Exposure of ligand-binding sites on platelet integrin α_{IIB}/β_3 by phosphorylation of the β_3 subunit

Gijsbert VAN WILLIGEN*, Ingeborg HERS, Gertie GORTER and Jan-Willem N. AKKERMAN
Department of Haematology, University Hospital Utrecht, P. O. Box 85.500, 3508 GA Utrecht, The Netherlands

The exposure of ligand-binding sites for adhesive proteins on platelet integrin α_{IIB}/β_3 (glycoprotein IIB/IIIA) by platelet-activating factor (PAF) is transient, whereas sites exposed by α-thrombin remain accessible. The same difference is seen in the phosphorylation of the β_3 subunit. Inhibition of protein kinases (1 μM staurosporine) and protein kinase C (10 μM bisindolylmaleimide) closes binding sites exposed by both agonists and induces dephosphorylation of β_3. Inhibition of Tyr-kinases (20 μM Herbimycin A) has only a slight effect. Inhibition of Ser/Thr-phosphatases (1 μM okadaic acid, 30 s preincubation) changes the transient exposure and β_3 phosphorylation by PAF into the 'permanent' patterns induced by α-thrombin. Inhibition of Tyr-phosphatases (100 μM vanadate) has little effect. Preincubation with okadaic acid makes exposed binding sites and phosphorylated β_3 insensitive to staurosporine, resulting in exposed α_{IIB}/β_3 independent of concurrent phosphorylation/dephosphorylation. The stoichiometry of β_3 phosphorylation by α-thrombin is 0.80 ± 0.10. Thus, one of the mechanisms that regulates exposure and closure of ligand-binding sites on the α_{IIB}/β_3 is phosphorylation/dephosphorylation of a Ser/Thr-residue in the β_3 subunit.

INTRODUCTION

The platelet integrin α_{IIB}/β_3 (glycoprotein IIB/IIIA) complex is a non-covalently linked Ca^{2+}-dependent heterodimer. The α_{IIB} subunit consists of a heavy chain (125 kDa) and a transmembrane light chain (23 kDa). The β_3 subunit (110 kDa) is a single, highly disulphide-bonded, transmembrane protein. Although the complex is present on the plasma membrane of resting platelets, it is inaccessible to ligands, but once the cells are activated, changes in the complex or its microenvironment make it a receptor for fibrinogen, fibronectin and other adhesive proteins (for reviews on integrin α_{IIB}/β_3 see [1,2]).

Stirring of platelet suspensions enables fibrinogen to bind one platelet to another and aggregates are formed. This process is accompanied by binding of α_{IIB}/β_3 to a Triton X-100-insoluble fraction containing actin filaments, Tyr-kinases such as $pp60^{c\text{-}src}$ and $pp62^{c\text{-}yes}$, GTPase activating proteins and protein kinase C (PKC), suggesting that multiple factors contribute to complex formation between α_{IIB}/β_3 and fibrinogen and subsequent coupling to the cytoskeleton [3,4]. So far, it has been difficult to determine how each of these factors relate to the different activation stages of α_{IIB}/β_3, such as exposure of binding sites, reversible ligand binding and formation of an irreversible fibrinogen–α_{IIB}/β_3 complex.

The exposure of ligand-binding sites on α_{IIB}/β_3 has been explained by (i) involvement of a regulatory protein, (ii) changes in lipid environment and (iii) phosphorylation of the β_3 subunit. O'Toole et al. [5,6] transfected Chinese hamster ovary cells with α_{IIB}/β_3 and found loss of ligand-binding capacity. Deletion of the cytoplasmic tail of α_{IIB} made the complex constitutively active [5,6]. The authors proposed a role for a regulatory protein that binds to both cytoplasmic tails, thereby activating the complex. Recently, Shattil et al. described a protein termed β_3-endonexin that binds to the cytosolic tail of β_3 and could serve as a regulatory protein [7]. Other proteins that may contribute to α_{IIB}/β_3 control are the GTP-binding protein Rho A [8] and another poorly defined low-molecular-mass GTP-binding protein [9]. Smyth et al. showed that (lyso)phosphatidic acid increased the ligand-binding affinity of α_{IIB}/β_3 [10]. These findings are best explained by assuming that the lipid environment has a major effect on the accessibility of α_{IIB}/β_3. Parise et al. demonstrated a parallel increase in fibrinogen binding and phosphorylation of the β_3 subunit upon stimulation with α-thrombin or phorbol ester [11]. A patient with an increased bleeding tendency and a defect in platelet–fibrinogen interaction had a mutation in a proposed phosphorylation site of β_3 [12]. A lymphocyte expression system containing α_{IIB}/β_3 shows that the cytoplasmic tail of β_3 is essential for PKC-induced phosphorylation and fibrinogen binding [13]. These findings suggest that the phosphorylation of a Ser/Thr on β_3 affects the ligand-binding capacity of $\alpha_{IIB}\beta_3$.

Earlier work showed that binding-site exposure on α_{IIB}/β_3 correlated closely with the activity of PKC. Phorbol esters exposed binding sites for fibrinogen and the activation-dependent antibody PAC-1 [14,15], whereas PKC inhibitors prevented fibrinogen binding and platelet aggregation [15–17]. PKC phosphorylates different proteins in platelets, such as the 47 kDa protein pleckstrin. Platelet-activating factor (PAF) induced a rapid increase in phosphorylated pleckstrin followed by a decline to control levels [15]. The exposure of binding sites was equally rapid, and exposed binding sites closed when [^{32}P]pleckstrin returned to pre-stimulation values. A stepwise increase in [^{32}P]pleckstrin by different combinations of PAF, Ca^{2+} ionophore and PKC inhibitors revealed an almost linear correlation with the number of exposed α_{IIB}/β_3 [15]. In contrast to the transient exposure by PAF, binding sites exposed by α-thrombin (0.1 unit/ml) remained accessible [17]. Again, [^{32}P]pleckstrin varied in parallel and remained elevated as long as the sites were

Abbreviations used: PAF, platelet-activating factor; PEP, phosphoenolpyruvate; PGI_2, prostacyclin/prostaglandin I_2; PKC, protein kinase C; PK, pyruvate kinase; PMA, phorbol 12-myristate 13-acetate; PP, protein phosphatase; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; PRP, platelet-rich plasma

* To whom correspondence should be addressed.

open. Thus, the balance between phosphorylation and dephosphorylation appears to be a major factor in the regulation of α_{IIB}/β_3.

Opposite to this view is the finding that PAF, ADP and adrenaline (epinephrine) induced different amounts of [^{32}P]pleckstrin although they exposed similar numbers of α_{IIB}/β_3 [15]. However, there is little evidence for a role of pleckstrin in α_{IIB}/β_3 control. In the present study we focused on the role of PKC in exposure of ligand-binding sites, making use of the fact that under ligand-free conditions, α_{IIB}/β_3 shifts between an exposed and closed conformation that is highly susceptible to modulations in cell-signalling mechanisms [15,17]. The results favour the concept that the phosphorylation state of the β_3 subunit is a major factor in the exposure of ligand-binding sites on α_{IIB}/β_3.

MATERIALS AND METHODS

Materials

Soybean trypsin inhibitor, PMSF, sodium orthovanadate, *N*-ethylmaleimide, benzamidine, trifluoroperazine, phorbol 12-myristate 13-acetate (PMA) and α-thrombin were purchased from Sigma (St. Louis, MO, U.S.A.). Okadaic acid, PAF, bisindolylmaleimide, phosphoenol pyruvate (PEP), pyruvate kinase (PK) and staurosporine were obtained from Boehringer (Mannheim, Germany) and fibrinogen (Grade L) from KABI (Stockholm, Sweden). Prostacyclin [prostaglandin I_2 (PGI_2)] was from Cayman Chemicals (Ann Arbor, MI, U.S.A.) and Sepharose 2B, Sepharose 4B and Protein G–Sepharose 4B from Pharmacia Biotech (Brussels, Belgium). Herbimycin A was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.) and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Bachem (Bubersdorf, Switzerland). Purified $\alpha_{IIb}\beta_3$ was obtained from Kordia (Leiden, The Netherlands) and the anti-phosphotyrosine antibody PY20 was from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). ^{125}I-Labelled Na (specific activity 629 GBq/mg) and 5-hydroxy(side chain-2-^{14}C)tryptamine creatinine sulphate ([^{14}C]serotonin, 1.85–2.29 GBq/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.) and [^{32}P]P_i (specific radioactivity 314 TBq/mmol) and the Renaissance chemiluminescence system were from New England Nuclear/Du Pont (Boston, MA, U.S.A.). All other chemicals were of analytical grade.

The monoclonal antibody 4A7 (directed against β_3 but precipitates the α_{IIb}/β_3 complex) was a gift from Dr. H. K. Nieuwenhuis (Department of Haematology, University of Utrecht, The Netherlands). For comparison, the P2 monoclonal antibody of Immunotech (Marseille, France) was used for precipitation of α_{IIb}/β_3.

^{125}I-Labelled fibrinogen and ^{125}I-labelled fibronectin were prepared as decscribed [18,19]. In short, fibrinogen was made fibrin- and fibronectin-free by passage through a gelatin–Sepharose 4B column. Fibronectin was isolated from fresh, frozen plasma by affinity chromatography on a gelatin–Sepharose 4B column. The isolated fibrinogen and fibronectin were radiolabelled with ^{125}I-labelled Na by a modified Iodogen method.

