Capture by chemical crosslinkers provides evidence that integrin α IIB β 3 forms a complex with protein tyrosine kinases in intact platelets

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Platelet activation is accompanied by a cascade of kinase reactions in which numerous specific proteins are phosphorylated on tyrosine. These events are strictly dependent upon functional activation of an integrin receptor, generally α IIB β 3 (also known as glycoprotein IIb–IIIa). It is not known how α IIB β 3 regulates protein tyrosine kinase activation and, in particular, neither this nor any other integrin has been shown to associate with a protein tyrosine kinase. We employed chemical crosslinking of intact platelets with the bifunctional reagents dithiobis(succinimidyl propionate) (DSP) (lipid-soluble) and dithiobis(sulphosuccinimidyl propionate) (DTSSP) (lipid-insoluble) followed by *in vitro* kinase assays of immunoprecipitated proteins to identify kinase activity associated with α IIB β 3 in intact platelets. It was found that DSP but not DTSSP crosslinked kinase activity to α IIB β 3, suggesting an internal association. In these immunoprecipitates from DSP-crosslinked platelets, the *in vitro* kinase reaction

revealed a complex of several phosphoproteins in association with α IIB β 3. This association was not seen when the resting platelets were lysed before crosslinking, indicating the specificity of the reaction in crosslinking only molecules in preformed spatial association. The β 3 subunit of α IIB β 3 was identified as one of the phosphoproteins in the complex obtained after subjecting anti- β 3 immunoprecipitates from lysates of DSP-treated platelets to an *in vitro* kinase reaction and SDS/PAGE analysis. Phosphorylation of this subunit is shown to be predominantly on tyrosine. Affinity purification of the crosslinked phosphoprotein complex with anti- β 3 followed by elution and re-precipitation identified pp60^{c-*src*} and pp54/58^{c-*lyn*} as two protein tyrosine kinases associating with the integrin. These results suggest that, upon platelet activation, α IIB β 3 may provide a transmembrane focus for proteins involved in signal transduction.

INTRODUCTION

The extracellular matrix exerts a major influence upon cell growth and differentiation. It is now becoming apparent that a major mechanism by which this influence is imparted is through signals delivered by a family of receptors termed integrins (reviewed in [1]). Integrin is the name given to members of a family of heterodimeric transmembrane glycoproteins which integrate the extracellular matrix with the internal cellular cytoskeleton. This is achieved by binding glycoprotein components of the matrix through their external domains, and by associating with cytoskeletal proteins through sequences in their short internal cytoplasmic domains [2–4]. In different systems it has been shown that several integrins are expressed on the cell surface in a ‘resting’ configuration, and in this form they are unable to bind to their extracellular ligand; upon cell stimulation, the integrin undergoes a conformational change to an ‘active’ configuration able to bind ligand [5–8].

Ligand binding by the activated integrin receptor can lead to marked changes in cellular morphology and behaviour, and there is considerable evidence to suggest that this is caused by integrin-mediated signal transduction [9–12]. Apparently in direct response to integrin–ligand interaction there can occur rapid phosphorylation of several specific proteins on tyrosine [9,13–15], accumulation of GTP-bound p21^{ras} [16] and consequential events

such as major cytoskeletal changes and gene transcription [17,18]. How integrins perform this signalling function is not known. When intact cells are plated on individual glycoprotein matrices, integrins that bind the matrix accumulate in areas of tight contact, termed focal adhesion plaques, and such plaques are greatly enriched in proteins phosphorylated on tyrosine [13,19,20]. The tyrosine-phosphorylated proteins thus located include paxillin and pp125 focal adhesion kinase (FAK), itself a kinase for cytoskeletal proteins [21,22]. Integrin binding to its ligand is considered to induce the phosphorylation of these proteins, but there have been no reports of integrin-associated tyrosine kinases. Activation of growth factor receptors that are themselves tyrosine kinases can disrupt adhesion plaques and cell adhesion to substrates, and Bartfeld et al. [23] demonstrated that after mouse 3T3 fibroblasts were stimulated with platelet-derived growth factor, the integrin α v β 3 (vitronectin receptor) associated with a tyrosine-phosphorylated protein of 190 kDa, and the tyrosine phosphorylation of this protein increased upon engagement of the vitronectin receptor with one of its ligands. The nature of this 190 kDa protein is, however, unknown.

Platelets have proved to be an extremely fruitful model for the study of cell adhesion and signal transduction [14,15,17,24–28]. The major integrin of platelets is α IIB β 3 [also known as glycoprotein (GP)IIb–IIIa], which functions as a primary receptor for fibrinogen [29,30]. The stimulation of platelets with an

Abbreviations used: DSP, dithiobis(succinimidyl propionate); DTSSP, 3,3′-dithiobis(sulphosuccinimidyl propionate); FAK, pp125 focal adhesion kinase; mAb, monoclonal antibody; RIPA buffer, radioimmuno-precipitation assay buffer; GPIIb–IIIa, glycoprotein IIb–IIIa; ECL, enhanced chemiluminescence.

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agonist such as thrombin leads directly to a conformational change of $\alpha\text{IIb}\beta_3$, enabling it to bind fibrinogen [6]. Binding of the integrin to fibrinogen, either as an accompaniment to platelet aggregation or used as a matrix substrate, leads to the phosphorylation of a number of specific platelet proteins on tyrosine [14,15,25]. This process, which is entirely dependent upon functional $\alpha\text{IIb}\beta_3$ integrin [14,15,25,28], leads to the activation of FAK, although FAK phosphorylation is preceded by the tyrosine phosphorylation of other, unknown, protein substrates [26,27]. Consequent upon platelet activation, much of the $\alpha\text{IIb}\beta_3$ together with the protein tyrosine kinases pp60^{c-src}, pp62^{c-yes} and the p21^{ras} GTPase-activating protein redistribute from the membrane skeleton to the cytoskeletal network of cytoplasmic actin filaments (the cytoskeleton), many of the components of which are substrates for tyrosine kinases [17].

In the present study we use chemical crosslinking with membrane-permeable and membrane-impermeable reversible homobifunctional crosslinking reagents to identify kinases associated with $\alpha\text{IIb}\beta_3$ in the intact platelet. It is shown that the integrin forms specific complexes with several proteins, including the protein tyrosine kinases pp60^{c-src} and pp54/58^{c-lyn}. The identification of such a physical association between the integrin and tyrosine kinases provides a possible means by which the integrin may directly regulate kinase function.

EXPERIMENTAL

Materials

The chemical crosslinking reagents dithiobis(succinimidyl propionate) (DSP) and 3,3'-dithiobis(sulphosuccinimidyl propionate) (DTSSP) were purchased from Pierce (Rockford, IL, U.S.A.). Sepharose-4B, Protein A and PD-10 buffer exchange columns were from Pharmacia (Uppsala, Sweden). Lactoperoxidase, octyl glucoside and CHAPS were from Boehringer-Mannheim (Mannheim, Germany). Na¹²⁵I was from Australian Radioisotopes (Sydney, N.S.W., Australia). [γ -³²P]ATP was from Bresatec (Adelaide, S.A., Australia). Dithiothreitol Ampholytes and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Bio-Rad (Hercules, CA, U.S.A.). Rabbit anti-mouse IgG was from Dako (Carpinteria, CA, U.S.A.). Enhanced chemiluminescence (ECL) detection reagents were from Du Pont NEN (Boston, MA, U.S.A.). Cellulose TLC plates were from Merck (Darmstadt, Germany). Prostaglandin E1, glucose oxidase, leupeptin, soybean trypsin inhibitor, Nonidet P-40, Triton X-100, β -mercaptoethanol, ninhydrin and PMSF were all purchased from Sigma (St. Louis, MO, U.S.A.) and were of the highest grade available.

