are released or become exposed during tissue damage results in adhesion to the vessel wall, cytoskeleton-driven shape changes, aggregation and ultimately release of granule contents [1].

Matrix receptors on the platelet membrane play a major role in activation and gpIIb-IIIa is the major platelet receptor for fibrinogen, although it will also bind to von Willebrand factor, fibronectin and vitronectin [1,2]. Lack of gpIIb-IIIa from platelet membranes leads to defective platelet function and bleeding disorders, such as Glanzmann's thrombosthenia [3]. GpIIb-IIIa is a complex of two transmembrane glycoprotein subunits and belongs to the integrin superfamily of adhesion receptors, other members of which have fundamental roles in cell-matrix and cell-cell interactions [2,4]. In the resting platelet, the intact heterodimer is present on the cell surface but does not bind fibrinogen. However the binding site becomes available after activation, probably due to a conformational change in the subunits [5]. Receptor occupancy is concurrent with the reorganisation of gpIIb-IIIa from a monodisperse to a clustered localisation within the plane of the membrane, and this appears to be an essential part of the pathway leading to full activation, including adhesive and cytoskeletal changes [6].

Phosphorylation events are crucial to platelet activation, and recently there have been several reports that many platelet proteins can be phosphorylated on tyrosine residues. The major tyrosine kinase in platelet membranes is $pp60^{c\text{-}src}$ which represents 0.2–0.4% of the total protein [7–10], however no substrates for this enzyme within the platelet have yet been identified. In other cells the β chain of another integrin, the fibronectin receptor, can be phosphorylated on tyrosine by the oncogenic homolog of $pp60^{c\text{-}src}$, $pp60^{v\text{-}src}$ [11]. GpIIIa has extensive homology with other integrin β chains and is therefore a potential target for the platelet enzyme. Since the phosphorylation of fibroblast integrin has been mapped to tyrosine 788, a residue conserved in the platelet gpIIIa chain [12], we have investigated whether there is any tyrosine-specific phosphorylation of gpIIb-IIIa in platelet membranes.

2. MATERIALS AND METHODS

2.1. *Materials*

All chemicals were Analar grade purchased from B.D.H. and all other materials were purchased from Sigma unless otherwise stated.

2.2. *Preparation of membranes*

Human platelets were isolated by differential centrifugation and different membrane fractions prepared by continuous flow electrophoresis as described in [13]. Three fractions were obtained: cytosol, intracellular membranes (ER and Golgi) and surface membranes. The intracellular and surface membrane components have been extensively characterised [13]. These were stored at −80°C until use. Protein determinations were performed using the BCA reagent (Pierce Ltd.) as described by the manufacturer.

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2.3. *Phosphorylation of membranes*

50 μg of protein were suspended in kinase buffer (20 mM Tris-HCl pH 7.5 containing 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 100 μM Na_3VO_4 and 5 μCi [^{32}P]ATP (3000 Ci/mmol) (Amersham) was added to initiate the reaction. After incubation at 37°C for 10 min the reaction was terminated either by addition of 50 μl twice strength SDS reducing Laemmli sample buffer [14] for immediate electrophoresis or by addition of 1 ml ice-cold extraction buffer (PBS containing 1% nonidet P40, 5 mM EDTA, 10 mM NaF, 1 mM PMSF and 100 μM Na_3VO_4) for subsequent immune precipitation.

2.4. *Alkali treatment of gels*

Following electrophoresis, the stained gels were autoradiographed and then treated with 1 M KOH for 2 h at 55°C as outlined [15].

2.5. *Immune precipitation*

After extraction on ice for 20 min the lysed membranes were centrifuged (14 000 × *g*) for 5 min. The supernatants were precleared with sepharose CL4B and then 2 μl of antibody were added and incubated for 1 h at 4°C. 50 μl of a 50% suspension of rabbit anti-mouse Ig complexed protein A-trisacryl (Pierce) was added for 1 h. The immune complexes were then washed 4 times and resuspended in SDS sample buffer for electrophoresis.