Methods

Platelet isolation

Freshly drawn venous blood from healthy volunteers (with informed consent), who claimed not to have taken any medication in the previous 10 days, was collected into 0.1 vol. of 3.8 % tri-sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation (200 ***g*** for 10 min at 22 °C). Platelets were isolated by gel-filtration or centrifugation. Gel-filtered platelets were prepared by placing PRP on a Sepharose 2B column equilibrated in Ca^{2+}-free Tyrode's solution (137 mM NaCl/2.68 mM KCl/0.42 mM NaH_2PO_4/1.7 mM $MgCl_2$/11.9 mM $NaHCO_3$, pH 7.25) or Hepes–Tyrode (145 mM NaCl/5 mM KCl/0.5 mM Na_2HPO_4/1 mM $MgSO_4$/10 mM Hepes, pH 7.2), both containing 0.1 % (w/v) glucose and 0.2 % (w/v) BSA. For isolating platelets by centrifugation, PRP was acidified to pH 6.5 with acid citrate dextrose (2.5 g of tri-sodium citrate, 1.5 g of citric acid and 2.0 g of D-glucose in 100 ml of distilled water) and centrifuged (700 ***g*** for 20 min at 22 °C). The platelet pellet was resuspended in the appropriate buffer.

In some experiments, Ser/Thr-phosphatases were inhibited with okadaic acid (1 μM for 30 s at 22 °C), Tyr-phosphatases with vanadate (100 μM for 30 min at 22 °C), PKC with bisindolylmaleimide (10 μM for 1 min at 22/37 °C), protein kinases with staurosporine (1 μM for 1 min at 22/37 °C) and Tyr-kinases with Herbimycin A (20 μM for 5 min at 22/37 °C).

Platelet aggregation

Gel-filtered platelets [(200–300) $\times 10^3$ platelets/μl] were preincubated for 1 min with Ca^{2+}-free Tyrode's solution, PEP/PK (14 mM PEP/150 units/ml PK), 20 μM Herbimycin A (5 min preincubation), 10 μM bisindolylmaleimide without or with the ADP scavenger PEP/PK, a combination of bisindolylmaleimide and Herbimycin A (5 min preincubation) and 1 μM staurosporine. Subsequently the platelets were stimulated with 0.1 unit/ml α-thrombin at 37 °C under continuous stirring at 900 rev./min at 37 °C in a Chronolog Lumiaggregometer (Chronolog Co., Havertown, PA, U.S.A.) and aggregation was recorded.

Binding of ^{125}I-fibrinogen and ^{125}I-fibronectin

Gel-filtered platelets [(200–300) $\times 10^3$ platelets/μl] were preincubated as described above. Subsequently these platelets were stimulated with 0.1 unit/ml α-thrombin in the absence of ^{125}I-fibrinogen and without stirring. Three minutes after stimulation 30 nM PPACK was added, and 1 μM ^{125}I-fibrinogen was added 2 min thereafter. Ten minutes after stimulation, samples were withdrawn (in triplicate) and analysed for specific binding as described below.

In an alternative approach, gel-filtered platelets [(200–300) $\times 10^3$ platelets/μl) were stimulated with 500 nM PAF, 0.1 unit/ml α-thrombin (all final concentrations) in the absence of ^{125}I-fibronectin/^{125}I-fibrinogen and without stirring. Samples were withdrawn at different times after stimulation and incubated with 1 μM ^{125}I-fibrinogen (platelets stimulated with PAF) or with 1 μM ^{125}I-fibronectin (platelets stimulated with α-thrombin) for 10 min without stirring. Under these conditions the binding of fibrinogen and fibronectin is a direct reflection of the number of exposed binding sites, as described in detail in earlier publications [15,20]. The data were interpreted by assuming that the number of exposed binding sites remained constant during the 10 min incubation period with ^{125}I-fibrinogen/^{125}I-fibronectin and taken to reflect exposed sites at the beginning of this period. Alternatively, platelets were stimulated with different concentrations of PMA (concentration range 0–100 nM) in the presence of ^{125}I-fibrinogen. The amount of fibrinogen binding was measured 10 min after stimulation. All incubations were carried out at 22 °C.

The binding of fibrinogen or fibronectin was measured by placing 200 μl samples of cell suspension (in triplicate) on top of

100 μl of 25 % (w/v) sucrose in Ca^{2+}-free Tyrode's solution in micro sedimentation tubes (Sarstedt, Vienna, Austria) and separating the cells from the medium by centrifugation (12000 ***g*** for 2 min at 22 °C) in a Beckmann Microfuge E. The tip of the tube (pellet fraction) was cut off and the pellet and supernatant were counted in a gamma-counter. The number of molecules bound per platelet was calculated from the radioactivity in the pellet fraction compared with the total activity in the pellet plus supernatant. The data were corrected for non-specific binding, defined as the binding of ^{125}I-fibronectin/^{125}I-fibrinogen to unstimulated platelets. The data are expressed as the number of exposed binding sites per platelet. Data are means ± S.D.

Control experiments revealed that when ^{125}I-fibrinogen was used as ligand for α_{IIB}/β_3 after α-thrombin (0.1 unit/ml) stimulation (coagulation was prevented by 30 nM PPACK, added 3 min after α-thrombin addition), the binding kinetics were identical with those with ^{125}I-fibronectin. Also, in PAF-stimulated platelets the closure of binding sites for ^{125}I-fibrinogen and ^{125}I-fibronectin was the same [17]. These data indicate that under the specific conditions of the present experiments the binding of both ligands reflects a property of the α_{IIB}/β_3 complex.

Phosphorylation of the β_3 subunit

Platelets were isolated by centrifugation and resuspended in Hepes–Tyrode buffer (pH 6.5), containing 0.1 % glucose (w/v) to a final platelet count of (200–350) × 10^3 platelets/μl. The cells were labelled with 37 MBq of carrier-free [^{32}P]P_i/ml for 1 h at 37 °C, collected by centrifugation and resuspended in Hepes–Tyrode buffer, pH 7.2. Labelled platelets were stimulated with 1 μM PAF, 0.2 unit/ml α-thrombin or different concentrations of PMA (range 0–100 nM) at 22 or 37 °C. Samples were drawn at 0, 2, 5, 10 and 20 min after stimulation and the α_{IIb}/β_3 was immunoprecipitated with the monoclonal antibody 4A7 or P2, as described below. The total yield varied between 10 and 20 % of total β_3 subunits, based on 100000 copies per platelet. The extracted proteins were separated by SDS/7.5 %-PAGE, according to the method of Laemmli [20a]. Gels were stained with Coomassie Brilliant Blue and the distribution of the radioactivity was determined by autoradiography of dried gels on Kodak Royal X-Omat. For determination of the ^{32}P radioactivity in β_3, the specific area was cut out of the dried gels and heated for 2 h at 80 °C in 30 % H_2O_2. The radioactivity was determined by liquid scintillation counting. The maximal count ranged from 150 to 2500 c.p.m. above background. The absolute amount of β_3 was determined by comparing the colour intensity of the β_3-band after Coomassie Brilliant Blue staining with that of known amounts of α_{IIB}/β_3.

The ^{32}P content of β_3 is expressed as a percentage of that of unstimulated platelets.

Tyr phosphorylation in the α_{IIB}/β_3 immune precipitate

Platelets were isolated by centrifugation, washed once with Hepes–Tyrode, pH 6.5, and resuspended in Hepes–Tyrode, pH 7.2, containing 0.1 % (w/v) D-glucose to a final platelet count of (200–350) × 10^3 platelets/μl. The platelets were stimulated with 0.2 unit/ml α-thrombin at 22 °C. Samples of 250 μl were drawn at 5 and 10 min after stimulation and platelets were solubilized at 4 °C with an equal volume of Immunomix: PBS, pH 7.4, containing 1 % (v/v) Nonidet P.40, 0.1 % (w/v) SDS, 0.5 % (w/v) *N*-octyl-glucoside, 1 % (w/v) BSA, supplemented with protease inhibitors (2 mM PMSF, 10 mM benzamidine, 20 μg/ml soybean trypsin inhibitor, 5 mM EDTA, 5 mM *N*-ethylmaleimide, 2 mM orthovanadate and 0.2 mM trifluoroperazine). The lysate was incubated for 16 h at 4 °C with the monoclonal antibody 4A7, which is directed against β_3 but precipitates the α_{IIB}/β_3 complex. Subsequently, the antigen–antibody complex was precipitated with 20 μl of protein G–Sepharose 4B beads (45 min, 22 °C). The beads were washed three times with Immunomix and once with PBS (pH 7.4). The bound protein was extracted from the Sepharose beads by boiling for 15 min in a 3-fold concentrated Laemmli electrophoresis sample buffer [0.003 % (w/v) Bromophenol Blue/15 % (v/v) 2-mercaptoethanol/30 % glycerol (v/v)/6 % (w/v) SDS/0.1875 M Tris, pH 6.8]. Proteins were separated on SDS/7.5 %-PAGE and electrophoretically transferred (1 h, 100 V) to Immobilon-P transfer membrane (Millipore, Milford, MA, U.S.A.) in 25 mM Tris/192 mM glycine (pH 8.3) and 20 % methanol (v/v) using a Bio-Rad mini trans-blot system (Bio-Rad, Richmond, CA, U.S.A.). The blots were blocked with PBS containing 5 % protifar (w/v) for 1 h at room temperature and subsequently incubated (16 h at 4 °C) with the anti-β_3 monoclonal antibody 4A7 or an anti-phosphotyrosine monoclonal antibody, PY20. Afterwards the blots were incubated with a rabbit anti-mouse peroxidase-labelled antibody (1 h at 22 °C). Subsequently the blots were treated with Renaissance chemiluminescence Western-blot reagent and exposed to Renaissance autoradiography film. Membranes were stripped of bound antibody and the blots were re-analysed with the other antibody.

Inhibition of Tyr phosphorylation in platelets by Herbimycin A

Gel-filtered platelets (200 × 10^3 platelets/μl) were incubated with different concentrations of Herbimycin A (0–20 μM) for 5 min at 22 °C. Subsequently these platelets were stimulated with α-thrombin (0.1 unit/ml) for 10 min. Aliquots of the stimulated platelets were lysed with 3-fold concentrated Laemmli sample buffer and proteins were separated on SDS/10 %-PAGE and electrophoretically transferred to Immobilon. Tyrosine phosphorylation was determined with PY20, as described above.

