Tyrosine phosphorylation of P-selectin in intact platelets and in a disulphide-linked complex with immunoprecipitated pp60$^{c\text{-}src}$

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P-selectin is a 140 kDa membrane glycoprotein found in secretory granules of platelets and endothelial cells where it is rapidly translocated to the plasma membrane upon cell activation. It then functions as a receptor for various types of leucocytes. Metabolic labelling of resting platelets with $^{32}P_i$ showed that P-selectin is primarily phosphorylated on serine residues, although some tyrosine phosphorylation was observed as well. However, tyrosine phosphorylation of P-selectin was greatly stimulated by treatment with the permeating phosphatase inhibitor, pervanadate. When P-selectin immunoprecipitates were incubated with [γ-^{32}P]ATP (*in vitro* kinase assay), a fraction of P-selectin was phosphorylated on its tyrosine residues by a co-precipitated kinase. P-selectin phosphorylated *in vitro* co-migrated with 140 kDa surface-labelled ^{125}I-P-selectin during SDS/PAGE under reducing conditions. Under non-reducing conditions, however, phosphorylated P-selectin was disulphide-linked to unknown protein(s) in a 205 kDa complex. *In vitro* kinase assays of the most abundant platelet tyrosine kinase, pp60$^{c\text{-}src}$, demonstrated the presence of similar 140 and 205 kDa phosphorylated proteins in SDS/PAGE under reducing and non-reducing conditions respectively. Extraction and reprecipitation studies with proteins phosphorylated *in vitro* indicated that P-selectin and pp60$^{c\text{-}src}$ form a 205 kDa 1:1 disulphide-linked complex. In the complex, pp60$^{c\text{-}src}$ autophosphorylation is inhibited and P-selectin is phosphorylated on tyrosine residues. As protein disulphides in the cytoplasm of intact cells are extremely rare, our results suggest that P-selectin and pp60$^{c\text{-}src}$, which co-localize in platelet dense granules, may be non-covalently associated and spontaneously form disulphide bridges during lysis. In addition, the observed tyrosine phosphorylation of P-selectin in intact platelets suggests that its function might be regulated by phosphorylation by pp60$^{c\text{-}src}$.

INTRODUCTION

P-selectin (GMP-140, PADGEM, or CD62 antigen) is a 140 kDa membrane glycoprotein present in the α and dense granules of blood platelets and in the Weibel–Palade bodies of endothelial cells [1–3]. Stimulation of these cells with agonists such as thrombin results in the rapid expression of P-selectin on the cell surface, where it serves as a Ca^{2+}-dependent receptor for ligands on various types of leucocytes [4–7]. The ligands for P-selectin on leucocytes are incompletely characterized, but are known to contain fucosylated lactosaminoglycans, including sialyl Lewis^x and sialyl Lewis^a [8]. A 120 kDa glycoprotein carrying sialyl Lewis^x has been identified as the P-selectin ligand on neutrophils and HL-60 cells [9,10].

P-selectin and two related molecules, L-selectin and E-selectin, all contain an N-terminal lectin domain, an epidermal growth factor (EGF)-like domain, a variable number of short consensus repeats, a transmembrane domain and a cytoplasmic domain [1]. L-selectin is constitutively present on the surface on many types of leucocytes, and E-selectin is synthesized by endothelial cells after induction by inflammatory cytokines or endotoxin; its expression peaks 4–6 h after stimulation [11]. All three selectins appear to mediate 'leucocyte rolling', the transient interactions of leucocytes with endothelial cells at sites of inflammation (reviewed in [12,13]). These interactions precede the tight adhesion and leucocyte extravasation mediated by the binding of activated leucocyte β2 integrins to endothelial ligands [14,15]. Endothelial P-selectin-mediated capture of neutrophils has been shown to enable juxtacrine neutrophil activation by membrane-bound endothelial platelet-activating factor [16]. A recent study with P-selectin-deficient mice showed that, in addition to mediating leucocyte rolling in inflammation, P-selectin is also required for the neutrophil extravasation that occurs in the absence of inflammation [17].

The binding of leucocytes to P-selectin on the surface of activated platelets in thrombi appears to be essential for the deposition of fibrin in such thrombi [18], but the mechanisms involved remain to be identified. It is expected that other platelet–leucocyte interactions, such as the recruitment of platelets by neutrophils during migration across inflamed endothelium [19], are also mediated by P-selectin.

Recently, regions in the lectin and EGF domains of P-selectin have been identified that are critical for binding to the leucocyte ligand(s) [20–23]. In addition, the P-selectin cytoplasmic domain has been shown to contain a signal for the targeting of the protein to secretory granules during biosynthesis [24]. In analogy with other membrane receptors, the cytoplasmic domain might have additional functions, such as modulation of receptor affinity, regulation of the expression of P-selectin on the cell surface, transduction of ligand-induced signals, etc. For L-selectin, deletion of the C-terminal 11 amino acids of the cytoplasmic domain did not interfere with recognition of the oligosaccharide moiety in the L-selectin ligand, but abolished leucocyte binding to endothelium, showing that the cytoplasmic domain is required for maximum receptor affinity [25].

For P-selectin, the 35-amino-acid cytoplasmic domain is highly

Abbreviations used: DTT, dithiothreitol; EGF, epidermal growth factor; mAb, monoclonal antibody; NP-40, Nonidet P-40; PMSF, phenylmethanesulphonyl fluoride.

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conserved in humans, mice and cows, but bears little sequence similarity to the cytoplasmic regions of the two other selectins [26–29]. In humans, P-selectin contains five potential phosphorylation sites (two serines, two threonines and one tyrosine; [26]). It has recently been shown that P-selectin in resting platelets is phosphorylated on serine residues and, very weakly, on threonine and tyrosine [30]. Platelet activation with thrombin strongly increased phosphorylation of all three types of residues, followed by a rapid and selective dephosphorylation of both threonine and tyrosine [30].

We have investigated whether platelet P-selectin is associated with a protein kinase by incubating P-selectin immunoprecipitates with [γ-^{32}P]ATP in *in vitro* kinase assays. This approach has previously been used to demonstrate the association between membrane receptors and cytoplasmic kinases that belong to the Src family of tyrosine kinases [31]. Platelets contain at least five Src family kinases [32], including large amounts of pp60$^{c\text{-}src}$, the prototype kinase [33].

Our studies confirm that P-selectin in resting intact platelets is predominantly phosphorylated on serine residues and also show that strong phosphorylation of P-selectin tyrosine residues in intact platelets can be induced by the phosphatase inhibitor, pervanadate. Unexpectedly, a fraction of immunoprecipitated P-selectin appeared to be disulphide-linked to pp60$^{c\text{-}src}$, both in P-selectin and pp60$^{c\text{-}src}$ immunoprecipitates. In this complex, P-selectin was phosphorylated on tyrosine residues, but autophosphorylation of pp60$^{c\text{-}src}$ appeared to be inhibited. These results suggest that P-selectin and pp60$^{c\text{-}src}$ might be closely associated in the membranes of intact platelets and raise the possibility that pp60$^{c\text{-}src}$ might be the kinase responsible for phosphorylation of P-selectin *in vivo*.