2.6. *Western blotting*

10 μg of purified platelet fractions were electrophoresed and transferred to nitrocellulose and then immunoblotted with rabbit anti-pp60 [16].

2.7. *Acid hydrolysis and phosphoamino acid analysis of GpIIIa*

GpIIb-IIIa was immunoprecipitated from ^{32}P-labelled membranes by MAb $MM_2/356$, electrophoresed and transferred to Immobilon-P membrane (Millipore) and autoradiographed to locate GpIIIa. The band was excised and the protein subjected to partial acid hydrolysis and thin layer electrophoresis at pH 3.5 [15].

2.8. *GpIIb-IIIa and synthetic peptide phosphorylation by immobilised pp60$^{c\text{-}src}$*

Following immunoprecipitation of pp60$^{c\text{-}src}$ from platelet membranes using the monoclonal antibody $^{v\text{-}src}$(Ab-1) (Oncogene Science), the immune complex was washed extensively with kinase buffer and then 5 μg of purified gpIIb-IIIa (a kind gift from Dr. J. Gonzales, Madrid) or 50 μg of 12-mer (NH_2-Ala-Ser-Lys-Tyr-Ile-Pro-Asn-Glu-Gly-Thr-Asp-Trp-COOH) were added. This is homologous to the region surrounding tyrosine 788, the phosphorylation site of integrin β_1 subunit which is partially conserved in gpIIIa. To initiate the reaction 35 μl of kinase buffer containing 2.5 μCi ^{32}P-γ-ATP was added. After incubation at 37°C for 20 min the sample was centrifuged and the supernatant subjected to SDS-PAGE (gpIIb-IIIa) or thin layer electrophoresis (12-mer peptide).

2.9. *Antibodies used*

Monoclonal anti-phosphotyrosine PY20 was purchased from ICN Biochemicals. Rabbit anti-pp60 was used as described [16] and monoclonal anti-pp60^{src}(Ab-1) was purchased from Oncogene Science. Monoclonal antibody MM2/356 which recognises the gpIIb-IIIa complex was generously provided by Dr. J.M. Wilkinson (this department).

3. RESULTS

3.1. *Phosphorylation of purified platelet membrane fractions*

Proteins could be phosphorylated in vitro in each of the subcellular membrane fractions, however the cytosol contained fewer phosphoproteins (Fig. 1A). After alkali hydrolysis there was a reduction in the number of phosphopeptides, however the surface membrane fraction contained several alkali resistant bands, the major band being 55–60 kDa which was probably autophosphorylated pp60$^{c\text{-}src}$. In addition other bands at 130, 100, 48, 40 and 36 kDa were visible (Fig. 1A). The presence of alkali-resistant phosphoproteins correlated with the presence of pp60$^{c\text{-}src}$ in the subcellular fractions, with the surface membrane containing most of the pp60$^{c\text{-}src}$ and the cytosol and intracellular membrane fractions containing barely detectable levels (Fig. 1B).

