Integrin-mediated Activation of Focal Adhesion Kinase Is Independent of Focal Adhesion Formation or Integrin Activation

STUDIES WITH ACTIVATED AND INHIBITORY \( \beta_3 \) CYTOPLASMIC DOMAIN MUTANTS

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Integrin \( \alpha_{IIb}\beta_3 \) functions as the fibronectin receptor on platelets and mediates platelet aggregation and clot retraction. Among the events that occur during either “inside-out” or “outside-in” signaling through \( \alpha_{IIb}\beta_3 \), is the phosphorylation of focal adhesion kinase (pp125FAK) and the association of pp125FAK with cytoskeletal components. To examine the role of pp125FAK in these integrin-mediated events, pp125FAK phosphorylation and association with the cytoskeleton was determined in cells expressing two mutant forms of \( \alpha_{IIb}\beta_3 \): \( \alpha_{IIb}\beta_3 \) (D723A/E726A), a constitutively active integrin in which the putative binding site for pp125FAK is altered, and \( \alpha_{IIb}\beta_3 \) (F727A/K729E/F730A), in which the putative binding site for \( \alpha \)-actinin is altered. Both mutants were expressed on the cell surface and were able to bind ligand, either spontaneously or upon activation. Whereas cells expressing \( \alpha_{IIb}\beta_3 \) (D723A/E726A) were able to form focal adhesions and stress fibers upon adherence to fibronectin, cells expressing \( \alpha_{IIb}\beta_3 \) (F727A/K729E/F730A) adhere to fibronectin, but had reduced focal adhesions and stress fibers. pp125FAK is recruited to focal adhesions in adherent cells expressing \( \alpha_{IIb}\beta_3 \) (D723A/E726A) and is phosphorylated in adherent cells or in cells in suspension in the presence of fibronectin. In adherent cells expressing \( \alpha_{IIb}\beta_3 \) (F727A/K729E/F730A), pp125FAK was phosphorylated despite reduced formation of focal adhesions and stress fibers. We conclude that activation of pp125FAK can be dissociated from two important events in integrin signaling, the assembly of focal adhesions in adherent cells and integrin activation following ligand occupation.

The cytoplasmic domains of integrins constitute an essential link between the extracellular, ligand-binding domain of the receptor and both signaling and structural mechanisms inside the cell. Ranging in length from 15 amino acids for the \( \alpha_1 \) subunit to over 1000 amino acids for the \( \beta_3 \) subunit, the cytoplasmic domains extend inward from the plasma membrane toward various cytosolic components known to be involved in integrin-mediated signaling such as heterotrimeric G proteins, proteins involved in phospholipid metabolism, serine-threonine kinases, tyrosine kinases, calcium transport systems, and cytoskeleton components (1–7). Although structurally distinct, the different \( \alpha \) and \( \beta \) cytoplasmic domains show areas of regional homology, suggesting a conserved tertiary structure. The nine known \( \beta \) subunits contain a highly conserved membrane-proximal polar region with the consensus sequence HDRERFAKEEK, which is separated by a stretch of nine amino acids from a central NPXY motif and a stretch of 16–25 amino acids from a distal NPXY motif. The NPXY sequence prescribes a signal for clathrin-coated pit-mediated internalization of integral membrane proteins (8) and is a recognition site for She, an SH2-containing adaptor protein (9). The spacing of the tyrosine residues in the \( \alpha_1 \), \( \beta_3 \), and \( \beta_6 \) cytoplasmic tails conforms to the spacing of tyrosine residues in immunoglobulin family tyrosine-based activation motifs, which represent coupling sequences for protein tyrosine kinases and adapter molecules (10). Like the \( \beta_3 \) subunits, the \( \alpha \) subunits also contain a conserved membrane-proximal region with the consensus sequence, KXGFFKR, followed in many cases by a sequence predicting a \( \beta \) turn. The structural diversity of the cytoplasmic domains is increased further by the expression of alternatively spliced forms of several subunits, including \( \beta_3 \) (11–13), \( \beta_3 \) (14), \( \alpha_3 \) (15), and \( \alpha_6 \) (16).

Several important integrin functions have been linked to sequences in the cytoplasmic domains. The recruitment of integrins into focal adhesions is mediated by the cytoplasmic domains. Solow ska and co-workers (17) provided early evidence that deletion of the \( \beta_3 \) subunit cytoplasmic domain blocked integrin association into focal adhesions. Subsequent deletion mapping and random mutagenesis studies have indicated that the sequences required for direction to focal adhesions are localized to several discrete regions in the \( \beta \) cytoplasmic domains, including the dibasic sequence in the membrane-proximal region that is highly conserved in those \( \beta \) cytoplasmic domains that localize to focal adhesions, the NPXY sequences, and the carboxyl terminus (18–23). Some of the same general regions required for direction to focal adhesions have been implicated in the interaction of \( \beta_3 \) subunit cytoplasmic domains with \( \alpha \)-actinin (24) and with talin (25–27), proteins that are components of the cellular cytoskeleton and are present in focal adhesions. Sequences in the \( \alpha \) subunit cytoplasmic domains also appear to play a role in normal ligand-dependent localization of integrins to focal adhesions (22, 28, 29). In addition, integrin cytoplasmic domains play an important role in regulating the affinity of the integrin for ligand. Both \( \alpha \) and \( \beta \) subunit cytoplasmic tails appear to be important for affinity modulation. O’Toole et al. (30) found that the affinity of \( \alpha_{IIb}\beta_3 \) for ligand was increased by truncation of the cytoplasmic domain of \( \alpha_{IIb} \). Activation correlated with removal of the highly conserved membrane-proximal GFFKR motif, suggesting that this motif was required to maintain the receptor in a low

