The Talin Rod IBS2 α-Helix Interacts with the β3 Integrin Cytoplasmic Tail Membrane-proximal Helix by Establishing Charge Complementary Salt Bridges*5

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Talin establishes a major link between integrins and actin filaments and contains two distinct integrin binding sites: one, IBS1, located in the talin head domain and involved in integrin activation and a second, IBS2, that maps to helix 50 of the talin rod domain and is essential for linking integrin β subunits to the cytoskeleton (Moes, M., Rodius, S., Coleman, S. J., Monkey, S. J., Goormaghtigh, E., Tremuth, L., Kox, C., van der Holst, P. P., Critchley, D. R., and Kieffer, N. (2007) J. Biol. Chem. 282, 17280–17288). Through the combined approach of mutational analysis of the β3 integrin cytoplasmic tail and the talin rod IBS2 site, SPR binding studies, as well as site-specific antibody inhibition experiments, we provide evidence that the integrin β3-talin rod interaction relies on a helix-helix association between α-helix 50 of the talin rod domain and the membrane-proximal α-helix of the β3 integrin cytoplasmic tail. Moreover, charge complementarity between the highly conserved talin rod IBS2 lysine residues and integrin β3 glutamic acid residues is necessary for this interaction. Our results support a model in which talin IBS2 binds to the same face of the β3 subunit cytoplasmic helix as the integrin αIIb cytoplasmic tail helix, suggesting that IBS2 can only interact with the β3 subunit following integrin activation.

Integrins are αβ heterodimeric receptors that mediate attachment of cells to the extracellular matrix (ECM) and therefore play important roles in cell adhesion, migration, proliferation, and survival (2, 3). Integrin clustering at sites of cellular attachment to the ECM triggers the assembly of large multifunctional submembrane protein complexes called focal adhesions (FAs),5 that orchestrate the two-directional processing of stimuli across the cell membrane (4, 5). Both the integrin α and β chains participate in regulating the integrin extracellular ligand binding capacity, while the β chain alone appears to link integrins to the actin cytoskeleton. Among the cytoskeletal proteins that directly interact with the β chain cytoplasmic domain, four proteins: talin, α-actinin, filamin, and tensin possess both integrin and actin binding affinities (6, 7). Talin and α-actinin also bind to vinculin, an additional major component of FAs with actin binding activity.

Talin and α-actinin are of particular interest as they have two integrin binding sites as well as multiple vinculin binding sites and display a similar structural organization comprising an extended rod domain composed of α-helical bundles. The α-actinin central rod domain, which is composed of 4 spectrin repeats of 3 α-helices (8, 9), associates with integrin β subunits as well as vinculin through distinct helix-helix interactions (10–16). Also, the 3 major talin rod-vinculin head (Vh) contacts have a similar architecture based on hydrophobic helix-helix interactions (17–19). Other FA proteins, such as members of the paxillin supergene family comprising paxillin, Hic-5, leupaxin, and PaxB, rely on an α-helical LD motif for their interaction with cognate partners, such as FAK, ILK, vinculin, or actopaxin, and establish a helix-helix interaction characterized by a hydrophobic patch between the two helices and surrounded by basic residues on one helix that interacts with the negatively charged residues of the second helix (20).

We have previously identified α-helix 50 of the talin rod domain as the minimal functional structure of the integrin binding site 2 (IBS2), able to interact with the β3 integrin cytoplasmic tail (1, 21). Here, we have further characterized the integrin β3-talin rod interaction. Based on β3 integrin and talin rod mutational analysis, SPR binding studies, as well as anti-

5 The abbreviations used are: FA, focal adhesion; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; GST, glutathione S-transferase; SPR, surface plasmon resonance; wt, wild type; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; FIB, fibrinogen.
body inhibition experiments, we provide evidence that the talin rod domain binds through its α-helix 50 to the membrane-proximal α-helix of the β3 integrin cytoplasmic tail. In addition, we provide evidence that this helix-helix association relies on charge complementarity between the highly conserved talin rod lysine residues and the integrin β3 glutamic acid residues.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following monoclonal antibodies (mAbs) were generous gifts: anti-αIIbβ3 mAb P12–73 (Dr. C. Kaplan, Institut National de la Transfusion Sanguine, Paris, France), anti-β3 mAbs P37 (Dr. J. Gonzalez-Rodriguez, Instituto de Quimica Fisica, Madrid, Spain), C3a and 4D10G3 (Dr. D. R. Phillips, Portola Pharmaceuticals, Inc., South San Francisco, CA), and anti-α1b mAb SZ22 (Dr. C. G. Ruan, Jiangsu Institute of Hematology, Suzhou, China). All other monoclonal or polyclonal antibodies were purchased: goat anti-β3 cytoplasmic tail C20 (Santa Cruz Biotechnology, Inc); anti-talin rod domain mAbs TD77 (Chemicon International, Inc., Temecula, CA) and 8d4 (Sigma); anti-vinculin mAb VIN-11–5 (Sigma); goat anti-GST Ab and horseradish peroxidase-conjugated goat anti-rabbit IgG; and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). TRITC-phalloidin was obtained from Molecular Probes BV, Leiden, The Netherlands.

Purified human fibrinogen (FIB) was purchased from Sigma. The αvβ3 inhibitor RO65–5233/001 was a generous gift of Dr. S. Reigner (Roche Applied Science, Basel, Switzerland). The β3 integrin cytoplasmic tail peptides (β3 wt, amino acids 716–762; β3 E726Q/E733Q mutant, amino acids 716–762) were purchased from Bio-Synthesis, Inc.

**Mammalian Cell cDNA Constructs, Cell Transfection, and Cell Culture**—QuikChange® site-directed mutagenesis (Stratagene, La Jolla, CA) of talin IBS2 or the β3 cytoplasmic tail was performed using the cDNA-encoding ΔsRed-tagged talin fragment containing the IBS2 site (amino acids 1977–2113) as a template to generate the single amino acid substitutions K2085D and K2089D, or pDNA3.1(−)/neo/β3 wt to introduce the β3 cytoplasmic tail mutations E726Q/E733Q or E726Q/E733Q. All constructs were verified by automated sequencing. The mutant β3 cDNA construct was transfected into Chinese hamster ovary (CHO) cells that had been pretransfected with human αIIb integrin cDNA using Lipofectamine (Life Technologies, Merelbeke, Belgium). The cDNA constructs encoding mutant talin fragment J were transiently transfected into CHO cells stably expressing recombinant wild-type αIIbβ3 (1). All CHO transfectants were cultured under standard tissue culture conditions in IMDM medium supplemented with 10% fetal bovine serum.