All other chemicals were from either Ajax Chemicals (Auburn, N.S.W., Australia) or BDH (Kilsyth, Victoria, Australia) and were of the highest quality available.

Antibodies

Monoclonal antibodies (mAbs) to GPIIb (SZ22), β_3 (1070; SZ21), GPIb-IX (AK2), platelet and T-cell activation antigen-1 (LeoA1), $\alpha_2\beta_1$ (AK7), P-selectin (AK6) and CD36 (5C8) were prepared in our laboratories and have been described previously [31–36], as were the affinity-purified rabbit antibodies to $\alpha\text{IIb}\beta_3$ and CD36 [37,38]. Other anti-CD36 mAbs (IA7; IE8) were kindly provided by Dr. A. W. Boyd, Walter & Eliza Hall Institute, Melbourne, Victoria, Australia, and an anti-CD9 mAb (FMC56) and anti-GPIb-IX (FMC25) were provided by Dr. Heddy Zola, Flinders Medical Centre, Adelaide, S.A., Australia. One mAb to pp60^{c-src} (src clone 327) was kindly provided by Dr. Ashley Dunn, Ludwig Institute, Melbourne, Victoria, Australia.

Other mAbs and polyclonal antibodies were purchased commercially: anti-CD4 (OKT4) from the American Type Culture Collection (Rockville, MD, U.S.A.); anti-phosphotyrosine (PY20) from ICN (Costa Mesa, CA, U.S.A.); anti-phosphotyrosine (4G10) from U.B.I. (Lake Placid, NY, U.S.A.); and rabbit anti-Lyn and anti-Src from Santa Cruz (Santa Cruz, CA, U.S.A.).

Platelet recovery

Normal human platelets were recovered from whole blood samples provided by the Hunter Area Health Service Blood Transfusion Unit. Blood samples were spun at 200 *g* for 20 min to prepare platelet-rich plasma. Prostaglandin E1 (1 μM) was added to the platelet-rich plasma and platelets were pelleted by centrifugation at 250 *g* for 20 min. Platelet pellets were washed in buffer A (138 mM NaCl, 2.9 mM KCl, 0.5 mM MgCl₂, 12 mM NaHCO₃, 0.3 mM NaH₂PO₄, 10 mM Hepes, pH 7.4) plus 2 μM prostaglandin E1 and resuspended in buffer A. In all experiments the washed platelets were allowed to stand at 37 °C for 30 min prior to use in order to allow recovery from the centrifugation steps.

Chemical crosslinking of platelet proteins

To determine spatial-functional protein associations within platelet membranes, platelets were exposed to bifunctional crosslinking reagents. Chemical crosslinker stock solutions were prepared immediately before use. DTSSP (in distilled water) and DSP (in dimethyl sulphoxide) were added to individual samples of 1×10^9 platelets to give final concentrations of 1 mM and 0.5 mM respectively, concentrations that were pre-determined to be optimal in preliminary experiments. The volume of stock solution of either crosslinking agent added to 0.5 ml of platelet suspension never exceeded 5 μl . The appropriate vehicle was added to control samples processed in parallel. Crosslinking was performed for 25 min at room temperature, since this time gave maximum crosslinking. The reaction was terminated and excess crosslinker simultaneously quenched by the addition of ice-cold 50 mM Tris/HCl (final concentration)-buffered solubilization buffers (see below). Samples were allowed to stand on ice for 15 min before further processing. In experiments in which DSP crosslinking was carried out after lysis of the resting platelets, the platelets were lysed [final concentrations in lysis buffer: 1% Triton X-100, 10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA plus proteinase inhibitors (see solubilization buffers)] and immediately crosslinked (0.5 mM DSP, 25 min). Tris (pH 7.4) was added to 100 mM to terminate crosslinking. The lysate was clarified by centrifugation at 15000 *g* for 15 min at 4 °C. $\alpha\text{IIb}\beta_3$ was immunoprecipitated and an *in vitro* kinase assay was performed on the radioimmuno-precipitation assay (RIPA) buffer-washed antigen-bead complexes (see below).

Solubilization of platelets

The relative strength of protein kinase associations within the intact platelet was ascertained by lysis using low- to high-stringency solubilization buffers, i.e. 0.5% CHAPS (0.5% CHAPS, 50 mM Tris, pH 7.6, 100 mM NaCl, 2 mM EDTA), 100 mM octyl glucoside (100 mM octyl glucoside, 50 mM Tris, pH 7.4, 150 mM NaCl) and 1% Triton X-100 (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA). All concentrations are final concentrations in whole lysate. All buffers included 2 mM PMSF, 10 $\mu\text{g/ml}$ leupeptin and 10 $\mu\text{g/ml}$ soybean trypsin inhibitor. Na₃VO₄ (2 mM) was added to buffers used where *in vitro* phosphorylation needed to be maintained.

Immunoprecipitation

Following platelet lysis, insoluble materials were pelleted by centrifugation at 15000 *g* for 15 min at 4 °C. Soluble lysates were precleared for 2 h at 4 °C with rabbit anti-mouse IgG coupled to Sepharose-4B beads if mAbs were to be subsequently used; or to Protein A-Sepharose-4B if polyclonal antibodies were used to precipitate the required antigen. Appropriate antibodies, either coupled directly to Sepharose beads or as free antibody (indirect), were added to the precleared lysates for 2 h at 4 °C. Indirect immunoprecipitations were completed by the addition of the corresponding bead-anti-species conjugate 30 min prior to completion of the procedure. All antigen-bead conjugates were washed twice in the original lysis buffer, except for 1 % Triton X-100-derived samples. These samples were washed in RIPA buffer: samples were washed with two consecutive cycles of RIPA buffer 1 (25 mM Tris, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.05 % SDS) and RIPA buffer 2 (25 mM Tris, pH 7.4, 500 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate). The washed immunoprecipitants were processed either immediately to SDS/PAGE or via additional procedures as described.

In vitro kinase assay

To determine the association of activated kinases with proteins of interest, immunoprecipitants of the primary protein were resuspended in 30 μ l of *in vitro* kinase buffer (1 mM dithiothreitol, 100 mM NaCl, 50 mM Hepes, pH 7.5, 10 mM MnCl₂, 10 mM MgCl₂, 0.5 % Nonidet P-40). [γ -³²P]ATP (10 μ Ci; specific radioactivity 4000 Ci/mmol) was added and the reaction was allowed to proceed for 12 min at 4 °C. The assay was stopped by the addition of 0.5 ml of *in vitro* kinase buffer. Antigen conjugates were recovered by up-down centrifugation at 15000 *g*, and reducing SDS/PAGE sample buffer was added to the bead pellets. Samples were processed by SDS/PAGE and ³²P incorporation detected by autoradiography. After autoradiography in several experiments the gel was treated with KOH and then re-autoradiographed as a measure of tyrosine phosphorylation [39]. For this, the autoradiographed SDS/PAGE gels were rehydrated into fresh 1 M KOH. Any backing paper was removed and the gel was allowed to stand for 2 h at 55 °C with occasional gentle agitation. The alkali was removed and the gel washed in 10 % acetic acid/10 % propan-2-ol solution before rinsing in water. The treated gel was re-dried under vacuum and re-autoradiographed.

