Primary arrest of circulating platelets on collagen involves phosphorylation of Syk, cortactin and focal adhesion kinase: studies under flow conditions

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After a vessel wall injury, platelets adhere to the subendothelium following a sequence of events: arrest of single platelets on the surface, progression to platelet spreading and final aggregation. Primary arrest of circulating platelets on subendothelial components occurs through platelet glycoprotein (GP) Ib and collagen receptors; then platelets spread and aggregate through a GPIIb-IIIa-dependent mechanism. A series of strategies were applied to analyse the tyrosine-phosphorylation mechanisms occurring at the different stages of platelet adhesion on subendothelial components under flow conditions, with special attention to primary arrest. To evaluate spread platelets, samples were exposed to acetylsalicylic acid, which blocks aggregate formation. To study single platelets in contact, a monoclonal antibody specific for GPIIb-IIIa was used to prevent platelet spreading and further aggregation. This experimental situation was also investigated using blood from two patients with Glanzmann's thrombasthenia (i.e. lacking GPIIb-IIIa). Results demonstrated that blockade of both spreading and aggregation results in significant changes in the tyrosine-phosphorylation patterns. Arrest of single platelets on collagen-rich surfaces resulted in phosphorylation of p125, identified as focal adhesion kinase (FAK), the 80/85 kDa doublet (cortactin), and p72, identified as Syk. Arrest of single platelets on von Willebrand factor as adhesive substrate showed that interaction through GPIb induces Syk phosphorylation, but not that of cortactin and FAK. Our data indicate that the initial arrest of platelets on subendothelial components involves Syk phosphorylation, which seems to be GPIb-dependent, and this is followed by activation and phosphorylation of cortactin and FAK. These processes seem to occur before GPIIb-IIIa becomes activated.

Key words: adhesion, glycoproteins, perfusion chamber, von Willebrand factor.

INTRODUCTION

In response to blood vessel injuries, platelets interact with sites of subendothelial exposure through primary adhesive mechanisms, thereby promoting activation, spreading and finally aggregation [1]. These processes take place under conditions of hydrodynamic stress, and require sufficient adhesive strength, especially during primary deposition of platelets [2,3]. During these events, different signalling mechanisms occur to amplify platelet responses [4], in order to bring about the formation of the haemostatic plug.

Platelets contain a large number of non-receptor tyrosine kinases that seem to be implicated in the regulation of agonist-induced activation and subsequent platelet responses [5]. Most of these mechanisms have been studied in platelets activated in suspension, thus bypassing the adhesive function of platelets. Under physiological conditions, platelets adhere to the exposed subendothelium, which consists primarily of fibrillar collagen and bound von Willebrand factor (vWF). The first contact of platelets basically occurs through the interaction of platelet glycoprotein (GP) Ib with vWF [6], which promotes the arrest of platelets on the damaged surface and opposes shear forces [7]. This first arrest, in which the platelet cytoskeleton plays a major role, facilitates the interaction of platelets with collagen receptors [8]. After primary arrest occurs, functionally active GPIIb-IIIa leads to platelet spreading and aggregate formation [9]. The significance of the signalling processes occurring during the progression of platelet interaction with adhesive substrates remains to be determined.

Upon stimulation of platelets, multiple proteins become phosphorylated at tyrosine residues [10]. This process is mediated by at least three families of tyrosine kinases, Src, Syk and focal adhesion kinase (FAK) [11]. It is currently accepted that tyrosine phosphorylation of proteins in platelets activated in suspension occurs in successive waves, depending on the activation state of GPIIb-IIIa [12], and consists of early activation, cross-linking of GPIIb-IIIa and final aggregation. Although the signalling processes involved in shape change and aggregation are beginning to be elucidated, the mechanisms that take place during primary arrest and further activation of platelets adhering on to substrates under flow conditions remain unclear.

The present study was undertaken to elucidate the signalling processes, occurring through tyrosine phosphorylation, during interaction of platelets with subendothelial components, under flow conditions. For this purpose, we investigated changes in tyrosine phosphorylation of proteins during primary arrest of platelets on extracellular matrix (ECM), generated by endothelial cells in culture, and on purified type-I tendon collagen (Col-I) by inhibiting both platelet spreading and aggregate formation. In some experiments, vWF was used as the adhesive substrate. To evaluate spread platelets, samples were exposed to acetylsalicylic acid (ASA), an agent that blocks aggregate formation. To study single platelets in primary arrest stages, samples were preincubated with an antibody to GPIIb-IIIa, since this GP is involved in platelet spreading and aggregation. Further studies were performed using blood samples from two patients with Glanzmann's thrombasthenia, whose platelets totally lacked GPIIb-IIIa.

Abbreviations used: ASA, acetylsalicylic acid; Col-I, purified type-I tendon collagen; ECL, enhanced chemiluminiscence; ECM, extracellular matrix; FAK, focal adhesion kinase; GP, glycoprotein; mAb, monoclonal antibody; vWF, von Willebrand factor.

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MATERIALS AND METHODS

Experimental design

The present study was designed to evaluate the signal transduction processes occurring through tyrosine phosphorylation after platelet activation by adhesion, under flow conditions, and to determine which phosphotyrosine proteins are implicated in primary arrest of platelets on adhesive surfaces. For this purpose, adhesion experiments were performed using different adhesive substrates: coverslips were coated with ECM generated by endothelial cells in culture, Col-I or vWF. Perfusions were carried out with blood samples containing platelets before and after incubation with ASA or an anti-GPIIb-IIIa monoclonal antibody (mAb), using a parallel-plate perfusion chamber. After 5 min of perfusion, adhered platelets were solubilized, and proteins were resolved by SDS/PAGE (8 % gels), followed by Western blotting to detect phosphotyrosine proteins. Tyrosine phosphorylation occurring in GPIIb-IIIa-deficient platelets adhered on collagen, under flow conditions, was also evaluated. Routine en face evaluation was performed to quantify the surface covered by platelets.

