Tyrosine Phosphorylation of the β3 Cytoplasmic Domain Mediates Integrin-Cytoskeletal Interactions*

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Tyrosine phosphorylation of the β3 subunit of the major platelet integrin αIIbβ3 has been shown to occur during thrombin-induced platelet aggregation (1). We now show that a wide variety of platelet stimuli induced β3 tyrosine phosphorylation, but that this phosphorylation occurred only following platelet aggregation. Several lines of evidence suggest that the β3 cytoplasmic domain tyrosine residues and/or their phosphorylation function to mediate interactions between β3 integrins and cytoskeletal proteins. First, phospho-β3 was retained preferentially in a Triton X-100 insoluble cytoskeletal fraction of thrombin-aggregated platelets. Second, in vitro experiments show that the cytoskeletal protein, myosin, associated in a phosphotyrosine-dependent manner with a diphosphorylated peptide corresponding to residues 740–762 of β3. Third, mutation of both tyrosines in the β3 cytoplasmic domain to phenylalanines markedly reduced β3-dependent fibrin clot retraction. Thus, our data indicate that platelet aggregation is both necessary and sufficient for β3 tyrosine phosphorylation, and that this phosphorylation results in the physical linkage of αIIbβ3 to the cytoskeleton. We hypothesize that this linkage may involve direct binding of the phosphorylated integrin to the contractile protein myosin in order to mediate transmission of force to the fibrin clot during the process of clot retraction.

Integrins are a family of heterodimeric transmembrane proteins that link the extracellular matrix to the cytoskeletal/contractile apparatus within a cell (2). These cytoskeletal linkages are characteristically induced by integrin clustering that can occur by the binding of multivalent or immobilized extracellular ligands, often resulting in the assembly of “focal contacts” in cultured cells. Several cytoskeletal proteins (e.g. talin, actin binding protein, α-actinin) directly bind to integrin cytoplasmic domains (3–6), indicating that integrins may interact by multiple mechanisms for focal contact assembly. Focal contact assembly is often followed by signal transduction events such as induction of gene transcription (7) and prevention of apoptosis (8, 9), regulating a diversity of cellular functions from embryonic development to hemostasis (10). Although cytoskeletal and signaling proteins have been identified which bind integrin cytoplasmic domains, major unsolved questions persist. For example, what is the identity of the proteins responsible for the initial interactions of integrins with the cytoskeletal structures? How are these interactions regulated? In this regard, it is relevant to the present study that focal contacts are major sites for protein tyrosine phosphorylation, one of the earliest signaling events observed upon integrin ligation (11).

On platelets, the interaction of the integrin αIIbβ3 with its adhesive ligands, fibrinogen, or von Willebrand factor leads to platelet aggregation and association of αIIbβ3 with the cytoskeleton (12, 13). Under normal conditions, platelet aggregation is the desired response to external trauma, allowing for hemostasis. However, inappropriate platelet aggregation does occur, as in ruptured atherosclerotic plaques, resulting in the formation of occlusive thrombi leading to myocardial infarction or thrombotic stroke (14). The importance of αIIbβ3-mediated events in both hemostasis and thrombosis is underscored in two ways. First, patients who lack, or have mutated, αIIbβ3, a condition known as Glanzmann’s thrombasthenia, have a bleeding disorder that arises from the failure of the platelets to aggregate (15). Second, clinical trials have shown that antagonists for αIIbβ3 ligand binding are effective antithrombotics (16).

αIIbβ3 is involved in both “inside-out” and “outside-in” signaling pathways during platelet aggregation (12). In order to bind soluble forms of its adhesive protein ligands, αIIbβ3 on resting platelets has to undergo a conformational change. This process, the consequence of “inside-out” αIIbβ3 signaling, occurs when agonists such as ADP or thrombin activate platelets. Binding of fibrinogen and von Willebrand factor to αIIbβ3 induces platelet aggregation and αIIbβ3 clustering; the signals transduced by this process are referred to as “outside-in” signaling events. The cytoplasmic domains of the integrin are thought to play a critical role in these signaling events (17–20). In addition, platelet aggregation induces the direct interaction of αIIbβ3 with the cytoskeleton (21, 22). The cytoskeletal proteins talin and α-actinin have been found to act directly with αIIbβ3 (4, 6). Along with αIIbβ3, many other intracellular proteins, including Src and FAK, redistribute to the cytoskeleton of aggregated platelets (22, 23). In these ways, the integrin may play a direct role not only in organizing the cytoskeleton but also in transducing signals to elicit cellular responses. Although it is clear that the cytoplasmic domains of αIIbβ3 are involved in signal transduction and cytoskeletal reorganization events, the precise mechanisms regulating these processes remain to be discovered.

Previously, we showed that tyrosine phosphorylation of β3 occurs upon thrombin-induced platelet aggregation, indicating a potential role for integrin cytoplasmic tyrosine residues in outside-in αIIbβ3 signaling (1). In support of this hypothesis, we...
observed that the signaling adaptor proteins SHC and Grb2 interacted with peptides corresponding to the tyrosine phosphorylated cytoplasmic domain of β₃. The present study shows that tyrosine phosphorylation of β₃ is a unifying event of platelet aggregation and provides in vitro evidence that tyrosine phosphorylation of this integrin subunit may direct its binding to myosin, a specific element contained within the platelet cytoskeleton.

