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Glycoprotein Ib_β is the only phosphorylated major membrane glycoprotein in human platelets

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Platelets were metabolically labelled with ^{32}P and the phosphoproteins examined by two-dimensional non-reduced/reduced gel electrophoresis and isoelectric-focusing/gel electrophoresis. Comparison with similar separations of surface-labelled platelets showed that the only major glycoprotein which is phosphorylated is the β-subunit of glycoprotein Ib, indicating that this subunit contains a cytoplasmic segment. The identification was confirmed using immunoblotting with an antibody to the β-subunit. Phosphoserine was the principal phosphorylation site, with some phosphothreonine, but phosphotyrosine was absent. No quantitative or qualitative differences could be detected in the phosphorylation of glycoprotein Ib_β from resting or activated platelets. These results exclude changes in phosphorylation of the major platelet membrane glycoproteins as a method of signal transmission by these receptors.

INTRODUCTION

Phosphorylation of membrane receptors after binding of an agonist is a common phenomenon in many cells and is probably important for signal transmission. Well-known examples are the insulin receptor (Avruch *et al.*, 1982) or the receptors for various growth factors (Hunter & Cooper, 1981; Ek *et al.*, 1982). Platelets respond to a wide variety of stimuli and contain a large number of phosphoproteins, some of which show remarkable changes in phosphorylation on activation (Haslam *et al.*, 1979). Little is known about the phosphorylation of platelet membrane glycoproteins, which are important receptors for activators, or about qualitative or quantitative changes resulting from platelet activation. Marchesi & Chasis (1979) extracted platelet glycoproteins with lithium di-iodosalicylate and also used wheat germ agglutinin affinity chromatography. They claimed that several major glycoproteins, including GPI, were phosphorylated. Solum & Olsen (1984) examined phosphorylated platelets by crossed immunoelectrophoresis and found only one phosphorylated precipitation arc corresponding to that containing GPIb. In order to clarify the situation we have examined phosphorylated platelets by two-dimensional gel electrophoresis (non-reduced/reduced and isoelectric-focusing/gel electrophoresis) using electroblotting to confirm the identity of the phosphorylated glycoprotein. Possible changes in phosphorylation states or distribution on activation have also been examined.

MATERIALS AND METHODS

Isolation of human blood platelets

Platelets were isolated from citrate-treated blood collected for the Central Laboratory of the Swiss Red Cross (Bettex-Galland & Lüscher, 1960). The buffy coats were transferred into a buffered glucose solution to give platelet-rich plasma containing about 4.8 mM-glucose, 30 mM- sodium citrate, 100 mM-NaCl buffer, pH 6.5, and about 4×10^9 platelets/ml (Massini & Lüscher, 1974). The platelets were isolated by centrifugation and were washed once with 30 mM-glucose/120 mM-NaCl/10 mM-EDTA/12 mM-sodium citrate, pH 6.5. Apyrase, prepared according to Traverso-Cori *et al.* (1965), was added to platelets (0.07 unit/ml) to hydrolyse ADP.

Surface labelling by periodate/NaB^3H_4 method

Platelets were isolated from platelet-rich plasma, washed and surface-labelled by the periodate/NaB^3H_4 method (Gahmberg & Andersson, 1977) as modified by Steiner *et al.* (1983).

Metabolic labelling with ^{32}P

Platelets (10^{10}/5 ml) were labelled with 1 mCi of ^{32}P as previously described (Clemetson *et al.*, 1984). Sodium [^{32}P]orthophosphate was from Amersham International or the Institut National des Radioelements, Brussels, Belgium.

Treatment of phosphorylated platelets

Platelets (2×10^9/ml) were treated with thrombin (Hoffmann–La Roche, Basle, Switzerland; 3 units/ml), forskolin (Calbiochem AG, Lucerne, Switzerland; 100 μM), bovine von Willebrand factor (4 μg/ml) prepared according to Furlan *et al.* (1979), human plasma and ristocetin (Lundbeck, Copenhagen, Denmark; 1.5 mg/ml) for 5 min at room temperature. The platelets were then centrifuged (1500 *g*, 10 min) and the pellet was solubilized in 500 μl of 2% (w/v) SDS/10 mM-EDTA/2 mM-phenylmethanesulphonyl fluoride (Fluka AG, Buchs, Switzerland)/100 μg of leupeptin (Sigma, St. Louis, MO, USA)/ml. In some experiments the SDS was replaced by 1% (v/v) Triton X-100 or X-114 (Sigma) or 1% (v/v) NP-40 (Shell, Zurich, Switzerland). When insoluble cytoskeleton was formed, solubilized platelets were centrifuged at 1500 *g* for 10 min. For non-reduced gel electrophoresis 10 mM-*N*-ethylmaleimide and for

Abbreviation used: GPIb, glycoprotein Ib.