The present study required blood samples from healthy volunteers and from two patients with type I Glanzmann thrombasthenia. Research was carried out in accordance with the Declaration of Helsinki of the World Medical Association, the Ethical Committee of the Institution approved it, and the subjects included gave informed consent to the work.

Antibodies and chemical reagents

Col-I was obtained from Chrono-log Corporation (Havertown, PA, U.S.A.), and Haemate-P, used as a source of vWF, was from Aventis Behring (Barcelona, Spain). Recombinant anti-phosphotyrosine (RC20) and anti-FAK mAbs were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-cortactin (p80/85) clone 4F11 and anti-Syk clone 4D10.1 mAbs were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Fab fragments of the chimaeric anti-GPIIb-IIIa mAb (7E3; ReoPro®) were from Lilly S.A. (Geneva, Switzerland). PBS, Medium 199, glutamine, penicillin and streptomycin were obtained from Gibco BRL, Life Technologies S.A. (Barcelona, Spain). ASA, adenosine, apyrase, benzamidine, EGTA, leupeptin, PMSF, pepstatin and orthovanadate were purchased from Sigma (St. Louis, MO, U.S.A.). Protein A–Sepharose was from Pierce (Rockford, IL, U.S.A.), and electrophoresis reagents and nitrocellulose membranes were from Bio-Rad Laboratories S.A. (Madrid, Spain). Enhanced chemiluminiscence (ECL) reagents and Hyperfilm-ECL were from Amersham Pharmacia Biotech Europe GmbH (Barcelona, Spain)

Preparation of the perfusates

Whole blood samples anti-coagulated with citrate/phosphate/dextrose (100 mM sodium citrate, 16 mM citric acid, 18 mM sodium hydrogen phosphate and 130 mM dextrose) (at a final citrate concentration of 19 mM) were obtained from healthy volunteers who had not taken any drugs affecting platelet function in the previous 10 days. Anti-coagulated blood samples were separated into plasma, washed red cells and washed platelets [13]. The washed platelets were then divided into three portions; one was kept untreated (control), a second one was incubated with 100 μM ASA (final concentration in blood), and a third one was incubated with the anti-GPIIb-IIIa mAb (10 μg/ml, final concentration in blood). Incubations were performed for 30 min at 37 °C. Plasma and washed red cells were then added back to each of these three platelet preparations to yield three samples of reconstituted whole blood (40 % haematocrit and 1.2×10^5 platelets/ml, final concentrations). In some experiments, apyrase was added to a final concentration of 1 unit/ml to remove ADP that might be released from platelets.

Aliquots (20 ml) of whole blood from healthy volunteers and from two patients with type I Glanzmann's thrombasthenia were used for the perfusion studies.

Isolation of endothelial cell ECMs

Endothelial cells were isolated from human umbilical veins with collagenase (0.2 % in PBS; for 15 min at 37 °C), as previously described [14], with minor modifications [15]. Cells were maintained and subcultured at 37 °C in a 5 % CO_2 humidified incubator in Medium 199 supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 20 % (v/v) pooled human serum. The culture medium was changed every 48 h. The cells were identified as endothelial cells both morphologically and by the presence of vWF, as detected by immunofluorescence. After the second passage, the cells were subcultured to confluence on 1 % (w/v) gelatin-coated glass coverslips. After confluence, cells were detached to obtain the ECM by treatment with 3 % (w/v) EGTA for 60 min at 37 °C.

Coating of coverslips with purified adhesive proteins

Col-I was sprayed with an air brush on to 18×18 mm² plastic coverslips in order to achieve a density of 100 μg/coverslip. vWF was sprayed in order to achieve a density of 1 unit/coverslip. Coverslips were kept at 4 °C overnight [13].

Perfusion studies

Adhesion studies were carried out using a parallel-plate perfusion chamber [16,17], using coverslips coated with the ECM produced by endothelial cells in culture, with Col-I or with vWF. For each perfusion, two coverslips were individually inserted into the separate receptacles of a parallel-plate perfusion chamber. Blood samples were recirculated through the perfusion chamber at a shear rate of 800 s^{-1} for 5 min. After perfusion, coverslips were rinsed with 0.15 M PBS. One of the two perfused coverslips was processed for light microscopy analysis. The remaining coverslip was incubated at 4 °C with Laemmli's buffer [125 mM Tris/HCl, 2 % (w/v) SDS, 5 % (v/v) glycerol and 0.003 % Bromophenol Blue] containing 2 mM orthovanadate and 0.625 mg/ml *N*-ethylmaleimide for 15 min. Platelets adhered on the perfused surface of the coverslip were then removed by scraping and the total lysate was collected in an Eppendorf tube and heated at 90 °C for 5 min. Samples were kept at −20 °C until electrophoretic analysis.

Morphometric analysis

For morphometric analysis, one of the two perfused coverslips was fixed with 0.5 % glutaraldehyde in 0.15 M PBS at 4 °C for 24 h, and then stained with 0.02 % Toluidine Blue and morphometrically evaluated. The degree of platelet deposition on the perfused surface was evaluated en face by means of an automated method [18]. Images from the perfused surface were captured by a video camera (Leica) connected to a light microscope (Polyvar; Reichert–Jung, Vienna, Austria). The video camera provided images to the automated image analysis system (SigmaScan; Jandel Scientific software) which was connected to a PC computer with a graphic card adapter. A software package provided specialized programs to determine the surface covered as a percentage of the total area screened.

