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Adhesion-Induced Unclasping of Cytoplasmic Tails of Integrin $\alpha_{IIb}\beta_3$[†,‡]

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Abstract

Integrin $\alpha_{IIb}\beta_3$ plays a pivotal role in hemostasis and thrombosis by mediating adhesive interactions of platelets. Binding of $\alpha_{IIb}\beta_3$ to its physiological ligands, immobilized fibrinogen and fibrin, induces outside-in signaling in platelets, leading to their adhesion and spreading even without prior stimulation by agonists. Implicit in these phenomena is a requirement for the linkage between integrins' cytoplasmic tails and intracellular proteins. However, the nature of the initiating signal has not been established. In this study, we examined whether binding of $\alpha_{IIb}\beta_3$ to immobilized fibrin(ogen), per se, triggers interaction of the integrin with cytoplasmic proteins. Using the integrin-binding skelemin fragment as a marker of exposure of residues involved in the clasp between α_{IIb} and β_3 cytoplasmic tails, we showed that its binding site in the membrane-proximal β_3 715–730 segment is cryptic and becomes exposed as a result of binding of isolated $\alpha_{IIb}\beta_3$ to immobilized ligands. Furthermore, the skelemin-like protein present in platelets and CHO cells does not associate with $\alpha_{IIb}\beta_3$ in resting platelets or suspended $\alpha_{IIb}\beta_3$-expressing CHO cells but is recruited to integrin during cell adhesion. In addition, not only β_3 but also the membrane-proximal 989–1000 segment of the α_{IIb} cytoplasmic tail binds the skelemin fragment. Finally, the same residues, α_{IIb} Val^{990}, α_{IIb} Arg^{995}, and β_3 His^{722}, involved in the formation of the clasp between the tails are also required for skelemin binding. These studies suggest that ligation of $\alpha_{IIb}\beta_3$ by immobilized ligands during platelet adhesion induces a transmembrane conformation change in the integrin, resulting in unclasping of the complex between the membrane-proximal parts of cytoplasmic tails, thereby unmasking residues involved in binding the skelemin-like

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SUPPORTING INFORMATION AVAILABLE

Substitutional peptide libraries derived from the sequence of the β_3 715–730 fragment. This material is available free of charge via the Internet at http://pubs.acs.org.

protein. Thus, the junction between α_{IIb} and β_3 cytoplasmic tails may contain the critical structural information for the initiation of outside-in signaling.

Integrins are noncovalently associated α–β heterodimer transmembrane receptors that mediate adhesive interactions of cells with the extracellular matrix and with other cells. Integrin-mediated adhesion initiates diverse intracellular reactions through a process known as “outside-in” signaling which results in changes in cellular behavior, including spreading, migration, differentiation, and altered gene expression. Intracellular reactions induced by these receptors are numerous and generally include coupling of integrins with the cytoskeleton and recruitment of signaling molecules. These processes are mediated by integrin cytoplasmic tails which thus serve as the focal points for initiation of intracellular signaling. Although their most conspicuous function is adhesion, integrins can also bind soluble ligands. However, this interaction is strictly dependent on the integrin’s prior activation, which is triggered by binding of agonists to their corresponding G-protein-coupled receptors or receptor tyrosine kinases, and then relayed to the integrin cytoplasmic tails. This process is known as “inside-out” signaling.

Among other family members, platelet integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa) is clearly distinguished by its ability to bind both soluble fibrinogen and the insoluble fibrin(ogen) matrix in the two physiologically relevant reactions, platelet aggregation and platelet adhesion, which occur at sites of vascular injury. It is well established that binding of soluble fibrinogen requires platelet stimulation and inside-out signaling. Thus, upon platelet activation by physiological stimuli (e.g., ADP or thrombin), integrin $\alpha_{IIb}\beta_3$ undergoes a conformational change from an inactive to an active state competent to bind soluble fibrinogen (1, 2). The conformational change is thought to be initiated at the cytoplasmic domain and then propagated via the transmembrane segments to the extracellular part of $\alpha_{IIb}\beta_3$ (reviewed in ref 3).

The cytoplasmic domain of $\alpha_{IIb}\beta_3$ is composed of the α_{IIb} (residues 989–1008) and β_3 (residues 716–762) cytoplasmic tails which form a complex (4–6) (Figure 1). Recent structural analyses revealed that the primary interface between the tails is formed by the helical membrane-proximal α_{IIb} Lys^{989}–Leu^{1000} and β_3 Lys^{716}–Glu^{726} segments and consists of bonds between electrostatic and hydrophobic residues (7). The electrostatic interface involves the side chain of α_{IIb} Arg^{995} which binds two acidic residues in the β_3 tail: Asp^{723} and Glu^{726}. The hydrophobic interface involves α_{IIb} Val^{990} and α_{IIb} Phe^{992} which interact with Leu^{718}, Ile^{719}, and Ile^{721} in the β_3 tail. The aromatic ring of α_{IIb} Phe^{992} also interacts with β_3 His^{722}. Mutations of several residues within the interface, including Arg^{995} and Phe^{992} in the α_{IIb} tail and Asp^{723} in the β_3 tail, have previously been shown to activate the receptor (8, 9). These findings suggest that the interaction between the α_{IIb} and β_3 tails in resting platelets maintains the receptor in a default inactive state and that disruption of the clasp as a result of intracellular signaling activates $\alpha_{IIb}\beta_3$, enabling fibrinogen binding.

Numerous studies have shown that whereas binding of soluble fibrinogen to $\alpha_{IIb}\beta_3$ requires platelet stimulation, fibrin and immobilized fibrinogen are capable of supporting $\alpha_{IIb}\beta_3$-mediated adhesion and spreading of unstimulated platelets even in the presence of inhibitors of platelet activation (10–13). In vivo, fibrin and immobilized fibrinogen are the principal

ligands known to mediate stable platelet adhesion at sites of vascular injury or to the surface of vascular grafts. However, the mechanisms involved in the initiation of adhesion-induced outside-in signaling via nonactivated $\alpha_{IIb}\beta_3$, as well as via other integrins, remain unclear. It is thought that an initial attachment of low-affinity "bent" integrin to immobilized ligands triggers the rearrangements in its extracellular portion leading to a high-affinity, extended conformer similar to that shown for the binding of soluble ligand-mimetic peptides to integrin $\alpha_v\beta_3$ (14). These extracellular conformational changes are proposed to be coupled with a separation of the α and β cytoplasmic tails as found for binding of soluble ICAM-1 to integrin $\alpha_L\beta_2$ (15). Since adhesion of platelets to immobilized fibrin(ogen) is initiated in the absence of agonist stimulation, it appears to begin when the cytoplasic domain of $\alpha_{IIb}\beta_3$ is still in the closed state. It is remarkable that several cytoplasmic proteins, including a skelemin-like protein, talin, and Rack1, have been shown to bind peptides which duplicate the membrane-proximal part of the β_3 tail exactly within the β_3 715–730 segment which is involved in the formation of the interface with the α_{IIb} segment (16–18). Although the docking residues within the β_3 715–730 segment for these proteins are not known, they are likely to be hidden within the interface. This would imply that $\alpha_{IIb}\beta_3$ in resting platelets does not associate with the intracellular molecules and that the conformational change triggered by platelet adhesion unclasps the interface and enables their binding.

In this study, we have used the skelemin-like protein to probe the conformational state of the cytoplasmic domain upon binding of isolated $\alpha_{IIb}\beta_3$ to immobilized fibrinogen, during $\alpha_{IIb}\beta_3$-mediated platelet adhesion and upon platelet stimulation with agonists (inside-out signaling). A member of the myomesin family of proteins (19), initially identified in non-muscle cells as a "skelemin-like protein" (16), this protein is deemed to be an appropriate molecule for examining the initial steps of outside-in signaling because it interacts with the cytoplasmic domain of $\alpha_{IIb}\beta_3$ (16) and colocalizes with this integrin from the very earliest stages of cell adhesion (20). We demonstrate that this protein does not bind $\alpha_{IIb}\beta_3$ in resting platelets but associates with this receptor during platelet adhesion. The results suggest that the membrane-proximal part of the cytoplasmic domain of $\alpha_{IIb}\beta_3$ contains the cryptic binding site for the skelemin-like protein which is unmasked by platelet adhesion. Thus, the membrane-proximal clasp between α_{IIb} and β_3 cytoplasmic tails may function as an intracellular switch which initiates outside-in signaling reactions in platelets.

EXPERIMENTAL PROCEDURES

Proteins, Peptides, and Monoclonal Antibodies

Human thrombin and fibrinogen, depleted of fibronectin and plasminogen, were obtained from Enzyme Research Laboratories (South Bend, IN). The fibrinogen D_{100} (100 kDa) fragment was produced as described previously (21, 22). Fibrin monomer with both fibrinopeptides A and B cleaved was prepared by clotting fibrinogen with thrombin and dissolving the fibrin clot in 0.02 M acetic acid (23). The platelet integrin $\alpha_{IIb}\beta_3$ was isolated from human blood platelets (The Blood Center, Hammond, LA) using affinity chromatography on Concanavalin A-agarose as previously described (24, 25). Approximately 85–90% of $\alpha_{IIb}\beta_3$ purified by this procedure is in the inactive state as verified by fractionation by affinity chromatography on KYGRGDSPK-Sepharose. Isolated

integrin was labeled with ^{125}I using IODO-GEN (Pierce, Rockford, IL). Iodinated protein was dialyzed against PBS[1] and stored at −20 °C. The recombinant fragment spanning immunoglobulin C2-like motifs 3–7 of mouse skelemin [residues 1113–1666 (GenBank entry NM_010867)] was prepared as a fusion protein with GST essentially as described previously (16). To cleave the skelemin fragment from the fusion part, human thrombin (5 units/mL) was added to glutathione–agarose beads bound with GST–skelemin. Analyses of the protein by SDS–PAGE demonstrated the presence of a pure protein with a molecular mass of ~60 kDa. Size-exclusion chromatography on Sephadex G-100 revealed a single peak corresponding to the monomeric form of skelemin. The protein was labeled with ^{125}I using IODO-GEN, dialyzed against PBS, and stored at −20 °C.