Measurement of PKC activity

Platelets were labelled with 3.7 MBq of carrier-free [^{32}P]P_i/ml of acidified PRP (pH 6.5) for 1 h at 37 °C. Platelets were isolated by centrifugation and resuspended in Hepes–Tyrode (pH 7.2) containing 0.1 % (w/v) glucose. Labelled platelets were stimulated with 500 nM PAF or 0.1 unit/ml α-thrombin at 22 °C. Samples were collected at 0, 5, 10 and 20 min after stimulation and the ^{32}P-radioactivity in the 47 kDa protein (pleckstrin), a major substrate for PKC in platelets, was determined as described elsewhere [21]. The ^{32}P content of pleckstrin is expressed as a percentage of that of unstimulated platelets.

In control experiments no differences in PKC activity could be detected between centrifuged and gel-filtered platelets (results not shown).

Stoichiometry of β_3 phosphorylation

Phosphorylation of the β_3 subunit was calculated by comparing the incorporation of ^{32}P label from [^{32}P]ATP with the amount of β_3 measured by densitometry. Since platelets have different compartments containing adenine nucleotides, the specific radioactivity of metabolic ATP was determined after correction for granule-stored, non-metabolic ATP. Two independent approaches were used: (i) measurement of the specific activity of actin-bound metabolic ADP, which is in isotopic equilibrium with metabolic ADP and ATP and can be isolated in an ethanol-insoluble trichloroacetic acid extract [22–24], and (ii) isolation of metabolic ADP–ATP by rapid, digitonin-induced permeabilization of the plasma membrane and isolation of the cytosol

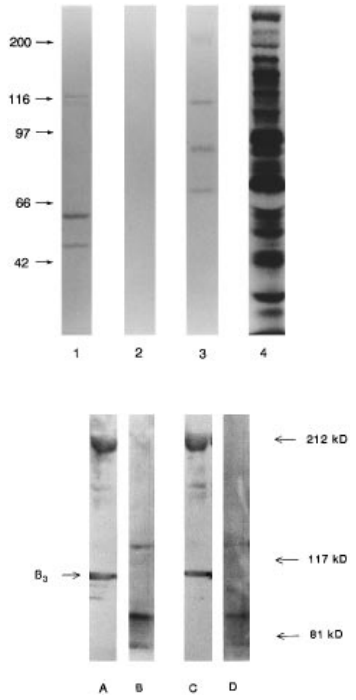

Figure 1 Phosphorylation of the β_3 subunit of integrin α_{IIB}/β_3

Top: α_{IIB}/β_3 was immunoprecipitated using unlabelled-and ^{32}P-labelled platelets with the 4A7 monoclonal antibody and the immunoprecipitate was electrophoresed by SDS/7.5%-PAGE. Lane 1 shows Coomassie Brilliant Blue staining of the immunoprecipitate. Lanes 2 and 3 are autoradiograms of an immunoprecipitate before (lane 2) and after (lane 3) 2 min stimulation with α-thrombin (0.2 unit/ml). Lane 4 shows an autoradiogram of a total lysate of α-thrombin-stimulated ^{32}P-labelled platelets electrophoresed by SDS/7.5%-PAGE to illustrate the presence of phosphorylated β_3 subunit in a total platelet lysate. The positions of molecular-mass markers are indicated on the left. Bottom: α_{IIB}/β_3 was immunoprecipitated, using the anti-β_3 monoclonal antibody 4A7, 5 min (lanes A and B) and 10 min (lanes C and D) after stimulation of washed platelets with 0.2 unit/ml α-thrombin. The immunoprecipitate was electrophoresed by SDS/7.5%-PAGE and proteins were transferred to a polyvinylidene difluoride membrane. The β_3 subunit was visualized with the anti-β_3 monoclonal antibody 4A7 (lanes A and C) and Tyr phosphorylation with an anti-phosphotyrosine monoclonal antibody PY20 (lanes B and D). Results are representative of three separate experiments. Abbreviation: kD, kDa.

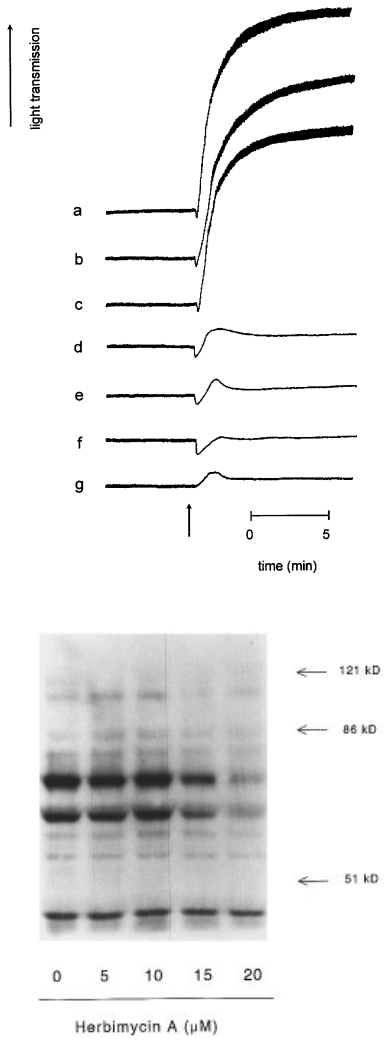

Figure 2 Role of PKC and Tyr-kinases in platelet aggregation

Top: gel-filtered platelets were preincubated for 1 min with (**a**) Ca^{2+}-free Tyrode's solution (control), (**b**) PEP/PK (14 mM PEP, 150 units/ml PK), (**c**) 20 μM Herbimycin A (5 min preincubation), (**d** and **e**) 10 μM bisindolylmaleimide without (**d**) or with (**e**) PEP/PK, (**f**) a combination of bisindolylmaleimide and Herbimycin A (5 min preincubation) and (**g**) 1 μM staurosporine. Subsequently the platelets were stimulated with 0.1 unit/ml α-thrombin at 37 °C under continuous stirring at 900 rev./min in a Chronolog Lumiaggregometer and aggregation was recorded. Bottom: gel-filtered platelets were preincubated with 0 (control), 5, 10, 15 or 20 μM Herbimycin A for 5 min at 22 °C. Subsequently the platelets were stimulated with 0.1 unit/ml α-thrombin. Ten minutes after stimulation samples were analysed for Tyr phosphorylation. A representative result is shown. Abbreviation: kD, kDa.

[25]. Platelets were ^{32}P-labelled as described for the phosphorylation of the β_3 subunit.

For method (i), the washed platelets were mixed with 2 vol. of freshly prepared ice-cold EDTA/ethanol (10 mM EDTA in 86% ethanol, pH 7.4). The extract was centrifuged (12000 ***g*** for 2 min at 22 °C) and the pellet fraction containing the ethanol-insoluble adenine nucleotides was washed in a 1:1 (v/v) solution of 150 mM NaCl and EDTA/ethanol. The pellet was extracted with 0.6 M $HClO_4$ and the supernatant was neutralized with 2 M K_2CO_3. Adenine nucleotides and metabolites were separated by high-voltage paper electrophoresis [26] and radioactivity in the spots was determined by liquid scintillation counting. The absolute amount of ADP in the $HClO_4$ extract was converted into ATP and measured by the luciferin/luciferase technique and a Packard Pico-lite luminometer (Packard Instruments Co., Donners Grove, IL, U.S.A.) [27].

For method (ii), platelets were radiolabelled with 1 μM [^{14}C]serotonin (60 min at 37 °C), a marker for the non-metabolic ATP–ADP-containing dense granules, and incubated for 7 s (room temp) with digitonin. The digitonin concentration was adjusted to the cell number [usually (200–300) $\times 10^3$ platelets/μl]) and induced approx. 90% liberation of the cytosol, 10–15% granule leakage and $< 5\%$ mitochondria lysis. Details of this method have been described previously [25]. Cytosol and membranes/granules were separated by centrifugation through a phthalate layer (5 min, 10000 ***g***, 22 °C). Adenine nucleotides in the supernatant were extracted with 1 vol. EDTA/ethanol (10 mM EDTA in 86% ethanol, pH 7.4). Concurrently, lactate dehydrogenase and [^{14}C]serotonin were measured in total suspension, supernatant and pellet and indicated that the supernatant contained $78 \pm 10\%$ of total cytosol and was contaminated with $8 \pm 2\%$ dense granule content ($n = 5$). [^{32}P]Adenine nucleotides and metabolites in the supernatant were separated by high-voltage paper electrophoresis [26] and the radioactivity in the spots was determined by liquid scintillation counting. The

absolute amount of ATP in the supernatant was measured by the luciferin/luciferase technique [27] and a Packard Pico-lite luminometer. Data were corrected for non-lysis (based on lactate dehydrogenase) and leakage of ATP from the dense granules (based on [^{14}C]serotonin).

The amount of β_3 subunit was determined after electrophoresis by densitometric comparison with serial dilutions of purified β_3 subunit.

Presentation of data

Data are expressed as means ± S.D. In some experiments data are expressed as means ± range. Statistical significance was determined by Student's *t*-test for paired data, where appropriate, or for unpaired data, and considered significant at $P < 0.05$.