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affinity activation state. Deletion of the membrane-proximal LLITIHID region of the β3 cytoplasmic domain also produced a high affinity receptor (31). Further studies have identified Asp-723 in β3 and Arg-995 in αIIb as important and suggested that these two residues form a molecular hinge that regulates receptor affinity (32). The NPYX motif in the β subunit cytoplasmic domain also appeared to be important in the regulation of the affinity state of αIIbβ3, since point mutations in this region inhibited activation through the α subunit (33). A naturally occurring mutation in the β cytoplasmic domain at serine 752 in a patient with Glanzmann’s thrombasthenia inhibited activation of αIIbβ3 (34).

While the association of integrins with focal adhesions has been well established, the factors regulating this association with focal adhesions and that activation of pp125FAK, which correlated with the assembly of focal adhesions and stress fibers (38, 41). Conversely, inhibition of tyrosine phosphatases results in increased phosphorylation of pp125FAK (35), is a component of focal adhesions and is phosphorylated following integrin-mediated adhesion or integrin clustering (36–40). Tyrosine phosphorylation of pp125FAK is an early event in adhesion and phosphorylation persists as long as cells remain attached to substrata (41). Inhibition of pp125FAK phosphorylation by tyrosine kinase inhibitors is associated with reduced formation of focal adhesions and stress fibers (38, 41). Conversely, inhibition of tyrosine phosphatases results in increased phosphorylation of pp125FAK, which correlated with the assembly of focal adhesions and actin stress fibers (42, 43). These observations led to a suggestion that tyrosine phosphorylation of pp125FAK might be implicated in cytoskeletal assembly and formation of focal adhesions.

The present studies were undertaken to examine further the role of pp125FAK in integrin function. We have used two unique mutants of the β3 cytoplasmic domain to examine the role of pp125FAK in assembly of focal adhesions and in integrin activation. One mutation is in the region of the β3 cytoplasmic domain, which has been suggested to interact with α-actinin and interrupts the association of αIIbβ3 with focal adhesions. The second, which is similar to a recent mutation reported by Hughes et al. (32), is in the putative pp125FAK binding site in the β3 cytoplasmic domain and results in a constitutively active integrin. To examine the role of pp125FAK phosphorylation in its association with focal adhesions and in integrin activation, we have examined the state of pp125FAK phosphorylation in cells expressing these mutant integrins. The results suggest that pp125FAK phosphorylation is independent of integrin association with focal adhesions and that activation of αIIbβ3 can occur without inducing pp125FAK phosphorylation.

MATERIALS AND METHODS

Antibodies and Reagents—AP2, an αIIbβ3 complex-specific monoclonal antibody (44), A2A9, a complex-specific monoclonal antibody (45), Tab, an αIIbβ3-specific monoclonal antibody (46), and PAC1, the activated αIIbβ3 complex-specific monoclonal antibody (47) were provided by Dr. Thomas Kuniczki (Scripps Research Institute, La Jolla, CA), Dr. Joel Bennett (Philadelphia, PA), the University Cell Center of Pennsylvania (Philadelphia, PA), and Dr. Rodger McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK), respectively. LlB6, a β3 monoclonal antibody which activates αIIbβ3 (48) was kindly provided by Dr. Mark Ginsberg (Scripps Research Institute, La Jolla, CA). LM609, an antibody to the vitronectin receptor (49), was from David Cheresh (Scripps Research Institute, La Jolla, CA). FITC-conjugated phalloidin, used for staining actin filaments, was purchased from Molecular Probes (Eugene, OR). Additional antibodies for staining focal adhesions included anti-vinculin, anti-paxillin, and anti-pp125FAK, all purchased from Transduction Laboratories (Lexington, KY). 2A7, an anti-pp125FAK monoclonal antibody used for immunoprecipitation studies, was generously provided by Dr. Tom Parsons (University of Virginia). A human polyclonal antibody against pp125FAK, HuB3, was provided by Dr. Michael Schaller (Chapel Hill, NC). Anti-phosphotyrosine monoclonal antibody, 4G10, was purchased from Transduction Laboratories, Inc. (Lake Placid, NY). Secondary antibodies, rhodamine-labeled donkey anti-mouse IgG and FITC-labeled donkey anti-rabbit IgG, were supplied by Chemicon International, Inc. (Temecula, CA). Goat anti-mouse IgG peroxidase conjugate was from Sigma. Fluorosave was purchased from Calbiochem. FITC-conjugated goat anti-mouse IgG, F(ab′)2 and rabbit anti-mouse IgM were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). The peptide, BRGDSP, was synthesized on an Applied Biosystems model 431A peptide synthesizer (Foster City, CA) by the University of North Carolina Protein Chemistry Laboratory (Chapel Hill, NC). Oligonucleotides were synthesized on a model 392–394 DNA synthesizer (Applied Biosystems, Foster City, CA) and purified by high performance liquid chromatography by the University of North Carolina Department of Pathology Oligonucleotide Facility (Chapel Hill, NC). Human fibrogenin, which was depleted of fibronectin by adsorption on gelatin-Sepharose, was a gift from Dr. L. Parise (University of North Carolina, Chapel Hill, NC). Human fibrogenin was purchased from Calbiochem. Plasmid vectors, pCDNAI/Amp and pRc/CMV, were obtained from Invitrogen (San Diego, CA), while pT7T7 was from Stratagene (La Jolla, CA). Human thrombin was a gift from Dr. Frank Church (University of North Carolina, Chapel Hill, NC).