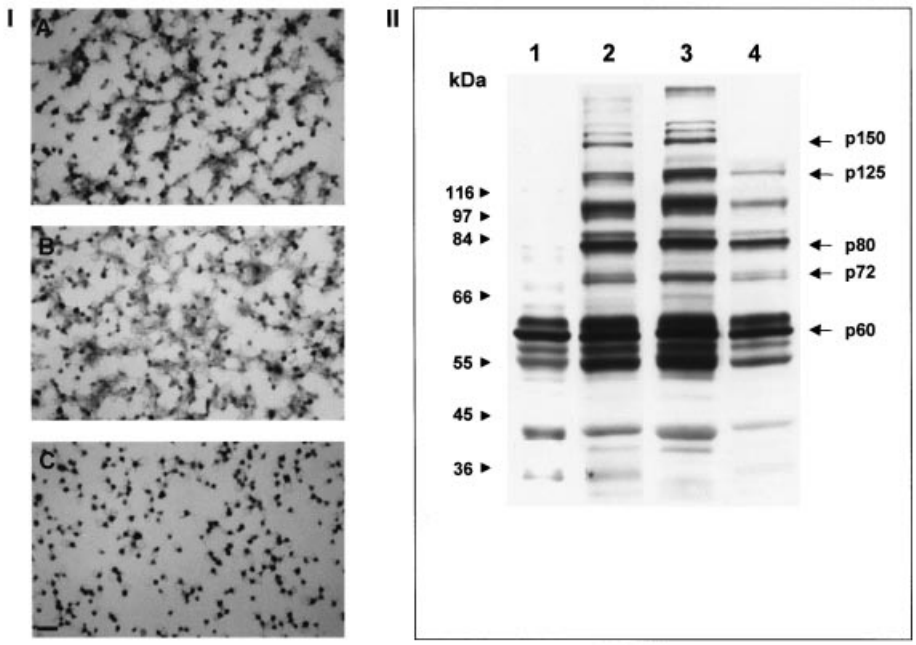

Figure 1 Platelet interaction with the ECM generated by endothelial cells in culture: effects of ASA and a mAb specific for GPIIb-IIIa (7E3)

Panel I: micrographs show grouped (aggregated) or single platelets, as they appear after en face visualization, adhered on ECM-coated coverslips after perfusion with blood samples at 800 s^{-1}, for 5 min. Adhered platelets were stained with Toluidine Blue. (**A**) Blood samples containing non-treated platelets, which were able to form aggregates on the ECM. (**B**) Blood samples containing platelets treated with ASA, which resulted in a decrease in aggregate formation with an increase in the surface covered by platelets. (**C**) Only single platelets in contact with the ECM were observed when blood samples contained platelets previously treated with Fab fragments of 7E3. Panel II: profiles of tyrosine-phosphorylated proteins from non-activated platelets in suspension (lane 1), and platelets interacting with ECM in the absence of anti-platelet agents (lane 2), in the presence of ASA (lane 3), and in the presence of Fab fragments of 7E3 (lane 4). Micrographs and profiles shown are representative of six different experiments. Scale bar = 15 μm.

Evaluation of phosphotyrosine proteins

Lysates of platelets activated by adhesion on ECM, Col-I or vWF were resolved by SDS/PAGE (8 % gels) [19]. Proteins present in the gels were transferred on to nitrocellulose membranes [20]. After blocking for non-specific binding, Western-blots were probed with a horseradish peroxidase-conjugated anti-phosphotyrosine recombinant antibody (RC20; 1:2500 dilution). Excess antibody was removed by extensive washing, and blots were developed by ECL [21].

Immunoprecipitation studies

Reconstituted blood samples containing platelets previously incubated with an antibody specific for GPIIb-IIIa were used as perfusates for immunoprecipitation studies. Samples were then perfused on coverslips coated with Col-I or vWF, as described before.

Adhered platelets were solubilized in ice-cold modified radioimmune precipitation buffer [50 mM Tris/HCl (pH 7.4), 1 % (v/v) Nonidet P40, 0.25 % sodium deoxycholate and 150 mM NaCl] containing 1 mM EGTA, 1 mM PMSF, 1 mM NaF, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM sodium orthovanadate, as protease and phosphatase inhibitors. Following removal of insoluble material by centrifugation at 14000 ***g*** for 1 min, at 4 °C, aliquots of the lysates containing equal amounts of protein were incubated overnight with either anti-FAK (p125) or anti-cortactin (p80/85) antibodies. Immune complexes were captured with affinity-purified rabbit anti-mouse IgG coupled to Protein A–agarose. Immunoprecipitates were recovered in Laemmli's buffer under reducing conditions, subjected to SDS/PAGE and transferred on to nitrocellulose membranes. To detect tyrosine phosphorylation of the immunoprecipitated proteins, membranes were probed with horseradish peroxidase-conjugated anti-phosphotyrosine antibodies (RC20; 1:2500 dilution). The presence of the blotted proteins was checked by probing the membranes with specific antibodies.

Statistics

Statistical analysis was performed using Student's t test; $P < 0.05$ was considered statistically significant.

RESULTS

Platelet activation by adhesion on endothelial cell ECM under flow conditions

Adhesion studies were performed using a parallel-plate perfusion chamber on coverslips with ECM at 800 s^{-1} for 5 min. After perfusions, coverslips were processed to analyse the surface covered by platelets, expressed as the percentage of surface coverage, and to assess changes in tyrosine phosphorylation of proteins in the adhered platelets.

The surface coverage on ECM after being perfused with control blood samples was 25.05 ± 1.8 % (mean ± S.E.M., n = 12) (Figure 1, panel IA). Treatment of perfusates with ASA resulted in a significant increase in the surface coverage to 51.8 ± 2.9 % (mean ± S.E.M., $n = 6$; $P < 0.001$) with a decrease in aggregate formation, as shown by the lower intensity of staining (Figure 1, panel IB). Results were significantly different when the perfusates were preincubated with an anti-GPIIb-IIIa