The peptides corresponding to the α_{IIb} and β_3 cytoplasmic tail sequences and their alanine-substituted derivatives were synthesized in the Biotechnology Core of the Cleveland Clinic Foundation. The peptides were purified by RP-HPLC on a preparative C18 Vydac column using a 5 to 90% linear gradient of acetonitrile in 0.1% TFA and analyzed by LC–MS. The following peptides were synthesized: α_{IIb} ^{989}KVGFFKRNRPPLEEDDEEGE^{1008}, α_{IIb} mutant ^{989}KVGFFKANAPALEEDDEEGE^{1008}, β_3 GY^{715}WKLLITIHDRKEFAKF^{730}, β_3 GY^{715} WALLITIADRKEFAKF^{730} (β_3 mutant 1), and β_3 GY^{715} WALLITIADAKEFAKF^{730} (β_3 mutant 2). The myristoylated α_{IIb} peptide (Myr-α_{IIb}) encompassing the sequence Lys^{989}–Glu^{1008} and its mutant derivative in which Arg^{995}, Arg^{997}, and Pro^{999} were substituted with Ala were synthesized and purified as described previously (26).

mAb AP3, directed against the β_3 integrin subunit, was from GTI (Brookfield, WI). mAb 3F5 (anti-LIBS$_{cyt}$1) which recognizes the neoepitope in the cytoplasmic tail of α_{IIb} was described previously (27). Purified mouse IgG was purchased from Sigma (St. Louis, MO). Polyclonal anti-skelemin antibody was generated by BioSource International Inc. (Camarillo, CA) using the recombinant skelemin fragment as an immunogen. The antibody was isolated from rabbit serum by precipitation with 35% ammonium sulfate. The antibody recognizes both human and mouse skelemins which have a high percent of interspecies homology (91% identical amino acid residues) as well as a high degree of homology between the different myomesin family members. The integrin-binding repeats Ig C4–5 within the recombinant skelemin fragment have 72% identical residues between the myomesin family members.

Synthesis of Cellulose-Bound Peptide Libraries and Screening for Skelemin Binding

The α_{IIb} and β_3 cytoplasmic domain-derived peptide libraries were prepared by parallel spot synthesis as described previously (28, 29). The 9-fluorenylmethoxycarbonyl (Fmoc)-protected and pentafluorophenyl (Pfp)-activated amino acids were purchased from Bachem (King of Prussia, PA). Pfp-activated Trp was obtained from Novabiochem (San Diego, CA). The following side chain protecting groups were used: trityl for Cys, His, Asn, and Gln; *tert*-butyl for Asp, Glu, Ser, and Thr; *tert*-butoxycarbonyl for Lys and Trp; and

[1]Abbreviations: CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; HBS-P, HEPES-buffered saline supplemented with 0.005% surfactant P20; DTT, dithiothreitol; SPR, surface plasmon resonance; mAb, monoclonal antibody.

pentamethylchroman-6-sulfonyl for Arg. All other reagents were of the highest quality and were used without further purification. Peptides were COOH-terminally attached to cellulose via a (β-Ala)$_2$ spacer and were acetylated N-terminally. The cellulose membranes with covalently coupled peptides were incubated for 1 min in methanol and then washed with TBS buffer. After being blocked with 1% BSA for 2 h at 22 °C, the membranes were incubated with 10 μg/mL [^{125}I]skelemin (10^5cpm/ml) in PBS for 3 h at 22 °C. After being washed with TBS containing 0.05% Tween 20, the membranes were dried. Skelemin binding was visualized by autoradiography and analyzed by densitometry.

Cells

Platelets were collected from fresh aspirin-free human blood in the presence of 2.8 μM prostaglandin E_1 and isolated by differential centrifugation followed by gel filtration on Sepharose 2B in divalent cation-free Tyrode's buffer (pH 7.2) containing 0.1% BSA. The $\alpha_{IIb}\beta_3$-expressing CHO cells (16) were maintained in DMEM/F-12 (GIBCO, Carlsbad, CA) supplemented with 10% FBS, 2 mM glutamine, 15 mM HEPES, 0.1 mg/mL streptomycin, and 0.1 unit/mL penicillin. The expression of $\alpha_{IIb}\beta_3$ on the surface of the cells was evaluated by FACS analyses using anti-β_3 mAb AP3 (10 μg/mL). FACS analyses were performed using a FAC-Scan (Beckton Dickinson) as previously described (30).

RT-PCR Analyses

To demonstrate a skelemin-like protein in platelets, total RNA was isolated from purified platelets as previously described (31). cDNA was synthesized from total cellular RNA with SuperScript III reverse transcriptase using 250 ng of random primers (Invitrogen). Total cDNA was amplified using Platinum Taq DNA polymerase (Invitrogen) and gene-specific primers. Synthetic primers for amplifying skelemin cDNA sequences TGCATATTGACCGAAACAC and CTCTGTTATAAGCAGGGTACA were constructed using the human skelemin sequence (GenBank entry NM_003803). These primers amplify a 350 bp product of the transcribed skelemin cDNA which was detected by agarose electrophoresis after staining with ethidium bromide.

Immunoprecipitation

The $\alpha_{IIb}\beta_3$-expressing CHO cells in DMEM/F12 medium were added to Petri dishes (100 mm × 15 mm) coated with fibrinogen (20 μg/mL) and allowed to attach for 15–120 min at 37 °C. Platelets in TBS containing 0.1% BSA, 1 mM Ca^{2+}, and 1 mM Mg^{2+} were added to the immobilized fibrinogen for 15–120 min at 37 °C. The nonadherent cells were removed, and adherent cells were solubilized with a lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM $CaCl_2$, 1 mM PMSF, 100 μg/mL leupeptin, and 10 mM benzamidine] for 30 min at 22 °C. After the removal of insoluble material by centrifugation at 12000*g* for 15 min, the lysates were incubated with 10 μg of normal mouse IgG (Sigma) and 50 μL of Zysorbin-G (Zymed, San Francisco, CA) for 2 h at 4 °C. After centrifugation, the supernatants were incubated with 1 μg of mAb AP3 (anti-β_3) for 2 h at 4 °C. The integrin–mAb complex was captured by incubation with 50 μL of Protein A-Sepharose (GE Healthcare, Piscataway, NJ) for 16 h at 4 °C. The immunoprecipitated proteins were eluted with SDS–PAGE loading buffer and analyzed by Western blotting for skelemin. Immobilon-

P membranes (Millipore, Billerica, MA) were incubated with either anti-skelemin antibody (1:40000 dilution) or anti-β_3 (1:4000 dilution) polyclonal antibodies and developed using SuperSignal West Pico substrate (Pierce).

Platelet Adhesion and Fibrin Clot Retraction Assays

Platelet adhesion was performed essentially as described previously (30). Briefly, the wells of 96-well tissue culture plates (Costar, Cambridge, MA) were coated with proteins overnight at 4 °C and postcoated with 1% BSA inactivated at 75 °C. Platelets were labeled with 10 μM calcein AM (Invitrogen) for 30 min at 37 °C, washed in isotonic HEPES buffer, and resuspended at a density of 1×10^8 cells/mL in the same buffer supplemented with 0.1% BSA, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. After aliquots (100 μL) of cells were added to the wells, the plates were briefly spun and incubated at 37 °C for 50 min. The nonadherent cells were removed by two washes with PBS, and fluorescence was measured in a Cytofluor II fluorescence plate reader (Applied Biosystems, Framingham, MA). The number of adherent cells was determined using the fluorescence of aliquots with a known number of labeled cells. Clot retraction assays were performed as described previously (30).

Surface Plasmon Resonance

The interactions of skelemin with $\alpha_{IIb}\beta_3$ or α_{IIb} peptides in fluid phase were tested by surface plasmon resonance using a BIAcore 3000 biosensor (Biacore AB, Uppsala, Sweden). The skelemin fragment was coupled to a CM5 sensor chip (Biacore) using the amine coupling kit according to the manufacturer's protocol. Fibrinogen was immobilized to a parallel cell of the same chip. The sensor chip surfaces contained ~900 and 1300 response units of skelemin and fibrinogen, respectively. Different concentrations of purified $\alpha_{IIb}\beta_3$ and the α_{IIb}-derived peptide in HBS-P buffer (BIAcore) containing 1 mM Ca^{2+} and 1 mM Mg^{2+} were passed over the flow cells, and the association between the immobilized and flowing proteins was detected as the change in the SPR response. The interaction of the α_{IIb} peptide with skelemin was examined by injecting different concentrations of α_{IIb} at a rate of 5 μL/min for 10 min. All data were corrected for the response obtained using a blank reference flow cell that was activated with EDC/NHS and then blocked with ethanolamine. The chip surface was regenerated with a 1 M NaCl/HBS-P mixture. The association rate constant (k_a) and the dissociation rate constant (k_d) were obtained by curve fitting of the association and dissociation phases of sensograms, respectively, with a two-state reaction model using BIAevaluation version 4.1 (BIAcore). K_d was then determined using BIAevaluation after obtaining the best fit to the interaction model.

Solid-Phase Binding Assays

The interaction of skelemin with $\alpha_{IIb}\beta_3$ prebound to various ligands was tested in solid-phase binding assays. The wells of 96-well breakable microtiter strips (Removawells, Immulon 4HBX) were coated with different proteins for 3 h at 37 °C and postcoated with 1% BSA for 1 h at 22 °C. The 0.1 mL aliquots of purified $\alpha_{IIb}\beta_3$ (20 or 50 μg/mL) in TBS containing 0.02% Triton X-100, 0.1% BSA, 1 mM Ca^{2+}, and 1 mM Mg^{2+} were added to immobilized ligands and incubated for 3 h at 22 °C. The wells were washed with the same buffer, and then different concentrations (0–200 μg/mL) of the [^{125}I]skelemin fragment with

a specific radioactivity of ~0.2–1 × 10^5 cpm/μg were added for 3 h at 22 °C. After the wells had been washed, bound radioactivity was quantified in a × counter. To measure the amount of integrin bound to each ligand, ^{125}I-labeled $\alpha_{IIb}\beta_3$ (50 μg/mL) with a specific radioactivity of 2 × 10^4 cpm/μg was added to the microtiter wells for 3 h and its binding was assessed. The level of nonspecific binding was measured in the presence of a 10-fold excess of the β_3 715–730 peptide or as binding of radiolabeled skelemin to each immobilized ligand postcoated with BSA. K_d and B_{max} values were derived by a curve fitting analyses of specific binding isotherms using SigmaPlot 8.0.