MATERIALS AND METHODS

Monoclonal antibodies

Anti-(P-selectin) monoclonal antibody (mAb) C2 (CLB-thromb/6) inhibits the binding of platelets to leucocytes [7]. C2 has been shown to bind to a determinant in the P-selectin lectin domain [22]. Anti-(P-selectin) mAb RUU-SP 1.18, which was kindly supplied by Dr. H. K. Nieuwenhuis (University Hospital, Utrecht, The Netherlands), does not affect the interaction between platelets and leucocytes [7]. Anti-pp60$^{c\text{-}src}$ mAb 327 [34] was kindly provided by Dr. P. van der Geer (Salk Institute, La Jolla, CA, U.S.A.). Anti-(PECAM-1) (CD31) mAb CLB-HEC-75 [35] and anti-(β3 integrin) (CD61) mAb Y2/51 [36] have been described previously.

Preparation of pervanadate

Pervanadate was freshly prepared by adding 1 mM H_2O_2 to a 5 mM solution of sodium orthovanadate (Sigma Chemical Co., St. Louis, MO, U.S.A.) and incubating the mixture for 15 min at room temperature [37]. Excess of H_2O_2 was removed by incubation with 200 μg/ml catalase. The concentration of pervanadate generated is denoted by the concentration of vanadate added to the mixture.

Immunoprecipitation from ^{32}P-labelled platelets

Human platelets were isolated as described [38], using 50 ng/ml prostaglandin I_2 (Sigma) during the washing procedure. The platelets were resuspended in Ca^{2+}/Mg^{2+}/phosphate-free Tyrode's buffer, containing 130 mM NaCl, 27 mM KCl, 16 mM $NaHCO_3$, 5.6 mM D-glucose, pH 7.35. Platelets at 2×10^9/ml were incubated for 3 h at 37 °C with 1.0 mCi/ml of $^{32}PO_4$ (carrier-free; Amersham International, Amersham, Bucks., U.K.). EDTA was then added to a concentration of 5 mM, and platelets were either left untreated or stimulated with 1 unit/ml of human thrombin (Sigma) for 5 min at 37 °C followed by incubation with 10 units/ml of hirudin (Sigma) for 5 min at 37 °C, or treated with 0.5 mM pervanadate for 20 min at 37 °C. The platelets were then pelleted (30 s, 12000 *g*) and lysed for 30 min at 0 °C in a buffer containing 1% (v/v) Nonidet P-40 (NP-40), 20 mM Tris/HCl (pH 7.5), 120 mM NaCl, 40 mM KH_2PO_4, 1 mM Na_3VO_4, 1 mM NaF, 5 mM EDTA, 0.1 mM leupeptin, 20 μg/ml soybean trypsin inhibitor and 1 mM phenylmethanesulphonyl fluoride (PMSF). The lysates were centrifuged at 12000 *g* for 30 min at 4 °C, to sediment insoluble material, and the supernatants were precleared by incubation with protein G–Sepharose (Pharmacia LKB, Uppsala, Sweden) for 30 min at 4 °C. Immunoprecipitation was performed by mixing the precleared lysates with protein G–Sepharose and antibodies [2 μl of ascites for anti-(platelet membrane glycoprotein) antibodies] and incubating for 2 h at 4 °C. The Sepharose beads were washed with a buffer containing 1% (v/v) NP-40, 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 mM Na_3VO_4, 1 mM EDTA and 1 mM PMSF. Bound antigens were analysed by SDS/PAGE [39].

Phophoamino acid analysis

^{32}P-labelled proteins were excised from polyacrylamide gels and homogenized in 50 mM NH_4HCO_3. After addition of SDS and 2-mercaptoethanol to 0.1% (w/v) and 1% (v/v) respectively, the homogenate was incubated for 5 min at 95 °C and extracted overnight at 37 °C. Proteins were precipitated with trichloroacetic acid (15%, v/v) after addition of carrier protein (20 μg/ml IgG). After washing in ice-cold ethanol and drying, the proteins were dissolved in 5.7 M HCl and hydrolysed for 1 h at 110 °C. The hydrolysate was resolved by electrophoresis on thin-layer cellulose plates in two dimensions, at pH 1.9 and pH 3.5, in the presence of unlabelled phosphoamino acids [40]. After autoradiography, the ^{32}P-labelled amino acids were identified by their co-migration with the unlabelled standards stained with ninhydrin.

In vitro kinase assay

Platelets were isolated as described [41], using prostaglandin E_1 (Sigma) at 100 nM during the washing procedure. The platelets were lysed by incubation on ice for 30 min in a buffer containing 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 0.1% SDS, 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 mM Na_3VO_4, 5 mM EDTA and 1 mM PMSF. Immunoprecipitation was then performed as described above for ^{32}P-labelled platelets, with the exception that only 0.1 μl of mAb 327 ascites was used for immunoprecipitation of pp60$^{c\text{-}src}$. The Sepharose beads were washed in lysis buffer containing EDTA at 1 mM rather than at 5 mM. The beads were then incubated in 50 μl of kinase reaction mixture, which contains 7.5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham), 10 mM Tris/HCl (pH 7.5), 5 mM $MnCl_2$, 5 mM $MgCl_2$, for 15 min at 4 °C, under constant agitation. Unless otherwise indicated, the beads were then washed once with 0.5 ml of lysis buffer before analysis by SDS/gel electrophoresis. Sequential precipitation experiments, followed by *in vitro* kinase assays, showed that the immunoprecipitation procedure described above removed more than 50% of P-selectin in the first round of precipitation.

Reprecipitation experiments

For some experiments, immunoprecipitated and *in vitro*-phosphorylated antigens were extracted and used for reprecipitation. To this end, protein G–Sepharose-bound antibodies used for the first round of precipitation were covalently coupled to the matrix by use of the cross-linker dimethyl pimelimidate (Sigma; [42]). After immunoprecipitation and phosphorylation in the *in vitro* kinase assay, the Sepharose beads were washed once in lysis buffer containing EDTA at 5 mM. Antigens were then extracted by incubation of the beads in 0.1% SDS, 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM PMSF, for 2 min at 95 °C. After elution, part of the eluate was immediately diluted 10-fold in a buffer containing 1% (v/v) NP-40, 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1 mM Na_3VO_4, 1 mM EDTA and 1 mM PMSF. The proteins in the remainder of the eluate were reduced by addition of dithiothreitol (DTT) to 10 mM and incubation at 95 °C for 2 min, which was followed by dilution in NP-40 buffer as above. Both non-reduced and reduced extracted antigens were then used for a second round of immunoprecipitation.

Immunoprecipitation from ^{125}I-labelled platelets

Platelets were isolated as described [41], resuspended in EDTA–Tyrode's buffer (130 mM NaCl, 27 mM KCl, 16 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.6 mM D-glucose, 5 mM EDTA, pH 7.35) and stimulated with thrombin as described above. After two additional washes, platelets were ^{125}I-labelled by the lactoperoxidase method [41]. Immunoprecipitation was performed as described for ^{32}P-labelled platelets, with omission of the phosphatase inhibitors, Na_3VO_4 and NaF, from the procedure.