EXPERIMENTAL PROCEDURES

Reagents—Aprotinin, apyrase, aspirin, prostaglandin I₂, phenylmethylsulfonyl fluoride, leupeptin, diisopropyl fluorophosphate, sodium orthovanadate, sodium pyrophosphate, the thromboxane A₂ agonist 9,11-diepoxy-11a-bromo-epoxymethanoprostaglandin F₂α (U46619), control mouse IgG, and Sigmoidate were all purchased from Sigma. The platelet aggregation reagents ADP, epinephrine, and collagen were purchased from Sigma Diagnostics. Thrombin receptor activating peptide (TRAP; Ref. 24) and the cyclic peptide αvβ₃ antagonist MP-9GDWP-Pen-NH₂ (25) were provided by COR Therapeutics, Inc., Medicinal Chemistry Department. Human α-thrombin was purchased from Hematologic Technologies, Inc., and human fibrinogen was from Chromogenix. The α₃β₁ antibody LM609 was generously provided by David Cheresh (Scripps Research Institute) (26). The anti-LIBS-6 monoclonal antibody has been described (27). Anti-human β₃ monoclonal antibody C3a.19.5, which recognizes the cytoplasmic tail of the β₃ subunit, was described previously (11). Anti-myosin monoclonal antibody was from Sigma. The anti-phosphotyrosine antibodies PY-20 and 4G10 were from Transduction Laboratories and Upstate Biotechnology, Inc., respectively. The horseradish peroxidase-conjugated secondary reagent sheep anti-mouse Ig and horseradish peroxidase-conjugated streptavidin were from Amersham Pharmacia Biotech. Hank’s buffered saline solution and Dulbecco’s modified Eagle’s medium were from Life Technologies, Inc. Goat anti-mouse fluorescein isothiocyanate-conjugated Fab’ was from Jackson and Gammunine N was from Miles. Biotinylated integrin cytoplasmic domain peptides, synthesized by SynPep Corporation using solid phase Fmoc (N(9-fluorenyl)methoxycarbonyl) chemistry, were dissolved at a concentration of 2 mg/ml in water and diluted as needed. Chymotrypsin and papain were from Boehringer Mannheim. 4–20% gradient SDS-PAGE gels were from Bio-Rad, and ECL Hyper nitrocellulose was from Amersham Pharmacia Biotech. See-blue molecular mass standards were purchased from Novex.

Platelet Preparation and Aggregation—Blood from healthy volunteers was drawn on the day of use, and washed platelets were prepared as described previously (28) except 0.6 units/ml apyrase and 50 mg/ml prostaglandin I₁ (final concentrations) were present in the collecting solution. Before storage in liquid nitrogen, the platelets (~4–8 × 10⁹/ml cell suspension) were incubated for 1 h at 37 °C in Tyrodes-HEPES buffer (12 m M NaHCO₃, 138 m M NaCl, 5.5 m M glucose, 2.9 m M KCl, 10 m M HEPES, pH 7.4, 1 m M CaCl₂, 0.5 m M MgCl₂) unless otherwise stated. Platelet samples of 0.5 ml were then stirred at 37 °C in a whole blood hematicregometer, and various agonists and conditions were examined. When platelet lysates were not prepared, 4% nonreducing Laemmli sample buffer containing vanadate (37 m M Tris, pH 6.8, 11.8% (v/v) glycerol, 2.36% (w/v) SDS, 2 m M sodium orthovanadate, and 0.002% (w/v) bromophenol blue (final concentration)) was added immediately after aggregation, and samples were boiled for 5 min.

Platelet Lysate Preparation—For two-dimensional gel analysis of detergent-soluble cytoskeletal fractions, platelet lysates were immediately after aggregation by the addition of an equal volume of ice-cold 2× Triton X-100 lysis buffer (1% (v/v) Triton X-100, 100 m M NaCl, 20 m M Tris, pH 7.0, 2 m M EDTA, 2 m M ethyleneglycol-bis(β-aminoethylether)-N,N,N’,N’-tetraacetic acid, 20 m M sodium orthovanadate, 0.01% (w/v) leupeptin, 1% (v/v) phenylmethylsulfonyl fluoride, 1% (v/v) sodium orthovanadate, 2 m M benzamidine, 0.27 m M diisopropyl fluorophosphate, 5 m M sodium pyrophosphate, and 2 m M sodium orthovanadate). The lysate was then centrifuged for 6 min at 15,000 × g to remove the Triton X-100 insoluble cytoskeletal forms during aggregation (22). The supernatant was reserved and 100 μl of 2× RIPA buffer (see below) was added to the pellet and sonicated for 20 min at room temperature in a Branson 5120 Sonicator to resolubilize the pellet. Nonreducing sample buffer (as described above) was added to each of the samples (supernatant and resolublized pellet) and boiled for 5 min.

For ligand blot analysis, platelets were lysed in RIPA buffer (1% (v/v) Triton X-100, 1% (v/v) deoxycholic acid, 0.1% sodium dodecyl sulfate, 5 m M ethylenediamine-tetraacetic acid, 20 m M Tris pH 7.5, 5 m M sodium orthovanadate, 1 m M phenylmethylsulfonyl fluoride, 75 μ M leupeptin, 20 μg/ml aprotinin (final concentrations)) and cytoplasmic actin filaments were sedimented by centrifugation at 15 000 × g for 15 min at 4 °C (22).

Decoration of β₃, Phosphorylation Level—Nonreduced-reduced two-dimensional gel electrophoresis was performed to visualize the characteristic migration of β₃ and assess its phosphorylation state as described previously (1, 29). The two-dimensional gels were transferred to nitrocellulose, and blots were probed with anti-phosphotyrosine antibodies PY-20 and 4G10. The blots were then stripped (according to ECL protocol, Amersham Pharmacia Biotech) and reprobed with the β₃ antibody C3a.19.5 to determine β₃ protein content for each sample. Phosphorylation results were normalized for the total amount of β₃ protein present.

