The Integrin Binding Site 2 (IBS2) in the Talin Rod Domain Is Essential for Linking Integrin β Subunits to the Cytoskeleton*

Received for publication, December 27, 2006, and in revised form, March 16, 2007. Published, JBC Papers in Press, April 11, 2007, DOI 10.1074/jbc.M611846200

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Talin1 is a large cytoskeletal protein that links integrins to actin filaments through two distinct integrin binding sites, one present in the talin head domain (IBS1) necessary for integrin activation and a second (IBS2) that we have previously mapped to talin residues 1984–2113 (fragment J) of the talin rod domain (1). From the crystal structure of the head domain, which interacts with the integrin extracellular ligands (8, 9), recent biochemical, cryo-electron microscopy, and NMR studies have mapped the integrin binding site in the talin head (7). The integrin binding site in the head domain (7) is still elusive. In some cells, integrin outside-in signaling leads to association of the large isoform of type 1 phosphatidylinositol phosphate kinase with talin. This activates the phosphatidylinositol phosphate kinase, and together they translocate to the plasma membrane (5). Local phosphatidylinositol 4,5-biphosphate production by the phosphatidylinositol phosphate kinase is then thought to activate talin (6), possibly by relieving an autoinhibitory head-tail association unmasking the integrin binding site in the talin head (IBS1). In platelets, talin becomes activated through a protein kinase C-dependent signaling pathway that stimulates association and translocation of the small GTPase Rap1 to the plasma membrane where it interacts with its effecter Riam, leading to the recruitment of talin and the unmasking of its integrin binding site in the head domain (7). Active talin, which can form anti-parallel homodimers (3), finally binds to and activates integrins allowing the exposure of a high affinity binding site for integrin extracellular ligands (8, 9). Recent biochemical, cryo-electron microscopy, and NMR studies have mapped the integrin binding site in the talin head to the F3 phosphotyrosine binding site of the FERM domain, which interacts with the NPXY motif in the β3 integrin cytoplasmic tail (10).

Talin can be cleaved by calpain into a 47-kDa head and a 220-kDa talin rod domain. The talin rod, which is composed of ~62 α-helical alanine- and leucine-rich repeats (11, 12), contains numerous functional domains. These include three well-characterized vinculin binding sites (VBS1–3) (13), as well as eight additional vinculin binding sequences (12) and a highly conserved actin binding site located at the C-terminal end upstream of a dimerization motif (14–16). It also contains an integrin binding site (IBS2) (1, 17, 18) whose functional role is still elusive.

We have previously localized IBS2 to residues 1984–2113 of the talin rod (talin rod fragment J) and demonstrated direct interaction of recombinant talin fragments containing IBS2 protein; GST, glutathione S-transferase; 4OHT, 4-hydroxytamoxifen; WT, wild type; ATR, attenuated total reflection; CIB, calcium and integrin-binding protein; CreER, Cre-estrogen receptor.

* This work was accomplished within a CNRS-sponsored European network “Integrins and Transfer of Information” (GDRE-ITI) and was supported by European Community Project HPRN-CT-2002-00253, the University of Luxembourg, and grants from the “Fonds National de la Recherche,” Luxembourg, and the “Fondation Luxembourgoise contre le Cancer,” Luxembourg. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Recipient of a doctoral fellowship from the Ministère de la Culture, de l’Enseignement Supérieur et de la Recherche, Luxembourg. Data presented here were obtained as part of a doctoral thesis to be submitted to the “Université Libre de Bruxelles,” Belgium.
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§ The abbreviations used are: aa, amino acid; CHO, Chinese hamster ovary; IBS, integrin binding site; VBS, vinculin binding site; GFP, green fluorescent protein; GST, glutathione S-transferase; 4OHT, 4-hydroxytamoxifen; WT, wild type; ATR, attenuated total reflection; CIB, calcium and integrin-binding protein; CreER, Cre-estrogen receptor.
with native platelet integrin αIIbβ3 or a recombinant β3 integrin subunit cytoplasmic tail, either in vivo, by intracellular fluorescence resonance energy transfer, or in vitro, by surface plasmon resonance and liquid phase pulldown assays (1). We now report the minimal structure of talin IBS2 and provide evidence that a 2-amino acid point mutation (L1/AA) in this minimal structure inactivates the integrin binding capacity of IBS2. In addition, we show that a full-length green fluorescent protein (GFP)-talin LI/AA mutant is unable to rescue the cell spreading-defective phenotype of talin1−/− cells, despite the presence of a functional IBS1 site. Our data provide the first direct evidence that IBS2 in the talin rod is essential to link integrins to the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following monoclonal and polyclonal antibodies were used: mouse anti-talin (TD77), rabbit anti-GST (Chemicon International, Inc., Temecula, CA); mouse anti-β3 (P37), a kind gift from Dr. J. Gonzalez-Rodriguez (Instituto de Quimica Fisica, Madrid, Spain); goat anti-β3 (C20) (Santa Cruz Biotechnology, Inc.); mouse anti-αIIbβ3 (PAC-1) (BD Biosciences); mouse anti-Myc tag (9B11) (Cell Signaling Technology, Inc., Danvers, MA); tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Amersham Biosciences). Rhodamine phalloidin was purchased from Molecular Probes. Diphenylcarbamyl chloride-treated trypsin was purchased from Sigma-Aldrich.