RESULTS

Phosphorylation of the β_3 subunit of integrin α_{IIB}/β_3

Figure 1 (top) illustrates that stimulation of platelets by α-thrombin is accompanied by phosphorylation of the β_3 subunit, confirming earlier observations [11]. Immunoprecipitation (Figure 1, top, lane 1) and electrophoresis showed the heavy chain of α_{IIB} (125 kDa) and the β_3 subunit (110 kDa) as well as the heavy and light chains of the 4A7 antibody. An autoradiogram of ^{32}P-labelled platelets lacked ^{32}P-labelled proteins in the immunoprecipitate before stimulation (Figure 1, top, lane 2), whereas a considerable ^{32}P-incorporation in the β_3 subunit was found after 2 min stimulation with 0.2 unit/ml α-thrombin (Figure 1, top, lane 3). Also in a total lysate (Figure 1, top, lane 4), [^{32}P]β_3 subunit could be detected together with numerous other ^{32}P-labelled proteins. The additional phosphorylated bands of about 70, 85 and 200 kDa are probably cytoskeletal proteins. Similar results were obtained after stimulation with 1 μM PAF (5 min) (results not shown). The same precipitation pattern was obtained with the commercially available P2 antibody (results not shown).

To investigate whether ^{32}P-incorporation in the β_3 subunit was the result of Tyr phosphorylation, immunoprecipitates were analysed with an anti-phosphotyrosine antibody (Figure 1, bottom). Western-blotting again revealed the β_3 subunit in platelets stimulated for 5 min (lane A) and 10 min (lane C) with 0.2 unit/ml α-thrombin, but no Tyr phosphorylation in β_3 was found (lanes B and D). This indicates that after stimulation with α-thrombin, β_3 is not phosphorylated on Tyr-residues.

Role of PKC and Tyr-kinases in platelet aggregation and ligand binding to integrin α_{IIB}/β_3 and phosphorylation of pleckstrin and β_3

Figure 2 (top) and Table 1 illustrate a comparison between aggregation studies (Figure 2, top), ligand-binding experiments, PKC activity, expressed as pleckstrin phosphorylation and β_3 phosphorylation (Table 1) following stimulation with 0.1 unit/ml α-thrombin. Compared with untreated platelets (curve a; control), suspensions treated with ADP scavenger mixture PEP/PK showed slightly lower aggregation (77 ± 13 % of control; $P < 0.05$, curve b) without changing ligand binding (92 ± 6 %; $P > 0.1$), pleckstrin phosphorylation (102 ± 7.2 %; mean ± range, $n = 2$) or β_3 phosphorylation (93 ± 9.2 %; mean ± range, $n = 2$). The Tyr-kinase inhibitor Herbimycin A (20 μM) left aggregation (curve c) and pleckstrin phosphorylation unchanged despite a weak inhibition of the ligand binding (70 ± 14 %, $P < 0.002$) and β_3 phosphorylation (83 ± 6.2 %; mean ± range, $n = 2$). In contrast to these minor changes, aggregation (curve d), pleckstrin phosphorylation and β_3 phosphorylation were almost completely abolished and ligand binding was reduced to 27 ± 8 % ($P < 0.001$) after pretreatment with the PKC inhibitor bisindolylmaleimide [28]. A combination of this inhibitor with PEP/PK (curve e) or Herbimycin A (curve f) suppressed aggregation and phosphorylation of pleckstrin and β_3 completely and reduced ligand binding slightly further to 16 ± 5 % and 6 ± 5 % ($P < 0.05$) respectively. Finally, incubation with staurosporine inhibited all responses completely (curve g). The concentration of Herbimycin A used inhibited Tyr phosphorylation completely after α-thrombin stimulation, while lower concentrations (5–15 μM) only partially inhibited α-thrombin-induced Tyr phosphorylation (Figure 2, bottom). Together these data reveal a major role for PKC in aggregation, ligand binding and β_3 phosphorylation in α-thrombin-stimulated platelets.

Exposure of integrin α_{IIB}/β_3 and phosphorylation of β_3

Stimulation with 500 nM PAF at 22 °C exposed 52880 ± 2485 fibrinogen binding sites/platelet, as described previously [15,17].

Table 1 Role of PKC and Tyr-kinases in exposure of integrin α_{IIB}/β_3, pleckstrin- and β_3-phosphorylation

Gel-filtered platelets [(200–250) × 10^3 platelets/μl] were preincubated under the conditions of the aggregation experiment (Figure 2, top), but at 22 instead of 37 °C. Subsequently these platelets were stimulated with 0.1 unit/ml α-thrombin in the absence of ^{125}I-fibrinogen and without stirring. Three minutes after stimulation, 30 nM PPACK was added, and 1 μM [^{25}I-fibrinogen 2 min thereafter. Ten minutes after stimulation, samples were withdrawn and analysed for specific binding. Data are expressed as a percentage of the maximal binding induced in the control situation. Data are means ± S.D., $n = 4$. To measure protein phosphorylation, ^{32}P-labelled platelets [(200–350) × 10^3 platelets/μl] were preincubated under the conditions of the aggregation experiment (Figure 2, top) and subsequently stimulated with 0.1 unit/ml α-thrombin (pleckstrin phosphrylation) and 0.2 unit/ml α-thrombin (β_3 phosphorylation) at 22 °C. Ten minutes after stimulation, samples were withdrawn and analysed for the phosphorylation of pleckstrin and β_3. Data are expressed as a percentage of [^{32}P]pleckstrin or [^{32}P]β_3 in the control. Data are means ± range, $n = 2$.

		Protein phosphorylation	
	Fibrinogen binding (%)	Pleckstrin phosphorylation (%)	β_3 phosphorylation (%)
Control	100 ± 6	100 ± 9.2	100 ± 6.7
PEP/PK	92 ± 6	102 ± 7.2	93 ± 9.2
Herbimycin A	70 ± 14	106 ± 4.1	83 ± 6.2
Bisindolylmaleimide	27 ± 8	2.5 ± 1.5	2.5 ± 7.7
PEP/PK + bisindolylmaleimide	16 ± 5	10 ± 4.1	2.5 ± 5.1
Herbimycin A + bisindolylmaleimide	6 ± 5	2.5 ± 3.1	7.8 ± 6
Staurosporine	2 ± 1	2.5 ± 2	2.5 ± 3.6

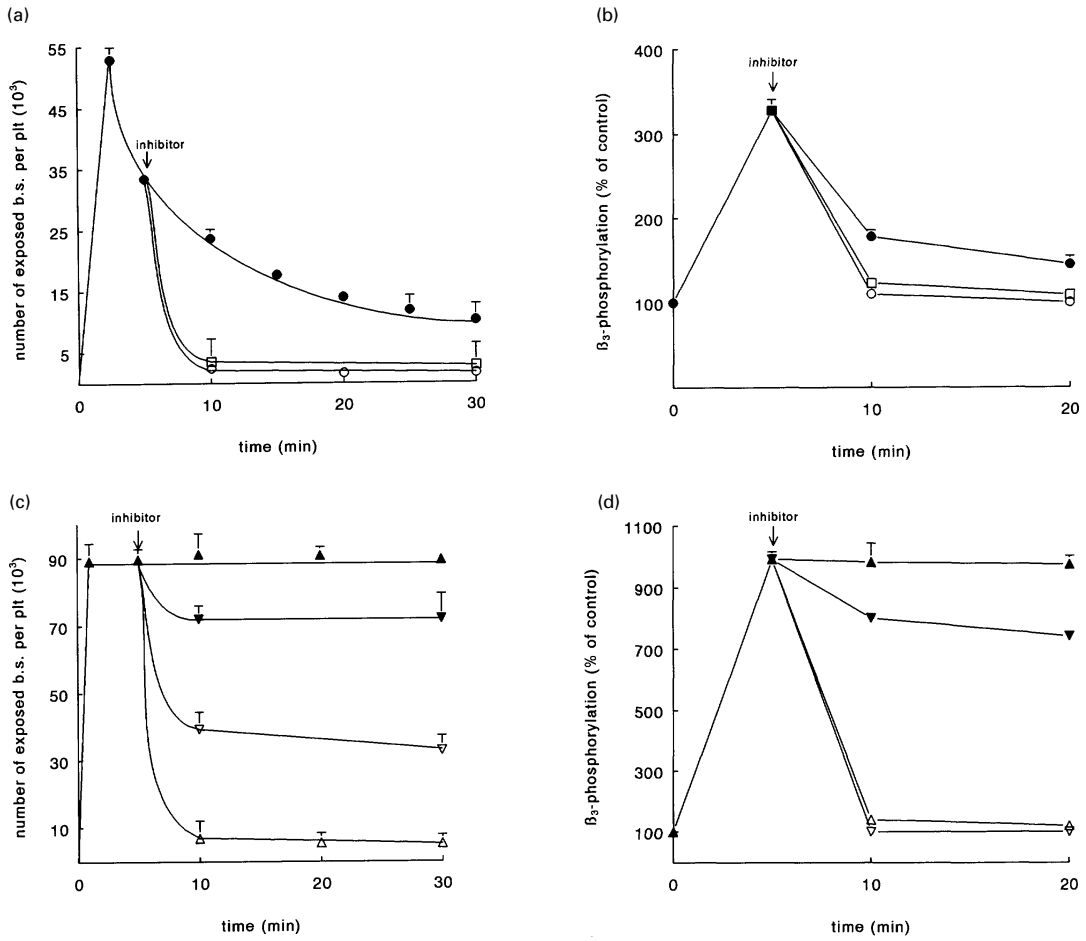

Figure 3 Role of PKC in exposure of integrin α_{IIB}/β_3 and phosphorylation of β_3

(**a**,**c**) Gel-filtered platelets [(200–250) $\times 10^3$ platelets/μl] were stimulated with 500 nM PAF (**a**) or 0.1 unit/ml α-thrombin (**c**) in the absence of radiolabelled ligand. Five minutes later the protein kinase inhibitor staurosporine (1 μM, ○,△), the PKC inhibitor bisindolylmaleimide (10 μM, □,▽), the Tyr-kinase inhibitor Herbimycin A [20 μM, ▼ (indicated in **c**)] or Tyrode solution (●,▲) was added. At different times after stimulation, samples were collected and incubated with ^{125}I-fibrinogen (PAF stimulation) or ^{125}I-fibronectin (α-thrombin stimulation) for 10 min at 22 °C to assess specific ligand-binding. Data are expressed as number of exposed binding sites (b.s.)/platelet (plt) (data are means ± S.D., $n = 3$; where no error bars are visible the S.D. is within the data point). (**b**,**d**) ^{32}P-Labelled platelets [(250–350) $\times 10^3$ platelets/μl] were stimulated with 1 μM PAF (**b**) or 0.2 unit/ml α-thrombin (**d**). Five minutes later the protein kinase inhibitor staurosporine (1 μM, ○,△), the PKC inhibitor bisindolylmaleimide (10 μM, □,▽), the Tyr-kinase inhibitor Herbimycin A [20 μM, ▼ (indicated in **d**)] or Tyrode solution (●,▲) was added. At the times indicated, samples were collected into ice-cold Immunomix. Integrin α_{IIB}/β_3 was immunoprecipitated and after electrophoresis on an SDS/7.5%-polyacrylamide gel, ^{32}P-radioactivity in β_3 was determined. Data are expressed as percentage of ^{32}P in β_3 of unstimulated platelets (means ± S.D., $n = 3$, where no error bars are visible the S.D. is within the data point; for bisindolylmaleimide and Herbimycin A, mean $n = 2$).