Preparation of Proteins—Myosin was purified from human platelets as described, and purification yielded myosin heavy chain and light chains (30). Controlled proteolytic digests of platelet myosin with papain or chymotrypsin were performed as described (31) except that myosin was not phosphorylated prior to chymotryptic digestion. Papain was activated according to the instructions of the manufacturer. Digests were run on 4–20% SDS-PAGE and subjected to Coomassie Blue staining or transferred to nitrocellulose for ligand blotting.

Ligand Blot Analysis—Platelet lysates or purified myosin were reduced, separated by SDS-PAGE, and transferred to nitrocellulose. The blots were then briefly equilibrated in HEPES blot buffer (25 m M HEPES, 25 m M NaCl, 5 m M MgCl₂, 1 m M dithiothreitol) at 4 °C. The transferred proteins were denatured by 6 m guanidine HCl in HBB for 10 min at 4 °C and renatured by 2-fold dilution of guanidine HCl (10-mi incubations each with 3, 1.5, 0.75 m M, 0.38 m M, 0.19 m M, and 0 m M guanidine HCl in HBB). The blot was blocked in HBB containing 4% bovine serum albumin overnight at 4 °C and probed with 1 μ M biotinylated peptide in HBB containing 0.5% bovine serum albumin for 3 h at room temperature. After washing in Tris-buffered saline (20 m M Tris, 150 m M NaCl, pH 7.4) 0.01% Nonidet P-40 three times at 4 °C, peptide-reactive bands were visualized by incubating the blots in horseradish peroxidase-conjugated streptavidin and employing ECL detection.

CHO Cell Generation and Flow Cytometry—β₃ (Y747F, Y759F) was generated and stably transfected into CHO cells as described (32). For flow cytometric analysis, cells were detached with trypsin, washed in Dulbecco’s modified Eagle’s medium + 25 m M HEPES once and resuspended at 3 × 10⁶ cells/ml in FACS buffer (Hanks buffered saline solution, 4% (v/v) fetal bovine serum, 1% (v/v) bovine serum albumin, 1% normal goat serum, 0.1% Gammunine N, 0.05% sodium azide). The cells were then seeded at 200 μ M/ml, pelleted, and incubated with 5 μ M primary antibodies LM609 or control mouse IgG for 1 h at 4 °C. After two washes, the cells were incubated with 1,200 goat anti-mouse fluorescein isothiocyanate-conjugated Fab’ for 30 min at 4 °C. The cells were washed and resuspended in FACS buffer, and the samples were analyzed by flow cytometry on a FACSort (Becton Dickinson).

Clot Retraction Assays—Clot retraction experiments were performed as described with minor modifications (33). In brief, cells were trypsinized, washed twice, and resuspended in Dulbecco’s modified Eagle’s medium + 25 m M HEPES. 0.5 m l of cell suspension containing 5 × 10⁶ cells was mixed with 0.1 m l of fibronecin-depleted plasma in a 12 × 70-mm glass tube treated with Sigmacoat. Fibrin clots were formed by adding 1 unit/ml thrombin and allowed to retract at 37 °C over a 2–3-h period. The extent of clot retraction was measured by removing and weighing the clot.
Tyrosine Phosphorylated $\beta_{3}$-Cytoskeletal Interactions

**TABLE I**

| Agonist       | Aggregation | $\beta_{3}$ Phosphorylation | Phosphotyrosine $\beta_{3}$ stimulated/control |
|---------------|-------------|----------------------------|---------------------------------------------|
| Thrombin      | Yes         | Yes                        | $++$                                        |
| Thrombin, cyclic | No         | No                         | 0                                           |
| RGD           | No          | No                         | 0                                           |
| TRAP          | Yes         | Yes                        | $++$                                        |
| ADP, fibrinogen | Yes       | Yes                        | $++$                                        |
| U44619, fibrinogen (unstirred) | No       | No                         | 0                                           |
| ADP, epinephrine, fibrinogen | Yes       | Yes                        | $++$                                        |
| Collagen      | Yes         | Yes                        | $++$                                        |
| LIBS6, fibrinogen | Yes     | Yes                        | 0                                           |
| LIBS6, fibrinogen, cyclic RGD | No        | No                         | 0                                           |
| U44619        | Yes         | Yes                        | $++$                                        |
| U44619, cyclic | No          | No                         | 0                                           |
| RGD           | No          | No                         | 0                                           |

The reactions were terminated by the addition of SDS-sample buffer to the aggregometer tubes either at maximal ADP-induced aggregation or after aggregation was fully reversed (Fig. 1C). As described above, ADP-induced aggregation led to an increase in $\beta_{3}$ phosphorylation, as well as the phosphorylation of a number of other substrates, similar to the response induced by thrombin (Fig. 1A). When samples were obtained in which ADP-induced platelet aggregation had reversed, no tyrosine phosphorylation of $\beta_{3}$ was observed (Fig. 1A). In contrast, when thrombin-induced platelet aggregates were maintained in suspension for up to 10 min prior to addition of SDS-sample buffer, no reversal of $\beta_{3}$ tyrosine phosphorylation was observed (data not shown).

Platelets aggregated in response to stimulation through the collagen or thromboxane $A_{2}$ receptors also showed a marked increase in $\beta_{3}$ tyrosine phosphorylation (Table I). However, if platelet aggregation was prevented by cyclic RGD peptide (a competitive inhibitor of $\alpha_{IIb}\beta_{3}$), $\beta_{3}$ phosphorylation was not observed (Table I). Together, these data indicate that many platelet agonists can induce aggregation-dependent tyrosine phosphorylation of $\beta_{3}$.