**CHO-αIIbβ3 Cell Culture and Transient Transfection**—Chinese hamster ovary cells (CHO) stably expressing recombinant αIIbβ3-GFP or wild type αIIbβ3 integrin (19) were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and transfected as previously described (1). For each cDNA construct, 1.5 × 10⁶ adherent cells were transfected with 3–15 μg of cDNA using Lipofectamine™ (Invitrogen) according to the manufacturer’s instructions. Fetal calf serum (10% final concentration) was added after 3 h of transfection.

**Generation of the Talin1 Null Mouse Embryo Fibroblasts**—Mice possessing a conditional allele of talin1 where the first 5 coding exons were flanked with loxP sites (Tln1fl) were crossed with mice possessing an allele encoding a Cre integrase-estrogen receptor (CreER) fusion protein that renders Cre activity tamoxifen (TM) inducible (20) in order to generate embryos and then mouse embryo fibroblasts with one talin1 null allele and one talin1 floxed allele (Tln1fl/−) along with one copy of the CreER transgene. These Tln1fl/− CreER mouse embryo fibroblasts were immortalized by transfection with a temperature-sensitive SV40 large T antigen construct. Clones were selected and grown routinely at 33 °C (permissive for T antigen expression). Treatment of these cells with 1 μM 4-hydroxytamoxifen (4OHT) deletes the “floxed” talin1 region, converting the cells to the talin1−/− genotype within 24 h. Cells were then transfected either with GFP-talin WT or mutant GFP-talin LI/AA, replated on glass coverslips, and analyzed for assembly of focal adhesions 24–48 h later.

**Mammalian Cell cDNA Expression Constructs**—The cDNAs encoding C-terminal DsRed-tagged talin fragments G (aa 1984–2344), J (aa 1977–2113), B (aa 430–1076), and Myc-tagged talin fragment J have been previously described (1). The cDNAs encoding talin residues 1977–2018 (11), 2010–2049 (J2), 2042–2080 (J3), and 2072–2113 (J4) were generated using talin fragment G cDNA as a template and were inserted into the mammalian cell expression vector pcDNA4/TO/myc-HIS (Invitrogen), generating recombinant polypeptides with a C-terminal Myc-His tag. For site-directed mutagenesis, the pDsRed-N1 vector with the cDNA encoding either talin fragment J (aa 1977–2113) or talin fragment B (aa 430–1076) was used as template (1). The single and double amino acid substitutions P2109A, L2094A/I2095A, T2098A/K2099A were introduced into talin fragment J and the double amino acid substitutions L622A/L623A and M870A/V871A into talin fragment B using the QuikChange™ site-directed mutagenesis kit according to the manufacturer’s protocol (Stratagene, La Jolla, CA). Full-length wild type mouse talin1 cDNA, a generous gift from Dr. A. Hutschenrecher (University of Wisconsin, Department of Pediatrics and Pharmacology, Madison, WI) was cloned into the pEGFP-C1 vector (Invitrogen) in-frame with the upstream GFP coding sequence to generate GFP-talin1 WT. GFP-talin L2094A/I2095A was subsequently generated by site-directed mutagenesis. Successful mutagenesis of all constructs was confirmed by automated sequencing (Applied Biosystems, Foster City, CA).