Two-dimensional SDS/PAGE analysis

Two-dimensional gel electrophoresis of α Ib β 3-kinase conjugates was performed as described by O'Farrell et al. [40] with modifications according to Bravo et al. [41].

Western blot analysis

In order to identify the presence of specific proteins in soluble/insoluble lysates or immunoprecipitations of crosslinked complexes, proteins were electrophoretically transferred to nitrocellulose membranes (Sartorius) to allow probing with specific antibodies. Detection of antigens was by ECL according to the manufacturer's instructions.

Quantification of β 3 and CD36 by densitometry

Densitometric analysis of immunoblotted purified protein samples was used to prepare a protein calibration curve to enable

quantification of α Ib β 3 and CD36 in crosslinked immunocomplexes. Briefly, 0, 2, 4 and 6 μ g of each purified protein was combined with 100 μ l of rabbit anti-mouse IgG bead slurry and 3 μ g of irrelevant IgG₁ antibody (to mimic the *in vitro* kinase experimental conditions). Reducing sample buffer was added and the samples were processed by SDS/PAGE. Separated proteins were electrophoretically transferred to nitrocellulose (38 V, 300 mA, 2 h). Detection of antigen was performed using polyclonal antibodies at concentrations determined to be optimal, with visualization by ECL. The exposure time for ECL was 30 s with Hyperfilm (Amersham). The density units of β 3 and CD36 at each concentration were determined using a Hoefer Scientific Instruments scanning densitometer (Model GS3100) and the GS370V2.3 software package. From these data, calibration curves for each purified protein were constructed using an equation for the curve of best fit determined using the Cricket Graph III V1.0 software package (Apple Computer Inc).

To utilize these curves for quantification, platelets (3×10^8 platelets/ml) were prepared, chemically crosslinked and lysed in 1 % Triton X-100 lysis buffer as described above. Indirect immunoprecipitations were performed on separate samples for α Ib β 3 (1070) and CD36 (IA7/IE8) using suboptimal amounts (3 μ g) of precipitating mAb. Samples were washed in RIPA and an *in vitro* kinase assay was performed on each sample. Directly coupled (Sepharose-4B) mouse IgG immunoprecipitants were processed in parallel. Samples were run under reducing conditions on SDS/PAGE and then electrophoretically transferred to nitrocellulose (38 V, 300 mA, 2 h). The membrane was autoradiographed for 5 days to reveal the phosphoprotein profile of each sample. Following autoradiography the membrane was sequentially immunoblotted with polyclonal antibodies to α Ib β 3 and CD36 under the precise conditions used to determine the quantification curves for these proteins, and the resultant bands were quantified by densitometry.

Low-pH elution of the integrin-kinase complex

Radiolabelled (*in vitro* kinase) α Ib β 3-kinase complexes were dissociated from primary antibody-bead conjugates by low-pH elution. Total conjugates were washed with 5 vol. of Tris buffer (50 mM Tris, pH 8.0, 0.1 % Triton X-100, 0.5 M NaCl); then with 5 vol. of sodium phosphate buffer (50 mM NaH₂PO₄, pH 6.3, 0.1 % Triton X-100, 0.5 M NaCl). The antigen complex was eluted from the beads with 5 vol. of glycine buffer (50 mM glycine, pH 2.5, 0.1 % Triton X-100, 150 mM NaCl). The glycine eluate was immediately neutralized by elution into 0.2 vol. of 1 M Tris, pH 9.0. Proteinase and phosphatase inhibitors were included in all solutions as per the initial lysis conditions. The eluates of the latter two buffers were combined and passed over a PD-10 desalting column equilibrated with the initial lysis buffer. Secondary immunoprecipitations were performed on the buffer-exchanged eluate to determine the presence of proteins complexed with the primary immunoprecipitated integrin.

Phosphoamino acid analysis

The phosphorylation profile of β 3 following the *in vitro* kinase assay was determined by recovering the ³²P-labelled protein from the SDS/PAGE gel. Briefly, the β 3 band (visualized by Coomassie Brilliant Blue staining of the gel) was excised from the reduced gel. The gel was re-autoradiographed to verify accurate removal of the single phosphorylated band corresponding to β 3, as revealed by the Coomassie Blue stain. The protein was eluted from the gel fragment in extraction buffer (0.05 mM NH₄HCO₃, 0.1 % SDS, 0.5 % β -mercaptoethanol). The sample was heated to

100 °C for 10 min and then allowed to stand for at least 12 h at 37 °C. Gel fragments were removed by centrifugation and 100 μ g of BSA was added to the supernatant as a carrier protein. Ice-cold trichloroacetic acid was added to 20 % (w/v) and the sample was allowed to stand at 4 °C for 4 h. Precipitated proteins were pelleted by centrifugation at 15000 g for 15 min at 4 °C. The pellet was washed once with ice-cold ethanol followed by ice-cold ethanol/ether (1:1, v/v). HCl (5.7 M; 50 μ l) was added to the dried protein pellet for 60 min at 110 °C to hydrolyse the protein. Following hydrolysis the sample was lyophilized. Phosphoamino acids were resolved in two dimensions on cellulose TLC plates by flat bed electrophoresis using the method of Cooper et al. [39]. Unlabelled phosphoamino acid standards run within each sample were detected with 0.2 % ninhydrin in acetone. 32 P-phospholabelled amino acids were detected by autoradiography.

RESULTS

In our initial attempts to identify kinase activity associated with α IIB β 3 we used the 'mild' zwitterionic and non-ionic detergents CHAPS and octyl glucoside, since these have been used successfully to identify protein complexes in other systems. Lysis in CHAPS, and to a lesser extent in octyl glucoside, followed by immunoprecipitation and washing in the same buffer before *in vitro* kinase and SDS/PAGE analysis did reveal a series of phosphoprotein bands associated with the α IIB β 3 integrin (Figure 1a), and this complex was dissociated by washing in the high-stringency RIPA buffer.

However, these experiments were generally unsatisfactory, since immunoprecipitation with antibodies to a range of glycoproteins also yielded associated phosphoprotein bands, and we questioned the efficacy of the washing procedure in the non-stringent detergent lysates. We therefore turned to the use of homobifunctional chemical crosslinking reagents which have also been used to identify associated proteins [42–44]. Two such crosslinkers were tested: DTSSP, which is membrane-impermeable, and DSP, which is lipid-soluble and readily permeates the lipid bilayer [45,46]. Crosslinking with DTSSP before lysis, immunoprecipitation and *in vitro* kinase analysis yielded very similar results to those obtained from platelets that had not been crosslinked. A series of phosphoprotein bands was seen in association with α IIB β 3 after lysis and washing with mild detergent (CHAPS), but these dissociated upon washing with RIPA buffer (Figure 1a). In contrast, crosslinking of the intact platelets with DSP before the same analysis yielded a different profile of associated phosphoproteins, and this complex remained associated after three washes in RIPA buffer. Figure 1(b) illustrates a typical experiment in which the platelets were left untreated or were crosslinked with DSP before lysis in 1 % Triton X-100; the α IIB β 3 immunoprecipitates were then washed in RIPA buffer before the *in vitro* kinase reaction and SDS/PAGE analysis. When analysed under reducing conditions (which cleaves the crosslinker), precipitates from the DSP-treated platelets showed the associated phosphoprotein bands in the autoradiograph. Precipitates from the non-crosslinked platelets showed little or no kinase activity or phosphoprotein bands, and such bands were not specifically associated with α IIB β 3. When the samples were run non-reduced, most of the phosphorylated protein from the DSP-treated platelets remained as high-molecular-mass material, indicating that it is crosslinked. Also shown (Figure 1b) is the corresponding Coomassie Blue-stained gel to illustrate that, in the reduced sample, a band in the position of β 3 corresponds to a band that is heavily phosphorylated after DSP crosslinking and *in vitro* kinase assay.