In the previous experiments, platelet agonists were used to induce fibrinogen binding and subsequent aggregation. To determine whether $\beta_{3}$ tyrosine phosphorylation can occur in the absence of an agonist, platelet aggregation was induced by anti-LIBS6, a $\beta_{3}$-specific antibody that, upon binding to $\beta_{3}$, activates the receptor such that it becomes competent to bind fibrinogen. This activation occurs even in the absence of detectable platelet stimulation (27). The LIBS6 antibody induced smaller platelet aggregates, and aggregation took slightly longer than that induced by classical platelet agonists. Nevertheless, an average 4-fold increase in the level of $\beta_{3}$ phosphorylation was still observed (Table I). Again, the addition of cyclic RGD peptide inhibited both platelet aggregation and $\beta_{3}$ phosphorylation. Thus, $\alpha_{IIb}\beta_{3}$-dependent platelet aggregation is both necessary and sufficient for tyrosine phosphorylation of $\beta_{3}$.

**RESULTS**

**$\beta_{3}$ Tyrosine Phosphorylation Is a General Consequence of Platelet Aggregation—**We previously reported that aggregation of platelets by thrombin in a stirred suspension induced a marked increase in the tyrosine phosphorylation of the $\beta_{3}$ subunit of $\alpha_{IIb}\beta_{3}$ (1). To determine whether this effect was thrombin-specific, we examined $\beta_{3}$ tyrosine phosphorylation in response to various agonists. Adding ADP or ADP + epinephrine to a stirred suspension of platelets in the presence of added fibrinogen induced platelet aggregation and an increase in $\beta_{3}$ phosphorylation, similar to that seen in thrombin-aggregated platelets (Table I). In contrast, when ADP was added in the absence of added fibrinogen or was added without stirring, no platelet aggregation occurred and no increase in $\beta_{3}$ tyrosine phosphorylation was seen. ADP added in this manner did, however, induce platelet stimulation since other substrates were tyrosine phosphorylated and fibrinogen binding on unstirred preparations occurred (data not shown). An illustration of the increase in $\beta_{3}$ tyrosine phosphorylation upon thrombin- or ADP-induced platelet aggregation is shown in Fig. 1A. Thus, ADP and ADP + epinephrine induced tyrosine phosphorylation of $\beta_{3}$ in an aggregation-dependent manner.

Threshold concentrations of ADP can result in reversible platelet aggregation (34, 35). We next determined the effect of reversal of ADP-induced aggregation on $\beta_{3}$ tyrosine phosphorylation. Washed platelets, resuspended in a buffer containing fibrinogen and Ca$^{2+}$, were stirred with ADP. Platelet aggregation occurred but, as illustrated in Fig. 1C, reversed with time.
it technically difficult to observe interactions between cytoskeletal and other proteins in vivo. Thus, to address this issue, we employed an in vitro ligand blotting approach in an attempt to identify candidate proteins that, by binding to tyrosine-phosphorylated cytoplasmic domain of $\beta_3$, could direct association of $\alpha_{IIb}^\beta_3$ to the cytoskeleton. Proteins from platelet lysates were separated by SDS-PAGE, transferred to nitrocellulose and renatured on the blot. Synthetic peptides corresponding to the cytoplasmic domain of $\beta_3$, which contained biotin at their amino termini, were used to probe the nitrocellulose blot (Fig. 3A). The direct binding of peptide to renatured proteins was visualized by the addition of streptavidin-horseradish peroxidase and detected by chemiluminescence. As illustrated in Fig. 3B, the $\beta_3$ peptide corresponding to residues 740–762 in the $\beta_3$ cytoplasmic domain bound to a 200-kDa protein in ligand blot analysis of platelet lysates. Binding was detected only when both $\beta_3$ tyrosine residues (Tyr-747 and Tyr-759) were phosphorylated; the nonphosphorylated $\beta_3$ peptide and singly phosphorylated peptides failed to bind the 200-kDa protein under the conditions of this assay (data not shown). To test the specificity of binding of the phosphorylated cytoplasmic domain of $\beta_3$ to this 200-kDa protein, a second, doubly phosphorylated $\beta_3$ peptide containing a naturally occurring single point mutation of $\beta_3$, found in a patient with Glanzmann’s thrombasthenia, was used (36). $\alpha_{IIb}\beta_3$ harboring this mutation does not support platelet aggregation (36) and is defective in signaling (37).
tyrosine phosphotyrosine-binding specificity of diphosphorylated β₃ peptide.

Binding of the diphosphorylated β₃ peptide to purified myosin in renatured blot (Fig. 4). A, CHO cells transfected with wild-type β₃ or Y(747F) and Y(759F) β₃ were incubated with control mouse IgG (control for wild-type β₃, shown in thin line, median channel 5.47; for mutant β₃, median channel was 5.86, not shown), or LM609 (thick line for wild-type β₃, median channel 108.45; dashed line for Y(747F,759F) β₃, median channel 128.64) and analyzed by flow cytometry. B, wild-type β₃ CHO transfectants and (Y(747F) and Y(759F)) β₃ CHO transfectants were subjected to the fibrin clot retraction assay as described under “Experimental Procedures.” The results from five experiments were expressed as clot weight ± S.E.