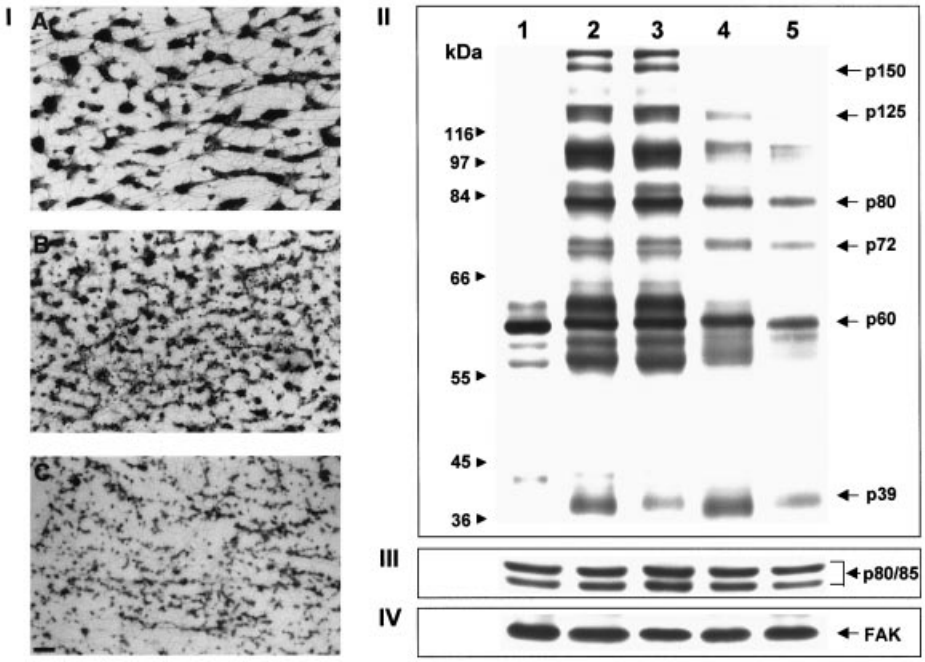

Figure 2 Platelet interaction with Col-I under flow conditions: effects of ASA and a mAb specific for GPIIb-IIIa (7E3)

Panel I: micrographs show grouped or single platelets, visualized en face after staining with Toluidine Blue, adhered on Col-I fibres perfused with blood samples at 800 s^{-1}, for 5 min. (**A**) The coverslips coated with Col-I fibres were perfused with blood samples containing non-treated platelets that formed aggregates on the surface. (**B**) Blood samples containing platelets treated with ASA, which resulted in a decrease in aggregate formation with an increase in the surface covered by platelets. (**C**) Only single platelets in contact with the Col-I fibres were observed when blood samples contained platelets previously treated with Fab fragments of 7E3. Panel II: profiles of tyrosine-phosphorylated proteins from non-activated platelets in suspension (lane 1), and platelets interacting with Col-I in the absence of antiplatelet agents (lane 2), in the presence of ASA (lane 3), and in the presence of an anti-GPIIb-IIIa mAb (lane 4). Lane 5 corresponds to platelets from a patient with Glanzmann's thrombasthenia interacting with Col-I. Panels III and IV: blots re-probed with anti-p80/85 or anti-pp125 antibodies respectively, to verify equal amounts of cortactin and FAK in all samples. Micrographs and profiles shown are representative of six different experiments. Scale bar = 15 μm.

mAb. The presence of the antibody completely inhibited aggregate formation, resulting in single platelets in contact (Figure 1, panel IC), and reaching a surface coverage of $12.7 \pm 2.5\,\%$ (mean $\pm$ S.E.M., $n = 6$; $P = 0.001$).

Interaction of platelets with ECM resulted in the phosphorylation of multiple proteins at tyrosine residues (Figure 1, panel II). Phosphorylation levels in ASA-treated platelets spread on ECM were qualitatively and quantitatively equal to those observed in non-treated samples (Figure 1, panel II, compare lanes 3 and 2). In contrast, tyrosine phosphorylation of proteins was partially inhibited by the presence of the anti-GPIIb-IIIa mAb. Only proteins p125, p80, p72 and p60 were consistently phosphorylated (Figure 1, panel II, compare lanes 4 and 2).

Platelet activation by adhesion on Col-I under flow conditions

A similar experimental approach was applied using Col-I as the adhesive substrate. The surface coverage on Col-I after perfusion with control samples was $34.2 \pm 1.7\,\%$ (mean $\pm$ S.E.M., $n = 12$), with formation of large aggregates (Figure 2, panel IA). Preincubation of perfusates with ASA (Figure 2, panel IB) resulted in a decrease in aggregate formation on Col-I, although the percentage of surface coverage was slightly higher than in controls due to an increase in platelet spreading ($37.1 \pm 1.6\,\%$ compared with $34.2 \pm 1.7\,\%$, $n = 6$). Pre-treatment of blood perfusates with an anti-GPIIb-IIIa mAb (Figure 2, panel IC), for 30 min before perfusion, resulted in a significant reduction in the surface of Col-I covered by platelets, from $34.2 \pm 1.7\,\%$ (in control experiments) to $19.7 \pm 0.8\,\%$ with single platelets in contact (mean $\pm$ S.E.M., $n = 6$, $P < 0.001$).

Under the experimental conditions applied, patterns of tyrosine-phosphorylated proteins observed in platelets interacting with Col-I were the same as those in platelets adhered on ECM. The presence of ASA did not modify these protein profiles (Figure 2, panel II, lane 3 compared with lane 2). However, perfusion of Col-I-coated coverslips with perfusates containing anti-GPIIb-IIIa mAb resulted in qualitative and quantitative differences in the tyrosine-phosphorylation patterns observed in the arrested platelets. Phosphorylation levels were in general weaker than in controls. Only proteins p125, p80, p72 and pp60 were clearly phosphorylated.

The effect of apyrase was assayed to evaluate the role of ADP released from platelets in this experimental model. No differences in the adhesion patterns or in the protein profiles were detected by the addition of apyrase to the platelet suspension buffers.

Tyrosine phosphorylation in GPIIb-IIIa-deficient platelets interacting with Col-I under flow conditions

Interaction of platelets from two patients with Glanzmann's thrombasthenia was also assessed on Col-I, under flow conditions. After 5 min of perfusion, only single platelets in contact with the collagen surface could be observed, leading to a surface coverage of $21 \pm 3.7\,\%$ (mean obtained from the analysis of 20 microscopic fields per coverslip, considering that the experiments were repeated three times).