Figure 1 *In vitro* kinase assay of α IIB β 3 immunoprecipitations from different detergent lysates of non-crosslinked and chemically crosslinked platelets showing the association of active protein kinase(s) with the integrin

Platelet suspensions (1×10^9 platelets/ml) were incubated in the presence or absence of chemical crosslinking agents. Incubations were terminated by the addition of an equal volume of lysis buffer. Lysates were centrifuged at 15000 g for 15 min and α IIB β 3 was immunoprecipitated from detergent-soluble supernatants. (a) Autoradiograph after 16 h exposure at -70 °C. The α IIB β 3 immunoprecipitants (with antibody 1070) derived from CHAPS and octyl-glucoside (OCTYL) lysates of platelets either non-crosslinked (NCL) or crosslinked with DTSSP prior to lysis were washed in the same lysis buffer; Triton X-100 immunoprecipitants were washed in RIPA buffers (RIPA). Washed samples were resuspended in assay buffer and the *in vitro* kinase assay was performed. The assay was stopped by the addition of 0.5 ml of assay buffer and the precipitated antigen complex was solubilized in reducing SDS sample buffer; solubilized proteins were electrophoresed by SDS/PAGE under reducing conditions. All gels were stained with Coomassie Brilliant Blue before autoradiography (not shown) to identify the positions of β 3 and of the antibody used for immunoprecipitation (mAb). In (b) is shown a separate experiment conducted under the same conditions in which the platelets were crosslinked with DSP (or non-crosslinked; NCL) prior to lysis in Triton X-100 and processed as above with washing in RIPA buffers. Shown are the Coomassie Brilliant Blue-stained gel and the corresponding autoradiographs (32 P) of the immunoprecipitations with anti- β 3 mAb (1070) run under reducing or non-reducing SDS/PAGE after the *in vitro* kinase assay. The position of β 3 is marked with an arrowhead, and in this experiment the autoradiograph was exposed for 17 h. The molecular mass standards shown apply to both the reduced and non-reduced samples. The experiments shown were reproduced in 10 similar experiments.

To determine whether the protein kinase activity was only associated with α IIB β 3, platelets were crosslinked with DSP, lysed and immunoprecipitated with a range of antibodies, and

Figure 2 Autoradiographs of an *in vitro* kinase assay of immunoprecipitants from lysates of platelets crosslinked with DSP before and after lysis showing the specificity of the phosphoproteins associating with α IIb β 3

(a) and (b) Platelet suspensions (1×10^9 platelets/ml) were left untreated (NCL) or chemically crosslinked with DSP and lysed in Triton X-100 lysis buffer. Insoluble material was removed by centrifugation at $15000g$ for 15 min at 4°C . Immunoprecipitations were performed on soluble lysates with antibodies against α IIb (a, lane 3), β 3 (a, lanes 2, antibody 1070 and 4, antibody SZ21), CD36 (a, lane 6), CD9 (a, lane 1) and αv (a, lane 5); and against GPIIb (Ib) or β 3 (1070) as indicated in (b). (c) Chemical crosslinking of platelets, an *in vitro* kinase assay and analysis were performed in the sequences described for (a) and (b), except in one sample (lysis/DSP) where the platelets were lysed before crosslinking of the soluble lysate with DSP. In this instance the lysis buffer was Hepes-buffered, and Tris/HCl was added post-crosslinking to terminate the reaction. Lysates were immunoprecipitated with antibodies against α IIb β 3 (1070), CD36 and the control antibody, CD4, as indicated. *In vitro* kinase assays were performed on RIPA-washed samples from all groups and protein complexes resolved by SDS/PAGE following solubilization of proteins in reducing SDS sample buffer. Gels were stained with Coomassie Brilliant Blue (not shown) to visualize the β 3, Ib and antibody bands. Phosphoproteins were detected by autoradiography for 16 h at -70°C . The electrophoretic mobility of molecular mass standards is indicated. All of the experiments shown were reproduced on at least two occasions.

subjected to an *in vitro* kinase reaction analysed by SDS/PAGE. The same phosphoprotein complex was identified with precipitates from two mAbs to β 3, one to α IIb (Figure 2a), and with a rabbit antiserum to α IIb β 3 (results not shown), but not with immunoprecipitates of several other platelet glycoproteins. Precipitates from antibodies to CD36, CD9, αv integrin (Figure 2a) and also PTA1 [34] and GMP140 [35] (results not shown) all showed no, or negligible, phosphoprotein bands. The crosslinked precipitate obtained using an anti-GPIIb antibody showed evidence of an associated kinase (Figure 2b). We have not investigated this further at this point, but the pattern of phosphoproteins associated in the complex is quite different from that seen with α IIb β 3. The great abundance of both α IIb β 3 and pp60^{c-*src*} within platelets [28] allowed the possibility that the apparent association between the integrin and kinase was an artefact of the crosslinking procedure, and that no such association exists in the intact platelet. This was addressed by lysing the platelets prior to DSP crosslinking and analysis as above. The results (Figure 2c) make it apparent that the complex exists in intact platelets but not in the platelet lysate, and is therefore not simply the result of non-specific crosslinking of abundant proteins.

The great sensitivity of the *in vitro* kinase reaction together with the relative abundance of α IIb β 3 still left the possibility that the immunoprecipitates from the crosslinked platelets revealed

kinase activity associated with the integrin and not other surface molecules simply because of the relatively greater amount of integrin that was precipitated. To test this, platelets crosslinked with DSP were immunoprecipitated with suboptimal concentrations of antibody and the amount of protein loaded was measured by densitometric analysis of Western blots related to a calibration curve of purified proteins. Figure 3 illustrates that, under conditions of equal protein loading, kinase activity was seen to associate with α IIb β 3 but not CD36. (The Western blots of these indirectly immunoprecipitated platelet proteins also illuminate the precipitating antibody, contrasting with the tracks using mouse IgG directly coupled to Sepharose beads, in which the antibody bands are absent.)