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reduced gel electrophoresis 1% (w/v) dithiothreitol was added. Solubilized platelets were boiled (100 °C, 2 min) and analysed by SDS/polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis

Two-dimensional polyacrylamide-gel electrophoresis [isoelectric-focusing/5–15% (w/v) or 5–20% (w/v) gradient polyacrylamide gel electrophoresis] was performed as described earlier (Clemetson *et al.*, 1980). The gels were prepared for fluorography (Laskey & Mills, 1975) or indirect autoradiography (Laskey & Mills, 1977). For non-reduced/reduced two-dimensional polyacrylamide-gel electrophoresis the method of Wang & Richards (1974) was used. First-dimension gels were reduced in 10% (v/v) 2-mercaptoethanol (Merck, Darmstadt, Germany) for 20 min at 60 °C.

Immunoblotting

Immunoblotting after two-dimensional SDS/polyacrylamide-gel electrophoresis (non-reduced/reduced) of platelet phosphoproteins was carried out by the method of Towbin *et al.* (1979). The nitrocellulose (Schleicher & Schüll, Dassel, Germany) was stained with a rabbit anti-GPIb$_\beta$ serum [kindly supplied by Mr. A. Wicki, Theodor Kocher Institute, Berne, Switzerland; diluted 1:50 in 10 mM-Tris/HCl/154 mM-NaCl/3% (w/v) bovine serum albumin (Sigma)/0.02% NaN_3, pH 7.6] and peroxidase-coupled goat anti-(rabbit IgG) [Bio-Science Products AG, Emmenbrücke, Switzerland, diluted 1:200 in 10 mM-Tris/HCl/154 mM-NaCl/3% (w/v) bovine serum albumin/0.2% NaN_3, pH 7.6]. After drying, the stained nitrocellulose was subjected to indirect autoradiography.

Phosphoamino acid analysis and tryptic peptide mapping

The ^{32}P-labelled GPIb$_\beta$ protein spots from six to eight gels (non-reduced/reduced) were cut out and electro-eluted into dialysis bags (150 V, 48 h) as described by Spiker & Isenberg (1983), with several changes of electrophoresis buffer. Bovine serum albumin (0.5 mg/ml) was added as carrier protein. Trichloroacetic acid (Fluka) was added [20% (w/v) final concentration] and the mixture was left at 0 °C for 4 h to precipitate the protein. The precipitate was washed and the protein isolated according to Beemon & Hunter (1978). It was then dissolved in about 1 ml of 6 M-HCl by boiling for 2 min, hydrolysed under Protectan (Tetenal-Photowerk, Hamburg and Berlin, Germany) for 2 h at 110 °C and afterwards centrifuged at 14000 ***g*** for 10 min. Phosphoserine, phosphothreonine and phosphotyrosine (Sigma; 200 μg) were added as internal standards. Phosphoaminoacid analysis was performed on two-dimensional cellulose thin-layer electrophoresis as described by Hunter & Sefton (1980). Tryptic peptide mapping of GPIb$_\beta$ was carried out as described by Beemon & Hunter (1978). Two-dimensional cellulose thin-layer electrophoresis/chromatography was carried out as described by Elder *et al.* (1977). Amino acids and tryptic peptides were detected by staining with ninhydrin (Merck; 0.1%). ^{32}P-phosphorylated amino acids and peptides were detected by indirect autoradiography (Laskey & Mills, 1977).

Alkaline treatment of the gels

The gels from two-dimensional electrophoresis (isoelectric-focusing/gel electrophoresis and non-reduced/reduced) were soaked in two changes of 10% (v/v) acetic acid/10% (v/v) propan-2-ol, dried and exposed for indirect autoradiography (Laskey & Mills, 1977). The same gels then were subjected to alkaline treatment as described by Cooper & Hunter (1981) and then dried again and exposed as before except that the exposure times were longer (8 days compared to 1 day) to get comparable exposures.

