Phosphorylation Sites in the Integrin β3 Cytoplasmic Domain in Intact Platelets*

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Protein seryl/threonyl phosphatase inhibitors such as calyculin A block inside-out and outside-in platelet signaling. Our studies demonstrate that the addition of calyculin A blocks platelet adhesion and spreading on fibrinogen, responses that depend on integrin αIIbβ3 signaling. We hypothesized that this reflects a change in αIIbβ3 structure caused by a specific state of phosphorylation. We showed that addition of calyculin A leads to increased phosphorylation of the β3 subunit, and phosphoamino acid analysis reveals that only threonine residues become phosphorylated; sequence analysis by Edman degradation established that threonine 753 became stoichiometrically phosphorylated during inhibition of platelet phosphatases by calyculin A. This region of β3 is linked to outside-in signaling such as platelet spreading responses. The effect of calyculin A on platelet adhesion and spreading and on the phosphorylation of T-753 in β3 is reversed by the calcium ionophore A23187, demonstrating that these effects of calyculin A are not generally toxic ones. We propose that phosphorylation of β3 on threonine 753, a region of β3 linked to outside-in signaling, may be a mechanism by which integrin αIIbβ3 function is regulated.

Effective hemostasis depends on three basic platelet responses (1). First, adhesion, which includes attachment and spreading reactions, initiates events that lead to formation of a hemostatic plug. Second, secretion of factors aid in the activation of additional platelets and in the repair of the damaged vessel wall. Third, aggregation of recruited platelets to each other and to adhered platelets results in growth of the hemostatic plug. Both adhesive and aggregatory responses are controlled by the coordinate actions of cell surface glycoprotein receptors. Many of these receptors are dimers of two different subunits (2). These include selective members of the integrin family of cell adhesion molecules such as αIIbβ1 (collagen-binding), αIIbβ2 (laminin), αIIbβ1 (fibronectin), and αIIbβ3 (fibrinogen and von Willebrand factor) (for review, see Ref. 1).

The αIIbβ3 complex (glycoprotein IIb–IIIa) is unique among integrin molecules expressed on platelets; it functions as a binary switch. On resting platelets, αIIbβ3 exists in an inactive conformation in which the binding pocket for soluble forms of fibrinogen and von Willebrand factor is cryptic. Thus, before activation, the interaction of αIIbβ3 is limited to surface-bound fibrinogen, which supports adhesion under low shear forces (2–5). As a consequence of platelet activation, αIIbβ3 converts to an active conformation (via a process referred to as inside-out signaling) and becomes capable of binding other surface-bound molecules and soluble forms of fibrinogen and von Willebrand factor (6). This latter activity facilitates platelet aggregation. The molecular events that switch αIIbβ3 into a high affinity state are not known. The structural correlations have identified potential cytoplasmic motifs in β3 that regulate its activation. First, a naturally occurring mutation in the cytoplasmic domain of αIIbβ3 negatively modulates its activity: serine 752 to proline mutation in the cytoplasmic portion of β3, a variant associated with Glanzmann’s thrombasthenia, inhibits αIIbβ3 signaling (7, 8). Second, site-directed mutagenesis of Asp276 in β3 effectively disrupts potential salt bridges between the α and β subunits and results in a constitutively active integrin molecule (9). Thus, it appears that changes in charge in the cytoplasmic portion of β3 may have important consequences with respect to integrin structure and function. Phosphorylation of β3 on a threonine residue, which would change the charge in β3, has been implicated in exposing binding sites on αIIbβ3 (10, 11), although the role that this phosphorylation plays in integrin function is unknown (12). Suggesting that a phosphorylation event controls αIIbβ3 activation is in accordance with protein phosphorylation events regulating numerous platelet responses, including adhesion and aggregation (13). Phosphorylation of proteins on serine, threonine, and tyrosine residues is controlled by the competing activities of protein kinases and phosphatases. Thus, increased phosphorylations can represent increased kinase activity or decreased phosphatase activity. Platelets contain numerous protein kinases (13) and protein seryl/threonyl phosphatase types 1, 2A, and 2B (14, 15). Type 1 and 2A protein phosphatases are active in resting and stimulated platelets (14) whereas type 2B, a Cu2+-dependent, calmodulin-stimulated enzyme, is only active after platelet activation (15). To understand the physiological significance of these enzymes in platelets, membrane-permeable inhibitors have been used. Type 1 and 2A phosphatases have been implicated in controlling platelet responses including aggregations (16–23). Very little is known, however, about how these enzymes modulate molecular events linked to the aggregatory response.