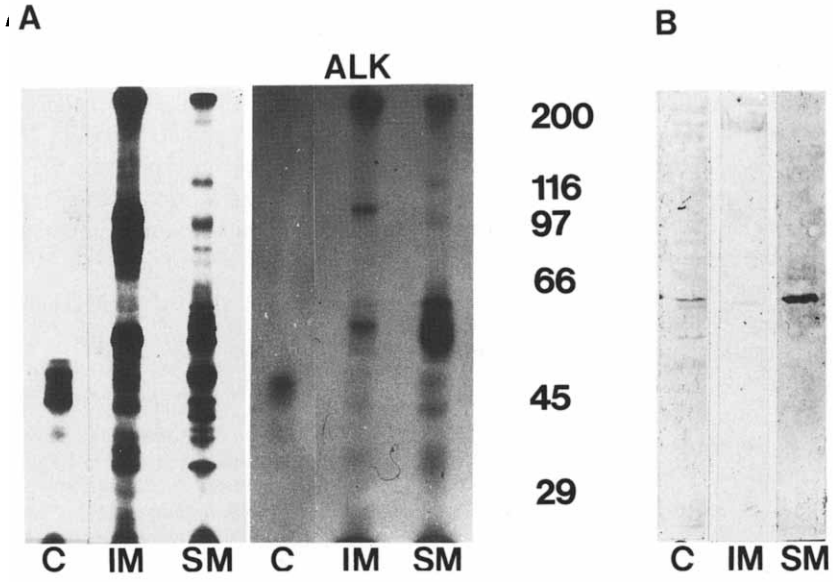

Fig. 1. (A) Autoradiographs of total membrane protein labelled with [^{32}P]ATP in purified platelet fractions before and after alkali treatment (alk). (B) Immunoblot of fractions transferred to nitrocellulose probed with anti-pp60. C, cytosol; IM, intracellular membranes; SM, surface membranes.

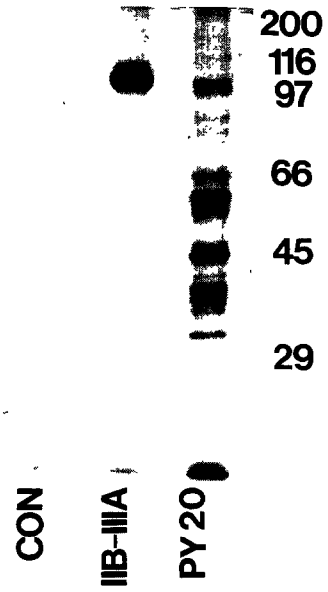

Fig. 2. Immunoprecipitation of gpIIb-IIIa (IIB-IIIA) and phosphotyrosine-containing proteins (PY20) from ^{32}P-labelled surface membranes. CON, non-immune Ig. 10% reducing gel.

The putative phosphotyrosine-containing proteins were further characterised in the plasma membrane fraction by immunoprecipitation using the antiphosphotyrosine antibody PY20. Major bands of molecular weights approximately 66, 60, 45 and 40 kDa and also a protein of molecular weight approximately 100 kDa were precipitated (Fig. 2). Immunoprecipitation of the membranes by anti-gpIIb-IIIa resulted in a phosphopeptide of about 100 kDa which had the same electrophoretic mobility as the tyrosine phosphorylated protein (Fig. 2), and there was a shift in the apparent molecular weight of the phosphorylated band which is characteristic of gpIIIa under nonreducing conditions (Fig. 3A). Coomassie blue staining showed that both gpIIb and gpIIIa were precipitated by the antibody, therefore the IIIa subunit of this complex was specifically phosphorylated in the membrane (Fig. 3A).

3.2. *Phosphoamino acid analysis of gpIIIa*

The previous experiments indicated that a protein which comigrated with gpIIIa contained phosphotyrosine. Phosphoamino acid analysis of ^{32}P-labelled gpIIIa revealed that the phosphorylation was exclusively on tyrosine (Fig. 3B).

3.3. *Phosphorylation of purified gpIIb-IIIa by isolated pp60$^{c\text{-}src}$*

To determine whether platelet pp60$^{c\text{-}src}$ was capable of phosphorylating gpIIb-IIIa, this enzyme was immune precipitated from membranes and the immobilised complex added to purified gpIIb-IIIa. After phosphorylation the complex was spun off and the supernatant containing gpIIb-IIIa subjected to SDS-PAGE. Unlike the situation in membranes where only gpIIIa was phosphorylated, this use of purified enzyme and substrate resulted in the phosphorylation of both the IIb and IIIa peptides in complexes containing anti-pp60, but not in complexes containing nonimmune Ig (Fig. 4). The 60 kDa phosphoprotein comigrated with autophosphorylated pp60$^{c\text{-}src}$.