RESULTS

Effect of Myrisoylated α_{IIb} Peptide on Platelet Adhesion

Previous studies suggested that agonist-induced platelet stimulation leads to unclasping of the interface between the membrane-proximal parts of the α_{IIb} and β_3 cytoplasmic tails converting the integrin to the active state and enabling fibrinogen binding (7). However, it is unknown whether the separation of tails occurs during platelet adhesion. To probe the conformation state of the $\alpha_{IIb}\beta_3$ cytoplasmic domain, we have initially examined the effect of the Myr-α_{IIb} peptide spanning the entire α_{IIb} tail (residues 989–1008) on platelet adhesion. The peptide has previously been shown to bind the β_3 tail and suppress integrin activation (26). We have tested the effect of Myr-α_{IIb} on platelet adhesion to the fibrinogen D_{100} fragment. The peptide was a strong inhibitor of adhesion of unstimulated platelets to the immobilized fragment (Figure 2). It inhibited adhesion in a concentration-dependent manner with an IC_{50} of 4.1 ± 0.65 μM. Myr-α_{IIb} was also a strong inhibitor of platelet-mediated fibrin clot retraction (not shown). As controls, the same concentrations of myristic acid and control nonmyristoylated α_{IIb} peptide were without effect. Furthermore, a control Myr-α_{IIb} in which residues critical for skelemin binding were substituted with Ala (see below) did not inhibit adhesion (Figure 2). These initial results suggested that Myr-α_{IIb} might inhibit adhesion by binding to the membrane-proximal part of the β_3 tail and, thus, compete with the critical cytoplasmic protein. If so, then the binding site(s) for Myr-α_{IIb} and/or other proteins might be unavailable in the closed integrin interface, and hence, the α_{IIb} and β_3 cytoplasmic tails must first unclasp to enable their binding.

The Binding Site for Skelemin Is Cryptic in Resting $\alpha_{IIb}\beta_3$

To test the idea that the binding site for any cytoplasmic protein which potentially can bind the inner face of the membrane-proximal part of β_3 involved in the clasp is cryptic, we selected skelemin. A skelemin fragment consisting of Ig C2-like motifs 3–7 has been shown to bind the β_3 peptide corresponding to the membrane-proximal β_3 715–730 segment (16). A skelemin-like protein has previously been identified by immunofluorescence in several non-muscle cells, including CHO cells, endothelial cells, and platelets (16). To demonstrate that the immunoreactive protein in platelets is a member of the myomesin family of homologous proteins to which skelemin belongs (19), lysates of platelets were subjected to immunoprecipitation with antibodies generated against the recombinant Ig C2 3–7 motifs skelemin fragment, and the immunoprecipitates were then probed with the same antibodies on Western blots. The antibodies immunoprecipitated a protein with an M_r of ~200 kDa (see below). The antibodies also immunoprecipitated an ~160 kDa protein from lysates of human

skeletal muscle, which, depending on the speed of the muscle, express primarily myomesin 1 (M_r = 175 kDa) or myomesin 2 (M_r = 165 kDa) family members (19). Thus, the protein that immunoprecipitated from platelet lysates migrated with a slightly higher molecular mass than that from muscle when two lysates are analyzed side by side. Using RT-PCR analysis of cDNA obtained from total platelet mRNA with primers that amplify sequences present in myomesin 1 isoforms, a product for a family member was detected (not shown). Given the high degree of homology between different members of the myomesin family, future studies will be needed to identify the member(s) expressed in platelets. However, taken together, these findings are consistent with the fact that a member of this family is expressed in platelets. As it was previously called a “skelemin-like protein” (16), for the sake of simplicity, in the rest of this work, we continue to follow the current practice (20, 32) of referring to this non-muscle myomesin family member as “skelemin”.

The interaction of $\alpha_{IIb}\beta_3$ with the recombinant skelemin fragment was initially tested using SPR. The skelemin fragment was coupled to a Biacore chip, and various concentrations of resting integrin were passed over the chip. To validate the resting state of $\alpha_{IIb}\beta_3$, fibrinogen was coupled to a parallel cell of the chip. No interaction was detected with either skelemin fragment or fibrinogen. To explore the possibility that conversion of $\alpha_{IIb}\beta_3$ to its active fibrinogen-binding form leads to skelemin binding, we treated the integrin with DTT as described previously (33, 34). As shown in Figure 3A, while DTT-treated $\alpha_{IIb}\beta_3$ was capable of fibrinogen binding, it did not bind the skelemin fragment (Figure 3B). Similar results were obtained in solid-phase binding assays using the skelemin fragment immobilized on microtiter wells: no interaction with either resting or DTT-activated soluble ^{125}I-labeled $\alpha_{IIb}\beta_3$ was detected (not shown). Thus, these results indicate that when integrin is present in solution (either in its resting or in its active state) it does not bind to immobilized skelemin.

Engagement of $\alpha_{IIb}\beta_3$ by Immobilized Ligands Induces Skelemin Binding

To explore the possibility that the engagement by $\alpha_{IIb}\beta_3$ of the immobilized ligand can induce skelemin binding, we have developed an assay in which purified resting integrin was first prebound to the surface-bound fibrin(ogen) and then its interaction with the ^{125}I-labeled skelemin fragment was tested. Such a format mimics the initial contact of integrin with the fibrin clot or the immobilized fibrinogen during adhesion of resting platelets. As shown in Figure 4A for immobilized fibrin, ligand-captured integrin was capable of skelemin binding. Skelemin binding was saturable and specific as unlabeled skelemin and the β_3 715–730 peptide inhibited binding in a concentration-dependent manner with an IC_{50} value of 0.5 ± 0.14 μM (Figure 4B, shown for the β_3 715–730 peptide). The control peptide was not active. The data from the binding isotherm in Figure 4A were graphed as a Scatchard plot (inset) which suggested that the interaction could be described by at least two classes of binding sites with estimated dissociation constants (K_d) of 123 ± 28 nM and 1.1 ± 0.13 μM. Skelemin binding to ligand-bound $\alpha_{IIb}\beta_3$ was observed to approach molar stoichiometries of ~1:1 and ~1:3 of skelemin to receptor for the high- and low-affinity interactions, respectively. Figure 5 shows that $\alpha_{IIb}\beta_3$ prebound to various immobilized fibrinogen ligands was capable of skelemin binding, with the D_{100} fragment being more effective compared to the fibrin monomer and fibrinogen. In contrast, integrin immobilized directly on plastic

bound a small amount of skelemin. In parallel experiments, we determined that equal amounts of ^{125}I-labeled $\alpha_{IIb}\beta_3$ bound to each substrate. Therefore, the difference in skelemin binding was not due to the quantities of integrin bound to various substrates. These results suggest that the interaction of $\alpha_{IIb}\beta_3$ with its immobilized ligands might induce the conformational change that leads to skelemin binding. To validate that our assay reports the conformational alterations occurring in the cytoplasmic domain, we have used mAb 3F5 (anti-LIBS$_{cyt}$1). This mAb is directed against the α_{IIb} tail and recognizes the epitope α_{IIb} Pro^{998}-Pro^{999} residing close to the α_{IIb}–β_3 interface and binds the ligand-occupied integrin only (27). As shown in Figure 5 (black bars), mAb 3F5 bound $\alpha_{IIb}\beta_3$ captured on all fibrin(ogen) ligands. Taken together, these results suggest that binding of $\alpha_{IIb}\beta_3$ to fibrin(ogen) via its ligand-binding domain propagates the conformational change to the integrin cytoplasmic domain, enabling skelemin binding.

The α_{IIb} Cytoplasmic Tail Binds Skelemin

The binding site for the skelemin fragment was identified previously in the peptide corresponding to the β_3 tail (16). Subsequent NMR studies have identified binding sites in both the β_3 and α_{IIb} subunits (32). Using an in vitro peptide binding assay and SPR, we have also obtained evidence for an interaction of the skelemin fragment with the α_{IIb} tail (residues 989–1008). In SPR experiments, the α_{IIb} peptide was passed over skelemin coupled to the Biacore sensor chip, and its concentration-dependent binding was detected (Figure 6). The interaction was also demonstrable in solid-phase binding assays in which the α_{IIb} peptide was immobilized on plastic and [^{125}I]skelemin was added (Figure 7B). The interaction between α_{IIb} and skelemin was specific as the mutant peptide with residues critical for skelemin binding mutated to Ala did not bind skelemin either in SPR or in solid-phase binding experiments. Furthermore, the α_{IIb} peptide, but not a control mutant peptide, inhibited the interaction of skelemin with $\alpha_{IIb}\beta_3$ prebound to its fibrinogen ligands with an IC$_{50}$ of ~15 μM (not shown). These results indicate that skelemin has the capacity to bind both α_{IIb} and β_3 tails.

Localization of Critical Residues in the α_{IIb} and β_3 Cytoplasmic Tails Involved in Skelemin Binding

To gain additional insight into the process responsible for the binding of skelemin to the α_{IIb}–β_3 cytoplasmic interface, we have identified the residues in each tail involved in its binding. Cellulose-bound peptide libraries spanning the sequences of α_{IIb} and β_3 tails were prepared by parallel spot synthesis and screened for skelemin binding. An overlapping library spanning the α_{IIb} tail (residues 989–1008) and consisting of 9-mer peptides with a one-residue offset is shown in Figure 7A (left panel). The membrane with covalently attached peptides was incubated with [^{125}I]skelemin, and the binding was detected using autoradiography. The results revealed that peptides 1–5 encompassing the membrane-proximal region, α_{IIb} ^{989}KVGFFKRNRPPLE^{1001}, bound skelemin. To identify critical residues, two mutational libraries were synthesized in which each residue in two overlapping peptides, ^{989}KVGFFKRNR^{997} (Figure 7A, middle panel) and ^{993}FKRNRPPLE^{1001} (Figure 7A, right panel), was consecutively substituted with Ala. On the basis of densitometry analyses, individual substitutions of Arg^{995}, Arg^{997}, and Pro^{999} reduced the level of skelemin binding by ~70%. In addition, mutations of Val^{990}, Phe^{992}, and Leu^{1000} lowered

the level of binding by ~40–50%. To test the functional role of the identified residues, the mutant α_{IIb} peptide with substitutions for three residues, Arg^{995}, Arg^{997}, and Pro^{999} (^{989}KVGFFKANAPALEEDDEEGE^{1008}), was prepared by traditional Fmoc chemistry. In solid-phase binding assays, the mutant peptide lost the ability to bind [^{125}I]skelemin (Figure 7B). The peptide also did not bind skelemin coupled to the chip in SPR experiments and did not inhibit skelemin binding to $\alpha_{IIb}\beta_3$ prebound to ligands (see above).