Synthetic peptide

A peptide, CPLNPHSHLGTYGVFTNA, corresponding to amino acids Cys^{766}–Ala^{783} of the cytoplasmic domain of P-selectin was synthesized by the solid-phase method using t-butoxycarbonyl (tBoc) methodology. The peptide was > 95% homogeneous as determined by h.p.l.c. and had the expected amino acid composition after acid hydrolysis, derivatization with phenyl isothiocyanate and C18 reverse-phase h.p.l.c.

RESULTS

Phosphorylation of P-selectin in intact platelets

Human platelets were metabolically labelled with $^{32}P_i$ and used for immunoprecipitation with a mAb raised against P-selectin. Figure 1 shows that in resting, untreated platelets a small amount of phosphate was incorporated into P-selectin (Figure 1a, lane 1). Stimulation of platelets with thrombin for 5 min, followed by a 5 min incubation with the thrombin inhibitor, hirudin, increased P-selectin phosphorylation (lane 2). Under both reducing (Figure 1) and non-reducing conditions, phosphorylated P-selectin co-migrated with P-selectin isolated from ^{125}I-surface-labelled platelets (results not shown). Phosphorylation of another platelet membrane glycoprotein, PECAM-1 (CD31 antigen), was also increased by thrombin (Figure 1b, lanes 4 and 5), consistent with previous reports [38,43]. The amount of platelet lysate used for the immunoprecipitation of PECAM-1 in this experiment was only one-tenth of that used for P-selectin. Moreover, the exposure time for Figure 1(a) was six times longer than that for Figure 1(b). Longer exposure showed a background of phosphorylated proteins identical to the ones seen in Figure 1(a). These results show that phosphorylation of P-selectin, with five potential phosphorylation sites [26], is much weaker than that of PECAM-1 with 22 potential phosphorylation sites [44].

Phosphoamino acid analysis of P-selectin and PECAM-1 isolated from resting platelets showed that both proteins were predominantly labelled on serine residues (Figures 2a and 2c), although small amounts of phosphotyrosine were detectable in both proteins. After thrombin/hirudin treatment as described above, only serine phosphorylation of both proteins was increased (results not shown).

Phosphorylation in the presence of the tyrosine phosphatase inhibitor, pervanadate

To investigate the possible significance of tyrosine phosphorylation in P-selectin and PECAM-1 further, ^{32}P metabolically labelled platelets were incubated with the potent cell-permeating tyrosine phosphatase inhibitor, pervanadate [37]. As shown in Figure 1, pervanadate induced a large increase of phosphorylation of both P-selectin (lane 3) and PECAM-1 (lane 6). Cerenkov counting of the excised bands showed that pervanadate increased total P-selectin phosphorylation more than 10-fold. For both P-selectin and PECAM-1, phosphoamino acid analysis showed a very large increase of phosphorylation of tyrosine residues (Figures 2b and 2d). These results suggest that tyrosine phosphorylation of both proteins is tightly regulated by a kinase/phosphatase system. In the case of P-selectin, some incorporation of phosphate into threonine was observed as well (Figure 2b). Additional stimulation of pervanadate-treated platelets with thrombin had little effect on total phosphorylation of either protein and did not affect the phosphoamino acid pattern (not shown).

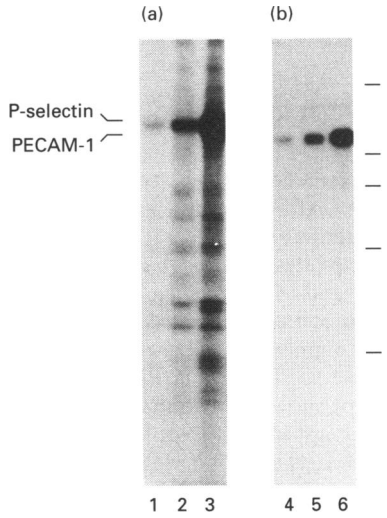

Figure 1 Metabolic phosphorylation of P-selectin

Washed platelets (2×10^9/ml) were incubated with [^{32}P]phosphate in Ca^{2+}/Mg^{2+}/phosphate-free Tyrode's buffer for 3 h at 37 °C. After addition of EDTA to 5 mM, aliquots of the platelets were either left untreated (lanes 1 and 4), stimulated with thrombin (1 unit/ml) for 5 min at 37 °C, followed by neutralization with hirudin (10 units/ml) (lanes 2 and 5) or treated with the tyrosine phosphatase inhibitor, pervanadate (0.5 mM), for 20 min at 37 °C (lanes 3 and 6). Platelets were then pelleted and lysed in the presence of inhibitors of proteases and phosphatases. Lysates were used for immunoprecipitation with C2, a mAb raised against P-selectin [lysates from 3×10^8 platelets; (**a**), lanes 1–3] and with HEC-75 against PECAM-1 [lysates from 3×10^7 platelets; (**b**), lanes 4–6]. The phosphorylated antigens were analysed by reducing SDS/PAGE on a 7.5% (w/v) polyacrylamide gel and by exposure of the dried gel to X-ray film for 12 h (**a**) and 2 h (**b**). Molecular-mass markers indicate 200, 116.25, 97.4, 66.2 and 42.7 kDa.

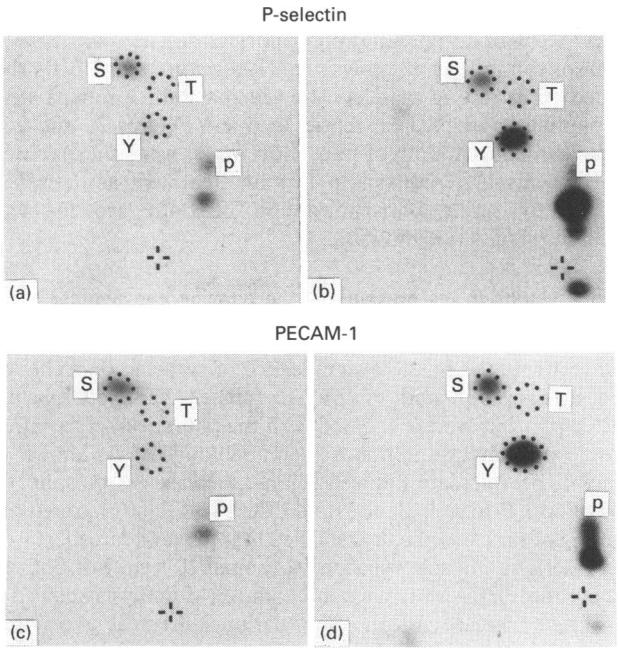

Figure 2 Phosphorylation of platelet P-selectin and PECAM-1 in resting and pervanadate-treated platelets detected by phosphoamino acid analysis

^{32}P-labelled proteins shown in Figure 1 were excised from the gel, extracted and submitted to partial acid hydrolysis. The hydrolysate containing ^{32}P-labelled free phosphoamino acids and peptides was mixed with unlabelled phosphoamino acids and resolved by two-dimensional thin-layer electrophoresis on cellulose plates. The positions of phosphopeptides (p), and of phosphoamino acids detected by ninhydrin staining, are indicated: S, phosphoserine; T, phosphothreonine and Y, phosphotyrosine. (**a**) P-selectin from resting platelets. (**b**) P-selectin from pervanadate-treated platelets. (**c**) PECAM-1 from resting platelets. (**d**) PECAM-1 from pervanadate-treated platelets.