Whether or not the complex that associates with α IIb β 3 is present in resting platelets or is induced upon platelet activation could not be determined using crosslinking of intact platelets. This is because we find that crosslinking of the platelet surface with DSP or DTSSP rapidly induces platelet activation. In aggregation studies, the crosslinkers themselves fail to induce significant aggregation: DTSSP induces no aggregation, and DSP induces only microaggregates; DSP also inhibits visible aggregation induced by thrombin (results not shown). However, activation is seen in a number of parameters. These include the phosphorylation of a number of proteins on tyrosine, increased autophosphorylation of pp60^{c-*src*} and the translocation of α IIb β 3

Figure 3 Autoradiograph and Western blot analysis of an *in vitro* kinase assay of immunoprecipitates of DSP-crosslinked platelets under conditions of equal protein loading

(a) Increasing quantities of purified α IIb β 3 and CD36 were each run under reducing conditions on SDS/PAGE and then transferred to nitrocellulose. Membranes were probed with polyclonal antibodies to each protein and visualized using ECL. A density gradient was derived and a calibration curve (protein concentration in pM versus density units) was constructed as described in the Experimental section. (b) Platelets (3×10^8 platelets/ml) were chemically crosslinked (DSP), lysed in Triton X-100 lysis buffer, and α IIb β 3 ($\beta 3$) (mAb 1070) and CD36 (mAb 1A7) were indirectly immunoprecipitated from the soluble lysate using suboptimal concentrations of antibody (3 μ g). Mouse-IgG-coupled Sepharose beads (mIgG) were immunoprecipitated in parallel samples as controls. Following RIPA washing, all samples were subjected to an *in vitro* kinase assay (IVK) and resolved by reducing SDS/PAGE. Proteins were transferred to nitrocellulose using conditions identical to those used to generate the calibration curve samples in (a). The membrane was autoradiographed for 5 days to reveal phosphoprotein profiles before probing for α IIb β 3 and CD36, again under conditions identical to those for the calibration curve. Densitometric analysis was used to determine relative amounts of $\beta 3$ and CD36 protein; in this experiment there was calculated to be 7.3 pM $\beta 3$ and 7.2 pM CD36. The experiment was repeated with similar results.

and pp60^{c-src} to the Triton-insoluble cytoskeleton (results not shown).

We have begun analysis of the phosphoprotein complex in association with α IIb β 3. Several of the phosphoprotein bands, including that in the position of $\beta 3$, largely resist hydrolysis with potassium hydroxide, suggesting possible tyrosine phosphorylation (results not shown). To substantiate phosphorylation of $\beta 3$, DSP was used to crosslink intact platelets and the lysate was immunoprecipitated with an anti- $\beta 3$ mAb. The washed pre-

Figure 4 Western blot of phosphate-labelled $\beta 3$ analysed by two-dimensional gel electrophoresis and phosphoamino acid analysis showing that $\beta 3$ is phosphorylated on tyrosine residues *in vitro*

Platelet suspensions (1×10^9 platelets/ml) were chemically crosslinked with DSP, lysed in Triton X-100 lysis buffer, and soluble lysates were immunoprecipitated with anti- α IIb β 3 (mAb 1070). *In vitro* kinase assays were performed on RIPA-washed immunocomplexes and the proteins were solubilized in reducing SDS sample buffer. In (a), solubilized proteins were resolved by two-dimensional gel electrophoresis, followed by transfer to nitrocellulose. 32 P-labelled proteins from the two-dimensional membrane were visualized by overnight autoradiography (left panel) and $\beta 3$ was detected by anti- α IIb β 3 probing of the membrane with ECL development and autoradiography (right panel). In (b) is shown the phosphoamino acid analysis of 32 P-labelled $\beta 3$. After crosslinking, immunoprecipitation and *in vitro* kinase reaction as above, the sample was run on reducing SDS/PAGE. The Coomassie Brilliant Blue-stained $\beta 3$ band was excised from the gel and two-dimensional phosphoamino acid analysis was performed as described in the Experimental section. Phosphoamino acids were detected by autoradiography and the phosphoamino acid standards [phosphotyrosine (PY), phosphothreonine (PT) and phosphoserine (PS)] were located by spraying the TLC plate with ninhydrin. Both experiments were repeated with the same result.

cipitate was subjected to an *in vitro* kinase reaction, and a portion was run on a two-dimensional O'Farrell gel and transferred to nitrocellulose. Brief exposure (5 min) of the paper revealed no 32 P spots on the autoradiograph, and the paper was exposed overnight to locate the phosphoprotein spots. The filter was then probed with a rabbit antibody specific for α IIb β 3 with ECL development and exposure for 3 min. The results (Figure 4a) identified $\beta 3$ as one of the prominent phosphoprotein bands. To determine the amino acids being phosphorylated on $\beta 3$, the Coomassie Blue-stained $\beta 3$ band was excised from a one-dimensional SDS/PAGE gel of an anti- $\beta 3$ immunoprecipitate of DSP-treated platelet lysate subjected to an *in vitro* kinase reaction. Phosphoamino acid analysis of this band revealed that serine, threonine and tyrosine residues on $\beta 3$ were all phosphorylated during the *in vitro* kinase reaction (Figure 4b), although, consistently in these experiments, the phosphorylation on tyrosine was proportionally much greater than that on serine or threonine.

These results suggested that there might be a tyrosine kinase(s) associating with the α IIb β 3 complex. Therefore, to seek to identify likely tyrosine kinases in the complex, $\beta 3$ immunoprecipitates from DSP-treated platelets were eluted with acid pH

Figure 5 Re-immunoprecipitation of α IIB β 3-associated complexes from crosslinked platelets showing the presence of pp60^{c-src} and pp54/58^{c-ly}

Platelet suspensions (1×10^9 platelets/ml) were crosslinked with DSP and lysed as described in Figure 3. α IIB β 3 immunoprecipitations were performed with Sepharose-4B-coupled mAb 1070 and *in vitro* kinase assays were carried out on RIPA-washed immunocomplexes. Radiolabelled α IIB β 3 complexes were dissociated from Sepharose-antibody conjugates by low-pH elution followed by re-establishment into lysis buffer via a desalting column. Secondary immunoprecipitations were performed on the eluate with rabbit polyclonal antibodies against α IIB β 3, p54/58^{c-ly} (LYN), pp60^{c-src} (SRC), CD36 and normal rabbit serum (NRS). Immunoprecipitants were washed with lysis buffer, solubilized in reducing SDS sample buffer and resolved by SDS/PAGE. Gels were stained with Coomassie Brilliant Blue to identify the position of β 3 (arrowhead) and shown are the phosphoproteins detected by autoradiography. In the autoradiographs shown the two left tracks [β 3 NCL (non-crosslinked) and β 3 DSP] were exposed for 4 h, and the tracks showing the reprecipitated acid eluates were exposed for 7 days. The experiment was repeated on three occasions with the same result.

after the *in vitro* kinase reaction as described in the Experimental section. The affinity-purified α IIB β 3 was then reprecipitated with rabbit antibodies to α IIB β 3 (positive control), pp60^{c-src}, pp54/58^{c-ly} and CD36, and analysed by SDS/PAGE and autoradiography. The results are shown in Figure 5. Reprecipitation of the eluate with anti- α IIB β 3 revealed the same pattern of phosphoprotein bands identified consistently throughout this study. Reprecipitation with normal rabbit serum (NRS in Figure 5) did not reveal any bands, and the anti-CD36 reprecipitate showed only a faint band at around 190 kDa that was much more prominent in the anti- α IIB β 3 precipitate. Reprecipitation of the eluate with anti-pp60^{c-src} antibodies showed a phosphoprotein band at 60 kDa, presumably pp60^{c-src}, a band in the position of β 3, and a prominent band at around 190 kDa. The anti-Lyn immunoprecipitates revealed the presumed pp54/58^{c-ly} complex and a band in the position of β 3, together with other bands shared with the anti- α IIB β 3 reprecipitate but absent from or only very weakly present in the pp60^{c-src} reprecipitate. These data provide evidence that pp60^{c-src} and pp54/58^{c-ly} associate with α IIB β 3 in the intact platelet. The different phosphoprotein profiles obtained with pp60^{c-src} and pp54/58^{c-ly} perhaps also indicate that α IIB β 3 forms distinct complexes with these two kinases.