Analyses of the overlapping peptide library derived from the β_3 715–762 fragment confirmed that the membrane-proximal β_3 715–725 segment binds skelemin (Figure 8A, left panel). Additional analyses of the substitutional peptide libraries derived from β_3 715–723 and β_3 715–730 fragments (Figure 8B, middle and right panel; see also Figure 1S of the Supporting Information) demonstrated that Lys^{716}, His^{722}, Arg^{724}, Lys^{725}, and Lys^{729} were critical for skelemin binding. Furthermore, other residues, including Leu^{717} and Phe^{727}, can contribute to binding. To validate the roles of the identified residues, two mutant peptides were synthesized. The substitution of Lys^{716} and His^{722} with Ala in the β_3 715–730 fragment yielded the peptide ^{715}WALLITIADRKEFAKF^{730} (β_3 mutant 1) which exhibited a reduced level of skelemin binding and the ability to inhibit the interaction between the β_3 peptide and skelemin (Figure 8B,C). The additional replacement of Arg^{724} with Ala yielding ^{715}WALLITIADAKEFAKF^{730} (β_3 mutant 2) resulted in a still further loss of inhibitory activity and the ability to bind skelemin. However, while β_3 mutant 2 was less active than its wild-type counterpart, it had residual activity which might be attributed to the contribution of other identified residues. Taken together, these analyses provide further evidence that the membrane-proximal parts of both α_{IIb} and β_3 are involved in skelemin binding and that several basic and hydrophobic residues in both tails are important for binding. Furthermore, since residues involved in skelemin binding are those that participate in the clasp between the α_{IIb} and β_3 tails, these finding suggest that for skelemin to associate with $\alpha_{IIb}\beta_3$, an adhesion-specific unclasping of the membrane-proximal region must occur.

Skelemin Binds the $\alpha_{IIb}\beta_3$ Cytoplasmic Domain during Cell Adhesion

To determine whether the binding site for the skelemin is cryptic in $\alpha_{IIb}\beta_3$ expressed in cells, lysates of suspended $\alpha_{IIb}\beta_3$-expressing CHO cells and resting platelets were subjected to immunoprecipitation with anti-β_3 mAb AP3. The immunoprecipitates were analyzed on Western blots using polyclonal anti-skelemin and polyclonal anti-β_3 antibodies. While AP3 immunoprecipitated a significant amount of the β3 subunit, no skelemin was detected in the immunoprecipitates (Figure 9A,B, lanes labeled 0). To determine whether adhesion can induce binding of skelemin to $\alpha_{IIb}\beta_3$, cells were allowed to adhere to the immobilized fibrinogen for selected periods of time (0–120 min). Adherent cells were solubilized; lysates were immunoprecipitated with mAb AP3, and the presence of the skelemin in immune complexes was analyzed. As shown in panels A and B of Figure 9, the protein was detected in complexes with β_3 as soon as 15 min after initiation of adhesion of platelets and 30 min after adhesion of the $\alpha_{IIb}\beta_3$-expressing CHO cells. Control mouse IgG did not immunoprecipitate skelemin from cell lysates. The increased level of recovery of skelemin in association with integrin was not due to an increased amount that becomes available in platelet lysates during adhesion since the same amount of protein was present in total lysates of nonadherent and adherent cells (Figure 9C). Furthermore, as shown in Figure 9D, almost

all endogenous skelemin is incorporated into the complex with $\alpha_{IIb}\beta_3$ within 60 min of adhesion, as evidenced by its absence in platelet lysates after the removal of immune complexes. Thus, the experiments clearly show that skelemin is not associated with integrin until after the cells adhere, at which time it is incorporated into integrin complexes. Furthermore, considering the previous studies in which a direct interaction of skelemin with the β_3 and α_{IIb} peptides was shown (16) and this study showing an interaction with isolated $\alpha_{IIb}\beta_3$, the most likely explanation for the co-immunoprecipitation is that skelemin interacts directly with the integrin.

It has previously been proposed that platelet activation with agonists results in disruption of the interface between the α_{IIb} and β_3 cytoplasmic tails (7, 8). To examine whether platelet activation induces skelemin binding, we performed immunoprecipitation analyses with platelets stimulated with different agonists. Platelets in suspension were activated for various periods of time with ADP or ADP and epinephrine in the absence or presence of exogenous soluble fibrinogen. Furthermore, after the addition of fibrinogen, platelets were stirred or not stirred. After Western blotting, densitometry analyses were performed to determine the maximal levels of bound skelemin. As shown in panels A and B of Figure 10, platelet stimulation with ADP resulted in a small amount of skelemin being immunoprecipitated with $\alpha_{IIb}\beta_3$, and the addition of epinephrine and fibrinogen did not further augment its association. Platelet stirring induced an approximately 2-fold increase in the level of skelemin binding. However, its levels were still ~2-fold lower than those in adherent cells. Control experiments demonstrated that platelet activation with agonists followed by the addition of fibrinogen did not change the amount of skelemin in total platelet lysates (Figure 10C), and the majority of the protein remained in lysates of agonist-activated platelets after the removal of immune complexes (Figure 10D). These experiments indicate that platelet activation with ADP and epinephrine followed by the addition of soluble fibrinogen results in only partial association of skelemin with $\alpha_{IIb}\beta_3$.

DISCUSSION

In this study, we have analyzed the binding of skelemin to platelet integrin $\alpha_{IIb}\beta_3$ and found that this interaction occurs only after the receptor engages the immobilized fibrin(ogen). Thus, the interaction of $\alpha_{IIb}\beta_3$ with the immobilized ligand induces the conformational change in the cytoplasmic domain of the receptor resulting in exposure of the binding site for skelemin. The following observations support this conclusion. First, skelemin does not interact with isolated intact $\alpha_{IIb}\beta_3$, but its binding is induced after integrin is prebound to immobilized fibrinogen, fibrin, or the fibrinogen D fragment. Second, skelemin does not associate with $\alpha_{IIb}\beta_3$ either in resting platelets or in $\alpha_{IIb}\beta_3$-expressing CHO cells in suspension but binds integrin during cell adhesion. Third, not only β_3 but also the α_{IIb} membrane-proximal segment is capable of skelemin binding. Fourth, the exact same residues in both cytoplasmic tails, α_{IIb} Arg^{995}, α_{IIb} Val^{990}, and β_3 His^{722}, which are involved in the formation of the α_{IIb}–β_3 interface are also required for skelemin binding. Thus, these data suggest that the membrane-proximal segments of the α_{IIb} and β_3 cytoplasmic tails, which in the resting integrin form a complex, unclasp during the $\alpha_{IIb}\beta_3$-mediated platelet adhesion and unmask the binding site for skelemin. In support of this conclusion, the membrane-permeable α_{IIb} peptide inhibited platelet adhesion which appears

to be possible only if the complementary binding residues in the β_3 tail become available as a result of separation of the tails. The regulation of skelemin binding by the conformational changes in the $\alpha_{IIb}\beta_3$ cytoplasmic domain implies that other proteins which bind the same region of $\alpha_{IIb}\beta_3$, including talin (35), can be recruited to integrin after unclasping of the interface during cell adhesion. Furthermore, since residues involved in the interface are highly conserved in the α and β subunits, unclasping of the interface upon adhesion may serve a regulatory role in the assembly of cytoskeletal proteins and signaling molecules in other integrins.

It is well-established that $\alpha_{IIb}\beta_3$ can undergo gross conformational alterations in its extracellular domain apparently similar to those found for $\alpha_V\beta_3$ (14). Numerous examples have been described in which ligand binding to $\alpha_{IIb}\beta_3$ induces both local and long-range conformational changes in the extracellular parts of both α_{IIb} and β_3 subunits. This results in the exposure of neoantigenic sites termed ligand-induced binding sites (LIBS), which are recognized by anti-LIBS mAbs (36–41). The conformational changes elicited by ligand binding are then propagated through the cell membrane to the cytoplasmic domain, inducing the exposure of the cryptic Pro^{998}-Pro^{999} epitope in the α_{IIb} cytoplasmic tail, as revealed by anti-$LIBS_{cyt1}$ mAb 3F5 (27). Furthermore, the conformational changes induced by ligand binding are thought to be coupled to the separation of the cytoplasmic tails as shown for integrin $\alpha_L\beta_2$ (15). It should be noted that exposure of LIBS in $\alpha_{IIb}\beta_3$ has been examined after binding of soluble ligands, either fibrinogen or the RGD peptide, and the separation of α_L and β_2 has been detected after binding of soluble ICAM-1. Our study is the first demonstration of the conformational change in the cytoplasmic domain of $\alpha_{IIb}\beta_3$ upon binding of integrin to immobilized ligands during adhesion. The finding that unclasping of the interface between the cytoplasmic tails occurs in the absence of prior platelet stimulation and ensuing inside-out signaling indicates that resting integrin may have the intrinsic capacity to undergo the conformational alterations upon engagement of the adhesive substratum. Moreover, the fact that isolated integrin prebound to immobilized fibrin(ogen) acquires the ability to interact with skelemin further supports the conclusion that unfolding of the integrin per se is responsible for this effect.