**Flow Cytometry**—Anti-αIIbβ3 antibody P12–73 binding to transfected CHO cells was measured on an Epics XL flow cytometer (Coulter Corp, Hialeah, FL) following R-phalloidin conjugated goat anti-mouse IgG labeling of the cells (Caltag Laboratories, Burlingame, CA).

**Cell Adhesion Assay**—Washed CHO cell transfectants (3 × 10^6) were preincubated for 30 min at room temperature with 10 μM of the αvβ3 inhibitor RO65–5233/001 to block αvβ3 receptor function and added to fibronogen-coated wells (20 μg/ml). Cell adhesion was allowed to occur at 37 °C for 2 h, and cell spreading was monitored by digital imaging of the cells in the wells without prior washing of the plates or discharge of non-adherent cells. Quantitation of spread cells versus non-spread round cells was performed according to the cell morphology. For each time point, 200 cells were counted, and the data reported as mean percent (± S.D.) of three independent experiments performed in triplicate.

**Immunofluorescence Staining**—For intracellular fluorescence staining, CHO cell transfectants adherent on fibronogen were fixed for 15 min at 4 °C with 3% paraformaldehyde, 2% sucrose in PBS, pH 7.4, rinsed with PBS, and permeabilized with 0.5% Triton X-100 in PBS, pH 7.4, containing 0.5% heat-denatured bovine serum albumin. Immunofluorescent staining was performed with antibodies to integrin β3 (P37), talin rod (TD77), or talin rod IBS2 (anti-IBS2 IgG). Cells were then incubated for 45 min with species-specific secondary antibodies linked to FITC. Polymerized actin was stained with TRITC-phalloidin. Slides were mounted in Mowiol 40–88/DABCO (Sigma) and analyzed with a Leica-DMRB fluorescence microscope using a ×63 or a ×100 oil immersion objective. Microphotographs were taken using a Leica DC 300F digital camera and the Leica IM1000 1.20 software.

**cDNA Constructs for Bacterial Protein Expression and Purification**—The pGEX-4T-2 plasmids with the cDNA constructs encoding either GST-talin G (amino acids 1984–2344), or the GST-tagged β3 cytoplasmic tail constructs β3 wt (amino acids 716–762), β3Δ744, β3Δ752, β3Δ754, β3Y747A, β3Y747F, β3Y759A, β3Y759F, β3S752P, β3S752A, β3Y747A/Y759F, and β3Y747F/Y759F have been previously described (21–23). cDNA constructs encoding the talin rod residues 1843–2108 and containing both the vinculin binding site VBS3 and the integrin binding site IBS2 (talin V3-I2) or residues 2071–2135 (talin rod helices 50–51) were amplified by PCR using full-length wild-type mouse talin 1 cDNA as template and cloned in-frame with GST into the pGEX-6P-1 expression vector, to generate GST-talin V3-I2 and GST-talin rod helices 50–51, respectively. The following single and double amino acid substitutions K2085D, K2089D, and K2085D/K2089D were subsequently introduced into the cDNA encoding talin V3-I2 by site-directed mutagenesis. The cDNA fragment encoding Vh (amino-acids 1–258) was amplified by PCR from full-length human vinculin cDNA and subcloned into the bacterial pET-15b expression vector (Invitrogen) to generate Vh-HisTag. All cloned cDNA fragments were verified by automated sequencing.

Expression of GST fusion proteins in *Escherichia coli* BL21 (DE3) bacteria was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. The bacterial pellet was lysed as previously described (24), and the fusion proteins were affinity-purified on glutathione-Sepharose 4B according to the manufacturer’s instructions (Amersham Biosciences). For talin G, removal of the GST tag was performed by thrombin digestion for 1 h at room temperature using 10 units of thrombin/mg
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of protein (Amersham Biosciences). Thrombin was neutralized with 1 mM phenylmethylsulfonyl fluoride, and the fragment was dialyzed against PBS. Removal of GST from talin rod helices 50–51 was performed on glutathione-Sepharose 4B beads using PreScission™ Protease (Amersham Biosciences) according to the manufacturer’s protocol. The GST-β3wt and GST-β3Y747A proteins used in NMR experiments were uniformly labeled with $^{15}$N by growing E. coli BL21 (DE3) bacteria in M9 minimal medium containing 1 g/liter $^{15}$NH$_4$Cl (Cambridge Isotope Laboratories), and induced and purified as described above. Expression of Vh-His$^*$Tag in E. coli BL21 (DE3) bacteria was induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside. The recombinant protein was affinity-purified on Ni-ni-trilotriacetic acid-agarose (Qiagen) according to the manufacturer’s instructions.

Generation of a Polyclonal Rabbit Anti-talin IBS2 Antibody—For the production of an anti-talin IBS2 polyclonal antibody, rabbits were immunized with the recombinant 7-kDa talin rod protein (helices 50–51) according to standard methods (AGRO-BIO s.a.s, La Ferté St. Aubin, France). Serum IgG from immunized rabbits was isolated by ammonium sulfate precipitation, dialyzed against PBS, and stored at 14 mg/ml at 4 °C.

Western Blot—CHO cells were detached, washed twice in PBS, and lysed 30 min on ice in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethyl maleimide (NEM), 1× protease inhibitor mixture (PIC). Lysates were clarified by centrifugation for 10 min at 13,000 rpm and 4 °C and denatured for 5 min at 95 °C in 1% SDS. The protein concentration was determined using the BCA protein assay kit (Pierce). Protein samples were denatured in SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Western blot was performed according to standard procedures. Antibodies C20, C3a, P37, and anti-IBS2 IgG were used as primary antibodies, and horseradish peroxidase-conjugated sheep anti-mouse, donkey anti-goat, or donkey anti-rabbit antibody (1:2500) as secondary antibodies. Antibody binding was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce), according to the manufacturer’s protocol.