**Indirect Immunofluorescence**— transiently transfected cells were detached with EDTA buffer (50 mM Hapes, 126 mM NaCl, 5 mM KCl, 1 mM EDTA, pH 7.5), washed, resuspended in serum-free Iscove’s modified Dulbecco’s medium, and seeded onto glass coverslips coated with fibronogen (20 μg/ml). After a 2-h adhesion at 37 °C, the cells were fixed for 15 min at 4 °C in fixation buffer (phosphate-buffered saline, pH 7.4, 3% paraformaldehyde, 2% sucrose) and washed four times in washing buffer (phosphate-buffered saline, pH 7.4, 0.5% Triton X-100, 0.5% bovine serum albumin). For indirect immunofluorescence staining of the Myc-tagged talin fragments J, J1, J2, J3, and J4, the fixed cells were incubated for 40 min with a monoclonal mouse anti-Myc antibody followed by a 30-min incubation with tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG. Immunofluorescent staining of β3 integrin was performed using the monoclonal antibody P37 and a fluorescein isothiocyanate-conjugated IgG antibody. Polymerized actin was stained with rhodamine phalloidin. After each incubation step the coverslips were washed three times for 5 min in washing buffer. Finally, the coverslips were mounted on microscopy glass slides using Mowiol/DABCO (Sigma). Single fluorescent images were collected on a conventional fluorescence microscope (Leica Leitz DMRB) with a ×63 oil immersion objective and a Leica DC 300F camera using the Leica IM1000 1.20 software. Images were processed digitally with Photoshop 6.0 (Adobe Systems).

**Western Blotting**—CHO-αIIbβ3 cells transfected with full-length GFP-talin WT or GFP-talin LI/AA were detached with EDTA buffer, washed three times in PBS washing buffer, and
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lysed on ice in buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 μg/ml pepstatin A, 5 μg/ml apronitin, and 2.5 μg/ml leupeptin, 7 μM E64d, and 25 μM 4-(2-aminoethyl)benzenesulfonyl fluoride. Lysates were clarified by centrifugation (4 °C, 10 min, 13,000 rpm), and protein concentration was determined using the BCA protein assay kit (Pierce). Equal amounts of total protein (100 μg) were denatured in 1% SDS sample buffer (95 °C, 5 min), resolved by 8% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane. Western blot analysis of the transferred proteins was performed with the monoclonal anti-talin rod antibody TD77 according to standard procedures.

Talin Peptide Dot Blot Overlay Assay—The cDNAs encoding the cytoplasmic tail of wild type β3 (β3cyto, aa 716–762) or calcium and integrin-binding protein (CIB) inserted in the pGEX-4T-2 bacterial expression vector have been previously described (1, 21). Expression of GST alone and GST fusion proteins in *Escherichia coli* BL21(DE3) was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Cells were lysed as previously described (34). Fusion proteins from the soluble fraction were affinity-purified on glutathione-Sepharose 4B beads according to the manufacturer’s instructions (Amersham Biosciences). Overlapping talin peptides 1 (aa 2077–2099), 2 (aa 2070–2091), and 3 (aa 2094–2115) were purchased from Neosystem SA, Strasbourg, France. The peptides were dotted on a nitrocellulose membrane with 0.5 or 1.5 nmol of peptide/spot. The membrane was blocked for 5 h in Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7) containing 1% of heat-inactivated fetal bovine serum and overlaid overnight at 4 °C with GST-β3cyto or GST-CIB at 150 nM in TBS-T (5% nonfat milk, 0.1% Tween 20). The membrane was then rinsed three times for 5 min with TBS-T (5% nonfat milk, 0.05% Tween 20) and blocked for 1 h in TBS-T (5% nonfat milk, 0.1% Tween 20). Bound GST-β3cyto or GST-CIB was detected using polyclonal antibodies to GST or the integrin β3 subunit cytoplasmic tail and horseradish peroxidase-conjugated secondary antibodies that were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce), according to the manufacturer’s procedure.

**Limited Proteolysis of Wild Type and Mutant Talin Fragment G**—The cDNA encoding talin fragment G was subcloned into the bacterial pET-29b+ vector (Invitrogen) to generate recombinant talin fragment G with a His tag at its C terminus (TalinG-His). Mutant L2094A/I2095A talinG-His fragment was generated by site-directed mutagenesis of recombinant talinG-His using the pET-29b+/talinG vector as a template. The L2094A/I2095A mutation was inserted as described above. Successful mutagenesis of the construct was confirmed by automated sequencing. Expression of Histagged proteins was induced in *E. coli* BL21(DE3) bacteria with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The recombinant proteins were affinity purified on nickel-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer’s protocol. Wild type and mutant L2094A/I2095A talinG-His fragments were subjected to limited trypsin proteolysis for 5 min, 30 min, or 4 h at room temperature using a trypsin/talin ratio of 1/100 (w/w). The proteolytic protein profile was resolved under reducing conditions on 12% SDS-PAGE and analyzed by Coomassie Blue staining and Western blotting using a polyclonal anti-talin rod antibody raised in-house against a recombinant 7-kDa talin rod fragment comprising helices 50 and 51 (aa 2071–2135).