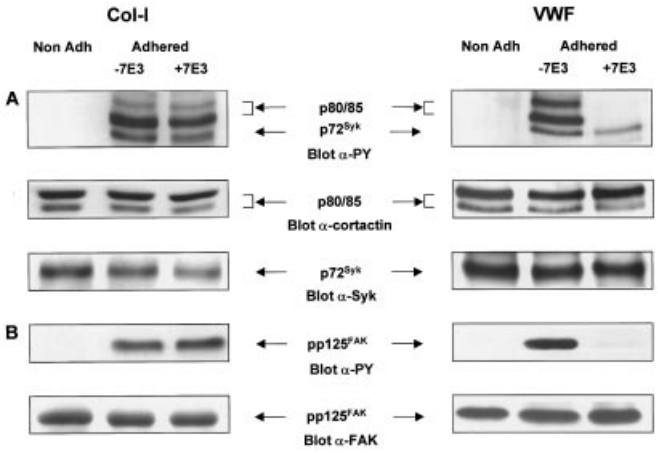

Figure 3 Identification of tyrosine-phosphorylated proteins in platelets interacting with Col-I and with vWF: effect of Fab fragments of a mAb specific for GPIIb-IIIa (7E3)

Immunoprecipitation experiments were performed using samples corresponding to non-activated platelets in suspension (Non Adh) and platelets adhered on Col-I or vWF, as indicated. Adhesion studies were performed under flow conditions using blood samples containing non-treated platelets (−7E3) or platelets previously incubated with Fab fragments of a mAb specific for GPII-IIIa (+7E3). (**A**) Samples were immunoprecipitated with an anti-p80/85 (cortactin) antibody. The immunoprecipitates were resolved by SDS/PAGE (8% gels) and proteins transferred on to nitrocellulose membranes to detect tyrosine phosphorylation of p80/85 cortactin by a specific antibody (blot α-PY). Presence of cortactin or Syk were checked in the membranes with the appropriate antibodies (blot α-cortactin and blot α-Syk). (**B**) Samples were immunoprecipitated with an anti-pp125^{Fak} antibody. The immunoprecipitates were treated as indicated previously to detect phosphorylation (blot α-PY) and the presence (blot α-FAK) of pp125^{FAK}. Profiles shown are representative of six different experiments.

The congenital absence of GPIIb-IIIa resulted in tyrosine-phosphorylation patterns comparable with those observed in control adhered platelets previously incubated with the anti-GPIIb-IIIa mAb. Proteins p80, p72 and pp60 appeared clearly phosphorylated at tyrosine residues. An increase in the time of exposure of the radiograph film to the nitrocellulose membrane did not modify the protein profiles (Figure 2, panel II, lane 5).

Immunoprecipitation of cortactin and FAK

When nitrocellulose membranes, containing phosphotyrosine protein profiles from adhered platelets either in the absence or in the presence of ASA, the anti-GPIIb-IIIa mAb or thrombasthenic platelets, were probed with antibodies against cortactin (p80/85) and FAK, both proteins were found to be present (Figure 2, panels III and IV).

To confirm that tyrosine-phosphorylated proteins p80 and p125 corresponded to cortactin and FAK, we performed immunoprecipitation studies with specific antibodies. Experiments were carried out using blood samples containing non-treated platelets or platelets previously incubated with an anti-GPIIb-IIIa mAb. Samples were perfused on to Col-I as the adhesive substrate. Both proteins, cortactin and FAK, were found to be present in platelets and became phosphorylated after adhesion to the collagen surface (Figure 3). Interestingly, when probing phosphorylation of cortactin, a protein of 72 kDa was also detected, which was identified as Syk by a specific antibody (Figure 3). The presence of the anti-GPIIb-IIIa mAb did not inhibit the phosphorylation of any of the previously mentioned proteins under these experimental conditions (Figure 3).

Immunoprecipitation of cortactin and FAK in platelets interacting with vWF

Adhesion studies were also performed using vWF as the adhesive substrate, applying the same experimental design as for Col-I. Perfusions were carried out with blood containing non-treated platelets or platelets previously incubated with an anti-GPIIb-IIIa mAb. Morphometric evaluation of the perfused coverslips was performed to verify the effect of the anti-GPIIb-IIIa mAb. The surface coverage reached on vWF was $34.9 \pm 1.9\,\%$ (mean $\pm$ S.E.M., $n = 4$) in the absence of the antibody, and $13.9 \pm 0.5\,\%$ ($n = 4$, $P < 0.001$) with single platelets in contact in the presence of the anti-GPIIb-IIIa mAb.

Immunoprecipitation of both cortactin and FAK were also performed (see Figure 3). Both proteins were present in lysates corresponding to platelets interacting with vWF in the absence and in the presence of anti-GPIIb-IIIa mAb. Immunoprecipitation of cortactin resulted in the co-immunoprecipitation of a protein of 72 kDa, identified as p72^{syk} (Figure 3). While cortactin and FAK appeared tyrosine-phosphorylated in the absence of the mAb, blockade of GPIIb-IIIa resulted in complete inhibition of the phosphorylation of both proteins at tyrosine residues. However, phosphorylated p72^{syk} was detected even in the presence of the anti-GPIIb-IIIa mAb (Figure 3).

DISCUSSION

Effective primary haemostasis depends on rapid recognition of the damaged surface by circulating platelets, thereby promoting initial platelet contact and further platelet activation [1,22]. These processes proceed through well synchronized signalling mechanisms to maintain platelet function [23]. We have found that the pattern of tyrosine phosphorylation in adherent platelets is determined by the nature of the adhesive substrate to which they are attached. Data from the present study indicate that arrest of single platelets on collagen triggers a series of signalling events differing from those observed by further activation to spreading and aggregation. Furthermore, our results indicate that phosphorylation of p72^{syk}, p80/85 (cortactin) and pp125^{FAK} is specifically involved in the initial arrest of platelets on collagen and seems to occur before GPIIb-IIIa becomes activated.