The exposure was followed by a rapid decrease in this number and after 25 min few accessible binding sites were available (Figure 3a). Concurrent measurement of phosphorylated β_3 showed a similar pattern, with a rapid increase to about 350% of initial radioactivity followed by a decline to prestimulation values after 20 min (Figure 3b). In contrast to the transient patterns induced by PAF, α-thrombin induced a 'permanent' exposure of binding sites on α_{IIB}/β_3 (Figure 3c). Again, phosphorylated β_3 increased in parallel with exposure of α_{IIB}/β_3 and remained constant thereafter (Figure 3d). Thus, exposure and closure of α_{IIB}/β_3 correlated closely with ^{32}P-radioactivity in β_3, suggesting that protein kinases contributed to the regulation of the α_{IIB}/β_3 complex.

When platelets were first stimulated with PAF to fully expose α_{IIB}/β_3, and thereafter treated with the PKC inhibitor bisindolylmaleimide (10 μM) or the protein kinase inhibitor staurosporine (1 μM), the closure of exposed α_{IIB}/β_3 (Figure 3a) and dephosphorylation of β_3 (Figure 3b) were much faster than with undisturbed PKC activity. Apparently active PKC was required to delay the decrease in accessible α_{IIB}/β_3. Despite the different regulation of α_{IIB}/β_3 in α-thrombin-treated platelets, the sensitivity to bisindolylmaleimide and staurosporine was the same, resulting in rapid closure of binding sites (Figure 3c) and dephosphorylation of β_3 (Figure 3d). When the Tyr-kinase inhibitor Herbimycin A (20 μM) was added after binding sites were exposed by α-thrombin, a small decrease in exposed binding sites and β_3 phosphorylation was observed [79 ± 4.5% ($P < 0.05$) and 80.2%, mean $n = 2$, respectively]. Thus, although α-thrombin-stimulated platelets could preserve the exposed configuration of α_{IIB}/β_3, continuous PKC activity was essential, while Tyr-kinases did not play a major role.

The exposure of α_{IIB}/β_3 was equally sensitive to modulation of phosphatase activity. Figure 4 illustrates that a short preincubation of platelets with okadaic acid (1 μM for 30 s at 22 °C), an inhibitor of the protein phosphatases PP-1 and PP-2A [29,30], changed the transient α_{IIB}/β_3 exposure by PAF into the

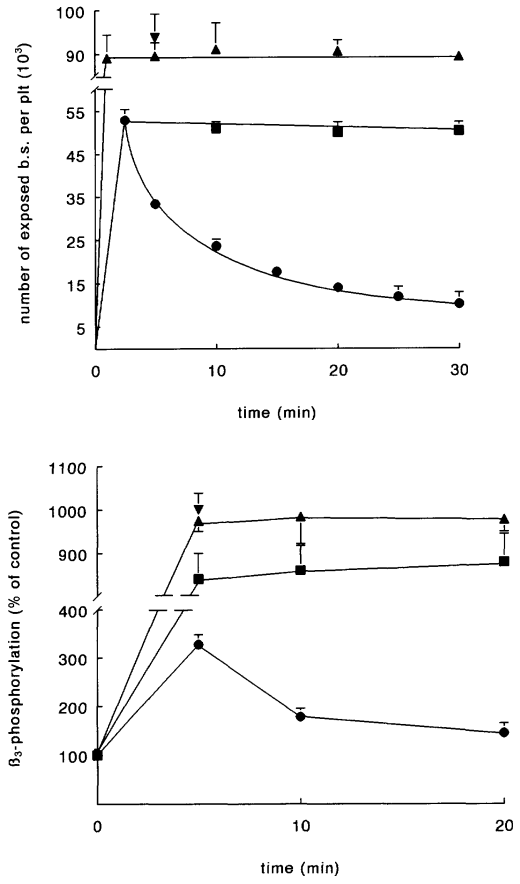

Figure 4 Role of Ser/Thr-phosphatases in integrin α_{IIB}/β_3 closure and dephosphorylation of β_3

Platelets without (Figure 4, top) and with (Figure 4, bottom) ^{32}P-labelling were preincubated with 1 μM okadaic acid (1 min, 22 °C, ■,▼) or Tyrode solution (●,▲). After stimulation with PAF or α-thrombin, samples were collected for analysis of ^{125}I-ligand-binding to exposed α_{IIB}/β_3 and [^{32}P]β_3 as described in the legend to Figure 3.

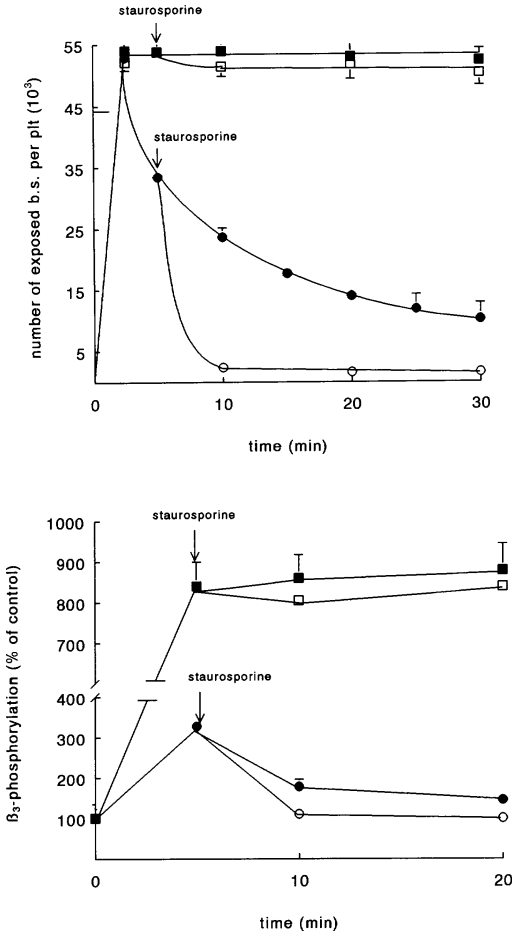

Figure 5 Step-wise inhibition of Ser/Thr-phosphatases and PKC

Platelets were preincubated with 1 μM okadaic acid (30 s, 22 °C, ■,□) or Tyrode solution (●,○). After stimulation with PAF, 1 μM staurosporine was added (arrow, open symbols) and binding of ^{125}I-fibrinogen to exposed α_{IIB}/β_3 (Figure 6, top) and [^{32}P]β_3 (Figure 6, bottom) were measured as described in the legend to Figure 3.

'permanent' exposure seen with α-thrombin, although the number of accessible binding sites did not change (Figure 4, top). The transient phosphorylation of β_3 was also changed into a more permanent ^{32}P-incorporation, which now reached the range found in α-thrombin-treated cells (Figure 4, bottom).

In contrast to the effect on platelets stimulated with PAF, pretreatment with okadaic acid did not change α_{IIB}/β_3 exposure and β_3 phosphorylation of α-thrombin-treated platelets (Figure 4, top and bottom). This suggests that, unlike PAF, α-thrombin delays the dephosphorylation of the β_3 subunit. It is noteworthy that addition of okadaic acid did not further increase the number of α_{IIB}/β_3 complexes exposed either by PAF or by α-thrombin. Orthovanadate, an inhibitor of Tyr-phosphatases, hardly changed the binding data, leading only to a slight delay in the closure of α_{IIB}/β_3 (results not shown). Therefore, preservation of exposed α_{IIB}/β_3 required that dephosphorylation of serine and/or threonine residues on the β_3 subunit was prevented.

We next investigated whether the concurrent phosphorylation of β_3 was essential for keeping α_{IIB}/β_3 in the exposed conformation. Platelets were first incubated with okadaic acid and thereafter stimulated with PAF (Figure 5). Subsequent addition of staurosporine, which closed α_{IIB}/β_3 and led to dephosphorylation of β_3 in untreated cells, had no effect on α_{IIB}/β_3 exposure and β_3 phosphorylation after pretreatment with okadaic acid. Thus, once β_3 was optimally phosphorylated and dephosphorylation was prevented, platelets maintained exposed α_{IIB}/β_3 independent of concurrent phosphorylation. Experiments in the absence of platelet activators showed that addition of okadaic acid alone did not expose binding sites or change the basal ^{32}P-radioactivity in β_3, indicating that in resting platelets ^{32}P-turnover in the β_3 subunit was minimal (results not shown).