DNA Constructs—β3 and αIIb cDNA were isolated from a cDNA library provided by Lawrence Brass (University of Pennsylvania, Philadelphia, PA). The β3 cDNA was mutated to eliminate the EcoRI site at nucleotide 2270 (β3ΔE). The cDNAs were subcloned into the EcoRI site in Bluescript II KS. The constructs were then subcloned into the HincII and XhoI sites in the multiple cloning region of pT7T7. A cytoplasmic domain cassette was created in β3ΔE using the molecular cloning strategy diagrammed in Fig. 1 with the oligonucleotides listed in Table I. All restriction sites, which were created or modified, resulted in silent mutations. A unique NdeI restriction site (C to A at nucleotide 2247) flanking the 5′ end of the cytoplasmic domain was introduced by PCR mutagenesis during an initial round of amplification using oligonucleotides 1 and 2 (Fig. 1, Step A). An existing BsoBI restriction site at base 2418, 24 bases downstream from the stop codon, was altered by a C to G base change at position 2417, which was introduced during a second round of amplification (Fig. 1, Step B). In this reaction, the initial PCR product from Step A was used as a megaprimer with oligonucleotide 3. The deletion of the EcoRI site removed a downstream EcoRI site, thereby making the construct susceptible to digestion with BsoBI when grown in standard host strains. Full-length β3 in pT7T7 was digested with Apol and XhoI. The digestion fragment containing the vector and the 5′ region of β3 up to the first Apol site at nucleotide 110 was isolated and ligated to the similarly digested PCR product (Fig. 1, Step C). Subsequently, the fragment from Apol 110 to Apol 2176 was cloned into the orientation (Fig. 1, Step C). The new construct lacked most of the 3′-untranslated region of β3 cDNA. The region of the construct from base 2176 to 2418, which was amplified by the polymerase chain reaction, was sequenced to confirm that there were no mutations. Three additional unique restriction sites, BstHII at base 2306 (A to G at 2304), AarII at base 2357 (C to G at 2355), and BglII, which is located at base 2405 in the 3′-untranslated region, were generated by PCR of the construct in Step D with oligonucleotides 4 and 5 (Fig. 1, Step E). The PCR product was digested with NdeI and XhoI and subcloned into the similarly digested BsoBI and pT7T7 construct (Fig. 1, Step F). The construct from Step F was digested with BstHII and AarII, isolated from an agarose gel, and ligated to annealed oligonucleotide pair 6 and 7. This completed the construction of the β3 cDNA with four unique, silent restriction sites in the cytoplasmic domain. Cytoplasmic domain microinjection was used with annealing specific oligonucleotide pairs at 85 °C for 5 min and allowing to cool to 22 °C over 1 h. Double-stranded fragments containing the base changes required for the generation of the desired amino acid substitutions were ligated to the appropriately digested construct (Fig. 1, Steps H, I, and J). These completed constructs were sequenced to verify that there were no synthesis errors and that the desired base changes were made. The constructs were digested with BssHII and XhoI, and the fragment was isolated and subcloned into similarly digested β3/pcDNAI/Amp. DNA for transfection was purified on a Qiagen-tip 100 (Qiagen, Chatsworth, CA).

Preparation of Cell Lines—CHO-K1 cells were obtained from the University of North Carolina Tissue Culture Facility and cultured in
and 9 and pair 10 and 11 were annealed, and the double-stranded fragments were ligated, purified on an agarose gel, and cloned into the NdeI and BssHII sites. J, the ligation product of oligonucleotide pair 12 and 13 to oligonucleotide pair 8 and 9 was subcloned into the NdeI and BssHII sites to generate mutant D723A/E726A. J, generation of mutant F727A/K729E/F730A with oligonucleotide pair 14 and 15.

Dulbecco's modified Eagle's medium (DMEM-H) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin, 100 \( \mu g/ml \) streptomycin, and non-essential amino acids (Life Technolo-
gies, Inc.). Adherent CHO-K1 cells were detached in 1 \( \times \) trypsin-EDTA (Boehringer Mannheim), washed, and resuspended in PBS containing 1 \( \mu g \) of linearized \( \alpha_{1b} \) cDNA in pReCMV-Neo and 10 \( \mu g \) linearized \( \beta_{3} \) cDNA constructs in pcDNAI/Amp. The cells were electroporated at 25 microfarads and 0.75 kV for 5 s in a gene pulser apparatus (Bio-Rad). After 48 h, cells were cultured in the presence of 0.5 mg/ml active Geneticin (Life Technologies, Inc.), and neomycin-resistant cells were selected for 2 weeks. Cells were harvested and immunostained with AP2 as described below, and single positive cells were sorted into individual wells in 96-well plates on an Epics model 753 cell sorter (Coulter Electronics, Hialeah, FL).