During the interaction of platelets with subendothelial structures, the intensity of platelet response depends on the severity of the vascular damage [24]. Primary haemostasis is related to the limited interaction of platelets with the ECM exposed after superficial endothelial denudation. However, platelet interactions with a collagen-rich surface are related to deep vascular damage and acute thrombotic events, which could trigger a more intense cascade of signalling mechanisms [23]. Tyrosine phosphorylation of proteins is one of the signalling mechanisms that regulates cell responses [25], and which has been extensively studied in platelets activated in suspension [5,26]. Our present study has considered that platelets fulfil their main function by interacting with the subendothelium under flow conditions. From this perspective, we have evaluated the signalling processes occurring during platelet interaction with different adhesive substrata under flow conditions, taking into consideration that this interaction occurs following a sequence of events that involves primary arrest, spreading and aggregation.

When evaluating platelet interaction with the ECM, we were able to stop platelets at arrest stages by using inhibitory strategies to block GPIIb-IIIa, resulting in dramatic changes in tyrosine phosphorylation of several proteins. Interestingly, when platelets were allowed to spread but not to aggregate by ASA treatment [27], the patterns of tyrosine phosphorylation found did not differ from those observed after aggregation on ECM. The ECM

is a complex matrix consisting of different adhesive proteins to which platelets adhere through multiple receptors. To discern the specific role of several primary platelet receptors in initiating signalling through tyrosine phosphorylation we used purified proteins, such as collagen and vWF, as adhesive substrates.

Collagen is the most important platelet-reactive protein present in basement membranes, including the subendothelium. Although many proteins have been proposed as collagen receptors, clinical and experimental evidence strongly supports roles for integrin $\alpha_2\beta_1$ (GPIa-IIa) [28] and the non-integrin receptors GPIV [29] and GPVI [30]. The interaction of platelets with collagen is complex and seems to occur following a biphasic process [31]. Collagen is thought to be the major binding site for vWF [32], which is recognized by platelet GPIb. Then, platelets interact more rapidly and permanently with collagen probably through GPIa-IIa. While GPIV seems to accelerate this process [13], GPVI is necessary for full activation and aggregation of platelets on the collagen surface under flow conditions [33].

In a previous study we demonstrated that activation of platelets by adhesion on collagen induces tyrosine phosphorylation of multiple proteins, which differ from those observed when activation is induced in platelets in suspension [34]. In the present study we observed that primary arrest of platelets, achieved in the presence of a specific GPIIb-IIIa blocking antibody, occurs with phosphorylation of only a few proteins: $p72^{syk}$, p80/85 (cortactin) and $pp125^{FAK}$. This experimental situation was reproduced in those experiments in which the perfusates consisted of blood samples from patients with a congenital deficiency of GPIIb-IIIa. Glanzmann's thrombasthenia not only provides a unique model to assess the involvement of GPIIb-IIIa in platelet function, but also to investigate the role of other receptors implicated in those processes occurring independently of GPIIb-IIIa. As expected, thrombasthenic platelets established contacts on Col-I fibres, although they were unable to spread and aggregate. Interestingly, proteins $p72^{syk}$, p80/85 cortactin and $p125^{FAK}$ also appeared in GPIIb-IIIa-deficient platelets in contact with collagen. On the other hand, when control platelets were allowed to spread in the presence of ASA no changes in terms of tyrosine phosphorylation were apparent. These data together with those observed on the ECM suggest that as spreading occurs multiple proteins become tyrosine phosphorylated and remain phosphorylated even when platelet-to-platelet interactions have been inhibited by the presence of ASA.

The rational for the experimental design applied in the present study was based on the fact that the main function of platelets starts by interacting with subendothelial surfaces. Therefore our main purpose was to investigate which signalling proteins are involved in the arrest of platelets on subendothelial components under flow conditions. Our results demonstrate that single platelets arrested on collagen show phosphorylation of $p72^{syk}$, p80/85 (cortactin) and $p125^{FAK}$. Cortactin has been identified as a cytoskeleton-associated substrate for c-Src in transformed cells [35]. In fact, phosphorylation of cortactin seems to play an important role in modulating cell shape [36], and in promoting platelet shape change in association with Syk [37]. Exposure of collagen to circulating blood is followed by immobilization of plasma vWF on the collagen surface by platelet GPIb, promoting temporary arrest of platelets [7]. Considering that platelet arrest on subendothelial surfaces is initially dependent on the interaction of GPIb and vWF, which is stabilized by the connection of GPIb to the cytoskeleton [39], it would have been reasonable to suggest that pp80/85, together with Syk, could be one of the earliest proteins involved in the signalling cascade during platelet interaction. However, results from our present studies using vWF as adhesive substrate, in the presence of anti-GPIIb-IIIa mAb, indicate that primary platelet interaction through GPIb induces phosphorylation of $p72^{syk}$ but not of cortactin. These results are supported by a previous study by other authors showing that vWF added to platelets in suspension in the presence of botrocetin induces $p72^{syk}$ activation [40].

On the other hand, cortactin is supposed to be a substrate for the tyrosine kinase Syk [37]. In our experimental setting, Syk associated with cortactin appeared phosphorylated in those platelets arrested on collagen even when GPIIb-IIIa was blocked. Platelets, slowed by the interaction with vWF, bind to collagen by collagen receptors, initially through the integrin GPIa-IIa [41,31]. Our results would, therefore, support a role for collagen receptors in further activation of protein $p72^{syk}$, followed by phosphorylation of cortactin, before GPIIb-IIIa becomes activated. These results would be in agreement with those from other authors [42,43] that have indicated the involvement of a GPIa-IIa-dependent pathway in signalling through Syk phosphorylation after collagen activation of platelets in suspension.