Densitometric analysis of the ^{32}P content of the GPIb$_\beta$ spot

The absorption of the GPIb$_\beta$ spots, as well as that of a reference phosphoprotein in the diagonal of the gel (with molecular mass of approx. 40 kDa) which remains virtually unchanged after thrombin-stimulation, on autoradiograms of two-dimensional gel electrophoretic separations from untreated and thrombin-treated platelets were measured by densitometry (model DD2; Kipp & Zonen, Delft, Holland). The area under the peaks from three measurements were cut out and weighed. Results are given in arbitrary units after correction with the reference protein. The ratio between resting and thrombin-treated platelets was calculated.

RESULTS

Indirect autoradiograms of two-dimensional gels (isoelectric-focusing/gel electrophoresis) of ^{32}P-labelled platelets were compared with previously obtained fluorograms from two-dimensional gels of platelets surface-labelled by the periodate/NaB^{3}H$_4$ method. However, because the labelling patterns are quite different it is difficult to find which components are labelled by both methods. Comparison using non-reduced/reduced two-dimensional gel electrophoresis (Figs. 1 and 2) provided a much clearer method. Most of the glycoproteins which are surface-labelled do not lie on the diagonal in this separation system and therefore contain disulphide bonds. In particular, the β-subunits of glycoproteins Ib, Ic and IIb characteristically lie well under the diagonal (Fig. 1). In the gel of the ^{32}P-labelled platelets separated by this method, nearly all the labelled components lie directly on the diagonal with only one spot in the region of the β-subunits. In some preparations of platelets, an additional faint spot was found (Fig. 2, arrow) at a lower apparent M_r in the first dimension, but at the same M_r as the GPIb$_\beta$ spot in the second dimension. A similar phenomenon is also found with platelets surface-labelled with ^{3}H (Fig. 1). Some other weaker spots were found under the diagonal which do not coincide with any platelet membrane glycoprotein; the positions of these are indicated in Fig. 2. When ^{32}P-labelled and ^{3}H-labelled platelets were mixed together and analysed by non-reduced/reduced gel electrophoresis, followed by fluorography, the ^{32}P-labelled spot was found to coincide with the ^{3}H-labelled GPIb$_\beta$ spot (results not shown). Platelets labelled with ^{32}P were separated by non-reduced/reduced gel electrophoresis, electrophoretically transferred to nitrocellulose and the resulting electroblot immunologically stained with a rabbit anti-GPIb$_\beta$ serum followed by peroxidase-coupled goat anti-(rabbit IgG) and developed with 4-chloro-1-naphthol. Two spots were stained (Fig. 3) which coincided with the position of the ^{32}P labelling determined by indirect autoradiography of the nitrocellulose sheet (results not shown) demonstrating that these are GPIb$_\beta$. Comparison

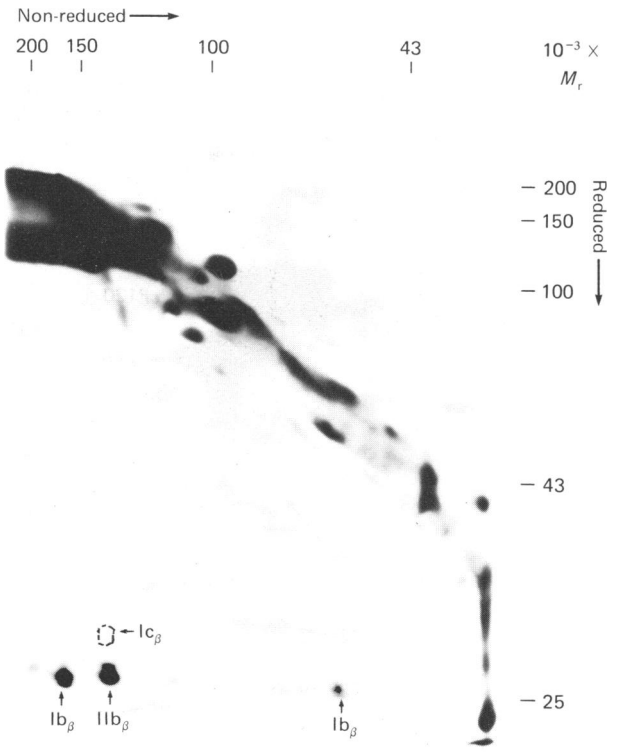

Fig. 1. Two-dimensional polyacrylamide-gel electrophoretic separations of platelet membrane glycoproteins labelled by the periodate/NaB^3H_4 method