Talin Immunoprecipitation Experiments—Platelet lysate (25 µg of protein) was incubated overnight at 4 °C with 20 µg of rabbit anti-IBS2 IgG or non-immune rabbit IgG in 100 µl of PBS. Protein A-Sepharose beads (Sigma-Aldrich) were added for 4 h at 4 °C, then washed with PBS prior to boiling in the presence of SDS sample buffer for 5 min. The eluted material was submitted to SDS-PAGE and Western blotting using anti-talin rod TD77 antibody (1:100) as primary antibody.

Surface Plasmon Resonance (SPR)—Measurements were performed on a Biacore 3000 instrument. Goat anti-GST antibodies were immobilized on a CM5 sensor chip according to the manufacturer’s instructions (Biacore). For kinetic analysis, two surfaces were pre-treated for 5 min at 2 µl/min with 400 nM recombinant GST (control channel) or GST-tagged proteins in HBS-P buffer (150 mM NaCl, 10 mM Hapes, and 0.005% NP20) provided by Biacore. Subsequently, purified recombinant talin G, Vh-His$^*$Tag, synthetic β3 wt, or β3 E726Q/E733Q peptides, monoclonal C3a, polyclonal anti-IBS2 or nonspecific rabbit or mouse antibodies at concentrations ranging from 20 µM to 6.6 nm in HBS-P, were allowed to react with the two channels at a flow rate of 20 µl/min for 3 min, followed by a dissociation phase of 3 min. In blocking experiments, rabbit anti-IBS2 IgG was allowed to react with talin V3-I2 at a concentration of 14 µg/ml before injection of the β3 peptide, while the C3a antibody was allowed to react with GST-β3 at a concentration of 1 µg/ml before injection of talin G. After each run, the channels were completely regenerated with 10 mM HCl. The sensorgrams were analyzed with Biacore 3.1 software (Biacore). Sensorgrams of the control channel were subtracted from the sensorgrams on the experimental channel and analyzed using nonlinear regression statistics to fit a simple Langmuir 1:1 binding model, taking into account the drifting base line, which is caused by the differential dissociation rate of GST alone and GST-tagged proteins from the anti-GST antibodies.

NMR Spectroscopy—Samples of $^{15}$N-labeled GST-β3 or of GST-β3 Y747A mutant were prepared in a buffer of phosphate 50 mM; NaCl 100 mM; EDTA 1 mM; dithiothreitol 1 mM, pH 6.6 at a final concentration of 400 µM. A standard HSQC spectrum (with 2048 × 256 points in the $t_1$, $t_2$ dimensions) was recorded at 293K on a Bruker 600 MHz spectrometer equipped with a cryogenic probe head. The resulting HSQC spectra were transformed with 2k × 1k complex points, after multiplication with a p/4 and p/2 squared sine bell function, respectively. In the spectra, only the flexible parts corresponding to the integrin tails are visible (25).

RESULTS

A Polyclonal Antibody Raised against Helices 50 and 51 of the Human Talin Rod Domain Inhibits the Talin IBS2-Integrin β3 Cytoplasmic Tail Interaction—We have previously identified the 23-residue amphipathic α-helix 50 of talin rod as the minimal functional IBS2 structure able to interact with the integrin β3 subunit cytoplasmic tail (1, 21). To further characterize this IBS2-β3 interaction, we have generated an anti-IBS2 polyclonal antibody (Ab) raised in rabbits against a recombinant 7-kDa GST-tagged talin rod fragment, corresponding to the α-helices 50 and 51 (residues 2071–2135). The purity of the recombinant talin rod fragment used as immunogen is shown in Fig. 1A. When the polyclonal Ab was tested in Western blot it reacted with the recombinant 39-kDa talin rod fragment G (helices 47–57), as well as full-length talin or the proteolytically cleaved 190-kDa talin rod fragment from SDS-denatured platelet lysate (Fig. 1B). In addition, this Ab also reacted with native platelet talin as shown by immunoprecipitation experiments using Triton X-100 platelet lysate (Fig. 1C). When tested in indirect immunofluorescence assays using CHO αIIbβ3 cells adherent on fibrinogen (Fig. 1D), the anti-IBS2 Ab reacted with endogenous hamster talin and preferentially labeled lamellipodia, where its staining overlapped with anti-talin rod or anti-β3 integrin labeling. In contrast, the anti-IBS2 Ab labeling was strongly reduced in mature FAs and was hardly seen at the tips of actin stress fibers, suggesting that in FA the talin IBS2 site is not accessible to the Ab, while it is freely exposed in lamellipodia.

To test whether this Ab was able to block the talin rod IBS2-β3 cytoplasmic tail interaction, we performed SPR analysis, using a recombinant GST-tagged 54-kDa talin rod fragment...
encompassing helices 43–50. This fragment, called GST-talin V3-I2 and containing the vinculin binding site 3 (VBS3) as well as the integrin binding site 2 (IBS2), was designed to allow simultaneous binding studies of two distinct talin ligands, integrin β3 and vinculin head (Vh). Fig. 2A shows the SDS-PAGE gel of the purified proteins used for SPR analysis. GST-talin V3-I2 or GST alone (used as a negative control) was captured on the sensor chip on immobilized anti-GST antibodies. The recombinant 31-kDa Vh protein or the 7-kDa β3 peptide was then injected as analytes. The sensograms are shown in Fig. 2, B and C. Interaction of increasing concentrations of Vh protein with talin fragment V3-I2 yielded a $K_D$ of $6.96 \times 10^{-9}$ M, a result close to the $K_D$ of $39 \times 10^{-9}$ M previously determined for the VBS3-Vh interaction by a pull-down assay (26), providing evidence that the immobilized talin fragment V3-I2 was functional for vinculin head binding. When the β3 peptide was tested (Fig. 2C), a direct interaction between the β3 integrin cytoplasmic tail and talin V3-I2 was also observed that yielded a $K_D$ of $3.45 \times 10^{-8}$ M, an affinity about 5 times lower than that observed for Vh binding. Interestingly, when the two peptides were injected simultaneously, they both interacted with talin V3-I2, providing evidence that the vinculin and integrin binding sites are both functional and are not mutually exclusive (data not shown). To test the effect of the anti-IBS2 antibody on the talin V3-I2-β3 peptide interaction, we first measured its binding affinity for GST-talin V3-I2 ($K_D$ of $1.54 \times 10^{-8}$ M) as compared with nonspecific rabbit IgG for which no binding could be observed (Fig. 2D). We next tested the ability of the anti-IBS2 Ab to inhibit this interaction (Fig. 2E). The tracing of the binding of the β3 peptide to talin V3-I2 in the absence (upper trace) or in the presence of rabbit anti-IBS2 IgG (lower trace) clearly demonstrates that the anti-IBS2 Ab strongly inhibited the interaction between the cytoplasmic tail of β3 and talin IBS2, by blocking the talin IBS2 binding site. It is noteworthy that the anti-IBS2 Ab had no inhibitory effect on the interaction between talin fragment V3-I2 and the Vh protein, underlining the specificity of the blocking effect of the antibody (supplemental Fig. S1A).