Exposure of integrin α_{IIB}/β_3 and β_3 phosphorylation by different agonists

Earlier indications for a role of PKC in exposure of α_{IIB}/β_3 were based on the parallel changes in fibrinogen or fibronectin binding and ^{32}P-labelled pleckstrin, which is a major substrate of PKC. Also the different exposure by PAF and α-thrombin were accompanied by similar differences in [^{32}P]pleckstrin [17]. However, the fact that almost the same numbers of α_{IIB}/β_3 complexes were exposed by PAF, ADP and adrenaline argued against a role for PKC, since PAF induced more pleckstrin phosphorylation than ADP, whereas pleckstrin phosphorylation by adrenaline

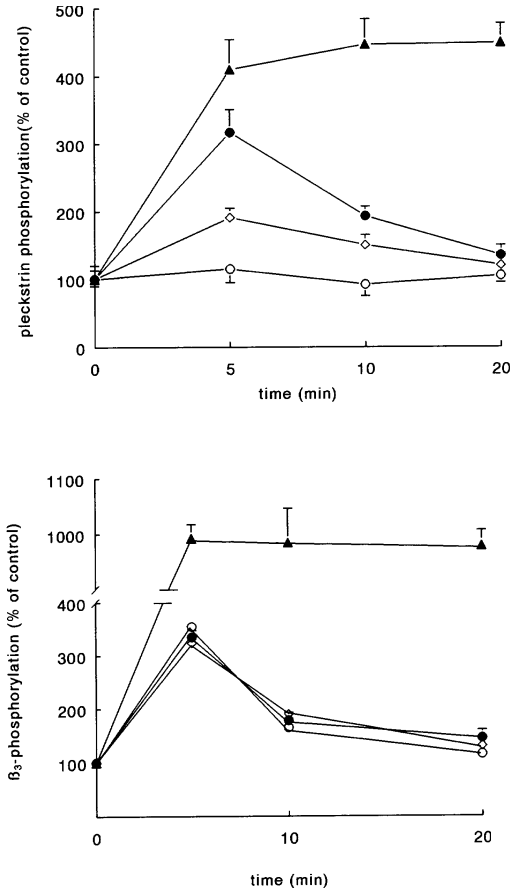

Figure 6 Comparison between phosphorylation of pleckstrin and β_3 induced by different agonists

Top: ^{32}P-labelled platelets were stimulated with 500 nM PAF (●), 0.1 unit/ml α-thrombin (▲), 10 μM ADP (◇) or 10 μM adrenaline (○). At the times indicated, the radioactivity in pleckstrin was measured as described in the Materials and methods section and expressed as the percentage of ^{32}P present in pleckstrin of unstimulated platelets (results are means ± S.D., $n = 3$). Bottom: ^{32}P-labelled platelets were stimulated with 1 μM PAF (●), 0.2 unit/ml α-thrombin (▲), 20 μM ADP (◇) and 20 μM adrenaline (○). At the times indicated, samples were collected and analysed for phosphorylated β_3, as described in the legend to Figures 3(b) and 3(d).

was low or virtually absent. This fact is illustrated in Figure 6 (top), which shows the different phosphorylation patterns of pleckstrin induced by PAF, ADP and adrenaline. Also shown is [^{32}P]pleckstrin in α-thrombin-stimulated platelets, illustrating the balance between phosphorylation and dephosphorylation also observed with [^{32}P]β_3. Under these conditions ADP and adrenaline exposed α_{IIB}/β_3 transiently, similar to the patterns induced by PAF [15]. Figure 6 (bottom) illustrates that PAF, ADP and adrenaline induced almost similar changes in [^{32}P]β_3. The increase in phosphorylated β_3 was transient and was followed by a decrease in prestimulation values, in contrast to the higher and permanent accumulation of [^{32}P]β_3 following stimulation with α-thrombin. These findings once more illustrate the close correlation between exposure of binding sites on α_{IIB}/β_3 and the ^{32}P-radioactivity of the β_3 subunit. Thus, the balance between phosphorylation and dephosphorylation is different between β_3 and pleckstrin, making the latter an inappropriate marker for the phosphorylation of the α_{IIB}/β_3 complex.

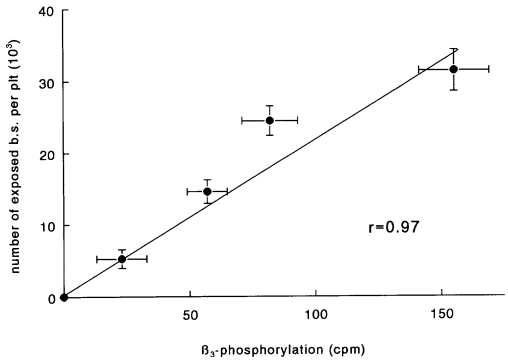

Figure 7 Comparison between number of exposed integrin α_{IIB}/β_3 and β_3 phosphorylation

Platelets were stimulated with different concentrations of PMA (0–100 nM) in the presence of 1 μM ^{125}I-fibrinogen. At $t = 10$ min the amount of fibrinogen molecules bound per platelet was measured (means ± S.D., $n = 3$). For the measurement of β_3 phosphorylation, ^{32}P-labelled platelets were stimulated with the same concentrations of PMA and β_3 phosphorylation was measured as described in the legend to Figures 3(b) and 3(d) (means with ranges of two separate experiments).

Comparison between [^{32}P]β_3 and the number of exposed integrin α_{IIB}/β_3 complexes

In an attempt to compare [^{32}P]β_3 and the number of exposed α_{IIB}/β_3 complexes in more detail, ^{32}P-labelled platelets were stimulated with different concentrations of PMA for 10 min to induce different levels of [^{32}P]β_3. Concurrently, the same platelets without preincubation with [^{32}P]P_i were used for analysis of PMA-exposed binding sites. Figure 7 illustrates the relation between exposed α_{IIB}/β_3 complexes, as assessed with ^{125}I-fibrinogen binding, and the levels of [^{32}P]β_3. It is noteworthy that in the range between 5000 and about 35000, exposed α_{IIB}/β_3 complexes and [^{32}P]β_3 correlated almost linearly. This is the range induced during stimulation by PAF, ADP and adrenaline.

Regulation of integrin α_{IIB}/β_3 by cyclic AMP

Previous studies have shown that an increase in cyclic AMP concentration induced immediate closure of binding sites on exposed α_{IIB}/β_3 [15,17]. To investigate whether this effect was due to interference with phosphorylation or dephosphorylation of the β_3 subunit, exposed binding sites were compared with [^{32}P]β_3 under different conditions of cyclic AMP-dependent α_{IIB}/β_3 control. Figure 8 (top) illustrates once more the exposure of binding sites induced by PAF followed by the decrease in accessible binding sites. Addition of PGI_2 at 5 min after exposure of the sites induced a rapid closure of α_{IIB}/β_3. Also, the permanent exposure of α_{IIB}/β_3 by α-thrombin was sensitive to PGI_2 addition, provided that after stimulation, α-thrombin had been removed from the receptors with an excess of hirudin, which is in agreement with earlier observations [17]. Figure 8, bottom, shows that none of these treatments changed [^{32}P]β_3. Hence, cyclic AMP did not affect the balance between phosphorylation and dephosphorylation of the β_3 subunit and triggered closure of binding sites via a second, independent mechanism.

Stoichiometry of β_3 phosphorylation

Table 2 lists the results of the stoichiometry measurements. In experiments 1–3 platelets were ^{32}P-labelled in phosphate-containing buffer and the specific radioactivity of actin-bound ADP

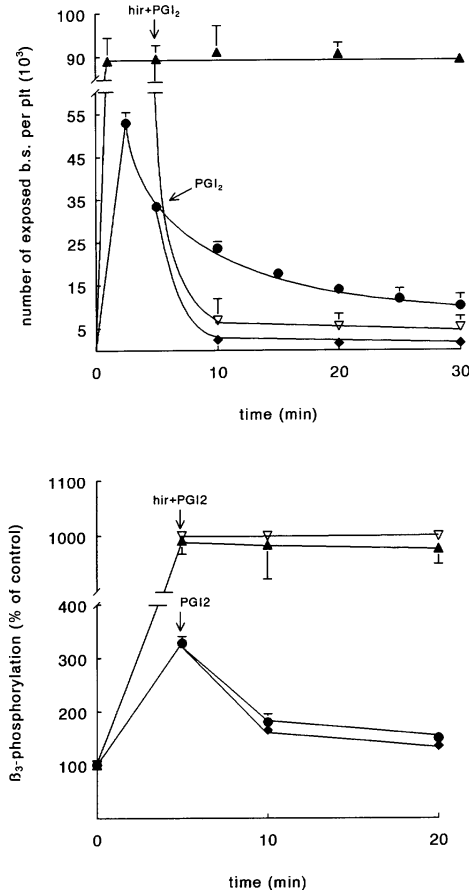

Figure 8 Effect of cyclic AMP on the exposure of integrin α_{IIB}/β_3 and β_3 phosphorylation

Top: platelets were stimulated with 500 nM PAF (●,◆) and 0.1 unit/ml α-thrombin (▲,▽) in the absence of radiolabelled ligands. Five minutes later 10 ng/ml PGI_2 (PAF stimulation, ◆) or a combination of PGI_2 + hirudin (10 ng/ml and 30 units/ml respectively, α-thrombin stimulation, ▽) was added. Binding of ^{125}I-fibrinogen (PAF stimulation) or ^{125}I-fibronectin (α-thrombin stimulation) to exposed α_{IIB}/β_3 was measured as described in Figure 4, top. Bottom: ^{32}P-labelled platelets were stimulated with 1 μM PAF (●,◆) and 0.2 unit/ml α-thrombin (▲,▽). Five minutes after stimulation 10 ng/ml PGI_2 (PAF stimulation, ◆) or a combination of PGI_2 + hirudin (10 ng/ml and 30 units/ml respectively, α-thrombin stimulation, ▽) was added. At the times indicated, samples were collected and analysed for $[^{32}P]\beta_3$ as described in the legend to Figures 3(b) and 3(d). Abbreviation: hir, hirudin.

was relatively low. Nevertheless, after stimulation with 0.2 unit/ml α-thrombin, a stoichiometry of 0.73 was found. Subsequent incubations in phosphate-free medium (see Methods section) gave a 2-fold increase in ^{32}P incorporation into metabolic ADP–ATP and in the β_3 subunit. Precipitation with a commercially available anti α_{IIB}/β_3 antibody (P2; Experiment No. 5, Table 2) led to a stoichiometry of 0.78, which was within the range of the findings with the 4A7 antibody. Approximately the same data were obtained at 22 and 37 °C, leading to an average stoichiometry of 0.05 ± 0.02 in resting platelets and of 0.80 ± 0.1 mol of phosphate/mol of β_3 following stimulation with α-thrombin.