The Tyrosine Residues within the β₃ Cytoplasmic Domain Are Important for β₃-dependent Fibrin Clot Retraction—The results reported above indicate that the phosphorylation of the β₃ cytoplasmic tyrosine residues might influence integrin-cytoskeletal interactions. This finding predicts that mutating the tyrosine residues of β₃ should affect cellular functions dependent upon integrin-cytoskeletal interactions. One such function is the β₃-dependent retraction of fibrin clots, where the integrin is believed to function as a transmembrane linkage between extracellular adhesion proteins and the cytoskeleton. Due to the difficulty of genetically manipulating platelets, we used a CHO cell system that has proven useful in the study of integrin function (19, 39) to directly address this issue. It has previously been shown that CHO cells transfected with wild-type β₃ will express the β₃ on the cell surface in conjunction with endogenous α₃ chains (19). In contrast to nontransfected CHO cells, the β₃-transfected CHO cells gain the ability to retract fibrin clots (Ref. 19, and data not shown). We generated stable CHO cell lines expressing either wild-type β₃ or β₃ bearing the con-
sorptive Y747F and Y759F mutations. As illustrated in Fig. 5A, FACS analysis with the αβ3-specific antibody LM609, confirmed that these transfectants expressed similar levels of αβ3 or αβ3(Y747F, Y759F) at the cell surface. When the two CHO cell lines were used in the fibrin clot retraction assay, it was found that clot retraction was reduced markedly in the Y747F, Y759F transfectants, as demonstrated by a 50 ± 11.9% increase in clot weight compared with the clot weights obtained with the wild-type β3-expressing CHO cells (Fig. 5B).

To assess whether the presence of αIIbβ3 would alter the effect of the double tyrosine to phenylalanine mutations on β3-mediated fibrin clot retraction, similar experiments were performed with CHO cells that had been co-transfected with αIIb in addition to the mutant or wild-type β3 cDNA. These cells expressed both αβ3 and αIIbβ3 on their surface, and essentially the same results were obtained: the cells bearing Y747F and Y759F β3 showed about a 50% decrease in their ability to retract fibrin clots when compared with those cells expressing wild-type β3 (data not shown). Thus, the tyrosine residues within β3 do indeed play a critical role in β3 integrin-mediated fibrin clot retraction.

**DISCUSSION**

A well established function of integrin cytoplasmic domains is as a bridge between extracellular matrix proteins and the cytoskeletal/contractile machinery within a cell. The data presented in this study indicate that the two tyrosine residues within the β3 cytoplasmic domain may be important in mediating some of these interactions and suggest a way in which a modification of these residues, namely by phosphorylation, may also be involved in these bridging processes. We have found that the tyrosine phosphorylation of the β3 subunit of αIIbβ3 occurs as a general consequence of platelet aggregation. That this phosphorylation may in turn affect the interaction of the β3 integrin with the platelet cytoskeleton is indicated by the following data. First, phosphorylated β3 is located preferentially within the detergent-insoluble cytoskeletal fraction of aggregated platelets; and second, the contractile protein myosin can bind directly, in a phosphotyrosine-dependent manner, to peptide corresponding to the cytoplasmic domain of β3. Furthermore, functional data obtained by analysis of CHO cells bearing a β3 in which both cytoplasmic tyrosine residues were mutated to phenylalanines also indicates the importance of these β3 tyrosines in the β3-dependent retraction of fibrin clots. We have previously demonstrated the interaction of tyrosine-phosphorylated β3 with the signaling proteins SHC and Grb2 (1) and hypothesized that tyrosine phosphorylation of β3 allowed for the recruitment of signaling complexes to the membrane. Our new studies indicate that in addition to the role of β3 tyrosine phosphorylation in binding signaling proteins, the phosphorylation may also influence the interaction of β3 with the myosin-based contractile apparatus and in doing so play an important role in integrin-dependent functions involving cytoskeletal reorganization.

The present data demonstrate that platelet aggregation is both necessary and sufficient to induce tyrosine phosphorylation of β3. First, conditions that only induced the active conformation of αIIbβ3 and did not allow for aggregation to occur, such as ADP stimulation in the absence of fibrinogen or in the presence of an inhibitory RGD peptide, did not induce β3 tyrosine phosphorylation. Second, platelet aggregation induced by LIBS6 independent of platelet stimulation also resulted in β3 tyrosine phosphorylation. Third, β3 tyrosine phosphorylation did not occur under conditions that induced ligand occupancy of αIIbβ3 but not platelet aggregation, such as the addition of ADP and fibrinogen in the absence of stirring. Last, a reversal of aggregation-induced β3 tyrosine phosphorylation was observed upon reversal of platelet aggregation. In all instances, platelet aggregation, with the subsequent platelet-platelet interactions, was absolutely required for β3 tyrosine phosphorylation.

Early studies established that a significant portion of αIIb and β3 could be isolated with cytoskeletal structures in thrombin-aggregated, but not activated, platelets (21), suggesting that platelet aggregation induces the association of αIIbβ3 with the platelet cytoskeleton. It was proposed that this integrin became Triton X-100 detergent-insoluble because of the macromolecular associations between the platelet membrane surfaces and actin filaments. Morphological studies have also demonstrated that fibrinogen binding to αIIbβ3 induced its interaction with the cytoskeleton since the membrane-bound integrin appeared to be co-aligned with cytoskeletal structures of the platelet (40). Further, Fox and co-workers demonstrated an aggregation-dependent redistribution of αIIbβ3 from the membrane skeleton to the Triton X-100-insoluble fraction of platelets (22). Several tyrosine kinases and other tyrosine-phosphorylated proteins also redistributed to the cytoskeleton upon platelet aggregation (22). The present data points to a possible mechanism for the redistribution of αIIbβ3 to the cytoskeleton in aggregated platelets. Examination of the phospho-β3 distribution between Triton X-100 soluble and insoluble fractions of aggregated platelets demonstrated that phosphorylated β3 preferentially redistributes to the cytoskeletal fraction. The observations that tyrosine phosphorylation of the β3 cytoplasmic domain is a common consequence of aggregation by a wide range of platelet agonists and is a potential player in driving the redistribution of β3 to the cytoskeleton prompted us to examine biochemically whether β3 phosphorylation plays a part in mediating novel interactions of the receptor with the platelet cytoskeleton.