*In Vitro allbβ3-Talin Fragment G Pulldown Assay*—The cDNA encoding talin fragment G inserted in the pGEX-4T-2 (GST-talin G) bacterial expression vector has been previously described (1). Mutations were introduced into GST-talin G cDNA by site-directed mutagenesis as described above, and successful mutagenesis was confirmed by automated sequencing. For direct protein-protein interaction assays, 200 μl of crude bacterial lysates containing GST-talin G WT, GST-talin G LI/AA, or GST alone were each mixed with 20 μl of glutathione-Sepharose beads overnight at 4 °C and subsequently washed four times with phosphate-buffered saline (0.05% Tween 20). The precoated beads were then incubated for 3 h at 4 °C with 15 μg of integrin allbβ3 that was purified from outdated platelets (provided by Dr. J.-C. Faber, Luxembourg Red Cross Blood Transfusion Center) by Concanavalin A affinity chromatography and activated with 0.5 mM MnCl2 and the monoclonal antibody PAC-1 (5 μg/ml). Unbound proteins were washed off and the trapped proteins extracted with 25 μl of denaturing SDS-PAGE loading buffer (5 min, 100 °C) and submitted to 10% polyacrylamide gel electrophoresis. Precipitated proteins were visualized by Western blot using antibodies to GST and integrin β3.

**Infrared Spectroscopy**—For infrared spectroscopy, synthetic peptides (Neosystem SA, Strasbourg, France) (23 mers) corresponding to wild type helix 50 (peptide 1, aa 2077–2099) or the L2094A/I2095A mutated helix 50 of the human talin rod domain were used. Attenuated total reflection infrared (ATR-FTIR) spectra were obtained on a Bruker IFS55 FTIR spectrophotometer (Ettlingen, Germany) equipped with a mercury-cadmium-telluride detector (broad band 12000–420 cm−1, liquid N2-cooled, 24-h hold time) at a resolution of 2 cm−1 with an aperture of 3.5 mm. The spectrometer was continuously purged with dry air (Whatman 75–62, Haverhill, MA). The internal reflection element was a 52 × 20 × 2-mm trapezoidal germanium ATR plate (ACM, Villiers St. Frédéric, France) with an aperture angle of 45° yielding 25 internal reflections. The germanium crystals were washed in Superdecontaminate (Interciences, AS, Brussels, Belgium), rinsed with distilled water, washed with methanol, then with chloroform, and finally placed for 2 min in a plasma cleaner PDC23G (Harrick, Ossining, NY) working under reduced air pressure. The peptides were dissolved in H2O at 10 mg/ml. Films were obtained by evaporating 5 μl (50 μg) or 20 μl of a 1 mg/ml solution (20 μg) on one side of the ATR plate under a stream of nitrogen. No difference related to the concentration could be detected. The germanium crystal was then placed in an ATR holder for liquid sample with an in- and outlet (Specac, Orpington, UK). The liquid cell was placed at 45° incidence on a Specac vertical ATR setup. For hydrogen/deuterium exchange, nitrogen gas was saturated with 2H2O by bubbling through a series of four vials containing 2H2O. The flow rate of 50 ml/min was controlled by a flowtube (Fisher Bioblock Scientific, Illkirch, France). Bubbling was started at least 1 h.
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before starting the experiments. At the zero time, the tubing was connected to the cavity of the liquid cell chamber surrounding the film. 20 scans were recorded and averaged for each time point. All the software used for data processing was written under MatLab 7.1 (Mathworks Inc., Natick, Ma).

RESULTS

Tentative Localization of the Talin IBS2 Site by a Bioinformatics Approach—Talin is an ancient, conserved cytoskeletal protein that is found in organisms ranging from the amoebozoan protozoa Dictyostelium discoideum to mammals (22, 23). Considering the similar functional properties and modular organization of invertebrate versus vertebrate talins, we hypothesized that functional domains in this protein including IBS2 should be conserved throughout evolution and that these domains could be identified by multiple sequence alignment.