Tyrosine kinase activity associated with immunoprecipitated P-selectin

The observed pervanadate-induced tyrosine phosphorylation of P-selectin and PECAM-1 prompted us to investigate whether these proteins might be associated with a tyrosine kinase. To this end, immunoprecipitates were incubated with [γ-^{32}P]ATP in the presence of Mn^{2+} and Mg^{2+} (*in vitro* kinase assays). Specifically phosphorylated proteins were not observed in the immunoprecipitates of a number of platelet glycoproteins, including those of PECAM-1, GPIIb/IIIa, GPIb and the $\alpha 2\beta 1$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrins (not shown). In contrast, precipitates of anti-(P-selectin) mAb C2 appeared to contain one protein that was specifically and strongly phosphorylated *in vitro*. Under reducing conditions, this protein co-migrated with P-selectin at 140 kDa (Figure 3a, lane 3) as judged by silver staining and by comparison with ^{125}I-labelled P-selectin from surface-labelled platelets (lanes 1 and 2). Under non-reducing conditions, a strongly phosphorylated protein was detected with an estimated molecular mass of 205 kDa (Figure 3b, lane 3), while a minor phosphoprotein co-migrated with ^{125}I-labelled P-selectin (lanes 1 and 2). The 205 kDa phosphorylated protein could not be visualized by silver staining (results not shown), indicating that P-selectin moiety in the 205 kDa protein represented only a minor fraction of total P-selectin. Identical results were obtained when an mAb (RUU-SP 1.18) raised against a different epitope on P-selectin [7] was used for immunoprecipitation (results not shown). Phosphoamino acid analysis (Figure 4) revealed that P-selectin was exclusively phosphorylated on tyrosine in the *in vitro* kinase assays, sug-

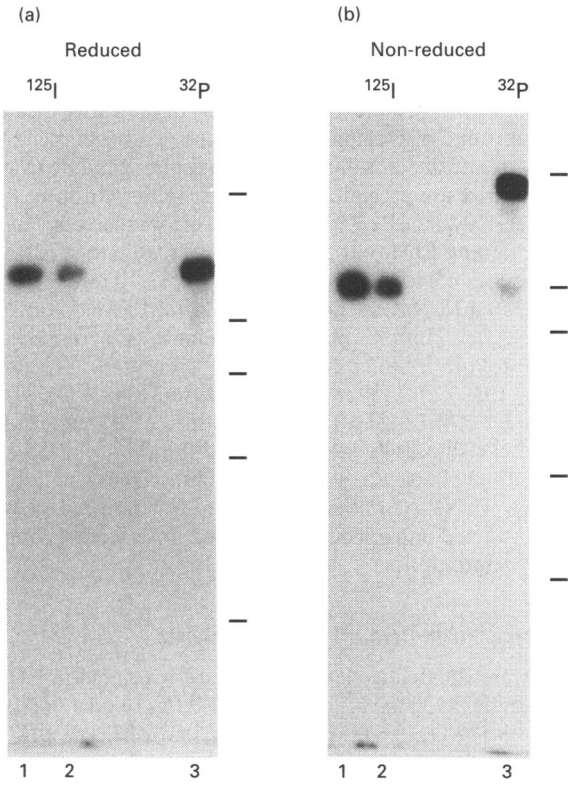

Figure 3 *In vitro* phosphorylation of P-selectin in a 205 kDa disulphide-linked complex in P-selectin immunoprecipitates

P-selectin was immunoprecipitated from resting washed platelets with mAb C2, using the stringent immunoprecipitation conditions described in the Materials and methods section. *In vitro* kinase assays were performed by incubating immunoprecipitates with [γ-^{32}P]ATP in the presence of Mn^{2+} and Mg^{2+} at 4 °C. P-selectin, labelled *in vitro* with ^{32}P (lanes 3), was compared with P-selectin from thrombin-stimulated, ^{125}I-surface-labelled platelets that was precipitated under stringent conditions (lanes 2) or from NP-40 lysates (lanes 1). The labelled antigens were detected by autoradiography after electrophoresis on 7.5% (w/v) polyacrylamide gels under reducing (**a**) and non-reducing (**b**) conditions. Molecular-mass markers are indicated as in the legend to Figure 1.

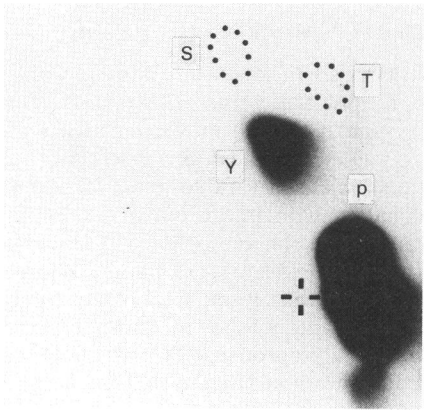

Figure 4 P-selectin is phosphorylated on tyrosine in the *in vitro* kinase assay

P-selectin immunoprecipitate was *in vitro*-phosphorylated as described in the legend to Figure 3. After SDS/PAGE under reducing conditions, phosphoamino acid analysis was performed as described in the legend to Figure 2.

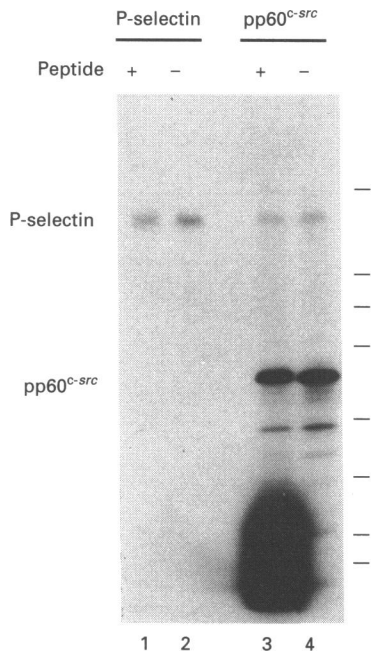

Figure 5 ***In vitro*** **phosphorylation of a P-selectin cytoplasmic domain synthetic peptide**

A phosphorylated protein with a mobility similar to that of P-selectin was detected in pp60$^{c\text{-}src}$ *in vitro* kinase assays. *In vitro* kinase assays were performed with immunoprecipitates of mAb C2 against P-selectin (lanes 1 and 2) and mAb 327 against pp60$^{c\text{-}src}$ (lanes 3 and 4). Assays were performed in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of a synthetic peptide corresponding to P-selectin cytoplasmic domain residues Cys^{766}–Ala^{783} (45 ng of peptide in a reaction volume of 25 μl). After incubation for 15 min at 4 °C, concentrated SDS-containing sample buffer was added to the reaction mixture without washing of the Sepharose beads, and phosphorylation was analysed by electrophoresis under reducing conditions on a 5–15% polyacrylamide gradient gel, followed by autoradiography. Molecular-mass markers indicate 200, 116.25, 97.4, 66.2, 42.7, 31, 21.5 and 14.4 kDa from top to bottom.

gesting that a platelet tyrosine kinase was co-precipitated with P-selectin. Stimulation of platelets with thrombin before platelet lysis had no effect on either electrophoretic mobility or intensity of phosphorylation of P-selectin in *in vitro* kinase assays (results not shown).