First dimension, 7.5% (w/v) acrylamide, non-reduced; second dimension, 10% (w/v) acrylamide, reduced. The broken line indicates the position of $GPIc_\beta$ which appears only on some autoradiograms.

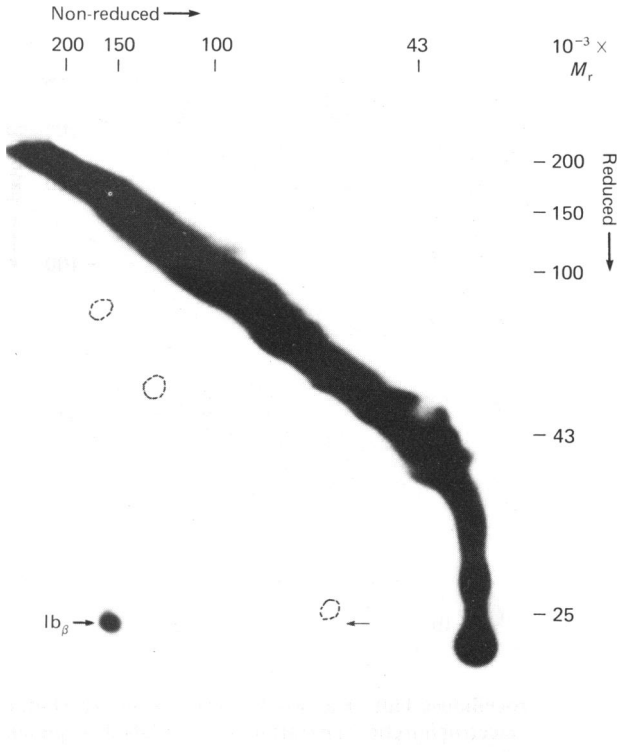

Fig. 2. Indirect autoradiogram of two-dimensional polyacrylamide-gel electrophoretic separations of platelet phosphoproteins metabolically labelled with ^{32}P

First dimension, 7.5% (w/v) acrylamide, non-reduced; second dimension, 10% (w/v) acrylamide, reduced. The arrow indicates the position of the $GPIb_\beta$ spot which migrates in the first dimension together with a split product of GPIb as described in the text. Broken lines indicate the positions of additional very weakly phosphorylated spots.

of indirect autoradiograms of two-dimensional (isoelectric-focusing/gel electrophoresis) polyacrylamide gels of ^{32}P-labelled platelets run under reducing or non-reducing conditions showed that, as expected, $GPIb_\beta$ could be detected on the reduced gel and not on the unreduced gel (results not shown). This was the only detectable difference between the patterns of the gels.

Within single platelet preparations, the amount of ^{32}P incorporated into $GPIb_\beta$ from resting and thrombin-stimulated platelets was the same (Table 1, last column). While no significant change was found in $GPIb_\beta$ phosphorylation between the two states on isoelectric focusing/gel-electrophoresis gels, P47 (a substrate for the Ca^{2+}/phospholipid-dependent protein kinase) incorporated more ^{32}P after stimulation with thrombin (Figs. 4*a* and 4*b*). No quantitative or qualitative changes in phosphorylation of $GPIb_\beta$ were detected either in ^{32}P-labelled platelets treated with bovine von Willebrand factor (4 μg/ml), human plasma (as source of von Willebrand factor) plus ristocetin (1.5 mg/ml) or forskolin (100 μM) compared with control platelets (results not shown).