Previous studies demonstrated that the treatment of $\alpha_{IIb}\beta_3$ with DTT leads to the rearrangement of disulfide bonds in the extracellular domain, resulting in integrin activation and fibrinogen binding (33, 34). Our analyses indicate that whereas DTT-treated integrin binds fibrinogen, it still does not bind skelemin. Uncoupling of ligand binding function from the conformational changes in the cytopalsmic domain has been noted for integrin $\alpha_L\beta_2$ as well: although Mn^{2+} enhanced integrin-mediated cell adhesion, it did not induce $\alpha_L\beta_2$ cytoplasmic domain separation (15). These data suggest that the conformational changes in the extracellular domain required for binding of soluble fibrinogen might not necessarily be associated with the separation of the cytoplasmic tails. This conclusion is supported by the data which show that certain anti-LIBS mAbs which expose the fibrinogen-binding site in the extracellular domain of $\alpha_{IIb}\beta_3$ do not themselves cause protein tyrosine phosphorylation and platelet activation (39, 42). Furthermore, although stimulation of platelets with ADP induces the conformational changes in the extracellular domain sufficient for fibrinogen binding (2), it does not result in significant skelemin recruitment (Figure 10). This

observation, in concert with previous data indicating that ADP-induced intracellular signaling does not result in the exposure of anti-$LIBS_{cyt1}$ (27), suggests that platelet stimulation with agonists does not lead to extensive tail separation. Furthermore, even though subsequent fibrinogen occupancy of the agonist-activated receptor and platelet stirring augment formation of the skelemin–$\alpha_{IIb}\beta_3$ complex (Figure 10), it still does not promote the extent of binding observed in adherent platelets. Inside-out signaling has previously been proposed to involve the "intermediate" integrin conformation. Takagi et al. have suggested that inside-out signaling should favor an extended conformer with a closed headpiece which should be sufficient to enhance the affinity for ligand (14). Whether the limited skelemin binding observed in inside-out signaling (agonist stimulation) is associated with this or other intermediate conformers remains to be determined.

In contrast to stimulation with agonists, platelet adhesion induces high levels of integrin–skelemin complexes indicative of rapid and massive unclasping of the cytoplasmic tails. These findings point to the more extensive nature of the conformational alterations in the cytoplasmic domain during platelet adhesion than upon binding of soluble ligand. Thus, outside-in signaling triggered by soluble and immobilized forms of fibrinogen may induce quantitatively different responses manifested in the different levels of receptors which undergo unclasping in their cytoplasmic domains. An alternative explanation is that the conformational changes initiated by soluble fibrinogen produce receptors with a partially open tail–tail interface which may prevent the binding of skelemin with high affinity. The fact that different molar ratios of skelemin to $\alpha_{IIb}\beta_3$ have been observed in this study (Figure 4) suggests that isolated receptors prebound to immobilized ligands may indeed represent a heterogeneous population with respect to unclasping in their cytoplasmic domains. In addition, it could be argued that intracellular molecules which are capable of binding the membrane-proximal segments of the β_3 cytoplasmic tail compete with skelemin. One of these molecules is talin which was shown to assist in $\alpha_{IIb}\beta_3$ activation (7, 43). However, this interpretation appears to be unlikely because talin exhibits a pattern of association with $\alpha_{IIb}\beta_3$, upon both agonist-induced platelet stimulation and platelet adhesion, which is similar to that of skelemin (unpublished data of N. P. Podolnikova, 2006). It remains to be determined which one of these possible scenarios works in platelets.

This study confirms that the binding site for skelemin interacts with the β_3 tail and further strengthens the observation that skelemin binds the α_{IIb} tail. The residues critical for skelemin binding have been localized to the membrane-proximal segments of both cytoplasmic tails and include Val^{990}, Phe^{992}, Arg^{995}, Arg^{997}, and Pro^{999} in the α_{IIb} subunit and Lys^{716}, His^{722}, Arg^{724}, Lys^{725}, and Lys^{729} in the β_3 subunit (Figure 1). Recent NMR studies have demonstrated that residues in the α_{IIb} peptide identified in this study as being critical for skelemin binding underwent perturbations upon addition of the skelemin fragment (32). Furthermore, although exact residues in the β_3 tail required for skelemin binding have not been determined, the involvement of the entire membrane-proximal region was also documented. An examination of residues critical for skelemin binding indicates that several of them, including α_{IIb} Val^{990}, α_{IIb} Phe^{992}, α_{IIb} Arg^{995}, and β_3 His^{722}, are involved in the formation of the clasp between the tails. Furthermore, α_{IIb} Pro^{999} resides in the immediate proximity of the interface and is part of the $LIBS_{cyt1}$ epitope not exposed in

the cytoplasmic tail of resting integrin (27). Since many residues critical for skelemin binding are the same as those involved in the formation of the α_{IIb}–β_3 complex, unclasping of the interface may be a prerequisite for subsequent high-affinity skelemin binding. Thus, the engagement of the immobilized ligand by $\alpha_{IIb}\beta_3$ appears to trigger the switch from intramolecular interactions which keep the cytoplasmic domain in the closed state to intermolecular interactions with cytoplasmic molecules.

Although skelemin has been examined in this study as a reporter of the conformational state of $\alpha_{IIb}\beta_3$, it is likely that other cytoplasmic proteins which bind the α_{IIb} and β_3 cytoplasmic tails exhibit the same behavior. Among these molecules, talin (17) and Rack1 (18) interact with the membrane-proximal part of the β_3 tail. The binding sites for the calcium and integrin-binding protein (CIB) (44) and ancient ubiquitous protein-1 (Aup-1) (45) are contained within the conserved membrane-proximal KVGFFKR sequence in the α_{IIb} tail. In addition, the residues in the β_3 727–730 segment, which is adjacent to the α_{IIb}–β_3 interface, have been reported to contribute to binding of the cytoskeletal protein α-actinin (46). We postulate that the binding sites for these molecules are cryptic in the closed interface and become unmasked during cell adhesion. In agreement with this idea, only a small portion of talin associates with $\alpha_{IIb}\beta_3$ in resting platelets and dramatically increases during platelet adhesion (unpublished data of N. P. Podolnikova, 2006). The two other proteins, CIB and Rack1, appear to exhibit a similar pattern of recruitment to the cytoplasmic tails. The binding site for CIB was localized in the α_{IIb} transmembrane and adjacent membrane-proximal Leu^{983}–Arg^{997} sequence, with Phe^{992}, Phe^{993} (44), and Arg^{995} (6) being critical for binding. It has been shown that the majority of CIB interacts with the α_{IIb} cytoplasmic tail only after adhesion of the platelet to fibrinogen as opposed to platelets seeded on albumin (47). Thus, it is tempting to speculate that the side chains of residues in the α_{IIb} tail which dock CIB face inwardly in the α_{IIb}–β_3 complex and are hidden from CIB binding in resting platelets. Likewise, another protein, Rack1, which interacts with the membrane-proximal regions of several β subunits (18), including β_3 (48), does not coprecipitate with integrins from resting suspended cells. As yet another example, the binding site for Aup-1 has been localized in the membrane-proximal sequence of α_{IIb} as well as many other α-integrin subunits (45). The finding that only a portion of the protein was immunoprecipitated with α_{IIb} from a megakaryocyte-derived cell line and no Aup-1 was found in association with other α-integrin subunits from various cells suggests that binding of this protein to the integrin cytoplasmic domains is also regulated. In contrast, both the membrane-proximal and membrane-distal sequences of the β_3 cytoplasmic tail were shown to be required for the interaction with endonexin, with the latter segment being more critical (49). In agreement with this localization, $\alpha_{IIb}\beta_3$ in lysates from platelets and $\alpha_{IIb}\beta_3$-expressing CHO cells attached to an affinity matrix made of endonexin (49, 50), suggesting that the binding site for this protein in the β_3 tail is constitutively available.

Numerous studies have documented the ability of $\alpha_{IIb}\beta_3$ on resting platelets to bind immobilized fibrinogen, resulting in cell adhesion and spreading (10–13). Likewise, ligation of other integrins by extracellular matrix proteins triggers intracellular reactions which lead to cell spreading. Although implicit in these observations is a requirement for the linkage between the integrins' cytoplasmic tails and the cytoskeleton, the nature of the initiating

signal remains elusive. On the basis of the data in this study, we propose a model of integrin-mediated adhesion in which the engagement by $\alpha_{IIb}\beta_3$ of the immobilized fibrinogen initiates unclasping of the α_{IIb} and β_3 tails. During this process, one or more cytoplasmic proteins which have the capacity to interact with the membrane-proximal regions of cytoplasmic tails and with the cytoskeleton might wedge between the tails. Once positioned between the tails, these proteins may connect with the cytoskeleton. Since cell adhesion and spreading induce integrin clustering, it is likely that the latter process is secondary to the initial tail separation. Among many molecules which bind the cytoplasmic tails (reviewed in ref 51), the skelemin-like protein and talin seem to be the best candidates to initiate these events. These proteins bind the membrane-proximal segments of both α_{IIb} and β_3 tails (refs 16, 17, and 52 and this study) and can associate with the cytoskeleton (53, 19). It is likely that a molecule with the highest affinity for the segments within the $\alpha_{IIb}\beta_3$ cytoplasmic clasp would be recruited first. It is of interest that skelemin associates with focal clusters, a type of integrin intracellular complex which appears during the very early stages of cell spreading, and is excluded from focal complexes and focal adhesions which form in more fully spread cells (20). Since both skelemin and talin interact with the same regions of the integrin α_{IIb} and β_3 cytoplasmic tails, future studies will be needed to investigate the spatial and temporary hierarchy of their recruitment to integrin complexes.

In conclusion, we have demonstrated that binding of $\alpha_{IIb}\beta_3$ to immobilized fibrinogen during platelet adhesion initiates unclasping of the complex between the α_{IIb} and β_3 cytoplasmic tails. The consequence of this conformational change is the binding of the cytoplasmic skelemin-like protein to both α_{IIb} and β_3 tails. Since the interface between the cytoplasmic tails is thought to be preserved across the integrin family, unclasping of the tails may represent a common mechanism for the initiation of cell adhesion and spreading.