Taken together, these results indicate that a minor fraction of immunoprecipitated P-selectin forms a 205 kDa disulphide-linked complex with (an) unknown protein(s) and that P-selectin in the complex, but not free uncomplexed P-selectin, is phosphorylated by a co-precipitated tyrosine kinase.

Phosphorylation of a P-selectin synthetic peptide in the *in vitro* kinase assay

To characterize the kinase responsible for P-selectin phosphorylation, the cation dependency of the reaction was determined. P-selectin phosphorylation in washed immunoprecipitates was strongly stimulated when Mn^{2+} (5 mM) was used as the only added bivalent cation. It proceeded weakly in the presence of Mg^{2+} (5 mM) only and was completely absent in the presence of either Ca^{2+} (5 mM) or EDTA (5 mM) (results not shown). P-selectin was also strongly phosphorylated when [γ-^{32}P]GTP rather than [γ-^{32}P]ATP was used as the phosphate donor in *in vitro* kinase assays (not shown). These characteristics resemble those of members of the Src family of tyrosine kinases [31]. To investigate whether the most abundant of these kinases, platelet pp60$^{c\text{-}src}$, could phosphorylate P-selectin, a synthetic peptide corresponding to a region (Cys^{766}–Ala^{783}) around Tyr^{777} in the P-selectin cytoplasmic domain was tested in *in vitro* kinase assays. When added to P-selectin immunoprecipitates in this assay, no phosphate was incorporated into the peptide (Figure 5, lane 1). However, immunoprecipitated platelet pp60$^{c\text{-}src}$ strongly phosphorylated the P-selectin peptide (Figure 5, lane 3). These results show that the tyrosine in the P-selectin peptide is available for phosphorylation by immunoprecipitated pp60$^{c\text{-}src}$, but not by the kinase in P-selectin immunoprecipitates.

Interestingly, the *in vitro* kinase assay of pp60$^{c\text{-}src}$ showed the presence of a phosphorylated protein with a mobility similar to that of P-selectin under reducing conditions (Figure 5, lanes 3 and 4). Under non-reducing conditions, a phosphorylated 205 kDa protein was observed similar to that seen in P-selectin *in vitro* kinase assays (not shown). Reprecipitation experiments, described below, confirmed the identity of the 140 kDa protein in lanes 3 and 4 as P-selectin.

Identification of a disulphide-linked complex of P-selectin and the tyrosine kinase, pp60$^{c\text{-}src}$

The results shown in Figure 5 suggested that the anti-pp60$^{c\text{-}src}$ antibody might either directly recognize the 205 kDa complex containing P-selectin or recognize a larger complex containing both pp60$^{c\text{-}src}$ and the 205 kDa complex. To examine these possibilities, P-selectin and pp60$^{c\text{-}src}$ immunoprecipitates were subjected to *in vitro* kinase reactions, followed by extraction of the phosphorylated antigens from Sepharose beads by incubation in SDS-containing buffer at 95 °C. The extracts were diluted in a buffer containing the non-ionic detergent, NP-40, and used for reprecipitation of the antigens with mAbs against P-selectin and pp60$^{c\text{-}src}$.

Figure 6(a), lanes 1 and 2, shows the *in vitro* kinase reactions of pp60$^{c\text{-}src}$ and P-selectin respectively. In addition to phosphorylated P-selectin, both assays also revealed a non-specific phosphorylated 125 kDa protein (non-reducing and reducing electrophoresis) that bound in various amounts to the affinity matrix (protein G–Sepharose) used for immunoprecipitation (compare Figures 6 and 7 to Figures 3 and 5). Lanes 3–6 show the results of reprecipitation experiments from untreated (non-reduced) extracts of *in vitro* phosphorylated P-selectin. In these extracts, P-selectin is present in a 205 kDa complex (cf. Figure 3b). Reprecipitated antigens were analysed by electrophoresis under reducing conditions, however, in which phosphorylated P-selectin runs as a 140 kDa protein (cf. Figure 3a). As shown in lanes 4 and 6, phosphorylated 140 kDa P-selectin was detected in immunocomplexes after reprecipitation with two anti-(P-selectin) antibodies, but not after reprecipitation with an antibody to platelet GPIIb/IIIa (lane 3). It was, however, present in immune complexes of antibody 327 against pp60$^{c\text{-}src}$ (lane 5). As the treatment with SDS at 95 °C used for extraction is known to dissociate virtually all non-covalent protein interactions, these results imply that antibody 327 to pp60$^{c\text{-}src}$ recognizes the 205 kDa complex in the extracts.

Figure 6(b), lanes 3–6, shows reprecipitation experiments from untreated (non-reduced) extracts of *in vitro* phosphorylated pp60$^{c\text{-}src}$. These extracts contain both phosphorylated pp60$^{c\text{-}src}$ and a number of other phosphorylated proteins, including a 205 kDa protein (not shown). Analysis of reprecipitated proteins under reducing conditions showed that immune complexes of mAb 327 contained both phosphorylated pp60$^{c\text{-}src}$ and phosphorylated 140 kDa P-selectin (lane 5). After reprecipitation