In the resting platelet, a proportion of α IIB β 3 appears to associate with the membrane skeleton of cytoskeletal proteins that remain Triton-soluble under conditions of low-speed centrifugation [17,47]. Upon platelet activation and aggregation, much of this membrane skeleton and associated proteins becomes incorporated into the Triton-insoluble cytoskeleton. Also associ-

Figure 6 Western blot analysis of α IIB β 3 precipitated from non-crosslinked and DSP-treated platelets showing the dissociation of the cytoskeletal proteins actin and α -actinin upon crosslinking

Platelet suspensions (1×10^9 platelets/ml) were either chemically crosslinked (DSP) or not (NCL), lysed in Triton X-100 lysis buffer, and α IIB β 3 was immunoprecipitated from the soluble lysate with mAb 1070. Following RIPA washing, proteins were solubilized in reducing SDS sample buffer and resolved by SDS/PAGE. Proteins were transferred to nitrocellulose and the membrane was probed sequentially with antibodies against α -actinin and actin. The membrane was not 'stripped' between probeds. Antigens were detected by ECL and autoradiography. The electrophoretic mobility of molecular mass markers is shown.

ated with the membrane skeleton are kinases, including pp60^{c-src} [17]. It is possible, therefore, that the association between α IIB β 3 and pp60^{c-src} (or pp54/58^{c-ly}) that we observe with DSP crosslinking is not the result of direct association between these molecules but, rather, is caused by an indirect association through cytoskeletal elements that have remained detergent-soluble. Since α IIB β 3, like other integrins, can bind α -actinin [47], a component of the membrane skeleton, it might be expected that α -actinin would be a primary candidate for involvement in such an indirect association. We consistently observed that β 3 precipitates from resting platelets run on SDS/PAGE and stained with Coomassie Brilliant Blue contained, in addition to α IIB β 3, prominent bands at the positions of myosin heavy chain, actin and a tight band under β 3 in the position of α -actinin (see Figure 1b). Upon crosslinking the intact platelet with DSP, however, β 3 immunoprecipitates from the lysate showed only α IIB β 3 and the precipitating antibody band in Coomassie Brilliant Blue-stained gels. To substantiate the loss of actin and α -actinin in β 3 immunoprecipitates from the soluble lysate upon DSP treatment of the platelets, we employed Western blotting. Lysates of platelets either untreated or crosslinked with DSP were immunoprecipitated with anti- β 3 mAb and sequentially blotted (without stripping) for α -actinin and actin. At this level of detection it is clear that, after DSP crosslinking of the intact platelet, neither α -actinin or actin remain associated with α IIB β 3 in the soluble lysate (Figure 6).

To further examine the association between the α IIB β 3 complex and cytoskeletal components, we treated the platelet lysate with DNase I, which binds actin monomer [50] and depolymerizes membrane skeletal actin [47,50,51]. Such treatment of the DSP-treated platelet lysate, before immunoprecipitation with anti- β 3 mAb and the *in vitro* kinase assay, had no effect either qualitatively or quantitatively on the phosphoprotein complex observed upon SDS/PAGE analysis (results not shown).

DISCUSSION

The present paper uses the homobifunctional chemical crosslinker DSP to capture molecules that are physically associated around the integrin $\alpha\text{IIb}\beta 3$ in the intact platelet. The technique was combined with an *in vitro* kinase assay to demonstrate kinase activity associated with the integrin, and two protein tyrosine kinases, pp60^{c-src} and pp54/58^{c-lyn}, were identified in the complex precipitated from lysates of the crosslinked platelets by an anti- $\beta 3$ antibody.

Chemical crosslinking with bifunctional crosslinking reagents has been used successfully to identify spatially associated cell surface molecules in a number of systems, and the specificity of this approach is well established [42–46]. For example, Brenner et al. [42] used DSP and other crosslinkers to demonstrate a spatial association between the T-cell receptor and CD3, results that have been amply confirmed by a number of techniques since then. It is interesting to note that, in their study, Brenner et al. [42] obtained little or no association if the cells were lysed in Nonidet P-40 before crosslinking. These authors suggested that the proteins are largely dissociated upon solubilization in detergent, such that they cannot readily be crosslinked [42]. This technique of using crosslinkers to 'capture' preformed spatial associations on the surface of the intact cell thus differs in principle from the use of crosslinkers to identify direct physical interactions in mixtures of proteins [52,53]. Feder and Bishop [52] used the latter technique to probe preparations of platelet membranes with myristylated N-terminal peptides of pp60^{c-src}, crosslinking specifically bound proteins with disuccinimidyl suberate to enable ready analysis. In this way they identified specific binding to proteins of 32, 50, 90 and 105 kDa in the platelet membrane preparation. The fact that they did not identify $\alpha\text{IIb}\beta 3$ as associating with the pp60^{c-src} peptide probe perhaps suggests that there is no direct association between these two molecules, since the integrin would be well represented in the membrane fraction used for binding. In the present study, the membrane-permeable crosslinker was used to capture molecules in a preformed spatial association within the intact cell. Included within the complex of proteins associating with $\alpha\text{IIb}\beta 3$ upon crosslinking, and revealed as phosphoprotein bands after an *in vitro* kinase reaction, were bands approximating in size to several of those identified as binding to pp60^{c-src} peptide by Feder and Bishop [52]. Any or all of these could provide an indirect association between the kinase and integrin, an association which could, nevertheless, be of functional significance.

The complex associated with $\alpha\text{IIb}\beta 3$ after capture with DSP within the intact platelet was specific to the integrin, and was demonstrably not an artefact of the crosslinking strategy. As visualized by SDS/PAGE and autoradiography after the *in vitro* kinase reaction, the profile of phosphoprotein bands that co-precipitated with $\alpha\text{IIb}\beta 3$ was highly reproducible and was not seen in precipitates of several other glycoproteins expressed on the platelet surface. When the resting platelets were lysed before crosslinking with DSP and then tested in the same way, no phosphoprotein bands were seen in the $\alpha\text{IIb}\beta 3$ precipitate, indicating that the integrin and kinase were not non-specifically crosslinked as a result of their relative abundance. In the same assay system, immunoprecipitates of GPIb-IX from the crosslinked platelets contained kinase activity, but the pattern of phosphoprotein bands that was associated with GPIb-IX was quite different from that associating with $\alpha\text{IIb}\beta 3$. We have not begun to analyse this complex, but it may be relevant that binding of GPIb-IX to its ligand von Willebrand Factor initiates platelet activation signals; in addition, we have recently shown that GPIb-IX forms a specific association with an isoform of the

multifunctional 14-3-3 family of proteins [54] that could conceivably provide a focus for a signalling complex. In contrast, immunoprecipitates of CD36 from the crosslinked platelets did not reveal any associated kinase activity. It has been reported that platelet CD36 associates with pp60^{c-tyr}, pp54/58^{c-lyn} and pp62^{c-yes} [55]; however, the same workers have also discussed their findings that CD36 dissociates from the Src-related kinases upon platelet activation with anti-CD36 antibody [56], and it may be that the activation induced by the DSP crosslinker causes such dissociation. Even in the absence of chemical crosslinking, we did not consistently observe kinase activity associated with CD36 immunoprecipitates; we cannot account for this discrepancy with the work of Huang et al. [55], although we were using different anti-CD36 antibodies from these authors.