Identification of Talin IBS2 Residues Involved in the Interaction with the β3 Cytoplasmic Tail—We have previously shown that alanine mutation of two highly conserved residues L2094A/L2095A within IBS2, which disrupted the α-helical structure of IBS2, was sufficient to inhibit the IBS2-integrin interaction (1), suggesting that this interaction relies, similar to the talin rod-vinculin or the actin-β1 integrin interaction, on a helix-helix protein-protein association. However, in contrast to the VBS sites that each correspond to hydrophobic residues exposed on one side of an amphipathic α-helix of the talin rod domain (17–19), our previously reported results (1) suggested that the highly conserved and charged residues clustered on the solvent face of the amphipathic talin rod α-helix 50 might be involved in the helix-helix interaction of IBS2 with β3. The sequence alignment of human talin helix 50 with the equivalent talin sequence of other species ranging from mouse to zebrafish is shown in Fig. 3. Five charged and conserved residues can be identified within the α-helix 50 sequence corresponding to Lys-2085, Asp-2086, Lys-2089, Asp-2093, and Lys-2099 (Fig. 3A). However, only residues Lys-2085 and Lys-2089 are positioned on the hydrophilic face of helix 50 (Fig. 3B) opposite to the hydrophobic face corresponding to the potential vinculin binding site (27), suggesting that they might function as direct contact sites in the IBS2-β3 integrin interaction. In addition, we have previously shown by site-directed mutagenesis that residue Lys-2099, located at the C-terminal end of helix 50, is not involved in this interaction (1).

Mutation of Residues Lys-2085 and Lys-2089 of Talin Rod Helix 50 Inhibits the Talin IBS2-β3 Integrin Interaction—To test our hypothesis, we used a charge reversal mutation approach as previously described for the identification of the HIP1R actin binding site (28) by mutating two positively charged Lys residues in talin V3-I2 into negatively charged Asp residues (Fig. 4A). The three following mutant proteins: talin V3-I2 K2085D, K2089D, and K2085D/K2089D were then tested for β3 binding (Fig. 4B) using SPR analysis. Interestingly, both the single and the double Lys/Asp mutations strongly

FIGURE 1. Specificity of the polyclonal rabbit Ab raised against the human talin rod IBS2 site. A, 7-kDa talin recombinant protein used to immunize rabbits and corresponding to residues 2071-2135 of the talin rod domain was produced and purified as indicated under “Experimental Procedures.” The protein (5 μg) was subjected to 15% SDS-PAGE electrophoresis and Coomassie Blue staining. (* indicates a contaminant and corresponds most likely to cleaved GST. B, Western blot analysis of purified recombinant talin fragment G (200 ng) or whole platelet lysate (10 μg) submitted to SDS-PAGE using the polyclonal rabbit anti-IBS2 Ab. C, immunoprecipitation experiments using platelet lysate (25 μg of protein) and rabbit anti-IBS2 IgG (lane 1) or preimmune rabbit IgG (lane 2). The precipitated immune complexes were resolved by SDS-PAGE and analyzed by Western blotting using the anti-talin rod TD77 mAb. Lane 3 corresponds to total platelet lysate (10 μg) used as positive control. D, two color immunofluorescence labeling of CHO cellβ3wt cells, plated for 2 h at 37°C on fibronectin (FIB), and stained with the anti-talin rod TD77 mAb (panel a), the β3-specific P37 mAb (panel c), or phalloidin (panel e) and co-labeled with the rabbit anti-IBS2 IgG (panels b, d, and f). The bar equals 10 μm.
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**FIGURE 2.** SPR analysis of the direct protein-protein interaction between the talin rod domain fragment V3-I2 and the synthetic β3 cytoplasmic tail peptide. 

A, recombinant proteins GST (26 kDa), GST-talin V3-I2 (54 kDa) as well as Vh-HisTag (31 kDa) were expressed in *E. coli* and purified from bacterial lysates by affinity chromatography. The synthetic 7-kDa β3 cytoplasmic tail peptide was purchased from BioSynthesis. Proteins were resolved by SDS-PAGE and stained with Coomassie Blue. 

B, sensorgrams of the binding of recombinant Vh (0.25, 0.5, and 1 μM) to recombinant GST-talin fragment V3-I2 (or GST alone used as negative control) that were captured on a sensor chip coated with anti-GST antibodies (Chi2 = 13.9). 

C, sensorgrams of the binding of the β3 peptide (45, 90, and 180 μM) to GST-talin V3-I2 (Chi2 = 3.7). 

D, left panel: sensorgram of the binding of the rabbit anti-IBS2 IgG (46–186 nM) to recombinant GST-talin fragment V3-I2 (or GST alone as negative control) captured on a sensor chip precoated with anti-GST antibodies (Chi2 = 0.14). Right panel: sensorgram of the binding of a nonspecific rabbit IgG (black trace: 2 μg/ml, gray trace: 20 μg/ml) to recombinant GST-talin V3-I2. The reference line is indicated by a dotted line. 