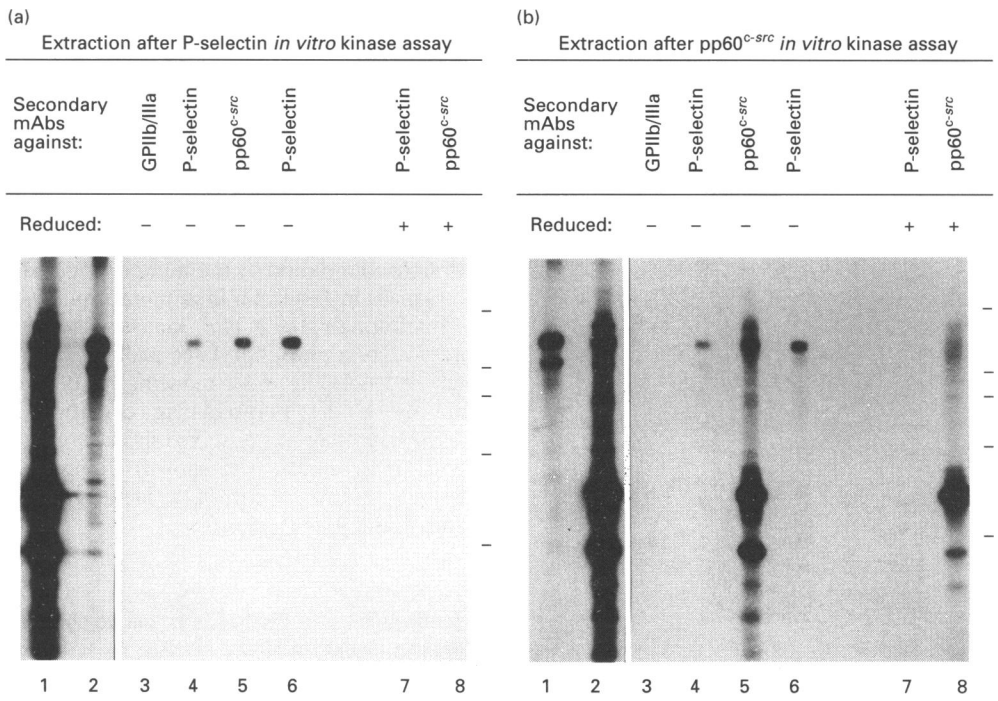

Figure 6 The 205 kDa complex containing *in vitro*-phosphorylated P-selectin is recognized by antibodies against P-selectin and pp60$^{c\text{-}src}$

Reprecipitation of phosphorylated antigens were released by treatment with SDS. Platelet P-selectin and pp60$^{c\text{-}src}$ were immunoprecipitated with mAbs covalently coupled to protein G–Sepharose. After *in vitro* phosphorylation, antigens were either analysed directly (lanes 1 and 2) or extracted by incubation for 2 min at 95 °C in a buffer containing phosphatase inhibitors and 0.1% SDS. Before reprecipitation, the extracted proteins were either directly diluted in NP-40-containing buffer (lanes 3–6) or first reduced by additional incubation for 2 min at 95 °C in the presence of 10 mM DTT (lanes 7 and 8). (**a**) Lane 1, pp60$^{c\text{-}src}$ *in vitro* kinase assay; lane 2, P-selectin *in vitro* kinase assay; lanes 3–6, reprecipitation from P-selectin *in vitro* kinase assay extracts with antibodies against GPIIb/IIIa (Y2/51; lane 3), P-selectin (C2, lane 4), pp60$^{c\text{-}src}$ (327, lane 5) and P-selectin (RUU SP-1.18, lane 6); lanes 7 and 8, reprecipitation from reduced P-selectin *in vitro* kinase assay extracts with antibodies against P-selectin (C2, lane 7) and pp60$^{c\text{-}src}$ (327, lane 8). (**b**) Lane 1, P-selectin *in vitro* kinase assay; lane 2, pp60$^{c\text{-}src}$ *in vitro* kinase assay; lanes 3–8, reprecipitation from pp60$^{c\text{-}src}$ *in vitro* kinase assay extracts with antibodies as described in (**a**), lanes 3–8. Immunoprecipitated antigens were electrophoresed under reducing conditions on an SDS/7.5% polyacrylamide gel. Molecular-mass markers are as indicated in the legend to Figure 1.

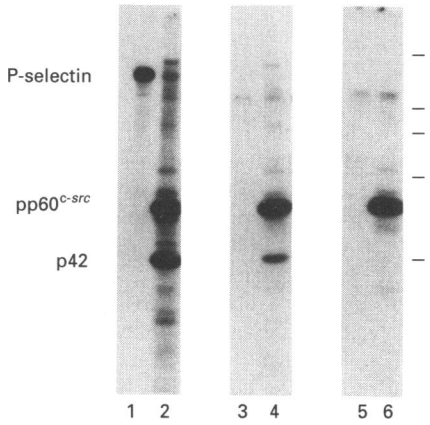

Figure 7 The disulphide bond between P-selectin and pp60$^{c\text{-}src}$ is required for *in vitro* phosphorylation of P-selectin

In vitro kinase assays were performed with immunoprecipitates of P-selectin (lanes 1, 3 and 5) and pp60$^{c\text{-}src}$ (lanes 2, 4 and 6) from normal platelet lysates (lanes 1–4) and from platelets that were lysed in the presence of DTT at 1 mM (lanes 5 and 6). Part of the immunoprecipitates from untreated platelet lysates were incubated for 15 min at 4 °C in *in vitro* kinase assay buffer containing 10 mM DTT before addition of [γ-^{32}P]ATP (lanes 3 and 4). Molecular mass markers are as indicated in the legend to Figure 1.

with anti-(P-selectin) antibodies, however, only phosphorylated 140 kDa P-selectin (lanes 4 and 6) was detectable. This result confirms that the 140 kDa protein in the pp60$^{c\text{-}src}$ *in vitro* kinase assay (e.g. Figure 5, lanes 3 and 4) is P-selectin.

When disulphide bonds in the extracted proteins were reduced before reprecipitation, anti-(P-selectin) mAb C2 no longer precipitated P-selectin from extracts of P-selectin (Figure 6a, lane 7) and pp60$^{c\text{-}src}$ (Figure 6b, lane 7) *in vitro* kinase assays. This is consistent with the finding that reactivity of ^{125}I-labelled C2 immunoglobulin with P-selectin in immunoblotting is lost after reduction (P. W. Modderman, unpublished work). In contrast, mAb 327 still precipitated phosphorylated pp60$^{c\text{-}src}$ from pp60$^{c\text{-}src}$ *in vitro* kinase assays after reduction (Figure 6b, lane 8). However, phosphorylated P-selectin was no longer present in the mAb 327-reprecipitation immunocomplexes (Figures 6a and 6b, lanes 8). These results show that reduction removes P-selectin from the complex with pp60$^{c\text{-}src}$ without destroying the mAb 327 epitope on pp60$^{c\text{-}src}$.

From these results we conclude that platelet lysates contain a 205 kDa disulphide-linked complex of P-selectin and pp60$^{c\text{-}src}$. In this complex, P-selectin, but not pp60$^{c\text{-}src}$, is phosphorylated on tyrosine residues in *in vitro* kinase assays.

In vitro phosphorylation of P-selectin is inhibited by prior reduction of protein disulphide bonds

To investigate whether the disulphide bond between P-selectin and pp60$^{c\text{-}src}$ is required for phosphorylation of P-selectin *in vitro*, DTT at 1 mM was added to the platelet lysis buffer. In

subsequent *in vitro* kinase assays with washed P-selectin immunoprecipitates, i.e. in the absence of DTT, P-selectin was not phosphorylated (Figure 7, compare lane 5 with lane 1). Similarly, the phosphorylation of a 42 kDa protein in the pp60$^{c\text{-}src}$ immunoprecipitate was strongly inhibited (compare lanes 6 and 2, see the Discussion). In contrast, autophosphorylation of pp60$^{c\text{-}src}$ was not affected (compare lanes 6 and 2). When P-selectin and pp60$^{c\text{-}src}$ immunoprecipitates from untreated lysates were preincubated for 15 min at 4 °C with DTT before addition of [γ-^{32}P]ATP in the *in vitro* kinase assay, P-selectin phosphorylation was completely inhibited by DTT at 10 mM (lane 3) and partially inhibited by DTT at 1 mM (results not shown). These results suggest that phosphorylation of P-selectin by pp60$^{c\text{-}src}$ requires that the two proteins be complexed through a disulphide bond. Electrophoretic analysis of the supernatants of the DTT-preincubated P-selectin *in vitro* kinase assays showed that pp60$^{c\text{-}src}$ released from the 205 kDa complex by reduction did not acquire the capacity to autophosphorylate under the conditions of the assay (results not shown).