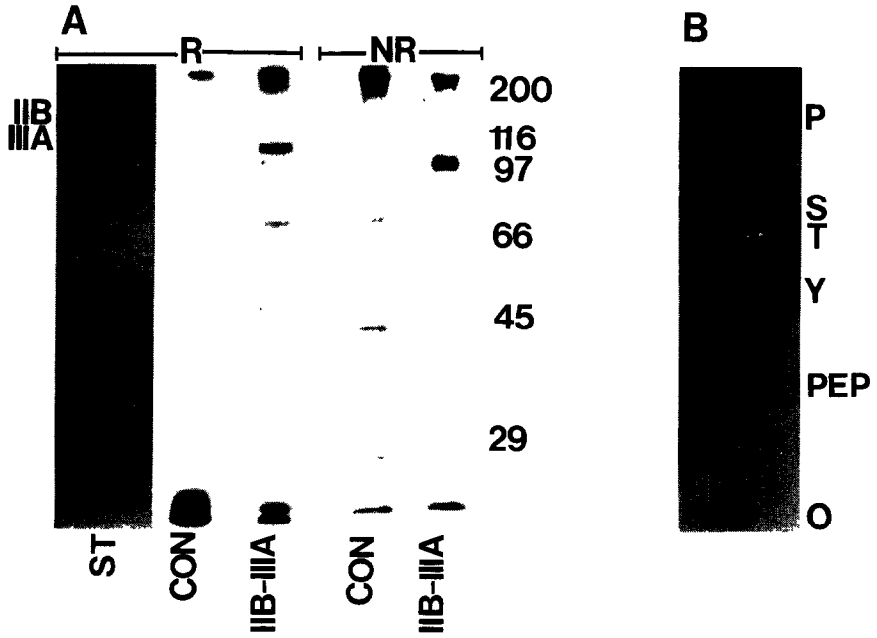

Fig. 3. (A) Immunoprecipitation of gpIIb-IIIa from ^{32}P-labelled platelet membranes under reducing (R) and non-reducing (NR) conditions. ST, Coomassie blue staining showing both IIb and IIIa subunits; CON, non-immune Ig. (B) Phosphoamino acid analysis of ^{32}P-labelled gpIIIa. O, origin; PEP, unhydrolysed peptide; Y, phosphotyrosine; T, phosphothreonine; S, phosphoserine; P, free phosphate.

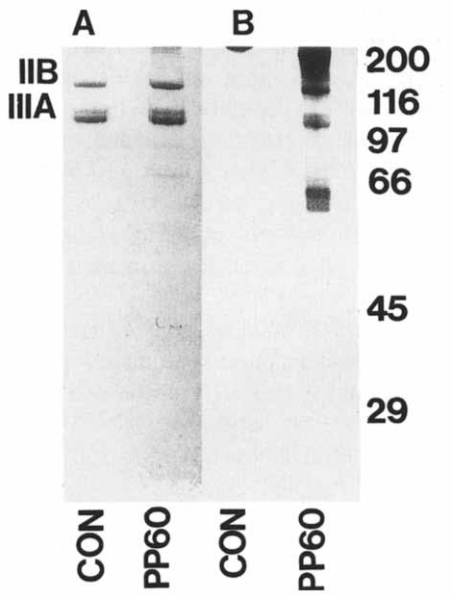

Fig. 4. Phosphorylation of purified gpIIb-IIIa by immunoprecipitated pp60$^{c\text{-}src}$ (A) Coomassie blue staining of gpIIb-IIIa in the phosphorylation reactions. (B) Autoradiograph of dried gel showing phosphorylation of both gpIIb and gpIIIa in pp60$^{c\text{-}src}$-containing immune complexes (PP60) but not in lysates precipitated with nonimmune Ig (CON).

3.4. *Phosphorylation of a synthetic peptide*

Since platelet gpIIIa has homology to β_1 integrins, and has a conserved putative tyrosine phosphorylation site for pp60$^{v\text{-}src}$ [17], we investigated whether pp60$^{c\text{-}src}$ can phosphorylate this site. pp60$^{c\text{-}src}$ was immune precipitated from platelet membranes and a 12-mer synthetic peptide homologous to the sequence surrounding integrin β chain tyrosine 788 used as a substrate in an in vitro phosphorylation reaction. This tyrosine residue is conserved in gpIIIa (tyrosine 747) [17]. Thin layer electrophoresis demonstrated that the peptide was phosphorylated by pp60$^{c\text{-}src}$ (Fig. 5). Control experiments demonstrated that this peptide was phosphorylated by pp60$^{v\text{-}src}$ precipitated from RSV-transformed fibroblasts but not from lysates immune precipitated with nonimmune serum (Fig. 5).