Supplementary Material

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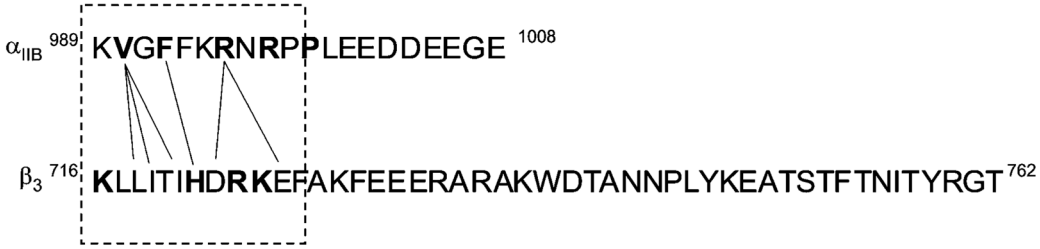

Figure 1.

Amino acid sequences of the α_{IIb} (residues 989–1008) and β_3 (residues 716–762) cytoplasmic tails. The membrane-proximal sequences of α_{IIb} 989–998 and β_3 716–727 which form the complex are boxed. Residues that are involved in the interface are connected by vertical lines. Residues critical for skelemin binding identified in this study are shown in bold.

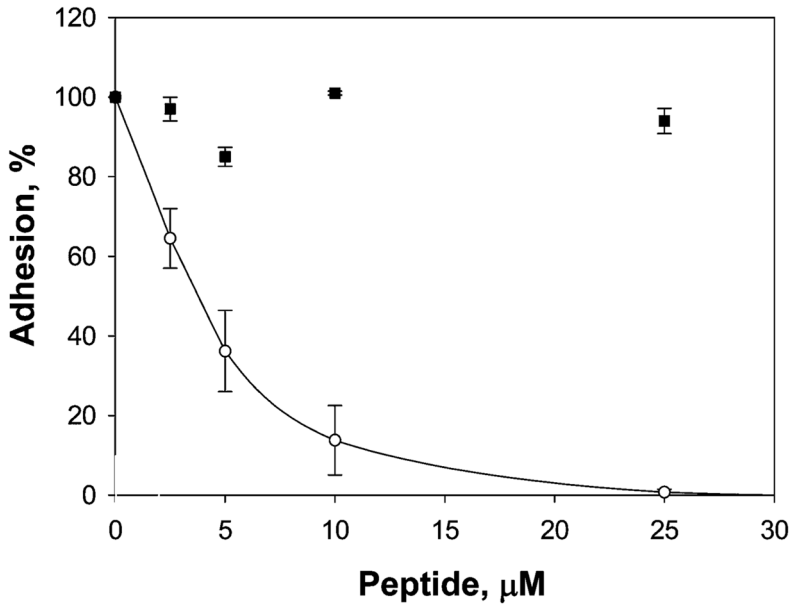

Figure 2.

Effect of the Myr-α_{IIb} peptides on platelet adhesion. Calcein-labeled platelets were preincubated with different concentrations of myristoylated wild-type α_{IIb} ^{989}KVGFFKRNRPPLEEDDEEGE^{1008} (○) and mutant α_{IIb} ^{989}KVGFFKANAPALEEDDEEGE^{1008} (■) peptides for 15 min at 22 °C and then added to the wells of microtiter plates coated with 2 μg/mL fibrinogen D_{100} fragment. The plates were briefly spun (1 min at 2200 rpm), and then platelets were allowed to adhere for an additional 50 min at 37 °C. Nonadherent platelets were removed by washing with PBS, and adherent cells were detected by measuring fluorescence. Data are expressed as the percentage of adhesion in the absence of the peptide and are the mean ± standard error of four individual experiments performed with triplicate determinations in each experiment.

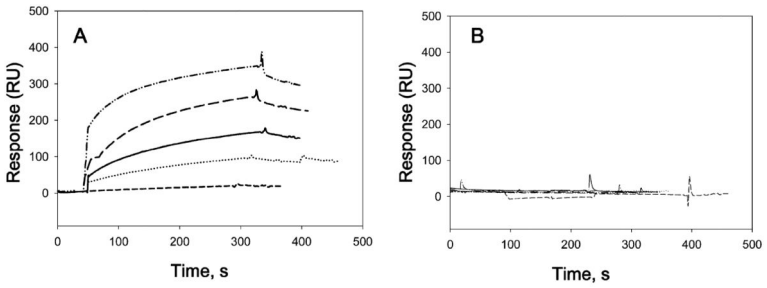

Figure 3.

Analyses of the interaction of isolated $\alpha_{IIb}\beta_3$ with the recombinant skelemin fragment by SPR. Representative sensograms of $\alpha_{IIb}\beta_3$ (0, 50, 100, 200, and 500 μg/mL) binding to fibrinogen (A) and skelemin (B) coupled on parallel cells of the sensor chip. To activate $\alpha_{IIb}\beta_3$, DTT was added to a final concentration of 3 mM for 15 min at 4 °C. Following incubation with DTT, the sample was extensively dialyzed. The DTT-activated integrin was passed over the chip for 5 min at a rate of 10 μL/min. RU, response units. No binding of $\alpha_{IIb}\beta_3$ to skelemin was detected.

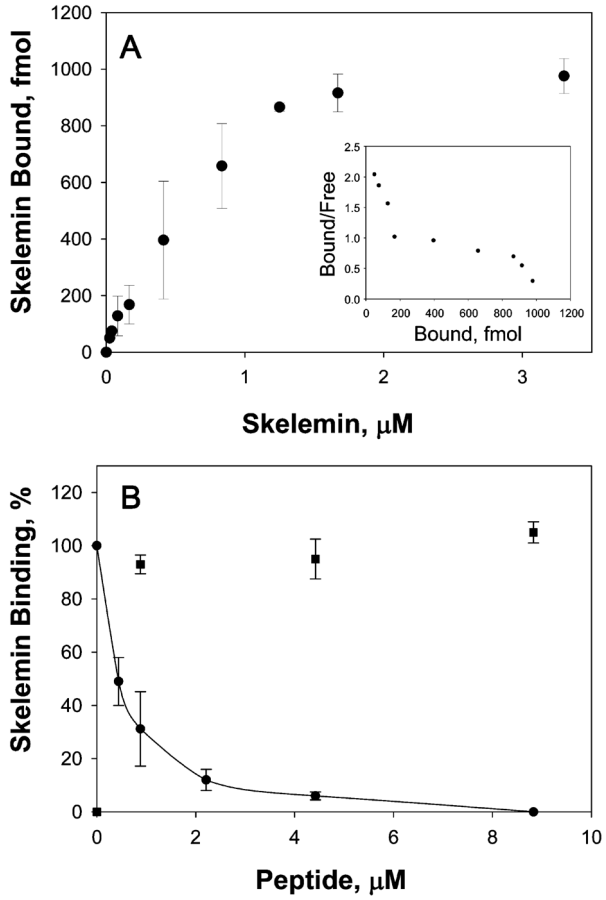

Figure 4.

Induction of the skelemin binding site in $\alpha_{IIb}\beta_3$ prebound to immobilized fibrin(ogen). (A) Purified $\alpha_{IIb}\beta_3$ (50 μg/mL) was added to the wells which were first coated with 20 μg/mL fibrin monomer and then postcoated with 1% BSA. After 3 h at 22 °C, the wells were washed and incubated with different concentrations of ^{125}I-labeled skelemin fragment for an additional 3 h at 22 °C. After being washed, the bound [^{125}I]skelemin fragment was counted in a γ counter. The level of nonspecific binding of the [^{125}I]skelemin fragment to immobilized fibrin with BSA was ~30%, and it was subtracted. Data are the means ± the standard error of six individual experiments performed with triplicate determinations in each experiment. The inset shows a Scatchard plot of [^{125}I]skelemin binding to $\alpha_{IIb}\beta_3$. (B) Inhibition of skelemin binding to $\alpha_{IIb}\beta_3$ by the β_3 715–730 peptide. Different concentrations of β_3 ^{715}WKLLITIHDRKEFAKF^{730} (●) or mutant α_{IIb} KVGFFKANAPALEEDDEEGE (■), which was used as a control peptide, were incubated with 100 μg/mL [^{125}I]skelemin fragment for 20 min at 22 °C and then added to the wells with $\alpha_{IIb}\beta_3$ captured on immobilized fibrin. Results are expressed as a percentage of binding in the absence of the peptide. Data shown are means ± the standard error from four individual experiments with triplicate determinations in each experiment.

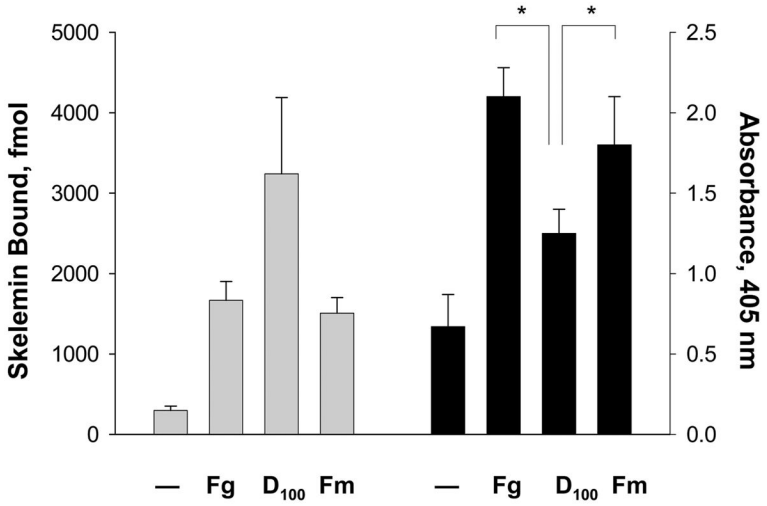

Figure 5.