Previous studies have shown that calyculin A, a serine/threonyl phosphatase inhibitor, inhibits platelet aggregation, which is dependent on αIIbβ3 function. The present study tests whether calyculin A affects other αIIbβ3 functions and tests the hypothesis that altered αIIbβ3 function is linked to altered structure of this integrin. The data show that, in addition to an effect on aggregation, calyculin A blocks platelet adhesion and spreading on fibrinogen, reactions that depend on αIIbβ3 out-
side-in signaling. Biochemical analysis links calyculin A effects to β₃ phosphorylation. Sequence analysis demonstrates that threonine 753, which is in the intracellular segment of β₃, becomes stoichiometrically phosphorylated. These data suggest that structural modification of β₃, through phosphorylation, may regulate outside-in, and perhaps inside-out, signaling through the α₁bβ₃ integrin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Prostaglandin E₁, apyrase, aprotinin, leupeptin, benzamidine, protein A-Sepharose, human fibrinogen, low molecular weight heparin (M₄, approx. 3,000), phenylmethylsulfonyl fluoride, sodium vanadate, and dithiothreitol were obtained from Sigma. [³²P]Orthophosphate was purchased from DuPont NEN. LC Services supplied calyculin A. Rhodamine-labeled phalloidin was purchased from Molecular Probes (Eugene, OR). Boehringer Mannheim supplied sequencing grade AspN protease and trypsin. Hirudin (BKHV recombinant) was obtained from Calbiochem. The C18 reverse phase TSK-GELOS-120T C18 reverse phase column (reverse phase HPLC), collecting fractions by peak detection (30). Isolated peptides were sequenced by automated Edman degradation on a model 477/120A pulse liquid sequencer (Applied Biosystems) using standard chemistry.

**Phosphoamino Acid Analysis**—Phosphoproteins extracted from SDS gels were hydrolyzed in 6 N HCl for 1 h at 110 °C, and phosphoamino acids were separated by two-dimensional electrophoresis on cellulose plates using 2.5% formic acid and 7.8% acetic acid, pH 1.9, in the first dimension and 0.5% pyridine and 5% acetic acid, pH 3.5, in the second dimension (24).

**Identification of Phosphorylation Sites**—Immunoprecipitated [³²P] phosphorylated β₃ was reduced and alkylated by the method of Talmadge et al. (29) before SDS-PAGE. The Cooamassie Blue-stained β₃ was cut from the gel using a scalpel and digested with AspN in situ and extracted (25). The extracts were concentrated under vacuum, resuspended in 0.1% trifluoroacetic acid in 6 M guanidine-HCl, and fractionated on a 120T C18 reverse phase column (reverse phase HPLC), collecting fractions by peak detection (30). Isolated peptides were sequenced by automated Edman degradation on a model 477/120A pulse liquid sequencer (Applied Biosystems) using standard chemistry.

**Static Platelet Adhesion Studies**—Washed platelets (5 x 10⁹/ml) were treated with either buffer or 100 nM calyculin A for 5 min at 21 °C. After the addition of buffer or A23187 (0.25 μM), the platelets were added to fibrinogen-coated slides and allowed to settle for 20–30 min. In some experiments, apyrase (0.5 units, Sigma) or ADP (10 μM, Sigma) were included in the incubations before adding the cells to the slides. Adhered platelets were processed for confocal microscopy as described below.