As shown in Fig. 1A, the overall sequence identity between human and Drosophila is only 47% but increases to 56% for the highly conserved C-terminal I/LWEQ actin binding domain (THATCH domain, aa 2339–2533) (23, 24). To test our hypothesis, we performed sequence alignment of human talin residues 1974–2276 with the equivalent talin sequence of different species.

Expression of Overlapping Myc Fusion Proteins Covering Residues 1977–2113 of Fragment J in CHO-αIIbβ3-GFP Cells—To extend these in silico data, talin fragment J and four overlapping peptides of fragment J with a Myc-His tag at the C-terminal end, corresponding to talin residues 1977–2276, were transiently expressed in CHO cells stably transfected with integrin αIIbβ3-GFP. The subcellular localization of the recombinant fragments was visualized with an anti-Myc antibody.

FIGURE 1. Pair-wise and multiple amino acid sequence alignments of human talin with talin from different species. Sequences of talin were obtained from the NCBI nucleotide data base (www.ncbi.nlm.nih.gov). Hs, Homo sapiens (human, accession number NM_006289); Gg, Gallus gallus (chicken, accession number AY150847); Dr, Danio rerio (zebrafish, accession number AY781336); Dm, Drosophila melanogaster (fruit fly, accession number AF299248); Ce, Caenorhabditis elegans (nematode, accession number L46861). A, sequence identity (%) as determined by pair-wise sequence alignment of full-length human talin with chicken (Gg), zebrafish (Dr), and Drosophila (Dm) talin. B, shown are multiple sequence alignments of human talin residues 1974–2276 corresponding to fragment J and L with the same talin sequence from different species. The alignment of the talin amino acid sequences was performed using the ClustalW algorithm. 100% conservation is indicated by yellow shading. Negatively charged amino acids are indicated in red, whereas positively charged residues are in blue and uncharged residues in black. The gray bars above the alignment represent the location of the α helices in mouse talin (12). Amino acid sequences of the recombinant human talin fragments J1, J2, J3, and J4 are indicated (black bars). C, diagram of the talin constructs used in this study. The structure of talin with the location of major functional domains is shown. The position of the two integrin binding sites (IBS1, IBS2), the three vinculin binding sites (VBS1, VBS2, VBS3), and the actin binding site (I/LWEQ, THATCH domain) are indicated. Horizontal bars represent the different recombinant human talin1 fragments generated. Numbers represent the first and last amino acid of each fragment.
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**A**

![β3-GFP](image1)

![myc-talin](image2)

**B**

![J1](image3)

![J2](image4)

![J3](image5)

![J4](image6)

**merge**

**FIGURE 2.** Subcellular localization of recombinant Myc-tagged talin fragments after transient expression in CHO-αIIbβ3-GFP cells. CHO cells expressing integrin αIIbβ3-GFP were transiently transfected with cDNA encoding talin fragment J and four overlapping talin fragments, J1, J2, J3, and J4, fused to a Myc-His tag. The transfected cells were detached, plated on fibrinogen-coated coverslips, and fixed with paraformaldehyde prior to staining of the recombinant Myc-His talin fragments with an anti-Myc monoclonal antibody and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG antibody. Images were acquired on a conventional fluorescence microscope. A, colocalization of Myc-talin fragment J (red) with the autofluorescent β3-GFP integrin (green) in focal adhesions. Scale bar, 5 μm. B, for each of the four overlapping talin J subfragments, β3-GFP (green) and Myc-talin (red) images were merged to visualize protein colocalization (white arrows). Scale bar, 10 μm.

**FIGURE 3.** Dot blot assay of recombinant β3 integrin cytoplasmic tail binding to immobilized talin peptides. A, sequence display of the talin peptides 1, 2, and 3, covering the highly conserved talin sequence (aa 2070–2115) and containing talin fragment J4 (2072–2113). B, the talin peptides 1, 2, and 3 were spotted onto a nitrocellulose membrane with 0.5 nmol peptide/spot, and bound GST-β3cyto fusion protein was detected with an anti-GST antibody or an antibody to the cytoplasmic tail of β3. Recombinant GST-CIB, detected with an anti-GST antibody, was used as a negative control.