DISCUSSION

Our ^{32}P metabolic-labelling studies showed that, in unstimulated platelets, the granule membrane protein P-selectin is predominantly phosphorylated on serine residues, with minor phosphorylation of its sole cytoplasmic tyrosine residue, Tyr^{777}. Analysis of P-selectin from platelets lysed 10 min after activation with thrombin showed increased phosphorylation of serine only. These results are consistent with recently published data showing that thrombin induces a sustained phosphorylation of P-selectin serine as well as a transient phosphorylation of threonine and tyrosine [30]. Phosphorylation of all three types of residues was found to peak 15–30 s after stimulation with thrombin, but 80–90 % of phosphothreonine and phosphotyrosine disappeared within 5 min [30]. The function of P-selectin phosphorylation remains to be determined.

A thrombin-induced increase of serine phosphorylation was also observed for PECAM-1. Although low levels of PECAM-1 phosphorylation in resting platelets have been previously described [38,43], the thrombin-induced increase of phosphorylation in previous studies appeared to be somewhat larger than that observed in our experiments. This may have been due to a partial premature activation of the platelets in our isolation procedure.

In our studies, both P-selectin and PECAM-1 were also phosphorylated on tyrosine residues, albeit at low levels, both in resting and thrombin-stimulated platelets. In addition, a very strong tyrosine phosphorylation of both proteins was induced by treatment of platelets with the permeating tyrosine phosphatase inhibitor, pervanadate. Weak phosphorylation of P-selectin threonine residues was observed as well. Previous studies have shown that pervanadate increases tyrosine phosphorylation of at least 27 unidentified platelet proteins, many of which were also phosphorylated after stimulation with thrombin [45]. In addition, pervanadate has been found to activate platelets, inducing many of the activation responses elicited by thrombin and other agonists [45–48]. It seems likely that pervanadate inhibits at least one or more key regulatory tyrosine phosphatases, indirectly inducing both platelet activation and the phosphorylation of a multitude of proteins. While thrombin causes only transient tyrosine phosphorylation of P-selectin [30], pervanadate-induced tyrosine phosphorylation of P-selectin (and PECAM-1) was very strong when determined at the end of a 20 min treatment with the inhibitor (Figure 1). This might indicate that pervanadate permanently stimulates an activating pathway by inhibiting a regulatory phosphatase. Moreover, in the case of P-selectin, pervanadate also caused some phosphorylation of threonine, which is also observed after activation with thrombin [30]. Nevertheless, pervanadate may also inhibit phosphatases that are normally regulated by signals other than platelet activation. This may explain the finding that PECAM-1 was strongly phosphorylated on tyrosine by pervanadate treatment (Figure 1), while thrombin only caused increased phosphorylation of serine [38,43]. Alternatively, PECAM-1 tyrosine residues might be very rapidly phosphorylated and dephosphorylated during platelet stimulation with thrombin, similar to the tyrosine phosphorylation of P-selectin.

In an attempt to identify the kinases responsible for tyrosine phosphorylation of P-selectin and PECAM-1, we found that a fraction of immunoprecipitated P-selectin was disulphide-linked to an unknown protein(s) in a 205 kDa complex. P-selectin in this complex, but not free uncomplexed P-selectin, was tyrosine-phosphorylated in the *in vitro* kinase assay. Identification of the kinase responsible for P-selectin phosphorylation was hampered by the absence of autophosphorylation of the kinase. However, it was found that the 205 kDa complex was also precipitated by an antibody against the platelet tyrosine kinase pp60$^{c\text{-}src}$. In addition, after treatment with SDS at 95 °C, the 205 kDa protein extracted from both P-selectin and pp60$^{c\text{-}src}$ *in vitro* kinase assays was reprecipitated by anti-(P-selectin) and anti-(pp60$^{c\text{-}src}$) antibodies.

Several arguments point to pp60$^{c\text{-}src}$ being disulphide-linked to P-selectin in the 205 kDa complex: (1) the 205 kDa complex is recognized by anti-(pp60$^{c\text{-}src}$) antibody; (2) the apparent molecular mass of the 205 kDa complex is consistent with a 1:1 complex between P-selectin and pp60$^{c\text{-}src}$; (3) P-selectin in the complex is tyrosine-phosphorylated by a co-precipitated kinase; (4) the cation dependency of P-selectin phosphorylation is similar to that observed for phosphorylation of exogeneous substrates by purified pp60$^{c\text{-}src}$ [49].

P-selectin Tyr^{777} occurs in a sequence, GTYGV, that is also present in p34^{cdc2}, a serine/threonine kinase, the activity of which is controlled by phosphorylation of the tyrosine in the GTYGV sequence [50]. Peptides containing GTYGV have previously been shown to be phosphorylated *in vitro* by the viral tyrosine kinase pp60$^{v\text{-}src}$ [50] as well as by cellular (platelet) pp60$^{c\text{-}src}$ [51]. Our results show that the P-selectin synthetic peptide was also phosphorylated by immunoprecipitated platelet pp60$^{c\text{-}src}$, although the context of the P-selectin GTYGV sequence differs from that of the p34^{cdc2} sequence.

Inhibition of autophosphorylation of pp60$^{c\text{-}src}$ in the complex with P-selectin may have resulted from modification of a critical pp60$^{c\text{-}src}$ cysteine residue through the binding of P-selectin. The sulphydryl reagent *N*-ethylmaleimide, which binds covalently to cysteines, also prevents pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ autophosphorylation, as well as the phosphorylation of some exogenous substrates [52,53]. The latter may explain the finding that the P-selection synthetic peptide was not phosphorylated by pp60$^{c\text{-}src}$ in the 205 kDa complex with P-selectin, although it was strongly phosphorylated in mAb 327 immunoprecipitates of pp60$^{c\text{-}src}$. The failure of pp60$^{c\text{-}src}$, in complex with P-selectin, to phosphorylate the P-selectin peptide was mirrored by the finding that the peptide did not inhibit P-selectin phosphorylation *in vitro*. In contrast, in some experiments, autophosphorylation of mAb 327-precipitated pp60$^{c\text{-}src}$ was partly inhibited by the P-selectin peptide, although this was not evident in the experiment shown in Figure 5.