The binding of integrin cytoplasmic domains to cytoskeletal proteins is not unprecedented, and although little in vivo data exist, due to the technical difficulties of working with poorly soluble cytoskeletal proteins, a variety of in vitro strategies have been used to discover and characterize such interactions. Interactions between talin and β3 integrin cytoplasmic domain were first studied using equilibrium gel filtration of purified proteins (3). In solid phase binding assays, talin was found to bind directly with αIIbβ3 integrin cytoplasmic tail sequences and to purified αIIbβ3 (4). α-Actinin has been shown to bind directly to the cytoplasmic domain of β3 as well as to purified αIIbβ3 (6). In another study, the cytoskeletal protein skelelin interacted with the β3 cytoplasmic domain in a yeast two-hybrid screen and with peptides corresponding to the membrane proximal regions of β3 and β4 (41). Actin binding protein has also been demonstrated to bind directly to the cytoskeletal domain of β3 integrin using peptide affinity chromatography (42) and to the dimerized β3 cytoplasmic domains using a novel experimental strategy (5). However, the mechanisms that regulate these integrin-cytoskeletal interactions are unknown.

Given that tyrosine phosphorylation of β3 is a general consequence of platelet aggregation and appears to direct the redistribution of αIIbβ3 to the cytoskeleton, we postulate that β3 tyrosine phosphorylation could be a general mechanism for regulating integrin-cytoskeletal interactions. Fittingly, members of the Src family of tyrosine kinases are known to selectively redistribute with a subpopulation of αIIbβ3 to the actin cytoskeleton in aggregated platelets (22, 23). This redistribution is reduced by treatment of platelets with tyrosine kinase inhibitors, suggesting that tyrosine kinases, either directly or through the phosphorylation of other proteins, may regulate the cytoskeletal attachment of αIIbβ3 (43). Further, αIIbβ3-mediated clot retraction is inhibited by tyrosine kinase inhibitors (43). Although there is circumstantial evidence that certain
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Integrin-cytoskeletal interactions are phosphotyrosine-dependent, experimental data directly supporting this hypothesis are lacking.

In the present work, we used in vitro ligand binding methodology to observe a novel interaction between myosin and a \( \beta_3 \) integrin cytoplasmic domain peptide that was regulated by tyrosine phosphorylation. Phosphorylated and nonphosphorylated integrin cytoplasmic domain peptides were synthesized and used to identify a direct and tyrosine phosphorylation-dependent interaction between the \( \beta_3 \) cytoplasmic domain peptide and platelet myosin heavy chain. Since the doubly phosphorylated \( \beta_3 \) peptide bound specifically to myosin, this contractile protein may possess tandem phosphotyrosine binding regions analogous to the tandem SH2 domains of the tyrosine kinases ZAP-70 or Syk, which bind ITAM domains in immune receptor complexes (44, 45). However, to our knowledge, classic phosphotyrosine binding motifs in platelet myosin heavy chain have not yet been identified. We further observed that the tail domain of myosin was responsible for its interaction with \( \beta_3 \). Interestingly, the tail region of myosin serves as an anchor so that it can translocate actin and has been hypothesized to bind certain myosin isoforms to cell or organellar membranes (46). Together these data suggest that the tyrosine phosphorylated \( \beta_3 \) binding domain of myosin exists on the tail region of myosin heavy chain and that this domain contains previously unrecognized phosphotyrosine binding motifs. These binding motifs may allow for the interaction of phosphorylated \( \beta_3 \) with the cytoskeletons of aggregated platelets, providing alignment for certain postaggregation contractile events, such as clot retraction, to occur.

Although our in vitro data strongly suggest that tyrosine phosphorylation of the \( \beta_3 \) cytoplasmic domain can allow for \( \alpha_{\text{IIb}}\beta_3 \) interaction with myosin, this conclusion was not possible to confirm in vivo because of the aforementioned problems associated with working with many cytoskeletal proteins. Indeed, myosin is insoluble at physiological salt concentrations; only highly stringent co-immunoprecipitation conditions could be employed using detergent lysates that are well known to disrupt protein-protein interactions. In this case, the problem is compounded by the fact that the major portion of tyrosine phosphorylated \( \alpha_{\text{IIb}}\beta_3 \) does itself translocate to the insoluble cytoskeleton in aggregated platelets. Also, robust tyrosine dephosphorylation mechanisms are present in platelets (47), which make it difficult to preserve tyrosine phosphorylation of \( \beta_3 \) except under denaturing conditions (e.g., by the addition of SDS-containing sample buffer) or in rapid postfocytic fractionations (e.g., cytoskeleton isolation) hampering immunoprecipitation experiments. Therefore, our data do not preclude other, possibly phosphotyrosine-independent, interactions between the cytoplasmic domains of \( \alpha_{\text{IIb}}\beta_3 \) and myosin. If other such interactions do indeed exist, it is attractive to hypothesize that tyrosine phosphorylation of \( \beta_3 \) cytoplasmic domain, possibly at only one of the tyrosine residues, could induce a more stable and avid interaction between previously-associated \( \alpha_{\text{IIb}}\beta_3 \) and myosin.