Whether or not the complex of proteins found in association with $\alpha\text{IIb}\beta 3$ after crosslinking with DSP is present in the resting platelet, or is induced upon platelet activation, could not be determined by crosslinking of the intact platelet. This is because the crosslinker itself induced platelet activation. That this was caused by crosslinking of membrane glycoproteins, rather than a non-specific event induced by the membrane-permeable DSP, was indicated by our finding of a similar induction of activation by the non-permeable crosslinker DTSSP. This finding was not unexpected. It has been recognized for some time that crosslinking of platelet surface glycoproteins with the multivalent lectin, concanavalin A, results in platelet activation and association of much of the surface $\alpha\text{IIb}\beta 3$ to the Triton-insoluble cytoskeleton [51,57,58]. In experiments using CHAPS detergent for lysis of the resting platelets and washing of the non-crosslinked immunoprecipitates, we did find kinase activity associating with $\alpha\text{IIb}\beta 3$; but after the *in vitro* kinase reaction, the pattern of associated phosphoproteins seen on SDS/PAGE was not the same as that seen with the DSP-crosslinked precipitates and, in particular, $\beta 3$ phosphorylation was not prominent. In preliminary experiments in which the platelets were activated (and aggregated) with thrombin for different periods of time and then lysed and immediately crosslinked with DSP, we have observed the progressive appearance of a phosphoprotein in the position of $\beta 3$ in the $\alpha\text{IIb}\beta 3$ immunoprecipitates (D. Dorahy, unpublished work), perhaps indicating that the integrin associates with the kinase upon activation of the platelet.

Upon platelet activation, $\alpha\text{IIb}\beta 3$ undergoes a conformational change converting it into a functional receptor for fibrinogen [6]. This event could possibly also promote association of the intracellular proteins with the integrin. Huang et al. [26] have proposed a model of integrin-mediated signalling in platelets in which binding of fibrinogen to the activated, functional receptor causes oligomerization of $\alpha\text{IIb}\beta 3$ within the plane of the plasma membrane, leading to actin assembly in proximity to these oligomers. It is also proposed that during this process one or more protein tyrosine kinases and their potential substrates localize to these regions through specific protein-protein interactions, and the complex continues to build as more enzymes and substrates become incorporated. Fox and colleagues [17] presented a detailed analysis of how this might occur, and provided evidence that $\alpha\text{IIb}\beta 3$, pp60^{c-src}, ras GTPase-activating protein, vinculin and several other enzymes and cytoskeletal proteins were to be found predominantly in association with the membrane skeleton, a lattice-like structure comprising short actin filaments, actin-binding proteins, spectrin, vinculin and other, unidentified, proteins, which may play an important role in regulating transmembrane signal transduction (see refs. within [17]). Technically, the membrane skeleton can be isolated from the actin cytoskeleton (detergent-insoluble) and the platelet cytosol by high-speed centrifugation of the soluble fraction of

detergent-lysed platelets which sediments the membrane skeleton and associated proteins. Fox et al. [17] then demonstrated that, upon platelet activation and aggregation, components of the membrane skeleton redistributed to the Triton-insoluble actin cytoskeleton in a co-ordinated way, and that this process was dependent upon α Ib β 3. It seems very likely that the complex of proteins found to associate with α Ib β 3 in our study of DSP-treated platelets represents a facet of the same process. The absence of detectable actin or α -actinin in the crosslinked complex (Figure 5) perhaps suggests that these components of the membrane skeleton are now firmly incorporated into the insoluble actin cytoskeleton. The α Ib β 3 and associated complex may be less firmly incorporated into the cytoskeletal network such that it can still be fragmented in detergent lysates. (It can be noted that DSP crosslinking does not inhibit redistribution of α Ib β 3 to the actin cytoskeleton and, in fact, induces this process.) If this is the case, it follows from our results that several of the redistributing proteins are spatially associated around α Ib β 3 and that the crosslinked complex is not dependent upon actin or α -actinin for its association.

The protein-protein interactions involved in the formation of the complex around the integrin are not known. Following an *in vitro* kinase reaction, we showed that the β 3 subunit becomes phosphorylated predominantly on tyrosine, with some phosphorylation also on serine and threonine (Figure 4b). Studies on α Ib β 3 phosphorylation *in vivo* have indicated that only a subpopulation of β 3 becomes phosphorylated, and such label is associated predominantly with threonine [59]. Others have shown previously that α Ib β 3 is a substrate for pp60^{c-src} *in vitro* [60] but, while β 3 has a potential tyrosine phosphorylation site within its short cytoplasmic tail [28,60], *in vivo* phosphorylation of this site in the integrin subunit has not been identified. It seems likely, therefore, that the integrin itself is not the primary substrate for associated tyrosine kinases; rather, the association between integrin and kinase may serve to regulate kinase activity during the processes of platelet activation and aggregation.

In summary, the present paper indicates that, within platelets, the integrin α Ib β 3 forms spatial associations with several proteins, at least two of which are protein tyrosine kinases. The technique employed to identify the complex or complexes, i.e. capture by chemical crosslinking with DSP, is highly reproducible and should prove a valuable tool for the identification of molecules forming the complex and further allow the elucidation of the role of this integrin in platelet activation.

We thank those mentioned in the Experimental section who generously provided antibodies for the study, and the members of staff of the Hunter Area Health Service Blood Transfusion Unit for their assistance in obtaining blood samples. We also thank Rob Andrews for assistance in the platelet aggregation studies, Dr. Judith Scott and Dr. Robert Burton for their critical comments on the manuscript, and Mrs. Lyndie Barrkman for secretarial assistance in the preparation of the manuscript. This work was supported by grants from the National Health and Medical Research Council and the National Heart Foundation (Australia).