The distribution of the phosphorylation within $GPIb_\beta$ was examined by cutting out the spots of ^{32}P-labelled $GPIb_\beta$ from non-reduced/reduced two-dimensional gels, eluting the protein, hydrolysing with acid and separating the amino acids by two-dimensional thin layer electrophoresis. This showed that the principal product was phosphoserine, with some phosphothreonine, but no phosphotyrosine was detected (Fig. 5). Analysis of tryptic peptides from ^{32}P-labelled $GPIb_\beta$ by two-dimensional thin layer electrophoresis/chromatography gave two principal spots which were identical whether from thrombin-treated (3 units/ml) or resting platelets (results not shown). Confirmation of the absence of phosphotyrosine from $GPIb_\beta$ was obtained by alkaline treatment of two-dimensional gels. Alkaline-resistant phosphoproteins remain as strong spots on autoradiograms and contain phosphotyrosine. The $GPIb_\beta$ spot disappears after this treatment (results not shown). P55 and parts of P120 (Fig. 6) still stand out strongly compared with a control gel (Fig. 4*a*). P47, a substrate of the Ca^{2+}/phospholid-dependent protein kinase (protein kinase C; Kawahara *et al.*, 1980) is still phosphorylated but markedly decreased. P20 (myosin light chain) disappears nearly completely and P80 is markedly decreased under these conditions, indicating that here again serine and threonine are the main phosphorylation sites (results not shown).

When resting ^{32}P-labelled platelets were solubilized with SDS, rather than non-ionic detergents such as Triton X-100 or NP-40, a higher level of phosphorylation of P20

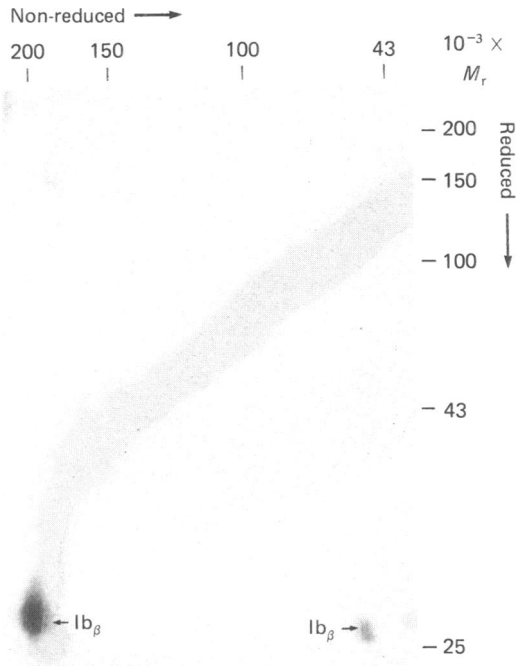

Fig. 3. Nitrocellulose blot of a two-dimensional polyacrylamide gel electrophoretic separation of ^{32}P-labelled platelet proteins

First dimension, 7.5% (w/v) acrylamide, non-reduced; second dimension, 10% (w/v) acrylamide, reduced. Spots were stained with rabbit anti-GPIb$_\beta$ serum and peroxidase-coupled goat anti-(rabbit IgG). Stained spots coincide with ^{32}P incorporated into GPIb$_\beta$.

Table 1. Densitometric analysis of the GPIb$_\beta$ spot on autoradiograms from two-dimensional gel electrophoretic separations (non-reduced/reduced)

Preparation	Density (arbitrary units) Untreated platelets	Thrombin-treated platelets	Ratio untreated/thrombin-treated
1	0.51	0.49	1.04
2	1.50	1.52	0.99
3	1.10	1.03	1.07
			Mean ±S.D.
			1.03±0.040

was found (results not shown). The reason for this may be the better solubilization of cytoskeletal proteins with this detergent.

DISCUSSION

The results obtained with autoradiography of two-dimensional gel electrophoresis separations of human platelets labelled metabolically with ^{32}P indicated that GPIb is the only major platelet membrane glycoprotein which is labelled and that the phosphorylation site(s) is (are) on the β-subunit. The second spot which appeared in some preparations under the diagonal (Fig. 2, arrow),