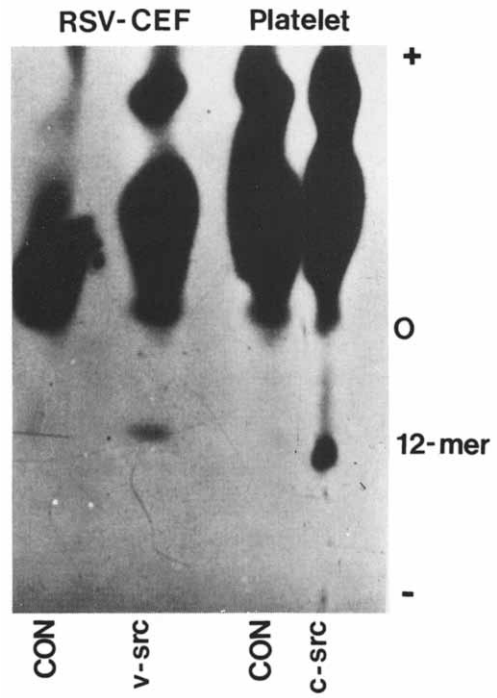

Fig. 5. Phosphorylation of a 12-mer synthetic peptide by immunoprecipitated pp60$^{v\text{-}src}$ from Rous sarcoma virus-transformed chick embryo fibroblasts (RSV-CEF) and by pp60$^{c\text{-}src}$ immunoprecipitated from platelet surface membranes. O, origin.

4. DISCUSSION

The rapid changes in platelet adhesiveness make it an excellent cell with which to investigate cell-matrix interactions. Whilst many of the integrin superfamily have been characterised in detail, there is still a paucity of information regarding the regulation of receptor-matrix interactions. It has been suggested that tyrosine-specific phosphorylation of the β_1 chain of the fibronectin receptor by pp60$^{v\text{-}src}$ leads to an alteration of the affinity of the receptor complex for fibronectin [12]. Platelets contain large amounts of the cellular homolog pp60$^{c\text{-}src}$, and the IIIa subunit contains a tyrosine phosphorylation site similar to that of the fibroblast β_1 integrin chain [17], therefore we have investigated whether gpIIb-IIIa in platelet membranes can be phosphorylated by pp60$^{c\text{-}src}$. Our results have demonstrated that in platelets most of the alkali-resistant bands and pp60$^{c\text{-}src}$ itself are in the plasma membrane fraction, indicating that this is the location of the major tyrosine kinase activity. The pattern of phosphorylation was similar to that previously reported for crude membranes, with pp60$^{c\text{-}src}$ the most intense band [7], although other higher molecular weight bands were also present. We have presented novel evidence that within membranes gpIIb-IIIa complex can be phosphorylated but only on the IIIa subunit, however in agreement with Findik et al. immunopurified pp60$^{c\text{-}src}$ phosphorylated both subunits of purified gpIIb-IIIa [18]. This may be due to a masking of the phosphorylation site of gpIIb in the membrane, or to a conformational change in the protein which exposes the phosphorylation site when purified or exposed to detergent. It is likely that the membrane phosphorylation of gpIIIa described here more closely mimics the situation within whole platelets, however it is possible that the phosphorylation of gpIIb may also occur. Phosphoamino acid analysis revealed that gpIIIa was phosphorylated exclusively on tyrosine. Furthermore, pp60$^{c\text{-}src}$ immunoprecipitated from platelet membranes phosphorylates a peptide similar to the putative tyrosine phosphorylation site of gpIIIa, indicating that tyrosine 747 in gpIIIa may be a phosphorylation site. These results indicate that gpIIIa can be phosphory-

lated by $pp60^{c\text{-}src}$ in platelet membranes and this may be an important regulatory process in platelet function.

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