E, sensorgrams of the binding of the β3 peptide to GST-talin V3-I2 in the absence (upper trace) or presence of rabbit anti-IBS2 IgG (lower trace). The slopes of both traces are indicated (Chi2 = 1.3).
decreased the binding affinity, with \( K_D \) of 7.33 \( \times 10^{-5} \) M for talin V3-I2 K2085D, 6.06 \( \times 10^{-5} \) M for talin V3-I2 K2089D, and 8.44 \( \times 10^{-5} \) M for talin V3-I2 K2085D/K2089D; affinities about 10^3 times lower than that measured for \( \beta 3 \) binding to wild-type talin V3-I2. In contrast, these mutations had no significant inhibitory effect on Vh binding to mutant talin V3-I2 (supplemental Fig. S1B). These results demonstrate that residues Lys-2085 and Lys-2089 of talin rod helix 50 are involved in mediating the talin IBS2-\( \beta 3 \) integrin interaction, most likely by establishing ionic salt bridges.

**Amino Acids Lys-2085 and Lys-2089 of Talin Rod Helix 50 Are Essential for the in Vivo Association of Recombinant Talin Fragment J with Integrin \( \beta 3 \) in Focal Adhesions**—To determine the in vivo relevance of the Lys-2085 and Lys-2089 residues in IBS2 integrin binding function, we transiently expressed the mutant DsRed-tagged fragments talin J K2085D and talin J K2089D in CHO cells and analyzed their subcellular localization (Fig. 5). In agreement with our previous data (1), the recombinant wild-type talin J fragment colocalized with integrin \( \beta 3 \) in FAs. In contrast in CHO cells expressing either talin J K2085D or talin J K2089D, the mutant recombinant proteins presented a diffuse staining and were absent from FAs identified with an anti-\( \beta 3 \) Ab. These data clearly indicate that both Lys-2085 and Lys-2089 residues of talin IBS2 are necessary for the recruitment of the talin rod domain to FAs.

**An Anti-\( \beta 3 \) Integrin Cytoplasmic Tail Monoclonal Ab Blocks the Talin IBS2-\( \beta 3 \) Integrin Interaction**—To identify the talin IBS2 contact site within the integrin \( \beta 3 \) subunit cytoplasmic tail, we used SPR analysis to investigate the potential inhibitory effect of available anti-\( \beta 3 \) cytoplasmic tail Ab on the \( \beta 3 \)-talin IBS2 interaction. In this experiment, GST-\( \beta 3 \)wt was immobilized on the sensor chip, and untagged talin fragment G was injected as analyte, either after the antibody injection or in its absence. Fig. 6 shows the reactivity of one selected mAb, C3a, that was raised against a synthetic peptide, corresponding to the full-length cytoplasmic tail of \( \beta 3 \) (residues 716–62) (29). C3a bound with high affinity to GST-\( \beta 3 \) immobilized on the
sensor chip ($K_d$ of $2.26 \times 10^{-11}$ M), while no interaction could be observed with nonspecific mouse IgG (Fig. 6B). Interestingly, this C3a antibody efficiently inhibited the binding of talin fragment G to GST-$\beta_3$ (Fig. 6C).

The $\beta_3$ Integrin Cytoplasmic Tail Epitope Identified by Monoclonal Antibody C3a Is Located in the $\alpha$-Helical Membrane-proximal Part of the $\beta_3$ Cytoplasmic Tail—To map the epitope identified by C3a, we performed Western blot analysis using a series of previously characterized mutant GST-$\beta_3$ cytoplasmic tail fusion proteins, together with well-documented $\beta_3$ deletion mutants expressed in CHO cells (23). In parallel, we also tested the reactivity of a polyclonal Ab (C20) raised against a synthetic peptide corresponding to the 20 C-terminal residues of the $\beta_3$ subunit cytoplasmic tail. As shown in Fig. 7, mAb C3a bound to all $\beta_3$ mutants including the deletion mutant $\beta_3\Delta744$, but did not react with the deletion mutant $\beta_3\Delta722$, providing evidence that the C3a epitope is contained within the $\beta_3$ residues 722–744, corresponding roughly to the $\beta_3$ membrane-proximal $\alpha$-helix (residues Lys-716 to Arg-734) (30). In contrast, the pAb C20 reacted with the most C-terminal residues of the $\beta_3$ subunit cytoplasmic tail, as it did not interact with the deletion mutant $\beta_3\Delta754$ and only weakly reacted with the $\beta_3Y759A$ mutant. The weak binding of this antibody to $\beta_3S752P$, a point mutation not located within the identified epitope, suggests that this substitution, but not the S752A substitution, has a structural effect on the extreme C terminus of $\beta_3$.

Identification of $\beta_3$ Cytoplasmic Tail Residues Involved in the Interaction with Talin IBS2—To identify residues located in the identified $\beta_3$ cytoplasmic tail membrane-proximal $\alpha$-helix that might establish contacts with the positively charged Lys-2085

**FIGURE 5.** Subcellular localization of recombinant wild type or mutant talin J-DsRed fragments (talin J wt, talin J K2085D, and talin J K2089D). CHO cells expressing wild-type were transiently transfected with cDNAs encoding the different talin J fragments, as described under “Experimental Procedures,” and plated for 2 h at 37 °C on fibronectin. Cells were subjected to immunofluorescence staining using P37 as anti-β3 mAb (green). The subcellular localization of talin J fragments fused to DsRed was compared with the localization of β3 integrins. The bar equals 10 μm.