REFERENCES

- Hynes, R. O. (1992) *Cell* **69**, 11–25
- Tamkun, J. W., De Simone, D. W., Fonda, D., Patel, R. S., Buck, C., Horwitz, A. F. and Hynes, R. O. (1986) *Cell* **46**, 271–282
- Santorio, S. A. and Lawing, W. J. (1987) *Cell* **48**, 867–873
- Horwitz, A., Duggan, K., Buck, C., Beckerle, M. C. and Burridge, K. (1986) *Nature (London)* **320**, 531–533
- Dustin, M. L. and Springer, T. A. (1989) *Nature (London)* **341**, 619–624
- Sims, P. J., Ginsberg, M. H., Plow, E. F. and Shattil, S. J. (1991) *J. Biol. Chem.* **266**, 7345–7352
- Faull, R. J., Kovach, N. L., Harlan, J. M. and Ginsberg, M. H. (1993) *J. Cell Biol.* **121**, 155–162
- Phillips, D. R., Charo, I. F., Parise, L. V. and Fitzgerald, L. A. (1988) *Blood* **71**, 831–843
- Kornberg, J. L., Earp, H. S., Turner, C. E., Prockop, C. and Juliano, R. L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8392–8396
- Nojima, Y., Humphries, M., Mould, A., Komoriya, A., Yamada, K., Schlossman, S. and Morimoto, C. (1990) *J. Exp. Med.* **172**, 1185–1192
- Adams, J. and Watt, F. (1989) *Nature (London)* **346**, 307–309
- Dedhar, S., Robertson, K. and Gray, V. (1991) *J. Biol. Chem.* **266**, 21846–21852
- Guan, J.-L., Trevithick, J. E. and Hynes, R. O. (1991) *Cell. Regul.* **2**, 951–964
- Golden, A., Brugge, J. S. and Shattil, S. J. (1990) *J. Cell Biol.* **111**, 3117–3127
- Ferrell, J. E. and Martin, G. S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2234–2238
- Kapron-Bras, C., Fitz-Gibbon, L., Jeevaratnam, P., Wilkins, J. and Dedhar, S. (1993) *J. Biol. Chem.* **268**, 20701–20704
- Fox, J. E. B., Lipfert, L., Clark, E. A., Reynolds, C. C., Austin, C. D. and Brugge, J. S. (1993) *J. Biol. Chem.* **268**, 25973–25984
- Yurochko, A., Liu, D., Eirman, D. and Haskill, S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9034–9038
- Turner, C. E. and Burridge, K. (1991) *Curr. Opin. Cell Biol.* **3**, 849–853
- Haimovich, B., Aneskievich, B. J. and Boettiger, D. (1991) *Cell. Regul.* **2**, 271–283
- Burridge, K., Turner, C. E. and Romer, L. H. (1992) *J. Cell Biol.* **119**, 893–903
- Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5192–5196
- Bartfeld, N. S., Pasquale, E. B., Geltosky, J. E. and Languino, L. R. (1993) *J. Biol. Chem.* **268**, 17270–17276
- Savage, B., Shattil, S. J. and Ruggeri, Z. M. (1992) *J. Biol. Chem.* **267**, 11300–11306
- Haimovich, B., Lipfert, L., Brugge, J. S. and Shattil, S. J. (1993) *J. Biol. Chem.* **268**, 15868–15877
- Huang, M.-M., Lipfert, L., Cunningham, M., Brugge, J. S., Ginsberg, M. H. and Shattil, S. J. (1993) *J. Cell Biol.* **122**, 473–483
- Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T. and Brugge, J. S. (1992) *J. Cell Biol.* **119**, 905–912
- Shattil, S. J. and Brugge, J. S. (1991) *Curr. Opin. Cell Biol.* **3**, 869–879
- Phillips, D. R. and Baughan, A. K. (1983) *J. Biol. Chem.* **258**, 10240–10246
- Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F. and Ruoslahti, E. (1986) *Science* **231**, 1559–1562
- Ruan, C., Du, X., Xi, X., Castaldi, P. A. and Berndt, M. C. (1987) *Blood* **69**, 570–577
- Berndt, M. C., Du, X. and Booth, W. J. (1988) *Biochemistry* **27**, 633–640
- Burns, G. F., Cosgrove, L., Triglia, T., Beall, J. A., Lopez, A. F., Werkmeister, J. A., Begley, C. G., Haddad, A. P., d'Apice, A. J. F., Vadas, M. A. and Cawley, J. C. (1986) *Cell* **45**, 269–280
- Scott, J. L., Dunn, S. M., Jin, B., Hillam, A. J., Walton, S., Berndt, M. C., Murray, A. W., Krissansen, G. W. and Burns, G. F. (1989) *J. Biol. Chem.* **264**, 13475–13482
- Skinner, M. P., Lucas, C. M., Burns, G. F., Chesterman, C. N. and Berndt, M. C. (1991) *J. Biol. Chem.* **266**, 5371–5374
- Mazurov, A. V., Vinogradov, D. V., Vlasik, T. N., Burns, G. F. and Berndt, M. C. (1992) *Platelets* **3**, 181–188
- Krissansen, G. W., Lucas, C. M., Stomski, F. C., Elliott, M. J., Berndt, M. C., Boyd, A. W., Horton, M. A., Cheresh, D. A., Vadas, M. A. and Burns, G. F. (1990) *Int. Immunol.* **2**, 267–277
- Berger, G., Caen, J. P., Berndt, M. C. and Cramer, E. M. (1993) *Blood* **82**, 3034–3044
- Cooper, J. A., Sefton, B. M. and Hunter, T. (1983) *Methods Enzymol.* **99**, 387–402
- O'Farrell, P. Z., Goodman, H. M. and O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142
- Bravo, R., Small, J. V., Fey, S. J., Mose-Larsen, P. and Celis, J. E. (1982) *J. Mol. Biol.* **159**, 121–143
- Brenner, M. B., Trowbridge, I. S. and Strominger, J. L. (1985) *Cell* **40**, 183–190
- Takeshita, T., Asao, H., Suzuki, J. and Sugamura, K. (1990) *Int. Immunol.* **2**, 477–480
- Slupsky, J. R., Sechafer, J. G., Tang, S.-C., Marcellis-Smith, A. and Shaw, A. R. E. (1989) *J. Biol. Chem.* **264**, 12289–12293
- Lomant, A. J. and Fairbanks, G. (1976) *J. Mol. Biol.* **104**, 243–261
- Jung, S. M. and Moroi, M. (1983) *Biochim. Biophys. Acta* **761**, 152–162
- Fox, J. E. B. (1985) *J. Clin. Invest.* **76**, 1673–1683
- Reference deleted
- Reference deleted
- Painter, R. G., Gaarde, W. and Ginsberg, M. H. (1985) *J. Cell. Biochem.* **27**, 277–290
- Zhang, J., Fry, M. J., Waterfield, M. D., Jaken, S., Liao, L., Fox, J. E. B. and Rittenhouse, S. E. (1992) *J. Biol. Chem.* **267**, 4686–4692
- Feder, D. and Bishop, J. M. (1991) *J. Biol. Chem.* **266**, 19040–19046
- Resh, M. D. and Ling, H.-p. (1990) *Nature (London)* **346**, 84–86

- 54 Du, X., Harris, S. J., Tetaz, T. A., Ginsberg, M. H. and Berndt, M. C. (1994) *J. Biol. Chem.* **269**, 18287–18290
- 55 Huang, M.-M., Bolen, J. B., Barnwell, J. W., Shattil, S. J. and Brugge, J. S. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7844–7848
- 56 Huang, M.-M., Indik, Z., Brass, L. F., Hoxie, J. A., Schreiber, A. D. and Brugge, J. S. (1992) *J. Biol. Chem.* **267**, 5467–5473
- 57 Painter, R. G. and Ginsberg, M. (1982) *J. Cell Biol.* **92**, 565–573
- 58 Wheeler, M. E., Gerrard, J. M. and Carroll, R. C. (1985) *J. Cell Biol.* **101**, 993–1000
- 59 Hillery, C. A., Smyth, S. S. and Parise, L. V. (1991) *J. Biol. Chem.* **266**, 14663–14669
- 60 Elmore, M. A., Anand, R., Horvath, A. R. and Kellie, S. (1990) *FEBS Lett.* **269**, 283–287

Received 15 November 1994/6 March 1995; accepted 22 March 1995