Binding of skelemin and mAb 3F5 to $\alpha_{IIb}\beta_3$ prebound to different ligands. Fibrinogen (Fg), fibrin monomer (Fm), and the D_{100} fragment were immobilized on microtiter wells at 6, 15, and 4 μg/mL, respectively. The wells were postcoated with 1% BSA, and 50 μg/mL $\alpha_{IIb}\beta_3$ (100 μL) was added for 3 h at 22 °C. These concentrations of fibrinogen ligands bind equal amounts of $\alpha_{IIb}\beta_3$ (76 ± 1.9 ng) and were determined in preliminary experiments with ^{125}I-labeled $\alpha_{IIb}\beta_3$. Alternatively, the same amount of $\alpha_{IIb}\beta_3$ was adsorbed directly onto plastic (−), and then the wells were postcoated with BSA. On the left ordinate, different concentrations of the ^{125}I-skelemin fragment (0–330 μM) were added to $\alpha_{IIb}\beta_3$ prebound to various ligands and incubated for 3 h at 22 °C. After the sample had been washed, the amount of bound skelemin was measured. The data shown represent the values of B_{max} which were determined from binding isotherms for each ligand (gray bars). Data are the means ± the standard error from four individual experiments each with triplicate measurements. No significant difference in skelemin binding to integrin prebound to different ligands was found. The right ordinate shows binding of mAb 3F5 (0.5 μg/mL) to $\alpha_{IIb}\beta_3$ prebound to various ligands or adsorbed directly on plastic (−). The concentrations of ligands used to coat plastic and the amount of $\alpha_{IIb}\beta_3$ bound to each ligand were as described above. mAb binding was detected using goat anti-mouse IgG conjugated to alkaline phosphatase with *p*-nitrophenyl phosphate as a substrate, measuring the absorbance at 405 nm (black bars). Asterisks denote $P < 0.05$.

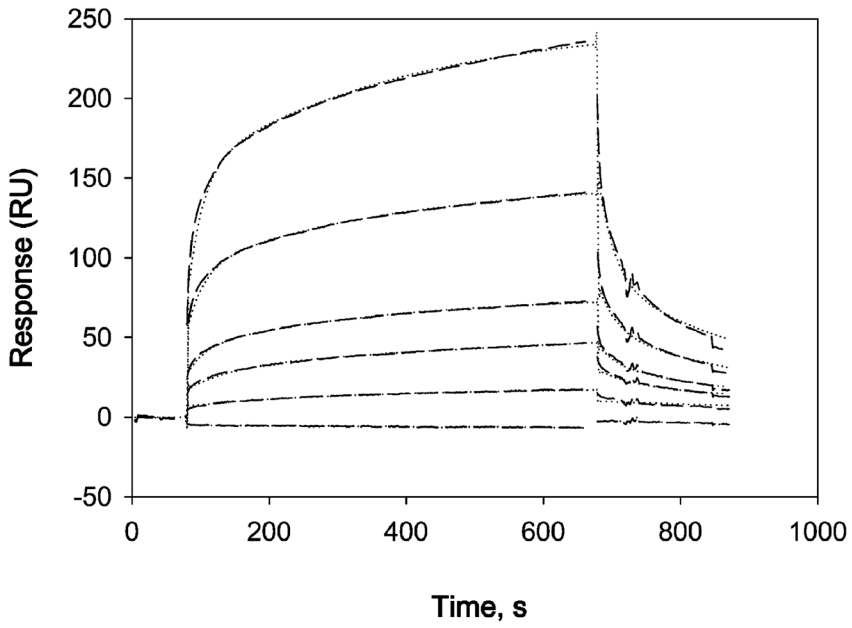

Figure 6.

Analysis of skelemin binding to the α_{IIb} peptide using SPR. Representative profiles of the SPR responses (---) for the interaction of α_{IIb} peptide (α_{IIb} 989–1008) with the skelemin fragment coupled to the sensor chip. Different concentrations of the peptide (0, 10, 20, 40, 100, and 200 μM) were passed over the chip for 10 min at a rate of 5 μL/min. The sensorgrams fit well to a two-state (conformational state) binding model obtained from analyses using BIAevaluation 4.1 (· · ·). The K_d determined from these analyses was 248 ± 62 μM (n = 3). RU, response units.

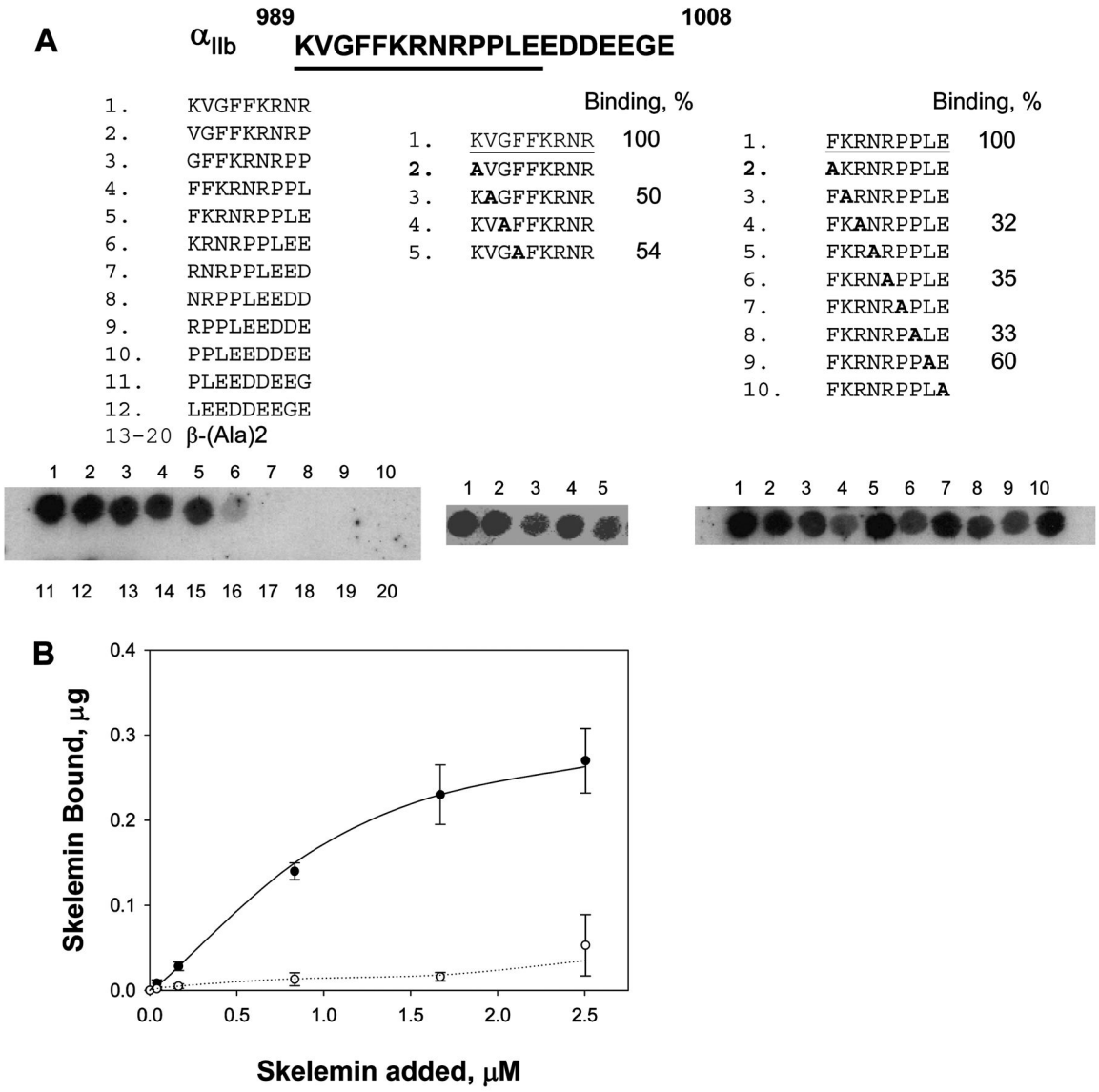

Figure 7.

Localization of residues in the α_{IIb} tail involved in skelemin binding. (A) Cellulose membranes with the assembled overlapping peptide library derived from the sequence of the α_{IIb} peptide (residues 989–1008) consisting of 9-mer peptides with a one-residue offset (left panel) and the substitutional libraries in which each residue in ^{989}KVGFFKRNR^{997} (middle panel) and ^{993}FKRNRPPLE^{1001} (right panel) was consecutively changed to Ala were incubated with the ^{125}I-labeled sekelemin fragment. After 3 h, the membranes were washed, dried, and subjected to autoradiography followed by densitometry. Skelemin binding is observed as black spots. Positions on the membrane containing the α_{IIb} overlapping peptide library (left panel) which correspond to spots 13–20 contain only the (β-Ala)$_2$ spacer. Skelemin binding to selected mutant peptides is expressed as a percentage of binding to wild-type peptides (which was assigned a value of 100%) and is shown as the numbers on the right of each sequence. The numbers are shown only for peptides with a reduced level of

skelemin binding. Wild-type sequences in each substitutional library are underlined. (B) Binding of ^{125}I-labeled skelemin to wild-type α_{IIb} ^{989}KVGFFKRNRPPLEEDDEEGE^{1008} (●) and mutant α_{IIb} peptide ^{989}KVGFFKANAPALEEDDEEGE^{1008} (○) was tested in solid-phase binding assays. The wells of Removawell strips were coated with peptides (20 μg/mL), postcoated with 1% BSA, and then incubated with various concentrations of ^{125}I-labeled skelemin. The result shown is representative of five independent experiments with triplicates at each experimental point.