**Perfusion Adhesion Studies**—Perfusions were conducted using one of three parallel plate perfusion chambers as described previously (26). Blood was drawn into acid-citrate-dextrose at a ratio of 1:10 (acid-citrate-dextrose:blood), hirudin (200 units/ml), or low molecular weight heparin (6 units/ml). Under the flow conditions described below, platelets adhered poorly in the presence of low molecular weight heparin, an anticoagulant that does not chelate calcium (ω = 3)," precluding its use in subsequent studies. This is analogous to its effect on platelet adhering to fibronectin (32). The blood (4 ml) was prewarmed at 37 °C for 10 min before the addition of dimethyl sulfoxide (Sigma, 0.001% final) or various concentrations of calyculin A. After an additional 5-min incubation at 37 °C, the blood was perfused through the chamber containing a fibrinogen-coated coverslip positioned 70 mm from the inlet valve. Whole blood was used within 3 h of withdrawal. Flow was conducted using two different approaches. In one approach, blood was allowed to recirculate for 10 min at 10 ml/min through an eight-roller precalibrated peristaltic pump (Cole Parmer). Under these conditions, wall shear rates of 650 and 100/s were achieved. Alternatively, whole blood passed through the chambers one time at a constant flow rate for 1–2 min. Flow rates of 1 and 2 ml/min produced 65 and 520/s shear rates, respectively, which corresponds to flow in veins and small arteries. When indicated, platelet-rich plasma was prepared by centrifugation at 1100 g for 3 min at 21 °C. Platelet counts in the platelet-rich plasma were standardized to 350,000/m³ using a modified Tyrode’s buffer. Calyculin A treatment and perfusion of platelet-rich plasma through the chamber were conducted as described for whole blood. For preparation of surfaces for flow studies, suspensions of 0.1 mg/ml fibrinogen, prewarmed in phosphate-buffered saline, were sprayed onto plastic coverslips using a retouching airbrush (model 100, Badger, Franklin Park, IL) at a nitrogen pressure of 1 atm as described previously (26). Coverslips (18 x 22 mm) were spray-coated with ~300 ul to a final surface density of ~5 μg/cm². The coverslips were kept at 21 °C for up to 12 h. Before perfusion with whole blood or platelet-rich plasma, coverslips were washed for 5 min with a modified Tyrode’s buffer, pH 7.4. For static adhesions, drops of the fibrinogen solution were pipetted onto glass slides and dried at room temperature. The matrix was washed with the modified Tyrode’s buffer prior to the adhesion assay. After perfusion with blood, the coverslips were immediately washed under flow conditions with the modified Tyrode’s buffer for 1 min. The coverslips were removed, fixed in 2.5% glutaraldehyde solution for 15 min, and washed twice with phosphate-buffered saline. After permeabilization with 0.1% Triton X-100 for 3 min, platelets were then washed twice with TBS, blocked for 3 min with 0.1% bovine serum albumin at 37 °C, and permeabilized with 0.1% Triton X-100 before the addition of 3% BSA. As the adherent platelets were processed for confocal microscopy, the coverslips were washed twice with TBS, blocked for 3 min with 0.1% bovine serum albumin at 37 °C, and permeabilized with 0.1% Triton X-100 before the addition of 3% BSA.
Albumin, and stained with rhodamine-conjugated phalloidin. Adhered platelets were imaged using a Bio-Rad MRC-1000 confocal microscope. Platelets were viewed after excitation with an argon laser beam using either an inverted- or upright-staged microscope equipped with a 60× oil immersion or 40× dry objective and an epifluorescent illumination attachment. The percentage of area covered by platelets was determined using a digitized palette and Sigmascan software.

RESULTS AND DISCUSSION

β3 Phosphorylation Correlates with Decreased Outside-in Signaling—Previous studies have shown that calyculin A, an inhibitor of protein seryl/threonyl phosphatase types 1 and 2A, markedly reduces platelet aggregation induced by collagen (18) and low doses of thrombin (17). These data support the idea that calyculin A blocks inside-out signaling linked to αIIbβ3. In the presence of high thrombin doses, calyculin A does not inhibit aggregation but does block cytoskeletal assembly that is usually coupled to an aggregatory response (17). This indicates that calyculin A blocks outside-in signaling by activated αIIbβ3.

To further test this hypothesis, we examined the effect of calyculin A on platelet adhesion to immobilized fibrinogen, a process that depends on outside-in αIIbβ3 signaling. Platelet adhesion was measured in both flow and static assays.