Thus, our data suggest that tyrosine phosphorylation of the \( \beta_3 \) cytoplasmic tail may regulate a direct association with myosin, providing anchorage of surface \( \beta_3 \) integrins to the contractile apparatus. A possible consequence of this interaction is to allow for \( \alpha_{\text{IIb}}\beta_3 \)-mediated clot retraction in platelets. We addressed the role of the \( \beta_3 \) cytoplasmic tyrosines in clot retraction using CHO cells transfected with \( \beta_3 \). Previous studies using such a CHO cell expression system have proven useful for analyzing the role of both \( \alpha_{\text{IIb}} \) and \( \beta_3 \) integrin cytoplasmic domains in \( \alpha_{\text{IIb}}\beta_3 \) signaling and adhesive functions (17–19, 36). In particular, CHO cells transfected with \( \alpha_{\text{IIb}}\beta_3 \) gain the ability to contract fibrin clots, whereas both untransfected CHO cells and cells expressing the S752P Glanzmann’s mutant \( \alpha_{\text{IIb}}\beta_3 \) fail to do so (36). Another expression system, in which CS-1 melanoma cells are transfected with a cDNA encoding the integrin \( \beta_3 \) subunit, has been used to characterize the roles of the \( \alpha_\beta_3 \) integrin cytoplasmic domains in adhesion, spreading, and migration on vitronectin (20). Although clot retraction was not addressed in this study, mutating either tyrosine 747 or 759 on \( \beta_3 \) to phenylalanine had little or no effect on other \( \alpha_\beta_3 \) adhesive events (20). In the present work, CHO cells bearing the Y747F and Y759F \( \beta_3 \) cDNA displayed a pronounced defect in fibrin clot retraction: the first demonstration of an effect of \( \beta_3 \) tyrosine to phenylalanine mutations on a biologically relevant event. Since integrins are believed to support clot retraction by providing the transmembrane linkage between extracellular adhesive proteins and the contractile cytoskeleton (19), it is interesting to hypothesize that, by mutating the tyrosine residues within the \( \beta_3 \) cytoplasmic domain, we have disrupted the phosphotyrosine-dependent integrin-myosin interaction and that this could account for the defective clot retraction observed in the mutant CHO cell transfectants.

Our working hypothesis of the role of \( \beta_3 \) cytoplasmic domain tyrosine phosphorylation in platelet function can be summarized as follows: the receptor is phosphorylated as a common consequence of aggregation in response to a number of platelet agonists. Although direct associations of known tyrosine kinases with \( \alpha_{\text{IIb}}\beta_3 \) have not yet been detected, members of the Src family of tyrosine kinases are capable of phosphorylating the receptor in vitro (1) and Src and Lyn can be cross-linked to \( \beta_3 \) in intact platelets treated with chemical cross-linking agents (48). Once phosphorylated, the \( \beta_3 \) integrin tails are capable of associating with signaling proteins SHC and Grb2 to potentially initiate outside-in signaling cascades (1). In addition to providing a scaffold for the recruitment of signaling complexes to the membrane, the doubly phosphorylated cytoplasmic domain of \( \beta_3 \) can also bind to cytoskeletal proteins. In particular, the present work demonstrates direct binding of a doubly tyrosine-phosphorylated \( \beta_3 \) integrin cytoplasmic domain peptide to myosin and further reveals that replacement of these tyrosine residues with phenylalanines in \( \alpha_{\text{IIb}}\beta_3 \)-transfected CHO cells results in defective \( \beta_3 \) integrin-mediated retraction of fibrin clots. In light of these data, we postulate that phosphorylation of \( \beta_3 \) integrin cytoplasmic domain may be an important mechanism for regulating a direct myosin-integrin interaction. Inhibition of this interaction may interfere with the transmission of the mechanical forces that regulate processes such as clot retraction and cell motility. Proving or disproving such hypotheses will be the focus of future work.

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Note Added in Proof—While this manuscript was in review, a manuscript was published which also reported effects of \( \beta_3 \) tyrosine mutations on clot retraction (Blystone, S. D., Williams, M. D., Slater, S. E., and Brown, E. J. (1997) J. Biol. Chem. 272, 28757–28761).