Surface Labeling and Immunoprecipitation—Adherent cells grown to confluence were rinsed with PBS, incubated on ice with 5 \( \mu M \) NHS-LC-biotin (Pierce) in PBS for 30 min, and rinsed with cold 5 \( \mu M \) Tris-Cl, pH 8, containing 1 \( \mu M \) MgCl\(_2\), 0.1 \( \mu M \) each MgCl\(_2\) and CaCl\(_2\), and 20 \( \mu M \) HEPES, pH 7.4 (PBC). Cells were incubated with AP2 ascites (1/16,000 dilution) for 30 min on ice, washed twice in PBS, and incubated as above with FITC-conjugated goat anti-mouse IgG. Cells were washed twice in PBS and resuspended in the buffers indicated below for the respective studies.

Flow Cytometry—Cells were harvested as described above and resus-
pended in PBS containing 2% BSA and 0.1 \( \mu M \) each MgCl\(_2\) and CaCl\(_2\) (PBC). Cells were incubated with AP2 ascites (1/16,000 dilution) for 30 min on ice, washed twice with cold PBS, and incubated as above with FITC-conjugated goat anti-mouse IgG. Cells were washed once and resuspended in 400 \( \mu l \) of PBS for fluorescence analysis on a FACScan (Becton Dickinson, San Jose, CA). For PAC1 staining, cells were incubated with purified IgM antibody at 1 \( \mu g/ml \) in Tyrode's solution containing 1 \( \mu g/ml \) BSA, 1 \( \mu M \) MgCl\(_2\), 0.1 \( \mu M \) CaCl\(_2\), and 20 \( \mu M \) HEPES, pH 7.4 (PBB), for 30 min at room temperature, then stained with FITC-conjugated rabbit anti-mouse IgM. Staining with PAC1 antibody was also performed in the presence of 1 \( \mu M \) GRGDSP. To detect acti-
vation of expressed \( \alpha_{\text{IIb}}\beta_{3} \) cells were preincubated with LIBS6 ascites for 30 min at 37 °C, washed once with cold PBS, and immunostained with PAC1 in the presence or absence of 1 \( \mu M \) GRGDSP.

Adhesion—96-well tissue culture plates (Costar, Cambridge, MA)
TABLE I

Oligonucleotides used in the molecular construction of the β2 cytoplasmic domain cassette and mutant cDNAs

| Oligonucleotide | Sequence 1 | Sequence 2 |
|-----------------|------------|------------|
| 1.              | 5'-AAAGACCTTAAAGGACCTGGCAGAGTGAC-3' | NdeI * |
| 2.              | 3'-CCGAGCGGGCCAGCGATATACCTTCTTGAG-5' | BsaI |
| 3.              | 3'-CGTCACTAGGAGGCTCCTAATATGCAATTGAGACGTTCCGCAATGC-5' | XbaI |
| 4.              | 5'-GCCGCGGATATGATGCTGGCTGCGTGTTGATGGTGATATTTCCGC-3' | NdeI BsaBI AatII |
| 5.              | 3'-ATTACTATCAGCTTCTCAGGGACTCTACATAGATTTAGTATCTGCAGGCGG-5' | BsaII |
| 6.              | 5'-CCGCGCGACGCGAAATGGGACACGCGACAAACCAACTGTAATAGAGGGGACG-3' | |
| 7.              | 3'-GGTGTGTTTACCTGTCGCGTGGTTGGGTGACATATTTCTCCGCGG-5' | AatII |
| 8.              | 5'-TTGCCGAACTCTCATCAGC-3' | |
| 9.              | 3'-ACCTTTGAGGAGTATGTTAG-5' | NdeI |
| 10.             | 5'-CATCCACGCGCAGAAGAAGATTTGGTAATGAGAGGACG-3' |
| 11.             | 3'-GGTGTGTTTACCTGTCGCGGAGCTTTGACATATTTCTCCGCGG-5' | BsaII |
| 12.             | 5'-CATCCACGCGCAGAAGAAGATTTGGTAATGAGAGGACG-3' |
| 13.             | 3'-GGTGTGTTTACCTGTCGCGGAGCTTTGACATATTTCTCCGCGG-5' | A A |
| 14.             | 5'-CATCCACGCGCAGAAGAAGATTTGGTAATGAGAGGACG-3' |
| 15.             | 3'-GGTGTGTTTACCTGTCGCGGAGCTTTGACATATTTCTCCGCGG-5' | A E A |

Note: Enzyme sites are indicated by the letters: **NdeI**, ***BsaBI***, **AatII**.