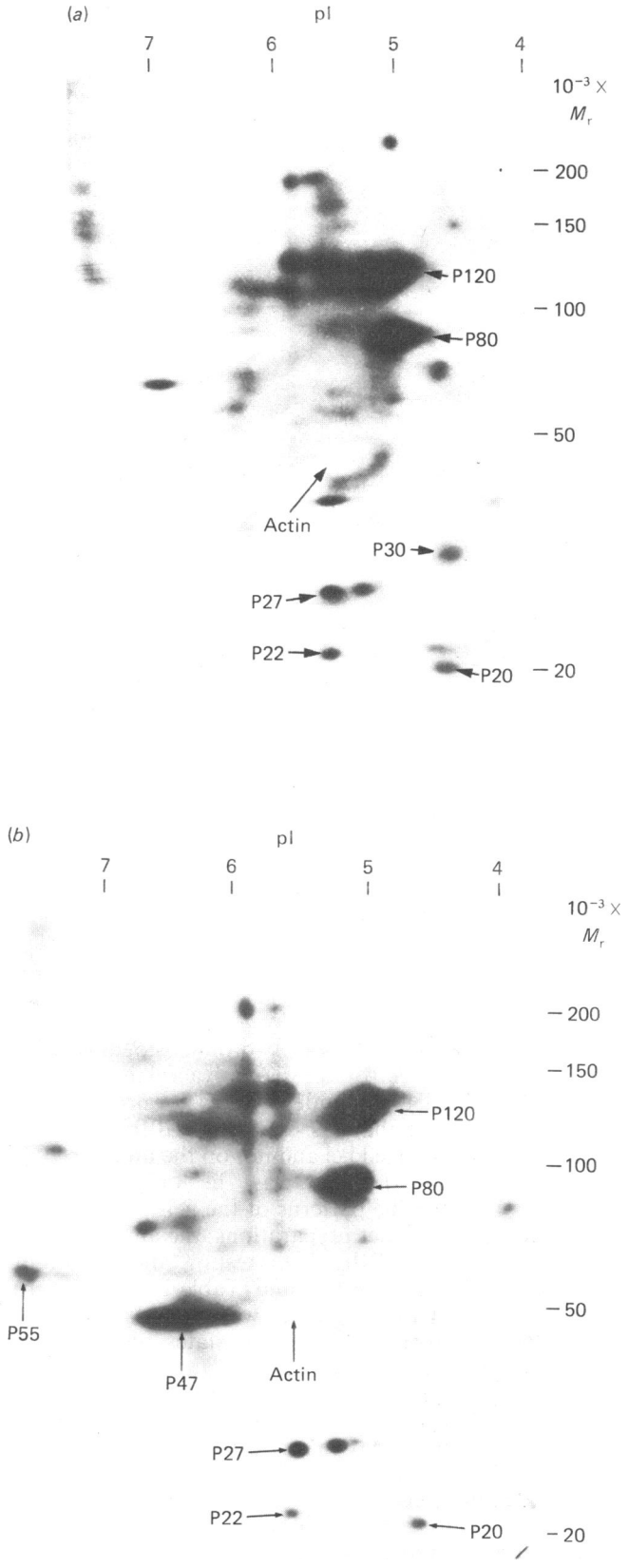

Fig. 4. Indirect autoradiograms of two-dimensional polyacrylamide-gel electrophoretic separations of platelet phosphoproteins metabolically labelled with ^{32}P

Isoelectric-focusing/5–15% (w/v) acrylamide gradient gel electrophoresis of (*a*) resting and (*b*) thrombin-activated (3 units/ml) platelets.