**FIGURE 6.** SPR analysis of the binding of the talin rod fragment G to captured GST-β3 in the presence or absence of the anti-β3 mAb C3a. A, recombinant proteins GST (26 kDa), GST-β3 (33 kDa), and GST-talin G (66 kDa) were expressed in E. coli and purified from bacterial lysates by affinity chromatography on glutathione-Sepharose. GST-talin G was cleaved by thrombin to give rise to talin G (39 kDa). Proteins were subjected to 10% SDS-PAGE electrophoresis and Coomassie Blue staining. B, upper panel: sensorogram of the binding of the anti-β3 C3a mAb (0.82–6.6 nM) to recombinant GST-β3 (or GST alone as negative control) captured on a sensor chip precoated with anti-GST antibodies (Ch2 = 0.56). Lower panel: sensorgrams of the binding of a nonspecific mouse IgG (black trace: 2 μg/ml, gray trace: 20 μg/ml) to recombinant GST-β3. The reference line is indicated by a dotted line. C, sensorgrams of the binding of the talin fragment G to GST-β3 in the absence (upper trace) or presence (lower trace) of the C3a mAb. The slopes of both curves are indicated (Ch2 = 3.3).

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where integrin αIIbβ3wt as well the cytoskeletal protein talin were localized in FAs at the tips of well-organized actin stress fibers (Fig. 9D). We next performed Western blot analysis to test whether these Glu residues are part of the C3a epitope (Fig. 9E). Interestingly, the C3a mAb did not react with the β3E/Q mutant, whereas it bound to β3wt and to the deletion mutant β3Δ744, used as positive controls. The affinity of C3a with mammalian β3 was however weaker than that observed with bacterially produced recombinant GST-β3.

Specific Mutation of Residues Glu-726 and Glu-733 of the β3 Cytoplasmic Tail Inhibits the Talin IBS2-β3 Integrin Interaction—To test which of the β3 Glu-726, Glu-731, and Glu-733 residues could interact with the talin IBS2 Lys residues, we performed computer modeling. Fig. 10 A shows the potential complex between the membrane-proximal helix of the β3 cytoplasmic tail and talin helix 50. Modeling indicates that residues Glu-726 and Glu-731, which are located on the same face of the β3 helix as residue Asp-723, are physically able to interact with amino acids Lys-2085 and Lys-2089 of talin helix 50, the distance between the carboxyl groups of β3 glutamic acid residues and the NH3 groups of talin lysine residues being smaller than 4 Å. Finally, to confirm that the β3 Glu-726 and Glu-733 residues are directly involved in the β3 cytoplasmic tail-talin IBS2 protein-protein interaction, we performed SPR analysis, using a synthetic β3 cytoplasmic tail peptide with residues Glu-726 and Glu-733 mutated into Gln. As shown in Fig. 10B, the β3 E726Q/E733Q double mutation strongly decreased the binding affinity of the β3 peptide for talin V3-I2, yielding a $K_D$ of $5.51 \times 10^{-5}$ M, an affinity very close to that measured for the β3wt-talin V3-I2 K2085D/K2089D interaction ($K_D$ of $8.44 \times 10^{-5}$ M), providing additional evidence that the binding inhibition is due to charge reversal rather than to a structural change in either β3 or talin IBS2. This result was also confirmed at the cellular level following transient transfection of the β3 E726Q/E733Q mutant in CHOαIIb cells, which were unable to spread on fibronectin (data not shown).

Previous results from our laboratory suggested an involvement of residue Tyr-747 within the highly conserved β3 cytoplasmic tail NPXY motif for talin rod IBS binding, because a Y747A substitution prevented the integrin β3-IBS interaction (21). In apparent contradiction, our present data underline the involvement of the β3 membrane-proximal α-helix in the interaction with IBS2. We therefore compared the NMR spectra of GST-β3wt and GST-β3Y747A to evaluate the effect of the Y747A mutation on the β3 cytoplasmic tail structure (see supplemental Fig. S2). Interestingly, in these spectra where we only observed the signals from the flexible integrin peptides (25), chemical shift differences for at least 10 correlation peaks could be identified. This indicates that the single Y747A residue mutation not only affects its immediate neighborhood, but equally more distal parts of the peptide. Based on previously published NMR data (32, 33), we could assign some peak shifts to residues C-terminal of the mutated Tyr-747, but equally to the upstream residues Asn-744 and Asn-742. This indicates that in the context of the GST fusion protein, the proline residue Pro-746 does not prevent propagation of the mutation-induced structural change toward the N-terminal part of the β3 tail.
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All together, our results provide evidence that the two highly conserved glutamic acid residues Glu-726 and Glu-733 of the β3 subunit cytoplasmic tail membrane-proximal helix, and the lysine residues Lys-2085 and Lys-2089 of talin IBS2 are directly involved in establishing ionic bonds allowing the integrin β3-talin IBS2 helix-helix interaction, necessary for connecting integrins to the actin cytoskeleton. Further structural data will however be necessary to precisely document this interaction.

DISCUSSION

Much effort has been devoted to the elucidation of the physical bonds that connect proteins in focal adhesions, and recent data have provided evidence that these protein-protein associations often rely on helix-helix interactions. Here we show that the talin IBS2-integrin cytoplasmic tail interaction also relies on a helix-helix association involving helix 50 of the talin rod domain and the membrane-proximal α-helix of the β3 integrin cytoplasmic tail. Using an antibody-based approach, we demonstrate that a polyclonal rabbit Ab, raised against repeats 50 and 51 of the talin rod domain, or a monoclonal Ab, C3a, directed against the membrane-proximal helix of the β3 integrin subunit, both inhibited the talin IBS2-integrin interaction. Furthermore, we have identified the residues that are directly involved in this interaction by site-directed mutagenesis and show that the positively charged lysine residues Lys-2085 and Lys-2089 of talin IBS2, and the negatively charged glutamic acid residues Glu-726 and Glu-733 of β3 are important for this interaction. These data provide evidence that the talin rod-β3 cytoplasmic tail association relies on ionic bonds established between the two protein helices.