Adhesion-dependent pp125FAK Phosphorylation—Plastic Petri dishes (10 cm) were precoated with 15 ml of either 50 μg/ml fibronectin in PBS or 2 μg/ml poly-d-lysine in PBS for 18 h at 4 °C. Before adherence, the plates were rinsed once with 5 ml of PBS. Cells were grown to confluence and then serum-starved in 0.5% fetal bovine serum in DMEM-H for 18 h. Cells from six 75-cm² flasks were harvested, washed twice in PBS and resuspended at 105 cells/ml in 20 ml of DMEM-H and maintained in suspension at 37 °C for 30 min. Cells (7.5 x 10⁴) were pipetted onto plates precoated with either fibronectin or poly-d-lysine or kept in suspension in 15-ml conical tubes and incubated for 25 min at 37 °C. Cells maintained in suspension were centrifuged for 5 min and lysed in 0.5 ml of lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris, pH 7.5, containing 1 mM sodium orthovanadate, 1 mM p-nitrophenyl phosphate, 10 μg/ml each leupeptin and pepstatin, 1 μg/ml aprotinin, and 0.5 mM Pefabloc). For adherent cells, medium was removed from plates, 0.5 ml of lysis buffer was added, and the plates were kept on ice for 10 min. The plates were then scraped and the lysates transferred to a 1.5-ml tube. All lysates were centrifuged at 12,000 x g for 10 min, and supernatants were transferred to a new tube. pp125FAK was immunoprecipitated with 1.5 μg of monoclonal antibody 2A7 at 1 h for 4 °C on a rocker. Immunoprecipitates were washed four times with lysis buffer, and beads were boiled in 50 μl of reducing buffer for 3 min. Samples were electrophoresed on a 7.5% acrylamide gel and blotted onto Immobilon-P. Blots were blocked for 1 h in TTBS (10 mM Tris, pH 7.4, 150 NaCl, 0.1% Tween 20) containing 3% BSA. Blots were incubated for 1 h with a 1:10000 dilution of polyclonal anti-FAK antibody (HuBS) in TTBS, washed extensively, and incubated for 1 h with anti-rabbit IgG (1:10,000) peroxidase conjugate. Phosphotyrosine was detected on a second blot with monoclonal antibody 4G10 (Upstate Biotechnology, Inc.) and 1:10,000 anti-mouse IgG peroxidase conjugate. Blots were washed and bands were detected with BM chemiluminescence (Boehringer Mannheim).
incubated on a gyratory shaker (New Brunswick Scientific, Edison, NJ) at 80 rpm for 20 min, and cell suspensions from each well were mounted on slides with coverslips and analyzed by brightfield photomicroscopy.

Clot Retraction—Harvested cells were resuspended in serum-free DMEM-H at 1 × 10^7/ml, and 200 µl was transferred to a glass aggregometry cuvette (Chrono-Log, Havertown, PA). Purified fibrinogen was diluted to 2 mg/ml in DMEM, and 100 µl was added to the cells with stirring. Clot formation was initiated by the addition of 100 µl thrombin (10 units/ml) in DMEM containing 28 mM CaCl2, and 25 mM HEPEs, pH 7.4. The stir bar was removed after stirring for 7 s, and tubes were incubated in a 37 °C water bath overnight and photographed.

RESULTS

The amino acid sequences of the wild-type and mutant cytoplasmic domains are shown in Fig. 2. Two mutant forms of β3 were constructed and expressed with wild type αIIb CHO cells, one termed D723A/E726A contained alanine substitutions in the membrane proximal region of the β3 cytoplasmic domain, which has been hypothesized to interact with α1b (51), and the other, termed F727A/K729E/F730A, contained substitutions in the region implicated in the binding of α-actinin (24).

Complex Formation and Surface Expression—To determine whether the α1bβ3 mutants formed heterodimeric complexes that were expressed on the cell surface, transfectants cultured in selective medium for 2 weeks were surface labeled with biotin, lysed, and immunoprecipitated with the α1b-specific antibody, Tab. As shown in Fig. 3, biotin-labeled α1b and β3 were recovered in the Tab immunoprecipitates from each cell line, indicating 1) that heterodimer formation had occurred, 2) that the complex was expressed on the cell surface, and 3) that the Tab epitope in the extracellular domain was intact. The heterodimer complex formation and surface expression of the α1bβ3 mutant constructs were confirmed by flow cytometry using AP2, a complex-specific monoclonal antibody. Like the epitope for Tab described above, the epitope for AP2 was preserved in the extracellular domains of the two mutants. All three constructs were expressed at similar levels on the cell surface as assayed semiquantitatively by florescence staining (see left column, Fig. 4).

Although agonist-stimulated mechanisms for activating α1bβ3 in CHO cells have not been identified, there are several methods that can be used to activate the complex externally to examine competence of the receptor to bind ligand. Partial reduction of disulfide bonds in α1b and/or β3 induces an activated conformation that is able to bind ligand (52). LIBS6 is a murine monoclonal antibody that binds to α1bβ3 and increases the affinity of the receptor for ligands such as fibrinogen or reporter antibodies like PAC1 (48). In response to LIBS6 (Fig. 4, third column from left), cells expressing the wild-type α1bβ3 showed increased binding of PAC1, a monoclonal antibody that recognizes the activated form of α1bβ3. Mutant F727A/K729E/F730A demonstrated a similar response to LIBS6 with increased binding of PAC1. In contrast, the mutant D723A/E726A complex bound increased PAC1, even in the absence of LIBS6 (Fig. 4, second column from left), suggesting that D723A/E726A was constitutively active. The addition of LIBS6 to cells expressing the mutant D723A/E726A complex induced additional binding of PAC1, implying that while the complex appears to be constitutively active, it may be only partially activated. D723A/E726A was not further activated by DTT (Fig. 4, right column).

To further determine if extracellular ligand binding domains of the mutant receptors were functionally intact, fibrinogen-dependent cell aggregation in response to DTT treatment was examined. As shown in Fig. 5, DTT-treated “activated” (A) wild-type cells formed large aggregates after 20 min, whereas the controls, which include DTT-treated cells in the absence of fibrinogen (not shown), and untreated cells in the presence (B) or absence (not shown) of fibrinogen, did not aggregate. Mutant D723A/E726A also formed large aggregates when activated, and, strikingly, also formed aggregates with no DTT pretreatment, a finding consistent with the demonstration of a constitutively active receptor by flow analysis with PAC1. DTT-treated mutant F727A/K729E/F730A also aggregated in response to DTT, which indicated that the extracellular region was functional.