DISCUSSION

The major findings of the present study are as follows: (i) modulation of PKC activity is accompanied by parallel changes in aggregation, ligand binding to α_{IIB}/β_3 and β_3 phosphorylation; (ii) exposure and closure of binding sites for fibrinogen and fibronectin on the α_{IIB}/β_3 complex are paralleled by qualitatively similar changes in $[^{32}P]\beta 3$; (iii) the 'permanent' exposure of α_{IIB}/β_3 by a high α-thrombin concentration is caused by α-thrombin's ability to bring phosphorylation and dephosphorylation of the β_3 subunit into equilibrium; (iv) exposure of α_{IIB}/β_3 and β_3 phosphorylation are fully sensitive to PKC inhibition and only slightly affected by Tyr-kinase inhibition; (v) once phosphorylation is completed, exposed α_{IIB}/β_3 can be preserved without concurrent phosphate turnover; (vi) step-wise activation of PKC by phorbol ester reveals a linear correlation between $[^{32}P]\beta_3$ and exposed binding sites in the range 0–35000 sites/platelet; and (vi) stimulation with α-thrombin raises the incorporation of phosphate from 0.05 ± 0.02 to 0.80 ± 0.1 mol of phosphate/mol of β_3.

The transient exposure of binding sites by PAF and the permanent exposure by α-thrombin are accompanied by similar differences in the accumulation of $[^{32}P]\beta_3$. Also, the level of [^{32}P]pleckstrin varies accordingly, suggesting that the balance between phosphorylation and dephosphorylation differs greatly between platelets stimulated by PAF and by α-thrombin. The presence of the phosphatase inhibitor okadaic acid changed the patterns seen with PAF to the characteristics of α-thrombin activation, both with respect to binding-site exposure and accumulation of $[^{32}P]\beta_3$. Although detection of exposed binding sites requires a 5 min [20] to 10 min (this study) incubation with ^{125}I-fibrinogen or ^{125}I-fibronectin, previous studies revealed

Table 2 Stoichiometry of β_3 phosphorylation

^{32}P-Labelled platelets [$(250–350) \times 10^3$ platelets/μl] were stimulated with 0.2 unit/ml α-thrombin and the specific activity of the β_3 subunit and of the metabolic ATP (Met. ATP) pool were determined as described in the Materials and methods section. In experiments 1–4 the α_{IIB}/β_3 complex was precipitated with the monoclonal antibody 4A7 and in experiment 5 with the monoclonal antibody P2. The specific activity of ATP in experiments 1–3 was based on actin-bound ADP and in experiments 3–5 on cytosolic ATP by digitonin permeabilization of the plasma membrane. Data are the means of three separate determinations within each experiment. The S.D.s were always less than 10% of the mean values. The mean stoichiometry is 0.80 ± 0.1 mol of phosphate/mol of β_3.

Experiment No.	Temperature (°C)	Specific activity: Actin–ADP (c.p.m./pmol)	Specific activity: Met. ATP (c.p.m./pmol)	Specific activity: β_3 subunit (c.p.m./pmol)	Stoichiometry of β_3 phosphorylation
1	22	126	–	103	0.82
2	22	814	–	565	0.70
	37	792	–	529	0.67
3	22	–	2784	1177	0.85
	37	–	2784	1268	0.91
4	22	–	2440	1084	0.81
5	22	–	2820	1093	0.78

maximal exposure within 2–5 min with a high concentration of PAF [20]. The effect of 0.1 unit/ml α-thrombin is probably equally rapid, since it is a more powerful agonist than PAF. Thus, exposure of α_{IIB}/β_3 occurs in a period when β_3 is maximally phosphorylated. In this stage there is little effect of okadaic acid and the activity of phosphatases must be low compared with the activity of PKC. However, addition of the protein kinase inhibitor staurosporine and the PKC inhibitor bisindolylmaleimide induced closure of α_{IIB}/β_3 and dephosphorylation of β_3, indicating that also at this stage some phosphatase activity remains ([15,17] and this study). When exposure of α_{IIB}/β_3 is completed, sites exposed by PAF gradually close while those exposed by a high concentration of α-thrombin remain open. Bisindolylmaleimide and staurosporine also induce closure of binding sites and dephosphorylation of β_3 at this stage, illustrating that phosphatases are still active. Hence, exposed α_{IIB}/β_3 depends on continuous phosphorylation/dephosphorylation. Earlier reports already indicated that exposure of α_{IIB}/β_3 required metabolic energy. Platelet aggregation depends on an intact energy metabolism [31] and exposed binding sites close immediately following abrupt arrest of ATP re-synthesis [32]. A patient with Friedreich's ataxia with impaired aggregation showed a defect in protein phosphorylation [33], and a few other patients with bleeding disorders had a similar defect [34]. The recent discovery of a patient with Glanzmann thrombasthenia with a Ser^{752}-to-Pro mutation in the cytoplasmic domain of the β_3 subunit [35], and the observation that the same mutation impairs bidirectional signalling [12], strongly support the concept that loss of a putative phosphorylation site in the α_{IIB}/β_3 complex can be the cause of impaired aggregation and an increased tendency to bleeding.

In previous studies the role of PKC in α_{IIB}/β_3 control was deduced from changes in [^{32}P]pleckstrin, which is a major substrate of PKC. In platelets stimulated with PAF, [^{32}P]pleckstrin and exposed α_{IIB}/β_3 correlated almost linearly at different rates of PKC activity [15]. However, when different agonists were compared, the correlation between [^{32}P]pleckstrin and exposed binding sites was lost. Our present findings reveal differences between the phosphorylation of pleckstrin and β_3, possibly because different PKC subtypes are involved.

The role of Tyr-kinases in α_{IIB}/β_3 exposure is minor. Preincubation of platelets with Herbimycin A, as well as addition of Herbimycin A after exposure of the α_{IIB}/β_3 was completed, only slightly reduced the number of exposed binding sites. These results are in line with the results of Schoenwaelder et al. who showed that preincubation with Herbimycin A had no effect on aggregation induced by several agonists, although clot-retraction and binding of the α_{IIB}/β_3 to the cytoskeleton were impaired [36].

Earlier work has shown that the exposure of α_{IIB}/β_3 by PAF, ADP, adrenaline [15], vasopressin and the thromboxane analogue U46619 (G. van Willigen and J. W. N. Akkerman, unpublished results) is transient and that sequential activation with these stimuli triggers a second and even a third exposure of binding sites [15]. The permanent exposure by a high concentration of α-thrombin appears therefore to be a unique property of this agonist. α-Thrombin-exposed sites remain sensitive to staurosporine, and the constant level of [^{32}P]β_3 reflects that phosphorylation and dephosphorylation are in equilibrium. Possibly, the activated α-thrombin receptor controls PKC activity via a mechanism that other receptors lack, but regulation via phosphatases is equally possible. The nature of the phosphatases that play a role in the regulation of α_{IIB}/β_3 can be deduced from several experiments. Vanadate, a Tyr-phosphatase inhibitor, had little effect. It slightly delayed the closure of binding sites, probably due to some secretion of α-granule contents (5–10 %, based on β-thromboglobulin secretion), thereby liberating platelet-derived growth factor [37]. Platelet-derived growth factor may further activate the platelets by binding to its receptor [38], which is known to depend on Tyr phosphorylation [32]. It is also possible that vanadate activates a GTP-binding protein which in turn activates platelets, as suggested by Paris and Pouyssegur [39]. In contrast, okadaic acid, a Ser/Thr-phosphatase inhibitor [29,30], abolished the dephosphorylation of β_3 in PAF-stimulated platelets provided that a high concentration (1 μM) of this inhibitor was used. This indicates that PP-1 is involved, in contrast to the PP-2A subtype, which is sensitive to low concentrations of okadaic acid (1 nM), and the PP-2B subtype, which is not inhibited by okadaic acid [29,30]. PP-1 is the most abundant Ser/Thr-phosphatase in platelets [40]. Okadaic acid-treated platelets, subsequently stimulated with PAF or α-thrombin, lost their sensitivity to staurosporine, which led to the peculiar observation of platelets with exposed α_{IIB}/β_3, independent of concurrent energy supply. If this state is maintained longer than the 30 min tested in the present study, these platelets would provide an attractive source for the purification of exposed α_{IIB}/β_3 or the generation of antibodies against this configuration.

A high concentration of cyclic AMP prevents exposure of binding sites on α_{IIB}/β_3 [41], and exposed sites rapidly become inaccessible upon addition of dibutyryl cyclic AMP or PGI_2 [15,17]. Our results show that cyclic AMP regulates α_{IIB}/β_3 via a mechanism that is independent of phosphorylation/dephosphorylation. Analysis of [^{32}P]pleckstrin shows that an increase in cyclic AMP concentration does not change PKC activation by a high α-thrombin concentration, in accordance with other observations [15,17], and also the phosphorylation of β_3 remains unchanged (Figure 8). This implies that cyclic AMP closes the binding sites via a different mechanism.