NMR studies have revealed multiple hydrophobic and electrostatic contacts between the membrane-proximal α-helices of the β3 and the αIIb cytoplasmic tails (30). These interactions contribute in the formation of a membrane-proximal clasp and, hence, in maintaining the default low affinity state of integrin αIIbβ3 (34, 35). In addition, the highly conserved membrane-proximal domain in the β3 tail has been shown to be important for regulating cell spreading (36). Interestingly, two of the three Glu residues identified here (Glu-726 and Glu-733) are located on the same face of the α-helix as residue Asp-723 (30), involved in the electrostatic interface with residue Arg-995 of the αIIb membrane-proximal α-helix, suggesting that the glutamic acids Glu-726 and Glu-733 are not accessible in the resting receptor because of the close contact with the αIIb subunit cytoplasmic tail. These results correlate with our previous pull-
study that talin IBS2 directly interacts with the membrane-proximal helix of β3 cytoplasmic tail. However, α-actinin, skelem, and more recently the talin head have also been shown to interact with the membrane-proximal part of the β3 cytoplasmic tail (10, 39, 40), suggesting that these different interactions might be sequential, as demonstrated for talin and filamin binding to the β integrin tail. In support of these results are the immunofluorescence data obtained with the anti-IBS2 Ab that strongly labeled lamellipodia while only very weakly focal adhesions, suggest that in lamellipodia, the IBS2-β3 interaction has not yet occurred, leaving the IBS2 site free to interact with the anti-IBS2 Ab, whereas in focal adhesions, the IBS2 site is in close contact with β3. On the other hand, the talin head has recently also been shown to interact with the membrane-proximal helix of the β3 cytoplasmic tail by establishing contacts with β3 residues Phe-727 and Phe-730 (40), located on the opposite face of the α-helix compared with residues Glu-726 and Glu-733 (30). Thus, the interactions of talin head and talin IBS2 with β3 might not be mutually exclusive, as previously suggested by Yan and Ginsberg (41), who demonstrated the cooperative binding of free talin head and talin rod to the β3 cytoplasmic tail.

Helix-helix interactions between FA proteins often require protein unfolding to unmask hidden binding sites. For example, the α-actinin spectrin repeats unfold when subjected to mechanical stress (42), which occurs following the formation of adhesion complexes, allowing the exposure of new docking sites for additional cytoskeletal proteins (9). Also, the C-terminal domains of FAK and vinculin have been reported to partially unfold to allow protein-protein interactions (20). Vinculin is autoinhibited by intramolecular interactions between its head and tail domains, and needs activation through a complex, combinatorial activation process involving talin as well as actin, to unfold and thereby unleash its scaffolding activity (43). Within the talin rod domain, the three vinculin binding sites are also cryptic, as they are located on the hydrophobic face of the amphipathic α-helices buried within helical bundles. It has therefore been suggested that a reshuffling in these α-helical bundles would be required to expose the

down data, showing that native integrin αIIbβ3 purified from platelets had to be activated with Mn²⁺ and the ligand mimetic mAb PAC-1 to allow its interaction with talin IBS2 (1). These data are also in agreement with the model of Tanentzapf and Brown (37), suggesting that the talin rod domain may only interact with high affinity, ligand-bound integrins.

Recent studies have indicated that talin and filamin have overlapping integrin β tail binding sites (38) that involve the NPXY motif, and that β tails adopt distinct conformations when bound to either filamin or to talin. In addition, in vitro pull-down assays have indicated that filamin and talin do cross-compete for integrin tail binding (38). We demonstrate in this

FIGURE 9. Immunofluorescence analysis of CHO cells expressing αIIbβ3wt (β3wt) or αIIbβ3E726Q733Q (β3E/Q). A, flow cytometry analysis of CHO cell clones expressing αIIbβ3wt or αIIbβ3E/Q. The cells were labeled with the complex-specific mAb PI2-73 (bold line) or control mouse IgG (thin line). B, Western blot analysis of CHO αIIbβ3wt (β3wt) or CHO αIIbβ3E/Q (β3E/Q) cell lysates (50 μg of protein) submitted to SDS-PAGE under non-reducing (NR) or reducing (R) conditions and hybridized with the anti-αIIb (SZ22) and the anti-β3 (4D10G3) mAbs. C, quantification of the adhesion of CHO αIIbβ3wt (β3wt) and CHO αIIbβ3E/Q (β3E/Q) cells to immobilized fibrin. The cells were microphotographed after a 2-h adhesion at 37 °C, and the percentage of spread versus the total number of cells was determined. Results were expressed as the means ± S.D. of three independent experiments performed in triplicate. D, immunofluorescence microphotographs of CHO αIIbβ3wt (β3wt) and CHO αIIbβ3E/Q (β3E/Q) cells plated on fibrin for 2 h at 37 °C and labeled with the anti-β3 mAb P37 (β3), the talin rod-specific mAb 8d4 (talin), and phalloidin to visualize polymerized actin (actin). The bar equals 10 μm. E, lysates of CHO cells expressing β3wt, β3Δ744, or β3E/Q (50 μg of protein) were submitted to Western blot analysis using the mAb C3a, and were further rehybridized with the anti-αIIb (SZ22) and anti-β3 (P37) mAbs.
molecular mechanisms that regulate integrin-dependent inside-out and outside-in signaling become even more complex and additional studies will be necessary to elucidate the temporal and spatial protein-protein interactions that take place to establish the link between integrins and the actin cytoskeleton.

Acknowledgments—We thank Dr. C. Kaplan, Dr. J. Gonzalez-Rodriguez, Dr. D. R. Phillips, and Dr C. G. Ruan for providing antibodies and are grateful to Dr. S. Reigner for the generous gift of the αvβ3 inhibitor.