Adhesion—Adherence of CHO cells expressing the various α1bβ3 constructs to fibrinogen was measured using a solid phase binding assay (Fig. 6). Wild-type and mutant D723A/E726A cells adhered to fibrinogen, and adhesion was inhibited by A2A9, a complex-specific antibody, indicating that adhesion was α1bβ3-specific. Whereas adhesion of wild-type cells was completely blocked by 1 mM GRGDSP, adhesion of mutant D723A/E726A cells was only partially blocked by 1 mM GRGDSP, but was completely blocked by 2 mM GRGDSP (not shown), suggesting that the receptor in these cells has a higher affinity for the ligand than wild-type. Mutant F727A/K729E/F730A cells adhered to fibrinogen, and adhesion was blocked with 1 mM GRGDSP, indicating that the extracellular portion of the receptor functions normally.

Clot Retraction—One of the functions of the cytoplasmic tails of integrins is their interaction with cytoskeletal components linking extracellular events with the cytoskeleton. Among other things, this interaction mediates retraction of a fibrin clot (22). To look at the interaction of the mutant cytoplasmic domains with cytoskeletal components, the effect of the β3 cyto-
plasmic domain amino acid substitutions on the integrin capacity to retract a fibrin clot was examined (Fig. 7). Harvested cells were mixed with purified human fibrinogen, and clot formation was initiated by the addition of thrombin. Wild type αIIbβ3 was able to efficiently retract a clot. In contrast, mutant F727A/K729E/F730A was unable to retract a clot. Mutant D723A/E726A retained the ability to retract a clot. Since transfection of CHO cells with β3 cDNA also results in complex formation between the β3 subunit and the endogenous α subunit to form αβ3 heterodimers, and since αβ3 can retract a fibrin clot in nucleated cells (53, 54), studies were performed to assess the relative contributions of αIIbβ3 and αβ3 in CHO cell-mediated retraction of the fibrin clot. Incubating cells with saturating amounts of AP2 inhibited clot retraction, whereas LM609 had no effect (not shown), suggesting that any vitronectin receptor expressed on the cell surface was not contributing significantly to clot retraction.

Formation of Focal Adhesions—During adhesion to extracellular matrix proteins, integrins become localized to focal adhesions at the ends of actin stress fibers. The interactions of the cytoplasmic domains of integrins with the cytoskeleton in focal adhesions involve the association of a number of proteins. We examined the ability of the wild-type or mutant αIIbβ3 heterodimers to form focal adhesions and to organize the actin cytoskeleton. Each of the CHO cell lines were treated with cycloheximide for 90 min to prevent matrix formation and plating onto fibrinogen-coated glass coverslips in serum-free media. Cells were fixed after 2 h, and the organization of actin was quantified by measuring the absorbance at 540 nm.

![Figure 4](image-url) **Fig. 4.** Flow cytometric analysis of cells expressing mutant β3. Mock-transfected CHO cells (A) or cells transfected with wild-type αIIb and either wild-type (B), F727A/K729E/F730A (C), or D723A/E726A (D) β3 cDNA were incubated with AP2 (left column), a monoclonal antibody specific for the complex, or PAC1 (second, third, and last columns), a monoclonal antibody specific for the activated state of the receptor, and analyzed on a FACScan. Cells were activated with anti-LIBS6 (third column) or 10 mM DTT (right column). Filled histograms, PAC1 binding in the absence of activation. Open histograms, PAC1 binding after activation.

![Figure 5](image-url) **Fig. 5.** Cell aggregation. Cells were resuspended in Tyrode’s solution containing 3.5 mg/ml albumin and treated with 10 mM DTT (A) or buffer (B) for 20 min at room temperature. After washing, cells were resuspended in Tyrode’s solution without albumin and mixed with fibrinogen and CaCl2, with rotating at 100 rpm for 20 min. Cell suspension were mounted on slides with coverslips and photographed.

![Figure 6](image-url) **Fig. 6.** Cell adhesion. Microtiter plates were coated with 4 μg/ml fibrinogen. Cells were incubated in the absence (filled bars) or presence of 1 mM GRGDSP (densely hatched bars) or 2.5 mg/ml A2A9 (open hatched bars), a complex-specific antibody. Cells were allowed to adhere to plates for 2 h, stained with 0.5% crystal violet, and cell binding was quantified by measuring the absorbance at 540 nm. A, mock; B, wild-type; C, F727A/K729E/F730A; D, D723A/E726A.

the cell, and stress fibers tended to extend between these focal adhesions with few stress fibers terminating within the center of the cell. There was little staining for α-actinin (Fig. 8h).
\( \alpha_{IIb}\beta_3 \) distribution appeared more diffuse throughout the cell. All three cell lines were able to spread equally on fibronectin-coated coverslips and form focal adhesions and stress fibers as judged by immunofluorescence localization of vinculin and actin (data not shown).

**pp125FAK Analysis**—

**pp125FAK** was normally distributed in focal adhesions in adherent cells expressing either wild-type (Fig. 9g) or mutant D723A/E726A (Fig. 9i). Mutant F727A/K729E/F730A (Fig. 9h) showed a more diffuse pattern of staining for pp125FAK. The pattern of tyrosine phosphorylation (Fig. 9, a–c) and paxillin (Fig. 9, d–f) staining was similar to that of pp125FAK. When plated onto fibronectin-coated coverslips, all three cell lines formed focal adhesions that stained positively for pp125FAK, paxillin, and phosphotyrosine, demonstrating that there was no defect in the capacity of these proteins to associate with focal adhesions (not shown).