We also investigated the role of FAK under our experimental conditions. Protein $pp125^{FAK}$ was initially shown to be localized in focal adhesions in fibroblasts [44]. In platelets, phosphorylation of $pp125^{FAK}$ has been associated with the aggregation process [45], and with platelet spreading mediated by GPIIb-IIIa [46]. In the present work, $pp125^{FAK}$ phosphorylation was inhibited by the presence of anti-GPIIb-IIIa mAb when vWF was used as adhesive substrate. In fact, platelet interaction with vWF occurs through GPIb, followed by activation of GPIIb-IIIa, which promotes platelet spreading and aggregation. However, when using collagen as adhesive substrate, $pp125^{FAK}$ phosphorylation occurred in GPIIb-IIIa-deficient platelets, again reinforcing a role for other integrin receptors such as GPIa-IIa [47], a functionally significant collagen receptor in platelets.

Our study indicates that the initial arrest of platelets though the interaction of GPIb with immobilized vWF involves phosphorylation of Syk. This first event is followed by activation and phosphorylation of cortactin and FAK, processes that seem to be dependent on collagen receptors. Both the binding of GPIb to vWF and the interaction with collagen through collagen receptors induces a conformational change in GPIIb-IIIa [38,48] that enable the complex to bind to vWF. Therefore a second step implies signalling during spreading and aggregate formation, with both dependent on GPIIb-IIIa. We have been able to identify specific signalling proteins during the primary arrest of platelets. However, further studies are required to investigate those proteins involved in signalling during platelet spreading and aggregation on adhesive surfaces under flow conditions.

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REFERENCES

1 Baumgartner, H. R. (1973) The role of blood flow in platelet adhesion, fibrin deposition, and formation of mural thrombi. Microvasc. Res. **5**, 167–179
2 Weiss, H. J., Turitto, V. T. and Baumgartner, H. R. (1978) Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. I. Shear rate-dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. J. Lab. Clin. Med. **92**, 750–764
3 Turitto, V. T. and Baumgartner, H. R. (1975) Platelet interaction with subendothelium in a perfusion system: physical role of red blood cells. Microvasc. Res. **9**, 335–344
4 Hawiger, J. (1989) Platelet secretory pathways: an overview. Methods Enzymol. **169**, 191–195
5 Clark, E. A. and Brugge, J. S. (1993) Tyrosine phosphorylation in platelets – potential roles in intracellular signal transduction. Trends Cardiovasc. Med. **3**, 218–227

6 Tschopp, T. B., Weiss, H. J. and Baumgartner, H. R. (1974) Decreased adhesion of platelets to subendothelium in von Willebrand's disease. J. Lab. Clin. Med. **83**, 296–300

7 Savage, B., Saldivar, E. and Ruggeri, Z. M. (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell (Cambridge, Mass.) **84**, 289–297

8 Santoro, S. A. (1988) Molecular basis of platelet adhesion to collagen. In Platelet Membrane Receptors: Molecular Biology, Immunology, Biochemestry and Pathology (Jamieson, G. A., ed.), pp. 291–314, Alan R. Liss, NY

9 Weiss, H. J., Turitto, V. T. and Baumgartner, H. R. (1991) Further evidence that glycoprotein IIb-IIIa mediates platelet spreading on subendothelium. Thromb. Haemostasis **65**, 202–205

10 Golden, A. and Brugge, J. S. (1989) Thrombin treatment induces rapid changes in tyrosine phosphorylation in platelets. Proc. Natl. Acad. Sci. U.S.A. **86**, 901–905

11 Levy-Toledano, S. (1999) Platelet signal transduction pathways: could we organize them into a 'hierarchy'? Haemostasis **29**, 4–15

12 Ferrell, Jr, J. E. and Martin, G. S. (1989) Tyrosine-specific protein phosphorylation is regulated by glycoprotein IIb-IIIa in platelets. Proc. Natl. Acad. Sci. U.S.A. **86**, 2234–2238

13 Diaz-Ricart, M., Tandon, N. N., Carretero, M., Ordinas, A., Bastida, E. and Jamieson, G. A. (1993) Platelets lacking functional CD36 (glycoprotein IV) show reduced adhesion to collagen in flowing whole blood. Blood **82**, 491–496

14 Jaffe, E. A., Nachman, R. L. and Becker, C. G. (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. **52**, 2745–2756

15 Aznar-Salatti, J., Bastida, E., Haas, T. A., Escolar, G., Ordinas, A., de Groot, P. G. and Buchanan, M. R. (1991) Platelet adhesion to exposed endothelial cell extracellular matrixes is influenced by the method of preparation. Arterioscler. Thromb. **11**, 436–442

16 Muggli, R., Baumgartner, H. R., Tschopp, T. B. and Keller, H. (1980) Automated microdensitometry and protein assays as a measure for platelet adhesion and aggregation on collagen-coated slides under controlled flow conditions. J. Lab. Clin. Med. **95**, 195–207

17 Sakariassen, K. S., Aarts, P. A., de Groot, P. G., Houdijk, W. P. and Sixma, J. J. (1983) A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. J. Lab. Clin. Med. **102**, 522–535

18 Diaz-Ricart, M., Carretero, M., Castillo, R., Ordinas, A. and Escolar, G. (1994) Digital image analysis of platelet-extracellular matrix interactions: studies in von Willebrand disease patients and aspirin-treated donors. Haemostasis **24**, 219–229

19 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685

20 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

21 Cichowski, K., McCormick, F. and Brugge, J. S. (1992) p21rasGAP association with Fyn, Lyn, and Yes in thrombin-activated platelets. J. Biol. Chem. **267**, 5025–5028

22 Weiss, H. J., Turitto, V. T. and Baumgartner, H. R. (1986) Platelet adhesion and thrombus formation on subendothelium in platelets deficient in glycoproteins IIb-IIIa, Ib, and storage granules. Blood **67**, 322–330

23 Kroll, M. H., Harris, T. S., Moake, J. L., Handin, R. I. and Schafer, A. I. (1991) von Willebrand factor binding to platelet GpIb initiates signals for platelet activation. J. Clin. Invest. **88**, 1568–1573