To determine the effect of calyculin A on platelet adhesion in a physiological setting, we used flow conditions. Citrated blood was treated with either vehicle or 100 nM calyculin A and recirculated through a parallel plate perfusion chamber at a wall shear rate of 650/s. The percent area covered by vehicle-treated platelets was ~40% (n = 5) on fibrinogen-coated surfaces (Fig. 1A). Adhesion was blocked by 80 ± 5% (mean ± S.E.; n = 4) by preincubating the blood with 100 nM calyculin A (Fig. 1B). Similarly, calyculin A added directly to platelet-rich plasma inhibited platelet deposition to fibrinogen, establishing that the drug was directly affecting platelets.

As seen in Fig. 1, substantial aggregation occurred when a recirculating system was used. This likely reflects the activation of platelets as a consequence of being exposed to the fibrinogen matrix and to activating agents release from adhered cells. To minimize this aggregatory response, whole blood was passed through the chambers one time at wall shear rates of 65 and 520/s. Under these conditions platelets adhered as singlets (Fig. 2, A and C). Regardless of the flow rate, a decrease in deposition by ~90% (n = 4) was observed by pretreating whole blood with calyculin A (Fig. 2, B and D). The decrease in platelet adhesion in citrated blood occurred in the presence of 100 nM calyculin A. As discussed later, the response to calyculin A occurs at a very sharp concentration threshold. These findings were confirmed using varying preparations of calyculin A.

The dramatic effect of phosphatase inhibitors on the adhesive properties of platelets observed in citrated blood might possibly be exaggerated by the effects of citrate itself in the experimental system. It is known that divalent cations modulate the binding of αIIbβ3 to fibrinogen, and it is possible that...
citrate, as a divalent cation chelator, is modifying platelet interactions that are exaggerating the effect of calyculin A (31). To determine whether the effect of phosphatase inhibition was dependent on citrate, we repeated the adhesion studies using the anticoagulant hirudin, which is not an ion chelator. Platelets from hirudin-treated blood adhered as singlets in the absence of type 1 and 2A phosphatases are inhibited, platelets attach but do not spread. Their ability to attach was further demonstrated by Western analysis of adhered "control" and "calyculin A-treated" platelets using β3 antibodies; similar levels of protein were shown. Increased spreading of calyculin A-treated platelets can be restored by applying calcium ionophore (A23187) to the platelet suspension before they settle onto fibrinogen. The addition of 0.25 μM A23187 to a suspension of calyculin A-treated platelets resulted in increased platelet spreading (Fig. 3C). The reversal of calyculin A inhibition by A23187 demonstrates that inhibition of phosphatases has a specific effect on platelet functions. The apparent effect of A23187 did not result from the release of ADP; the addition of apyrases did not
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reverse the ionophore effect, and the addition of 10 μM ADP did not overcome the effect of calyculin A. The effect of the calcium ionophore appears to be calmodulin-dependent; trifluoperazine prevents the effect of A23187 (Fig. 3D).

Identifying Phosphorylation Sites in αIIbβ3—Because the platelet responses affected by calyculin A are mediated by αIIbβ3, we hypothesized that hyperphosphorylation of αIIbβ3 could be the structural change that modifies αIIbβ3 function. Thus, studies were undertaken to determine the phosphorylation state of αIIbβ3 in calyculin A-treated platelets. The cytoplasmic portion of β3 (residues 716–762) contains 8 potential serine and threonine phosphorylation sites: 7 threonines and 1 serine (27). To determine its phosphorylation state, β3 was immunoprecipitated from platelets that had been labeled with [32P]orthophosphate and treated with calyculin A. Immunoprecipitated β3 was analyzed by SDS-PAGE followed by autoradiography. As seen in Fig. 3, little β3 was phosphorylated in the absence of calyculin A (Fig. 3A), whereas a loss of phosphatase activity by calyculin A treatment led to increased phosphorylation of β3 (Fig. 3B). This correlated with a decrease in spreading. The addition of a calcium ionophore, which partially restores spreading, caused a corresponding dephosphorylation in β3 (Fig. 3C). The major protein band that becomes phosphorylated by calyculin A treatment is β3 as identified by Western analysis (Fig. 4A). Maximal phosphorylation occurred at 100 nM calyculin A (Fig. 5; n = 3), a concentration that inhibits adherence to fibrinogen (as shown in Figs. 1, 2, and 5) and collagen-induced aggregations (18). The same concentration of ionophore that reversed the effect of calyculin A on platelet spreading decreased the phosphorylation of β3 (shown in Fig. 3), which is likely attributable to the activation of the calmodulin-dependent phosphatase, protein phosphatase 2B. Together, the data indicate that an inverse relationship exists between β3 phosphorylation and platelet adhesion reactions.