To determine if the pp125FAK in focal adhesions was phosphorylated, cell lysates were immunoprecipitated with anti-pp125FAK. Samples were divided in half, electrophoresed, and transferred to nitrocellulose. Blots were incubated with either anti-pp125FAK or anti-phosphotyrosine (Fig. 10). pp125FAK immunoprecipitated from wild-type cells maintained in suspension or incubated in poly-D-lysine-coated flasks was minimally phosphorylated. As expected, integrin engagement on a fibrinogen matrix resulted in increased tyrosine phosphorylation of pp125FAK in wild-type cells. Cells expressing mutant D723A/E726A also showed no evidence for constitutive phosphorylation in cells maintained in suspension but displayed phosphorylation in cells plated on fibronogen. The addition of fibronogen to mutant D723A/E726A cells in suspension without an activating agent resulted in phosphorylation of pp125FAK, and this was blocked by 1 mM GRGDSP. Interestingly, adhesion of mutant F727A/K729E/F730A cells to fibrinogen also resulted in phosphorylation of pp125FAK, despite the failure of these cells to form significant focal adhesions or stress fibers. Semiquantitation of pp125FAK phosphorylation, expressed as the ratio of phosphorylated pp125FAK to pp125FAK antigen, indicated that pp125FAK phosphorylation in mutant F727A/K729E/F730A was approximately 85% that in wild-type cells (Table II).

**DISCUSSION**

These results provide evidence that activation of pp125FAK can be dissociated from two important events in integrin signaling. First, activation of pp125FAK can occur despite inhibition of integrin association with and assembly of focal adhesions and stress fibers. Second, pp125FAK was not activated in cells in which the \( \alpha_{IIb}\beta_3 \) receptor was constitutively active but not occupied by ligand. These observations suggest that events other than pp125FAK phosphorylation are needed for assembly of focal adhesions and that events other than integrin activation are needed for phosphorylation of pp125FAK.

The finding that phosphorylation of pp125FAK occurred only after ligand binding and was not observed in cells expressing the activated form of the receptor without ligand binding fits well with observations by Shattil and co-workers (40) that...
phosphorylation of pp125FAK during "outside-in" signaling did not occur with simple activation of \( \alpha_{IIb} \beta_3 \) but required a co-stimulatory signal. Based on the data of Shattil and others, it has been suggested that \( \alpha_{IIb} \beta_3 \) exists in several states of activation: a resting state, which is unable to bind fibrinogen or other ligands, a state of partial activation in which the receptor has undergone a conformational change which makes it able to bind ligand, and a state of activation in which ligand is bound and additional ligand-induced conformational changes have occurred. The results presented here are consistent with a multistate receptor and imply that the mutant receptor is only partially activated. Full activation of the receptor and generation of inwardly directed signals to pp125FAK occur after binding of ligand.

This behavior of the partially activated mutant form of \( \alpha_{IIb} \beta_3 \) appears to be similar to that reported by Hughes et al. (32) using a \( \beta_3 \) mutation in the same region. They suggested that the membrane-proximal regions of both \( \alpha \) and \( \beta \) subunits act as a molecular hinge. In their model, the charged residue, Asp-723, in \( \beta_3 \) cytoplasmic domain interacted with Arg-995 of the \( \alpha_{IIb} \) cytoplasmic domain to maintain the receptor in an inactive state. Mutation of either charged residue resulted in activation of the receptor, perhaps by breaking the molecular hinge. However, in contrast to our findings with mutant D723A/E726A in which phosphorylation of pp125FAK occurred only after ligand binding, Hughes et al. (32) found that constitutively active \( \alpha_{IIb}(F992A) \beta_3 \) or \( \alpha_{IIb}\beta_3(D723A) \) mediated both ligand-dependent and ligand-independent phosphorylation of pp125FAK. Whether these represent different effects of different mutations remains unclear.

One can speculate on the mechanism of activation of \( \alpha_{IIb} \beta_3 \) in the mutant D723A/E726A cell line. Perhaps the simplest explanation is that the mutations in the cytoplasmic tail induce local conformational changes that are transmitted to the extracellular domains, increasing affinity for ligand. In this case, the change in the extracellular domain should be a direct consequence of the cytoplasmic domain structural change and inde-
dependent of cellular energy requirements. However, we found that PAC1 did not bind to these cells in the presence of inhibitors of oxidative phosphorylation,\(^2\) raising the likelihood that some intracellular intermediate may be involved in receptor activation. Another possibility is that the mutation in the cytoplasmic tail affects the interaction of the \(\beta_3\) tail with cytosolic signaling molecules. In addition to \(\alpha\)-actinin (24) and pp125\(^{FAK}\) (51), several proteins have been identified that interact in vitro with either the \(\beta_3\) or the \(\beta_3\) cytoplasmic domains, including paxillin (51), talin (25–27), integrin-associated protein (55), \(\beta_3\)-endonexin (56), and an integrin-linked protein kinase (57).