REFERENCES

1. Law, D. A., Nannizzi-Alaimo, L., and Phillips, D. R. (1996) J. Biol. Chem. 271, 10811–10815
2. Hynes, R. O. (1992) Cell 69, 11–25
3. Horwitz, A., Duggan, K., Buck, C., Beckerle, M. C., and Burridge, K. (1986) Nature 320, 531–533
4. Knezevic, I., Leisner, T. M., and Lam, S. C. T. (1996) J. Biol. Chem. 271, 16416–16421
5. Plaff, M., Liu, S., Erle, D., Burrell, K., Campbell, I., and Ginsberg, M. H. (1997) J. Biol. Chem. 273, 6104–6109
6. Otey, C. A., Pavalko, F. M., and Burrell, K. (1990) J. Cell Biol. 111, 721–729
7. Werb, Z., Treimle, P. M., Behrendt, O., Crowley, E., and Damsky, C. H. (1989) J. Cell Biol. 109, 877–889
8. Meredith, J. E., Fazeli, B., and Schwartz, M. A. (1993) Mol. Biol. Cell. 4, 953–961
9. Frisch, M. S., and Francis, H. (1994) J. Cell Biol. 124, 619–626
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10. Clark, E. A., and Brugge, J. S. (1995) *Science* **268**, 233–238
11. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) *J. Biol. Chem.* **267**, 23439–23442
12. Shattil, S. J., Ginsberg, M. H., and Brugge, J. S. (1994) *Curr. Opin. Cell Biol.* **6**, 695–704
13. Phillips, D. R., Charo, I. F., Parise, L. V., and Fitzgerald, L. A. (1988) *Blood* **71**, 831–843
14. Davies, M. J. (1996) *Circulation* **94**, 2013–2020
15. George, J. N., Caen, J. P., and Nurden, A. T. (1990) *Blood* **75**, 1383–1395
16. Lefkovits, J., and Topol, E. J. (1996) *Eur. Heart J.* **17**, 9–18
17. Hughes, P. E., O'Toole, T. E., Ylanne, J., Shattil, S. J., and Ginsberg, M. H. (1995) *J. Biol. Chem.* **270**, 12411–12417
18. Ylanne, J., Huuskonen, J., O'Toole, T. E., Ginsberg, M. H., Virtanen, I., and Gahmberg, C. G. (1995) *J. Biol. Chem.* **270**, 9550–9557
19. Ylanne, J., Chen, Y., O'Toole, T. E., Loftus, J. C., Takada, Y., and Ginsberg, M. H. (1993) *Cell Biol.** 86**, 77–86
20. Fox, J. E., Lipfert, L., Clark, E. A., Reynolds, C. C., Austin, C. D., and Brugge, J. S. (1993) *J. Biol. Chem.* **268**, 25973–25984
21. Fox, J. E., and Phillips, D. R. (1982) *J. Biol. Chem.* **257**, 4120–4126
22. Phillips, D. R., Naughton, M. A., Teng, W., Rose, J. W., Phillips, D. R., Nannizzi, L., Arfsten, A., Campbell, A. M., and Charo, I. F. (1993) *J. Biol. Chem.* **265**, 1066–1073
23. Chen, Y. P., Djaffar, I., Pidard, D., Steiner, B., Ciutat, A. M., Caen, J. P., and Rosa, J. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10169–10173
24. Chen, Y. P., O'Toole, T. E., Ylanne, J., Rosa, J. P., and Ginsberg, M. H. (1994) *Blood* **84**, 1857–1865
25. Glenney, J. R., Jr., Zokas, L., and Kamps, M. P. (1988) *J. Immunol. Methods* **109**, 277–285
26. Simmons, S. R., and Albrecht, R. M. (1997) *J. Lab. Clin. Med.* **128**, 39–50
27. Sharma, C. P., Ezrell, R. M., and Arnaout, M. A. (1995) *J. Immunol.* **154**, 3461–3470
28. Schoenwaelder, S. M., Jackson, S. P., Yuan, Y., Teasdale, M. S., Salem, H. H., and Mitchell, C. A. (1994) *J. Biol. Chem.* **269**, 32479–32487
29. Ishiwashima, M., Irving, B. A. van Oers, N. S., Chan, A. C., and Weiss, A. (1994) *Science* **263**, 1136–1139
30. Rowley, R. B., Burkhardt, A. L., Chao, H. G., Matsueda, G. R., and Bolen, J. B. (1988) *J. Biol. Chem.* **263**, 11590–11594
31. Sellers, J. R., Soheir, M. S., Faust, K., Bungur, A. R., and Harvey, E. V. (1988) *Biochemistry* **27**, 6977–6982
32. Hughes, P. E., Renshaw, M. W., Piaff, M., Forreth, J., Keivens, V. M., Schwartz, M. A., and Ginsberg, M. H. (1997) *Cell* **86**, 521–530
33. Chen, Y. P., O'Toole, T. E., Lesng, L., Liu, B. Q., Diaz-Gonzalez, F., and Ginsberg, M. H. (1995) *Blood* **86**, 2606–2615
34. Marguerie, G. A., Plow, E. F., and Edgington, T. S. (1979) *J. Biol. Chem.* **254**, 5357–5363
35. Mustard, J. F., Perry, D. W., Kinlough-Rathbone, R. L., and Packham, M. A. (1975) *Am. J. Physiol.* **228**, 1757–1765
36. Chen, Y. P., Djaffar, I., Pidard, D., Steiner, B., Ciutat, A. M., Caen, J. P., and Rosa, J. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10169–10173
37. Chen, Y. P., O'Toole, T. E., Ylanne, J., Rosa, J. P., and Ginsberg, M. H. (1994) *Blood* **84**, 1857–1865
38. Marguerie, G. A., Plow, E. F., and Edgington, T. S. (1979) *J. Biol. Chem.* **254**, 5357–5363
39. Mustard, J. F., Perry, D. W., Kinlough-Rathbone, R. L., and Packham, M. A. (1975) *Am. J. Physiol.* **228**, 1757–1765
40. Simmons, S. R., and Albrecht, R. M. (1997) *J. Lab. Clin. Med.* **128**, 39–50
41. Reddy, R. B., Gascard, P., Price, M. G., and Fox, J. E. (1996) *Circ. Suppl. I* **94**, 1–88
42. Sharma, C. P., Ezrell, R. M., and Arnaout, M. A. (1995) *J. Immunol.* **154**, 3461–3470
43. Schoenwaelder, S. M., Jackson, S. P., Yuan, Y., Teasdale, M. S., Salem, H. H., and Mitchell, C. A. (1994) *J. Biol. Chem.* **269**, 32479–32487
44. Ishiwashima, M., Irving, B. A. van Oers, N. S., Chan, A. C., and Weiss, A. (1994) *Science* **263**, 1136–1139
45. Rowley, R. B., Burkhardt, A. L., Chao, H. G., Matsueda, G. R., and Bolen, J. B. (1988) *J. Biol. Chem.* **263**, 11590–11594
46. Sellers, J. R., and Goodson, H. V. (1995) *Protein Profile* **2**, 1323–1423
47. Frangioni, J. V., Oda, A., Smith, M., Salzman, E. W., and Neel, B. G. (1993) *EMBO J.* **12**, 4843–4856
48. Dorathy, D. J., Berndt, M. C., and Burns, G. F. (1995) *Biochem. J.* **309**, Pt. 2, 481–490