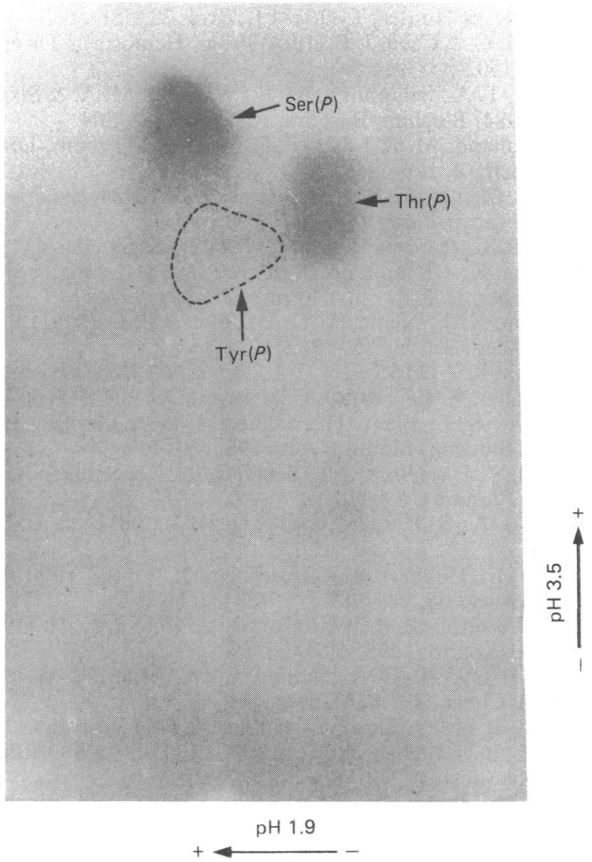

Fig. 5. Indirect autoradiogram of two-dimensional cellulose thin-layer electrophoresis (first dimension pH 1.9, second dimension pH 3.5) of partially hydrolysed GPIb$_\beta$

Hydrolysis was 6 M-HCl, 110 °C, 1 h. The positions of phosphoserine and phosphothreonine are marked with arrows. The broken line indicates the position of the phosphotyrosine internal standard stained with ninhydrin.

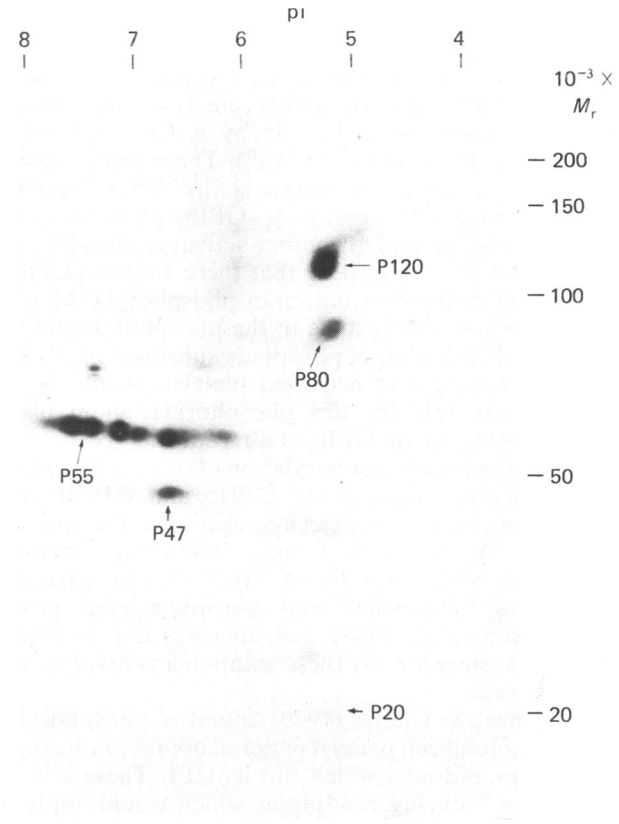

Fig. 6. Indirect autoradiogram of two-dimensional polyacrylamide-gel electrophoretic separation of platelet phosphoproteins

Isoelectric-focusing/5–15% (w/v) acrylamide gradient gel electrophoresis after alkaline treatment of the gel.

is most likely GPIb$_\beta$ which migrates together with a fragment of GPIb$_\alpha$, after glycocalicin (a water-soluble, heavily glycosylated cleavage product of GPIb; Clemetson *et al.*, 1981) has been split off by endogenous calcium-activated proteinase (Solum *et al.*, 1980). This spot corresponds well with a spot found with surface labelling technique (Fig. 1, indicated also as GPIb$_\beta$). It cannot be excluded that other minor surface components are phosphorylated, but if this is the case they either do not contain disulphide bridges, unlike the majority of the membrane glycoproteins, or else show such rapid phosphorylation/dephosphorylation that the phosphorylated state is not detected. Solum & Olsen (1984) were also only able to show phosphorylation associated with GPIb, using crossed immunoelectrophoresis techniques. It is not possible, using this technique, to exclude the presence of other phosphorylated proteins, such as actin-binding protein, which forms a complex with GPIb.

Comparison of levels of phosphorylation of GPIb$_\beta$ in resting and thrombin-activated platelets did not show any significant difference, whereas P47 and other phosphoproteins were more phosphorylated after thrombin treatment. The glycocalicin piece of GPIb$_\alpha$ contains binding sites for thrombin (Okumura *et al.*, 1978) and von Willebrand factor (Jamieson *et al.*, 1979). Treatment of platelets with bovine von Willebrand factor or with forskolin, which inhibits platelet activation, also caused no significant difference in GPIb$_\beta$ phosphorylation.