In addition, a recent study of activated \(\alpha_\text{IIb}\beta_3\) receptor provides evidence for association with pp60\(^{src}\) and pp54/58\(^{fyn}\) (58). As a result of an altered interaction with such molecules, changes may occur in the extracellular domain to increase affinity for ligand. Since the mutation is in a putative binding site for pp125\(^{FAK}\), it is tempting to speculate that activation may be caused by an altered interaction with pp125\(^{FAK}\). Finally, because CHO cells do not possess the same mechanisms for activating \(\alpha_\text{IIb}\beta_3\) that are present in platelets, it is possible that the mutant \(\beta_3\) interacts with a CHO signaling protein that does not normally appear to be available in platelets.

Based on early results from several laboratories suggesting that tyrosine phosphorylation of pp125\(^{FAK}\) occurs coincident with focal adhesion and stress fiber assembly and that inhibition of phosphorylation of pp125\(^{FAK}\) correlated with decreased focal adhesion and stress fiber assembly (38, 41), it has been suggested that phosphorylation of pp125\(^{FAK}\) precedes and is necessary for focal adhesion and stress fiber formation. Since pp125\(^{FAK}\) was phosphorylated in adherent cells expressing mutant F727A/K729E/F730A, which had reduced formation of focal adhesions, our studies suggest that it is a change related to the integrin receptor itself and not subsequent cytoskeletal assembly into focal adhesions, which leads to phosphorylation of pp125\(^{FAK}\). This apparent dissociation of p125\(^{FAK}\) phosphorylation from focal adhesion formation mirrors recent findings showing that pp125\(^{FAK}\) can be displaced from focal adhesions and the tyrosine phosphorylation in focal adhesions reduced to an undetectable level without affecting the assembly or stability of these structures (59). Interestingly, although our results indicate that fibrinogen binding was associated with phosphorylation of pp125\(^{FAK}\), occupation by small peptide ligands was not sufficient for phosphorylation. This difference between fibrinogen and peptide ligands suggests that receptor clustering may be crucial for phosphorylation of pp125\(^{FAK}\).

The disruption of adhesion-dependent formation of focal adhesions and stress fibers with mutation of membrane proximal sequences may have several explanations. First, the mutated sequences are part of a region in \(\beta_3\) identified in peptide studies as an \(\alpha\)-actinin binding site (24). Mutations in this region might inhibit the interaction of \(\alpha\)-actinin with \(\beta_3\) and therefore prevent the clustering of \(\alpha_\text{IIb}\beta_3\) that generates focal adhesions. Second, the mutated sequences are also part of a region in \(\beta_3\) identified as a talin binding site (27). Work showing that talin can associate with actin directly (60) or indirectly through an interaction with vinculin (61–63) suggests another possible mechanism by inhibition of the interaction of \(\beta_3\) with talin. Further studies using this mutant to examine the interaction with \(\alpha\)-actinin and talin may help clarify the relative roles of these two proteins in the association of \(\alpha_\text{IIb}\beta_3\) with focal adhesions. A third possibility is that the mutated region interacts with other as yet unidentified cytoskeletal components.

Interestingly, mutant D723A/E728A cells adhere to immobilized fibrinogen formed focal adhesions that stained positively by immunofluorescence for \(\alpha_\text{IIb}\beta_3\), phosphotyrosine, and pp125\(^{FAK}\), indicating that pp125\(^{FAK}\) is recruited into focal adhesions despite mutation of a putative pp125\(^{FAK}\) binding site within the \(\beta_3\) cytoplasmic domain. On the surface, this appears inconsistent with the findings of Schaller et al. (51) who reported that pp125\(^{FAK}\) binds to a synthetic peptide corresponding to the membrane-proximal region of \(\beta_3\). When the aspartic acid and glutamic acid were substituted with alanines, pp125\(^{FAK}\) no longer bound to the peptide. However, there are several potential explanations for our results. First, Hildebrand et al. (64) identified a focal adhesion-targeting sequence in the COOH-terminal region of pp125\(^{FAK}\), but it is the NH\(_2\)-terminal region of the molecule that interacts with the integrin. As a consequence, it should be possible to block pp125\(^{FAK}\) binding to integrin and still observe incorporation of pp125\(^{FAK}\) into focal adhesions. Consistent with this, introduction of the carboxyl-terminal focal adhesion-targeting sequence of pp125\(^{FAK}\) into cells displaces pp125\(^{FAK}\) from focal adhesions (59, 65). Second, the previously demonstrated interaction between purified pp125\(^{FAK}\) and the cytoplasmic domain peptide may not actually represent the physiological interaction. Third, the mutations in the \(\beta_3\) cytoplasmic tail, although within the region implicated in pp125\(^{FAK}\) binding may not prevent pp125\(^{FAK}\) binding. It is possible that a second region in the \(\beta_3\) cytoplasmic domain may contribute to pp125\(^{FAK}\) binding. The results of truncation studies indicating that the COOH-terminal amino acids are required for pp125\(^{FAK}\) association with integrin (66) are consistent with this explanation. Finally, recent work has shown an interaction of talin and paxillin with pp125\(^{FAK}\) (67–70). Thus, pp125\(^{FAK}\) may be recruited to focal adhesions in association with talin or paxillin and not with the \(\beta_3\) cytoplasmic domain.

In summary, these results document the importance of membrane proximal cytoplasmic domain sequences in integrin function. Studies to further define the sites of interaction, the proteins with which these sites interact, and the molecular events that attend these interactions will significantly enhance our understanding of integrins. The constructs we have presented here may be useful in this regard.

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\(^2\) S. Lyman, S. Gidwitz, and G. C. White II, unpublished observations.