REFERENCES

1. Moes, M., Rodius, S., Coleman, S. J., Monley, S. J., Goormaghtigh, E., Tremeth, L., Kox, C., van der Holst, P. F., Critchley, D. R., and Kieffer, N. (2007) J. Biol. Chem. 282, 17280–17288
2. Hynes, R. O. (2002) Cell 110, 673–687
3. Giancotti, F. G. (2000) Nat. Cell Biol. 2, E13–14
4. Burridge, K., and Connell, L. (1983) Cell Motil. 3, 405–417
5. Craig, S. W., and Johnson, R. P. (1996) Curr. Opin. Cell Biol. 8, 74–85
6. Zhao, R., Pathak, A. S., and Stouffer, G. A. (2004) Arch. Immunol. Ther. Exp. (Warsz) 52, 348–355
7. Liu, S., Calderwood, D. A., and Ginsberg, M. H. (2000) J. Cell Sci. 113, 3563–3571
8. Broderick, M. J., and Winder, S. I. (2002) J. Struct. Biol. 137, 184–193
9. Djovic-Carugo, K., Gauthier, M., Ylanne, J., and Young, P. (2002) FEBS Lett. 513, 119–123
10. Otey, C. A., Vasquez, G. B., Burridge, K., and Erickson, B. W. (1993) J. Biol. Chem. 268, 21193–21197
11. Otey, C. A., and Carpen, O. (2004) Cell Motil. Cytoskeleton 58, 104–111
12. Ylanne, J., Scheffzek, K., Young, P., and Saraste, M. (2001) Cell Mol. Life Sci. 6, 234
13. Ylanne, J., Scheffzek, K., Young, P., and Saraste, M. (2001) Structure 9, 597–604
14. Jockusch, B. M., Babucke, P., Giehl, K., Kroemker, M., Moschener, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. (1995) Annu. Rev. Cell Dev. Biol. 11, 379–416
15. Kroemker, M., Rudiger, A. H., Jockusch, B. M., and Rudiger, M. (1994) FEBS Lett. 355, 259–262
16. McGregor, A., Blanchard, A. D., Rowe, A. J., and Critchley, D. R. (1994) Biochem. J. 301 (Pt 1), 225–233
17. Papaprigoriou, E., Gingras, A. R., Barsukov, I. L., Bate, N., Fillingham, I. J., Patel, B., Frank, R., Ziegler, W. H., Roberts, G. C., Critchley, D. R., and Emsley, J. (2004) EMBO J. 23, 2942–2951
18. Fillingham, I., Gingras, A. R., Papaprigoriou, E., Patel, B., Emsley, J., Critchley, D. R., Roberts, G. C., and Barsukov, I. L. (2005) Structure 13, 65–74
19. Gingras, A. R., Vogel, K. P., Steinhoff, H. J., Ziegler, W. H., Patel, B., Emsley, J., Critchley, D. R., Roberts, G. C., and Barsukov, I. L. (2006) Biochemistry 45, 1805–1817
20. Hoellerer, M. K., Noble, M. E., Labesse, G., Campbell, I. D., Werner, J. M., and Arolf, S. T. (2003) Structure 11, 1207–1217
21. Tremeth, L., Kreis, S., Melchior, C., Hoebeke, J., Ronde, P., Plancon, S., Takeda, K., and Kieffer, N. (2004) J. Biol. Chem. 279, 22258–22266
22. Valler, L., Melchior, C., Plancon, S., Droboch, H., Lippens, G., Regnault, V., and Kieffer, N. (1999) J. Biol. Chem. 274, 17257–17266
23. Schaffner-Keckinger, E., Gouon, V., Melchior, C., Plancon, S., and Kieffer, N. (1998) J. Biol. Chem. 273, 12623–12632
24. Woodside, D. G., Obergeff, A., Talapatra, A., Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2002) J. Biol. Chem. 277, 39401–39408
25. Pierce, M. M., Baxa, U., Steven, A. C., Bax, A., and Wickner, R. B. (2005) Biochemistry 44, 321–328
26. Bass, M. D., Patel, B., Barsukov, I. G., Fillingham, I. J., Mason, R., Smith, B. J., Bagshaw, C. R., and Critchley, D. R. (2002) Biochem. J. 362, 761–768
27. Gingras, A. R., Ziegler, W. H., Frank, R., Barsukov, I. L., Roberts, G. C., Critchley, D. R., and Emsley, J. (2005) J. Biol. Chem. 280, 37217–37224
28. Brett, T. J., Legendre-Guillemin, V., McPherson, P. S., and Fremont, D. H.
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(2006) Nat. Struct. Mol. Biol. 13, 121–130
29. Law, D. A., Nannizzi-Alaimo, L., and Phillips, D. R. (1996) J. Biol. Chem. 271, 10811–10815
30. Vinogradova, O., Vaynberg, J., Kong, X., Haas, T. A., Plow, E. F., and Qin, J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 4094–4099
31. Hughes, P. E., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J. A., Shattil, S. J., and Ginsberg, M. H. (1996) J. Biol. Chem. 271, 6571–6574
32. Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin, J. (2002) Cell 110, 587–597
33. Ulmer, T. S., Calderwood, D. A., Ginsberg, M. H., and Campbell, I. D. (2003) Biochemistry 42, 8307–8312
34. Kim, M., Carman, C. V., Yang, W., Salas, A., and Springer, T. A. (2004) J. Cell Biol. 167, 1241–1253
35. Ma, Y. Q., Yang, J., Pesho, M. M., Vinogradova, O., Qin, J., and Plow, E. F. (2006) Biochemistry 45, 6656–6662
36. Bodeau, A. L., Berrier, A. L., Mastrangelo, A. M., Martinez, R., and LaFlamme, S. E. (2001) J. Cell Sci. 114, 2795–2807
37. Tanentzapf, G., and Brown, N. H. (2006) Nat. Cell Biol. 8, 601–606
38. Kiema, T., Lad, Y., Jiang, P., Oxley, C. L., Baldassarre, M., Wegener, K. L., Campbell, I. D., Ylanne, J., and Calderwood, D. A. (2006) Mol. Cell 21, 337–347
39. Reddy, K. B., Gascard, P., Price, M. G., Negrescu, E. V., and Fox, J. E. (1998) J. Biol. Chem. 273, 35039–35047
40. Wegener, K. L., Partridge, A. W., Han, J., Pickford, A. R., Liddington, R. C., Ginsberg, M. H., and Campbell, I. D. (2007) Cell 128, 171–182
41. Yan, B., Calderwood, D. A., Yaspan, B., and Ginsberg, M. H. (2001) J. Biol. Chem. 276, 28164–28170
42. Altmann, S. M., Grunberg, R. G., Lenne, P. F., Ylanne, J., Raae, A., Herbert, K., Saraste, M., Nilges, M., and Horber, J. K. (2002) Structure 10, 1085–1096
43. Chen, H., Choudhury, D. M., and Craig, S. W. (2006) J. Biol. Chem. 281, 40389–40398