When *N*-ethylmaleimide (5 mM) was included in the platelet lysis buffer, it completely inhibited the *in vitro* autophosphoryl-

ation of platelet pp60$^{c\text{-}src}$. Surprisingly, *N*-ethylmaleimide also inhibited *in vitro* phosphorylation of P-selectin, even when added 2 h after initiation of lysis, showing that the effect did not depend on inhibition of disulphide-bond formation between P-selectin and pp60$^{c\text{-}src}$ (P. W. Modderman, unpublished work). This finding suggests that *N*-ethylmaleimide modifies pp60$^{c\text{-}src}$ cysteines in addition to the one involved in binding to P-selectin.

The complex of P-selectin and pp60$^{c\text{-}src}$ may have been previously described. First, McEver and Martin [54] found that, in addition to 148 kDa P-selectin, preparations of affinity-purified P-selectin contained a protein that, in non-reducing SDS/gel electrophoresis, had an apparent molecular mass greater than 200 kDa. Under reducing conditions, however, only P-selectin was detectable. Secondly, Grandori and Hanafusa [55] reported that, in platelets, a fraction of pp60$^{c\text{-}src}$ is associated with a 150 kDa protein in a complex that was sensitive to reducing agents. Their experiments were designed to determine whether platelets contain a complex similar to the one they had identified between pp60$^{c\text{-}src}$ and a 38 kDa protein (p38) in chromaffin granules from adrenal medulla. In this complex, pp60$^{c\text{-}src}$ and p38 were disulphide-linked and both were tyrosine phosphorylated in *in vitro* kinase assays of pp60$^{c\text{-}src}$ immunoprecipitates. It was noted that binding of p38 to pp60$^{c\text{-}src}$ caused a decreased ability of pp60$^{c\text{-}src}$ to phosphorylate itself. In platelets, however, a similar complex between pp60$^{c\text{-}src}$ and a 38 kDa protein was not detected [55].

In contrast, in our own studies, in addition to P-selectin, a 42 kDa protein was co-precipitated with pp60$^{c\text{-}src}$ that was strongly phosphorylated in the *in vitro* kinase assays. As with P-selectin, phosphorylation of this protein was not apparent when the reducing agent, DTT, was included in the platelet lysis buffer (Figure 7). *In vitro* phosphorylation of a platelet protein of similar molecular mass by pp60$^{c\text{-}src}$ was recently described by Huang et al. [56]. The 40 kDa platelet Fc receptor FcRγII appeared to be tyrosine-phosphorylated when immunocomplexes of both pp60$^{c\text{-}src}$ and FcRγII were bound to the same immunoadsorbent [56]. However, phosphorylation of FcRγII was not observed in immunoprecipitates containing only pp60$^{c\text{-}src}$ immunocomplexes, suggesting that the 42 kDa phosphoprotein observed in our experiments may be different from FcRγII.

The complex of chromaffin granule pp60$^{c\text{-}src}$ and p38 studied by Grandori and Hanafusa (see above) appeared to be stable to reducing agents as long as the granule membranes were not solubilized in detergents [55]. As disulphide bonds are rarely found among cytoplasmic proteins, due to the high concentration of glutathione in the cell, the authors suspected that pp60$^{c\text{-}src}$ and p38 interact non-covalently in intact granules, and that spontaneous disulphide bonding occurs upon lysis.

A similar non-covalent association may exist between pp60$^{c\text{-}src}$ and P-selectin. P-selectin contains a single cytoplasmic cysteine residue (Cys^{766}, [26]), which was recently shown to be modified post-translationally by thioester linkages with two fatty acids, palmitic acid and stearic acid [57]. The presence of these stable modifications of Cys^{766} would also exclude the possibility of disulphide bonding to other molecules. However, as the stoichiometry of acylation of P-selectin could not be determined [57], the possibility remains that a fraction of the P-selectin molecules could have a free cysteine available for disulphide bonding during platelet lysis.

While pp60$^{c\text{-}src}$ has been observed in association with the platelet plasma membrane and the membranes of the surface-connected cannalicular system [58], the bulk of the enzyme appears to be associated with platelet dense granules [59]. P-selectin, on the other hand, was initially described to be present in α granules only [1], but was recently found in dense granules as well [3]. The co-localization of P-selectin and pp60$^{c\text{-}src}$ with dense granules raises the possibility that the complexes between them derive from the proteins in these granules. Platelet activation by thrombin did not change P-selectin phosphorylation in *in vitro* kinase assays, suggesting that the presumed association between P-selectin and pp60$^{c\text{-}src}$ is maintained during translocation of dense granules and expression of P-selectin on the plasma membrane.

The hypothesized association between pp60$^{c\text{-}src}$ and P-selectin might serve to anchor pp60$^{c\text{-}src}$ to membranes. Although it is known that a myristate residue on the N-terminal glycine residue of pp60$^{c\text{-}src}$ is necessary for association of the kinase with membranes [60,61], the mechanism of association is unknown. The presence of fatty acids on both P-selectin and pp60$^{c\text{-}src}$ raises the possibility that interactions between these components may be instrumental in anchoring pp60$^{c\text{-}src}$ to membranes.

The observed tyrosine phosphorylation of P-selectin in platelets and its complexation with pp60$^{c\text{-}src}$ in immunoprecipitates suggests that pp60$^{c\text{-}src}$ might phosphorylate P-selectin Tyr^{777} in intact platelets. Although *in vitro* phosphorylation of P-selectin by pp60$^{c\text{-}src}$ requires the presence of a disulphide bond between the two proteins, which is not likely to occur in the cytoplasmic milieu, the requirements for P-selectin phosphorylation in intact cells might be different. The finding that metabolically tyrosine-phosphorylated P-selectin did not appear to be complexed with pp60$^{c\text{-}src}$, i.e. had a normal electrophoretic mobility under non-reducing conditions, may indicate that phosphorylation of P-selectin Tyr^{777} in intact platelets prevents disulphide bonding between the two molecules during subsequent lysis. As yet, attempts to co-precipitate a P-selectin-phosphorylating kinase with P-selectin in the presence of DTT under non-stringent conditions (i.e. in the presence of NP-40 or digitonin) have been unsuccessful (P. W. Modderman, unpublished work). Therefore, definitive identification of the tyrosine kinase responsible for phosphorylation of P-selectin may require other techniques, such as the use of platelets from animals in which the genes for Src family kinases have been deleted by homologous recombination, as described by Soriano et al. for pp60$^{c\text{-}src}$ [62].

In conclusion, our studies show that the platelet receptor for leucocytes, P-selectin, is phosphorylated on serine and tyrosine in platelets. Serine phosphorylation of P-selectin is increased by platelet activation with thrombin. The effect of the tyrosine phosphatase inhibitor pervanadate indicates that tyrosine phosphorylation of P-selectin is tightly regulated by both tyrosine kinase and phosphatase activities. The finding that a fraction of P-selectin is both disulphide-linked with pp60$^{c\text{-}src}$ and tyrosine-phosphorylated in immunoprecipitates suggests that pp60$^{c\text{-}src}$ may be involved in the regulation of P-selectin function.

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