Comparison of the phosphoamino acid composition of GPIb$_\beta$ in resting and thrombin-activated platelets showed in both cases predominantly phosphoserine, with some phosphothreonine and no detectable phosphotyrosine. This finding was confirmed by alkaline treatment of two-dimensional gels of ^{32}P-labelled platelets. Both phosphoserine and phosphothreonine are alkali-labile and are readily hydrolysed by this treatment (Cooper & Hunter, 1981). The radioactivity associated with the GPIb$_\beta$ spot and with many of the other phosphoproteins was quickly lost. However, other phosphoproteins, in particular P55 and parts of P120, probably composed of different phosphoproteins, remained relatively strongly labelled, indicating that they are rich in phosphotyrosine. P47, which is strongly labelled only in activated platelets (Lyons *et al.*, 1975), also retained some label after alkaline treatment, indicating that it is not phosphorylated only by Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) which is serine/threonine-specific (Imaoka *et al.*, 1983), but that there is a possible involvement of a tyrosine kinase also. On the other hand, the phosphate in P20 (myosin light chain), which is also strongly labelled only in activated platelets (Daniel *et al.*, 1977), was completely hydrolysed by this treatment. Myosin light chain is phosphorylated either by a

Ca^{2+}/calmodulin-dependent myosin light chain kinase (Hathaway & Adelstein, 1979), by a Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C; Naka *et al.*, 1983), which in smooth muscle cells is serine/threonine-specific (Adelstein, 1983), or by a Ca^{2+}-independent mechanism (Hallam *et al.*, 1985). These results indicate that myosin light chain kinase is also serine/threonine-specific. Tryptic mapping of $GPIb_\beta$-phosphopeptides from untreated and thrombin-activated platelets gave similar patterns, indicating that there are no qualitative differences in the distribution of phosphorylation sites.

This lack of any change in the phosphorylation state or in the distribution of phosphate substitution of $GPIb_\beta$ between resting and activated platelets would tend to exclude any role for this phosphorylation in platelet activation involving GPIb as a receptor.

The absence of phosphorylation of other major platelet glycoproteins, in particular GPIIb and GPIIIa which have a major role in platelet aggregation as the fibrinogen receptor (Nachman & Leung, 1982), was previously suggested by the results of Triton X-114 separation, comparing ^3H-labelled and phosphorylated proteins (Clemetson *et al.*, 1984), and indicates that in platelets signal transmission via these components involves other mechanisms.

Marchesi & Chasis (1979) found a phosphorylated band in solubilized platelet material bound to wheat germ agglutinin and interpreted this as GPI. These gels were run under reducing conditions, which would imply that this band was $GPIb_\alpha$. However, in the present study $GPIb_\alpha$ was not labelled. Attempts on our part to isolate ^{32}P-labelled GPIb by wheat germ agglutinin affinity chromatography gave several phosphorylated bands not related to GPIb, but with similar M_r values, which may be phosphoproteins complexed with GPIb (results not shown). This may provide an explanation for the apparent contradiction above. There is now considerable evidence that GPIb is complexed with other proteins, including actin-binding protein (Fox *et al.*, 1983; Fox, 1985; Okita *et al.*, 1985; Solum & Olsen, 1985) and a 22 kDa glycoprotein (Coller *et al.*, 1983) also known as GPIX (Berndt & Caen, 1984) or GP17 (Clemetson *et al.*, 1982). Actin-binding protein is phosphorylated on activation of platelets (Carroll & Gerrard, 1982) and these changes may be important for signal transmission via GPIb. Changes may also occur in the phosphorylation state of other phosphoproteins complexed with GPIb.

The fact that $GPIb_\beta$ is phosphorylated provides additional evidence that it passes through the membrane and contains a cytoplasmic sequence. Berkhout *et al.* (1984) have shown that both GPIb subunits were labelled by a hydrophobic photoactivable probe and are therefore integral membrane proteins.

Part of this work has been previously published in abstract form (Bienz *et al.*, 1984; Wyler & Clemetson, 1985).

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