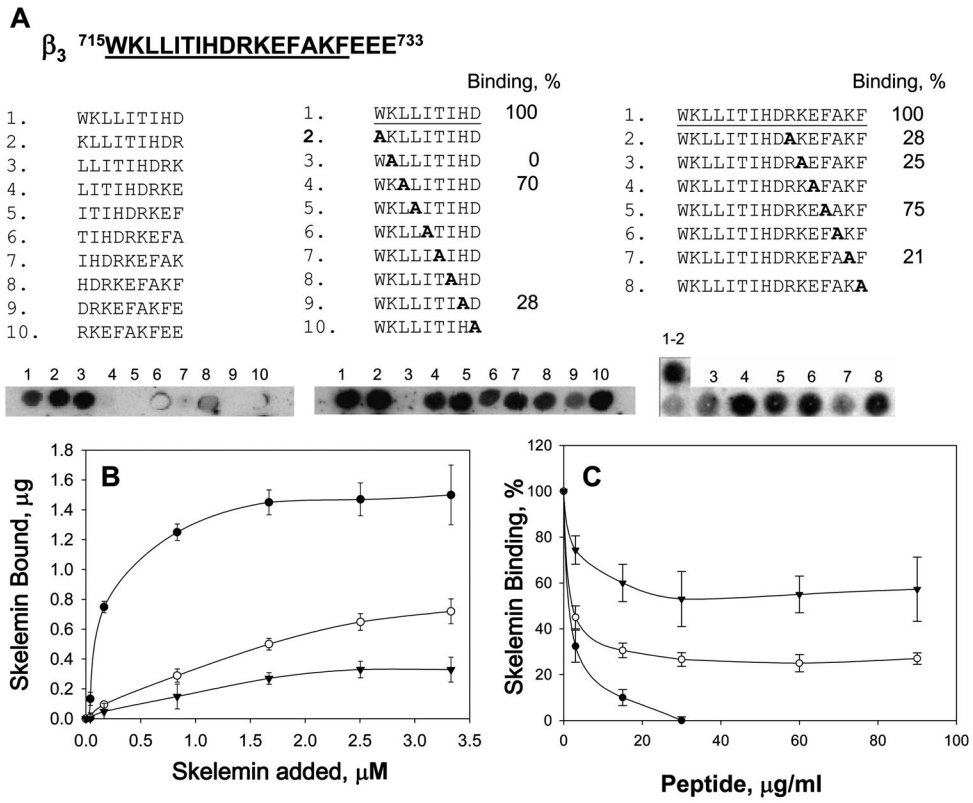

Figure 8.

Identification of residues in the β_3 715–733 segment critical for skelemin binding. (A) Cellulose membranes with the overlapping (left panel) and substitutional (middle and right panels) peptide libraries derived from β_3 cytoplasmic tail residues 715–733 were incubated with the [^{125}I]skelemin fragment. Binding was detected by autoradiography followed by densitometry analyses. Skelemin binding to selected mutant peptides is expressed as a percentage of binding to wild-type peptides (numbers on the right) and is shown only for peptides with a reduced level of skelemin binding. Wild-type sequences in each substitutional library are underlined. (B) Different concentrations of the [^{125}I]skelemin fragment were incubated with wild-type β_3 ^{715}WKLLITIHDRKEFAKF^{730} (●), β_3 mutant 1 ^{715}WALLITIADRKEFAKF^{730} (○), and β_3 mutant 2 ^{715}WALLITIADAKEFAKF^{730} (▼) peptides coated on the wells of microtiter plates at 20 μg/mL. (C) Inhibition of skelemin binding by wild-type and mutant β_3 715–730 peptides. Microtiter wells were coated with the D_{100} fragment, and then $\alpha_{IIb}\beta_3$ (10 μg/mL) was added for 3 h at 22 °C. After the samples had been washed, aliquots of the [^{125}I]skelemin fragment (100 μg/mL) preincubated for 10 min with different concentrations (0–90 μg/mL) of wild-type β_3 (●), β_3 mutant 1 (○), and β_3 mutant 2 (▼) peptides were added to the wells. Binding is expressed as the percent of the control without inhibitor.

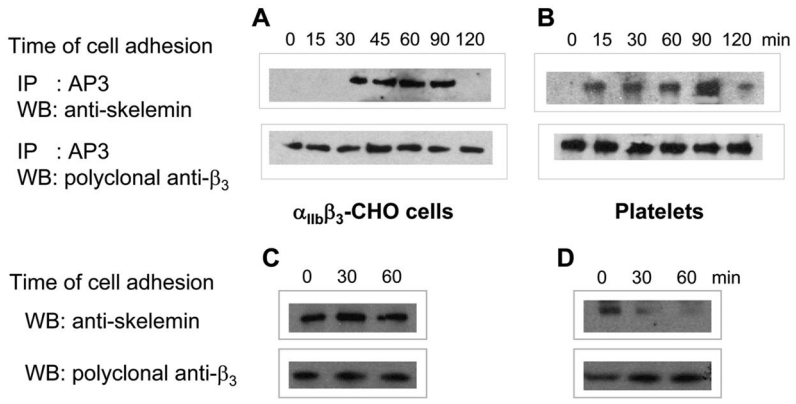

Figure 9.

Characterization of skelemin–$\alpha_{IIb}\beta_3$ interactions in suspended and adherent cells by immunoblotting. (A and B) Western blot analyses of immunoprecipitates obtained from lysates of suspended nonadherent (denoted as 0) and adherent cells (times of adhesion of 15–120 min). Lysates of the $\alpha_{IIb}\beta_3$-expressing CHO cells or platelets were subjected to immunoprecipitation with mAb AP3. The proteins in the immunoprecipitates were resolved on 7.5% SDS–polyacrylamide gels, transferred to an Immobilon P membrane, and probed with anti-β_3 (1:4000 dilution) and anti-skelemin (1:40000 dilution) polyclonal antibodies. Equal amounts of the β_3 integrin subunit were applied to each well during electrophoresis. A band disclosed with the anti-skelemin antibody has a molecular mass of ~200 kDa which corresponds to the endogenous skelemin-like protein. Representative Western blot analyses of samples obtained from three and five separate experiments with $\alpha_{IIb}\beta_3$-expressing CHO cells and platelets, respectively, are shown. (C) Analyses of total skelemin-like protein in lysates of nonadherent and adherent platelets. Lysates obtained from cells in suspension (0) or 30 and 60 min after adhesion were analyzed by Western blotting using anti-skelemin antibodies (1:40000). The proportion of protein in each sample was equalized on the basis of the amount of β_3 integrin subunit. (D) Analyses of skelemin in lysates of nonadherent (0) and adherent (30 and 60 min) platelets after the removal of skelemin–integrin complexes. Platelet lysates obtained from nonadherent (0) and platelets adherent for various times were first incubated with anti-β_3 mAb AP3. Skelemin–$\alpha_{IIb}\beta_3$ complexes were then removed using Protein A–agarose beads, and the skelemin remaining in the lysates was analyzed by Western blot analyses using anti-skelemin antibody.

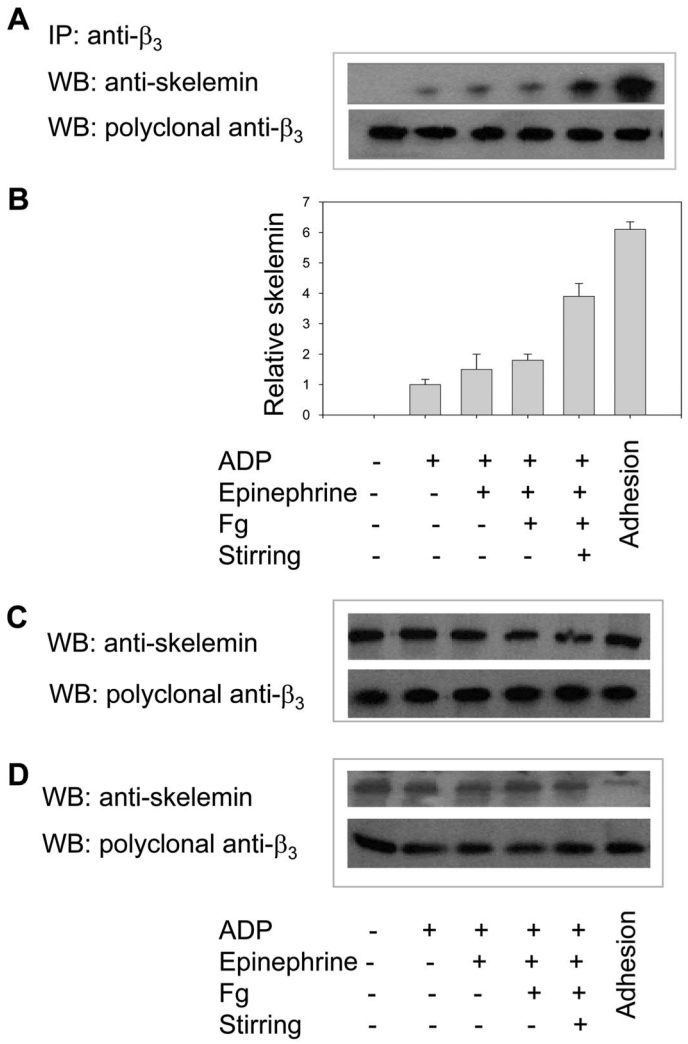

Figure 10.

Immunoblot characterization of skelemin–$\alpha_{IIb}\beta_3$ interactions in agonist-stimulated and adherent platelets. Platelets in suspension (5×10^8 per milliliter) were incubated for various periods of time (0–60 min) in the presence of 10 μM ADP alone or 10 μM ADP and epinephrine. In addition, platelets stimulated with both agonists were incubated for 15–60 min in the presence of fibrinogen (3 μM). Platelet lysates were immunoprecipitated with mAb AP3, and skelemin in the immune complexes was detected by Western blotting using anti-skelemin polyclonal antibodies (1: 40000 dilution). The amount of skelemin immunoprecipitated under each condition was determined by densitometry after equalizing the amount of β_3 subunit in each sample. The maximal amount of skelemin in complex with $\alpha_{IIb}\beta_3$ was observed after 60 min in the presence of ADP or ADP and epinephrine (shown). In the presence of fibrinogen, the maximal amount of skelemin immunoprecipitated with $\alpha_{IIb}\beta_3$ was detected after 15 min (shown) and then declined. (A) The indicated lysates of agonist-stimulated platelets containing approximately equal amounts of β_3 integrins were compared with the lysate obtained from adherent (60 min) platelets. A representative experiment of nine individual Western blot analyses of samples obtained from three

independent platelet preparations is shown. (B) Densitometry analyses of skelemin in the samples (n = 9). (C) The amount of total skelemin present in lysates of platelets treated as described for panel A was assessed by Western blot analyses using the anti-skelemin antibody. The samples contained approximately equal amounts of β_3 integrins. (D) Platelet lysates prepared as described for panel A were immunoprecipitated with mAb AP3; immune complexes were removed with Protein A–agarose beads, and the amount of skelemin remaining in the lysates was analyzed by Western blotting with the anti-skelemin antibody.