Phosphoamino acid analysis of β3 showed the presence of only phosphothreonine (Fig. 4B). The sites of phosphorylation of β3 were determined directly by a two-dimensional peptide isolation and sequencing approach. β3 was immunoprecipitated from [32P]-labeled platelets exposed to 100 nM calyculin A, reduced, alkylated, and separated from coprecipitating polypeptides by SDS-PAGE. The Coomassie Blue-stained β3 band was excised and digested in situ with AspN. Proteolytic fragments were separated by reverse phase HPLC. Peptide peaks (and gaps between peaks) were collected, and their content of [32P] was determined by Cerenkov counting. Two prominent [32P]-labeled peptides were recovered (Fig. 6A, N-1 and N-2). Approximately 40% of the [32P] was recovered in N-1, and 60% was recovered in N-2. These radiolabeled peptide fractions were digested with trypsin and rechromatographed by reverse phase HPLC. All the radioactivity from each digest was recovered in single peaks, indicating the presence of only one phosphopeptide from each digest. Both of these phosphopeptides were sequenced, and the resulting analysis revealed that both peptides were residues 749–760 of β3 (Fig. 6A). The sequences of these peptides differed in that the peptide generated from N-1 was missing a detectable signal at threonine 753, whereas the peptide generated from N-2 was missing the threonine residues at positions 751 and 753. Because a phosphorylated residue is not released for detection in the Applied Biosystems protein sequencers, the absence of a signal at these positions is consistent with these threonine residues being the sites of phosphorylation. Using quantitative regression to extrapolate the initial yield of each peptide (Fig. 6B), the singly (Thr753) phosphorylated peptide, N-1, was ~3 pmol, and the doubly (Thr751 and Thr753) phosphorylated peptide, N-2, was ~2 pmol. The amount of peptide [32P] sequenced was initially 160 cpm for N-1.
peptide and 230 cpm for N-2 peptide, and, assuming equal efficiencies for initial coupling during sequencing of these samples, the specific activity for N-1 is 53 cpm/pmol, and that for N-2 is 115 cpm/pmol. These results are consistent with one phosphorylation site in peptide N-1 and two in peptide N-2; this independently confirms the phosphothreonine assignment derived from direct Edman sequencing.

Direct sequence analyses of other peptide peaks obtained from the same AspN digests gave recoveries of ~5–8 pmol (data not shown). Because these peptides represented extracellular regions of $\beta_3$ not related to phosphorylation, the recovery of phosphorylated peptides (~5 pmol) is consistent with a nearly stoichiometric phosphorylation (63–100%) of platelet $\beta_3$. Thus, inhibiting protein serine/threonine phosphatase activity in platelets results in the phosphorylation of almost all $\beta_3$ molecules at Thr753 and the phosphorylation at Thr751 in ~50% of $\beta_3$ molecules.

Conclusion—The present study tests the hypothesis that decreased type 1 and 2A phosphatase activity affects platelet responses by altering the structure of $\alpha_{IIb}\beta_3$. Indeed, the addition of calyculin A decreases platelet adhesion and spreading on fibrinogen. A major finding of these studies is that phosphatase inhibition leads to increased phosphorylation of $\beta_3$ on threonine residues, linking this phosphorylation event to decreased outside-in signaling. These data suggest that protein phosphatases are critical for regulating $\alpha_{IIb}\beta_3$ activity. The phosphorylation of $\beta_3$ occurs on threonine residues adjacent to serine 752. Given that a point mutation of this serine to proline adversely affects $\alpha_{IIb}\beta_3$ function and is linked to reduced aggregation (7), outside-in signaling (8), and ability of $\beta_3$ to recruit signaling proteins (33), it is tempting to speculate that phosphorylation of these serine residues may be a common mechanism to negatively modulate integrin adhesiveness to ligands. In support of this, preliminary studies indicate that calyculin A blocks platelet adhesion to collagen and laminin, which bind via integrins that contain $\beta_1$ subunits.

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