24 Ip, J. H., Fuster, V., Israel, D., Badimon, L., Badimon, J. and Chesebro, J. H. (1991) The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty. J. Am. Coll. Cardiol. **17**, B77–B88

25 Hunter, T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell (Cambridge, Mass.) **80**, 225–236

26 Levy-Toledano, S., Gallet, C., Nadal, F., Bryckaert, M., MacLouf, J. and Rosa, J. P. (1997) Phosphorylation and dephosphorylation mechanisms in platelet function: a tightly regulated balance. Thromb. Haemostasis **78**, 226–233

27 Roux, S. P., Sakariassen, K. S., Turitto, V. T. and Baumgartner, H. R. (1991) Effect of aspirin and epinephrine on experimentally induced thrombogenesis in dogs. A parallelism between *in vivo* and *ex vivo* thrombosis models. Arterioscler. Thromb. **11**, 1182–1191

28 Nieuwenhuis, H. K., Akkerman, J. W., Houdijk, W. P. and Sixma, J. J. (1985) Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. Nature (London) **318**, 470–472

29 Tandon, N. N., Kralisz, U. and Jamieson, G. A. (1989) Identification of glycoprotein IV (CD36) as a primary receptor for platelet-collagen adhesion. J. Biol. Chem. **264**, 7576–7583

30 Sugiyama, T., Okuma, M., Ushikubi, F., Sensaki, S., Kanaji, K. and Uchino, H. (1987) A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. Blood **69**, 1712–1720

31 Moroi, M., Jung, S. M., Nomura, S., Sekiguchi, S., Ordinas, A. and Díaz-Ricart, M. (1997) Analysis of the involvement of the von Willebrand factor-glycoprotein Ib interaction in platelet adhesion to a collagen-coated surface under flow conditions. Blood **90**, 4413–4424

32 Sakariassen, K. S., Bolhuis, P. A. and Sixma, J. J. (1979) Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-Von Willebrand factor bound to the subendothelium. Nature (London) **279**, 636–638

33 Moroi, M., Jung, S. M., Shinmyozu, K., Tomiyama, Y., Ordinas, A. and Díaz-Ricart, M. (1996) Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion. Blood **88**, 2081–2092

34 Arderiu, G., Estebanell, E., Pujol-Moix, N., Escolar, G., Ordinas, A. and Díaz-Ricart, M. (2000) Adherence of platelets under flow conditions results in specific phosphorylation of proteins at tyrosine residues. Cell. Adhes. Commun. **7**, 349–358

35 Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R. and Parsons, J. T. (1991) Identification and characterization of a novel cytoskeleton-associated pp60src substrate. Mol. Cell. Biol. **11**, 5113–5124

36 McNiven, M. A., Kim, L., Krueger, E. W., Orth, J. D., Cao, H. and Wong, T. W. (2000) Regulated interactions between dynamin and the actin-binding protein cortactin modulate cell shape. J. Cell Biol. **151**, 187–198

37 Gallet, C., Rosa, J. P., Habib, A., Lebret, M., Levy-Toledano, S. and Maclouf, J. (1999) Tyrosine phosphorylation of cortactin associated with Syk accompanies thromboxane analogue-induced platelet shape change. J. Biol. Chem. **274**, 23610–23616

38 Reference deleted

39 Fox, J. E. (1985) Linkage of a membrane skeleton to integral membrane glycoproteins in human platelets. Identification of one of the glycoproteins as glycoprotein Ib. J. Clin. Invest. **76**, 1673–1683

40 Asazuma, N., Ozaki, Y., Satoh, K., Yatomi, Y., Handa, M., Fujimura, Y., Miura, S. and Kume, S. (1997) Glycoprotein Ib-von Willebrand factor interactions activate tyrosine kinases in human platelets. Blood **90**, 4789–4798

41 Nieuwenhuis, H. K., Sakariassen, K. S., Houdijk, W. P., Nievelstein, P. F. E. M. and Sixma, J. J. (1986) Deficiency of platelet membrane glycoprotein Ia associated with a decreased platelet adhesion to subendothelium: a defect in platelet spreading. Blood **68**, 692–695

42 Keely, P. J. and Parise, L. V. (1996) The $\alpha_2\beta_1$ integrin is a necessary co-receptor for collagen-induced activation of Syk and the subsequent phosphorylation of phospholipase Cγ2 in platelets. J. Biol. Chem. **271**, 26668–26676

43 Asazuma, N., Yatomi, Y., Ozaki, Y., Qi, R. M., Kuroda, K., Satoh, K. and Kume, S. (1996) Protein-tyrosine phosphorylation and p72syk activation in human platelets stimulated with collagen is dependent upon glycoprotein Ia/IIa and actin polymerization. Thromb. Haemostasis **75**, 648–654

44 Bockholt, S. M. and Burridge, K. (1995) An examination of focal adhesion formation and tyrosine phosphorylation in fibroblasts isolated from src-, fyn-, and yes-mice. Cell. Adhes. Commun. **3**, 91–100

45 Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T. and Brugge, J. S. (1992) Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. J. Cell Biol. **119**, 905–912

46 Ji, P. and Haimovich, B. (1999) Integrin αIIbβ3-mediated pp125FAK phosphorylation and platelet spreading on fibrinogen are regulated by PI 3-kinase. Biochim. Biophys. Acta **1448**, 543–552

47 Polanowska-Grabowska, R., Geanacopoulos, M. and Gear, A. R. (1993) Platelet adhesion to collagen via the $\alpha_2\beta_1$ integrin under arterial flow conditions causes rapid tyrosine phosphorylation of pp125FAK. Biochem. J. **296**, 543–547

48 Nakamura, T., Kambayashi, J., Okuma, M. and Tandon, N. N. (1999) Activation of the GP IIb-IIIa complex induced by platelet adhesion to collagen is mediated by both $\alpha_2\beta_1$ integrin and GP VI. J. Biol. Chem. **274**, 11897–11903

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