Phosphorylation is a major factor in the control of other integrin receptors, such as LFA-1 (α_L/β_2) [42], α_v/β_s [43], CSAT (fibronectin receptor) [44] and α_6/β_1 [45]. Phosphorylation of the β-chains changes the adhesive properties of these receptors and is almost complete (80–100 %). Our results are in line with these observations and indicate that phosphorylation of the β_3 subunit, which is virtually absent in resting platelets, increases to about 80 % after stimulation with α-thrombin. This value is much higher than the 5 % phosphorylation reported by Hillery et al. [46] for α-thrombin-stimulated platelets. A higher yield after the precipitation step, increased ^{32}P incorporation and two alternative means to determine the specific radioactivity of metabolic ATP may account for the higher stoichiometry found in the present study.

The high stoichiometry suggests almost complete phosphorylation of the available β_3 subunits after stimulation with α-thrombin. The close correlation between integrin exposure and β_3 phosphorylation observed with PAF, ADP and adrenaline, which is about 50 % of responses induced by α-thrombin, suggests that about half of the available integrin molecules are phosphorylated. An apparent discrepancy between α_{IIB}/β_3 exposure and β_3 phosphorylation is observed in PAF-stimulated platelets, where pretreatment with okadaic acid increases [^{32}P]β_3 2–3-fold without inducing more ^{125}I-fibrinogen binding. This may be explained by PAF's inability to induce α-granule secretion and opening of the canalicular system, making this population of α_{IIB}/β_3 molecules inaccessible to ^{125}I-fibrinogen although they might be in the exposed, phosphorylated configuration.

Although platelets in suspensions must be stimulated before binding sites on α_{IIB}/β_3 are exposed (inside-out signalling), they bind to surface-bound fibrinogen via α_{IIB}/β_3 without prior stimulation (outside-in signalling) [47,48]. Furthermore, α_{IIB}/β_3-dependent Tyr-kinase activities are found in aggregating platelets

but not in unstirred suspensions, indicating that platelet–platelet contact contributes to outside-in signal transduction via this integrin [49,50]. Thus, exposure of ligand-binding sites by β_3 phosphorylation is only one of the steps in α_{IIB}/β_3 regulation during the formation of a platelet aggregate.

In conclusion, our study indicates that the balance between phosphorylation of β_3 by PKC and dephosphorylation by the Ser/Thr-phosphatase PP-1 is a major factor in the control of exposure of binding sites for adhesive proteins on the α_{IIB}/β_3 complex.

This study was supported by The Netherlands Heart Foundation/Netherlands Organization for Scientific Research (grant 940-50-102) and The Netherlands Thrombosis Foundation (grant 92.002)

REFERENCES

1 Bennet, J. S. and Poncz, M. (1995) in Molecular Basis of Thrombosis and Hemostasis (High, K. A. and Roberts, H. R., eds.), pp. 579–602, Marcel Dekker Inc., New York
2 Calvete, J. J. (1994) Thromb. Haemostasis **72**, 1–15
3 Fox, J. E. B. (1993) Thromb. Haemostasis **70**, 884–893
4 Fox, J. E. B. (1993) Adv. Exp. Med. Biol. **344**, 175–185
5 O'Toole, T. E., Mandelman, D., Forsyth, J., Shattil, S. J., Plow, E. F. and Ginsberg, M. H. (1991) Science **254**, 845–847
6 O'Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Loftus, J. C., Shattil, S. J. and Ginsberg, M. H. (1994) J. Cell Biol. **124**, 1047–1059
7 Shattil, S., O'Toole, T., Eigenthaler, M., Thon, V., Williams, M., Babior, B. and Ginsberg, M. (1995) Thromb. Haemostasis **73**, 1190 (abstract)
8 Morii, N., Teru-uchi, T., Tominaga, T., Kumagai, N., Kozaki, S., Ushikubi, F. and Narimiya, S. (1992) J. Biol. Chem. **267**, 20921–20926
9 Torti, M., Sinigaglia, F., Ramaschi, G. and Balduini, C. (1991) Biochim. Biophys. Acta Biomembr. **1070**, 20–26
10 Smyth, S. S., Hillery, C. A. and Parise, L. V. (1992) J. Biol. Chem. **267**, 15568–15577
11 Parise, L. V., Criss, A. B., Nannizzi, L. and Wardell, M. R. (1990) Blood **75**, 2363–2368
12 Chen, Y.-P., O'Toole, T. E., Ylänne, J., Rosa, J.-P. and Ginsberg, M. H. (1994) Blood **84**, 1857–1865
13 Loh, E., Beaverson, K., Vilaire, G., Qi, W., Poncz, M. and Bennett, J. S. (1995) J. Biol. Chem. **270**, 18631–18636
14 Shattil, S. J. and Brass, L. F. (1987) J. Biol. Chem. **262**, 992–1000
15 Van Willigen, G. and Akkerman, J.-W. N. (1991) Biochem. J. **273**, 115–120
16 Hannun, Y. A., Greenberg, C. S. and Bell, R. M. (1987) J. Biol. Chem. **262**, 13620–13626
17 Van Willigen, G. and Akkerman, J.-W. N. (1992) Blood **79**, 82–90
18 Kloprogge, E. and Akkerman, J.-W. N. (1986) Biochem. J. **240**, 403–412
19 Nievelstein, P. F. E. M., D'Alessio, P. A. and Sixma, J. J. (1988) Arteriosclerosis **8**, 200–206
20 Kloprogge, E., Mommersteeg, M. and Akkerman, J.-W. N. (1986) J. Biol. Chem. **261**, 11071–11076
20a Laemmli, U. K. (1970) Nature (London) **227**, 680–685
21 Kloprogge, E., Hasselaar, P., Gorter, G. and Akkerman, J.-W. N. (1986) Biochim. Biophys. Acta **883**, 127–137
22 Daniel, J. L., Robkin, L., Molish, I. R. and Holmsen, H. (1979) J. Biol. Chem. **254**, 7870–7873
23 Verhoeven, A. J. M., Cook, C. A. and Holmsen, H. (1988) Anal. Biochem. **174**, 672–678
24 Daniel, J. L., Molish, I. R. and Holmsen, H. (1980) Biochim. Biophys. Acta **632**, 444–453
25 Akkerman, J.-W. N., Ebberink, R. H. M., Lips, J. P. M. and Christiaens, G. C. M. L. (1980) J. Haematol. **44**, 291–300
26 Holmsen, H., Setkowsky, C. A. and Day, H. J. (1974) Biochem. J. **144**, 385–396
27 Holmsen, H., Storm, E. and Day, H. J. (1972) Anal. Biochem. **46**, 489–501
28 Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. and Kirilovsky, J. (1991) J. Biol. Chem. **266**, 15771–15781
29 Haystead, T. A. J., Sim, A. T. R., Carling, B., Honnor, R. C., Tsukitani, Y., Cohen, P. and Hardie, D. G. (1989) Nature (London) **337**, 78–81
30 Cohen, P. (1989) Annu. Rev. Biochem. **58**, 453–508
31 Verhoeven, A. J. M., Mommersteeg, M. E. and Akkerman, J. W. N. (1984) Biochem. Biophys. Acta **800**, 242–250
32 Kloprogge, E., Hasselaar, P. and Akkerman, J.-W. N. (1986) Biochem. J. **238**, 885–891
33 Kornecki, E., Cooper, B. A. and Ehrlich, Y. G. (1988) J. Lab. Clin. Med. **111**, 618–626
34 Speiser-Ellerton, S. and Weiss, H. J. (1990) J. Lab. Clin. Med. **115**, 104–111
35 Chen, Y.-P., Djaffar, I., Pidard, D., Steiner, B., Cieutat, A.-M., Caen, J. P. and Rosa, J.-P. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10169–10173
36 Schoenwaelder, S. M., Jackson, S. P., Yuan, Y., Teasdale, M. S., Salem, H. H. and Mitchell, C. A. (1994) J. Biol. Chem. **269**, 32479–32487
37 Lindner, B. L., Chernoff, A., Kaplan, K. L. and Goodman, D. S. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4107–4111
38 Bryckaert, M. C., Rendu, F., Tobelem, G. and Wasteson, A. (1989) J. Biol. Chem. **264**, 4336–4341
39 Paris, S. and Pouyssegur, J. (1987) J. Biol. Chem. **262**, 1970–1976
40 Lerea, K. M., Tonks, N. K., Krebs, E. G., Fischer, E. H. and Glomset, J. A. (1989) Biochemistry **28**, 9286–9292
41 Hawiger, J., Parkinson, S. and Timmons, S. (1980) Nature (London) **283**, 195–197
42 Chatila, T. A., Geha, R. S. and Arnaout, M. A. (1989) J. Cell Biol. **109**, 3435–3444
43 Freed, E., Gailit, J., Van der Geer, P., Ruoslahti, E. and Hunter, T. (1989) EMBO J. **8**, 2955–2965
44 Dahl, S. C. and Grabel, L. B. (1989) J. Cell Biol. **108**, 183–190
45 Shaw, L. M., Messier, J. M. and Mercurio, A. M. (1990) J. Cell Biol. **110**, 2167–2174
46 Hillery, C. A., Smyth, S. S. and Parise, L. V. (1991) J. Biol. Chem. **266**, 14663–14669
47 Du, X., Plow, E. F., Frelinger, A. L., III, O'Toole, T. E., Loftus, J. C. and Ginsberg, M. H. (1991) Cell **65**, 409–416
48 Haimovich, B., Lipfert, L., Brugge, J. S. and Shattil, S. J. (1993) J. Biol. Chem. **268**, 15868–15877
49 Clark, E. A., Trikha, M., Markland, F. S. and Brugge, J. S. (1994) J. Biol. Chem. **269**, 21940–21943
50 Clark, E. A., Shattil, S. J. and Brugge, J. S. (1994) Trends Biochem. Sci. **19**, 464–469

Received 9 October 1995; accepted 30 October 1995