Reported by us, talin fragment J colocalized with integrin αIIbβ3 in focal adhesions (Fig. 2A) (1). When talin fragments J1, J2, J3, and J4 were tested, only fragment J4 exhibited a punctate fluorescence (red) that clearly colocalized with autofluorescent αIIbβ3-GFP (green) as demonstrated by the shift in color of the dots from green to yellow in the merged fluorescent picture, whereas fragments J1, J2, and J3 revealed a diffuse overall cytoplasmic staining and absent colocalization with β3-GFP (Fig. 2B). These data provide evidence that talin fragment J4 contains IBS2. Not surprisingly, fragment J4 corresponds to a highly conserved sequence identified by our bioinformatics approach and overlaps with α helix 50 of the talin rod domain (12).

**Delineation of the Integrin Binding Site in Talin Fragment J4 to the α Helix 50**—To further delineate IBS2, three synthetic peptides covering the amino acid sequence of talin fragment J4 were used in an in vitro dot blot assay (Fig. 3). The synthetic peptides were spotted on a nitrocellulose membrane and overlaid with the recombinant integrin β3 subunit cytoplasmic tail fused to GST. Bound GST-β3cyto was detected using a polyclonal antibody to GST or to the β3-cytoplasmic tail. A specific interaction was observed for talin peptide 1, but not for peptide 2, whereas a weak interaction was seen for peptide 3, which overlaps for 6 residues with peptide 1 but has no sequence identity with peptide 2. No antibody binding was observed when the spotted membrane was overlaid with GST-CIB, a protein interacting with the integrin αIIb subunit cytoplasmic tail and used here as a negative control. Increased antibody binding was only observed for peptides 1 and 3 with an increase of peptide concentration (result not shown). These data provide evidence that talin peptide 1 corresponding to residues 2077–2099 of the talin rod domain interacts with the integrin β3 subunit cytoplasmic tail and thus contains the IBS2 site. This minimal IBS2 sequence corresponds exactly to the α helix 50 of the talin rod (12).

**Disruption of the IBS2 Site by Site-directed Mutagenesis**—To further characterize the talin IBS2 site, the conserved residues common to peptides 1 and 3 (see Figs. 1 and 3), namely L2094A/I2095A (LI/AA) and T2098A/K2099A (TK/AA), and located within the α helix 50 as well as a highly conserved proline residue in the non-helical sequence linking helix 50 to 51 (P2109A, P/A) were mutated and the DsRed-tagged talin J mutants expressed in CHO cells stably transfected with integrin αIIbβ3. As shown in Fig. 4, similar to wild type fragment J, the talin J mutants TK/AA and P/A localized to focal adhesions as visualized with an anti-β3 antibody. In contrast, fragment J with the
LI/AA mutation exhibited diffuse staining, providing evidence that the LI/AA mutation disrupted the integrin binding site.

**The LI/AA Mutation Disrupts the α-Helical Structure of IBS2**—To determine the effect of the LI/AA mutation on the α-helical structure of the IBS2 peptide, we performed infrared spectroscopy using two synthetic peptides (23 mers), one corresponding to wild type helix 50 and the second to helix 50 with the LI/AA mutation. The infrared spectrum of peptides and proteins is dominated by the Amide I band (1700–1600 cm$^{-1}$) and the amide II band (1600–1500 cm$^{-1}$). The former is essentially due to amide carbonyl stretching (C=O) and the latter to amide N-H bending. Amide I is particularly sensitive to the secondary structure of the backbone and used to get information on the secondary structure of proteins (25, 26). Fig. 5 demonstrates the large difference in the conformation of these two peptides. For the wild type peptide, amide I absorbs at 1655 cm$^{-1}$, a wave number typical of α-helical structures, whereas the LI/AA mutant displays a major band at 1628 cm$^{-1}$ typical of an anti-parallel β-sheet structure and a shoulder at 1659 cm$^{-1}$ indicating significant amounts of turns or helices. Upon hydrogen/deuterium exchange of the amide proton, amide II band is supposed to disappear completely and a new band called amide II’ arise near 1450 cm$^{-1}$ (27, 28). It is remarkable that for both peptides the extent of exchange is very weak after 280 min (Fig. 5). In agreement with this observation, the major bands present in amide I do not shift by more than 2 cm$^{-1}$. This behavior indicates a well defined, stable secondary structure because transient opening of the H bonds is required for exchange with the external medium.

To provide additional proof that the LI/AA mutation affected the α-helical structure, resulting in a conformational change of IBS2, we performed limited trypsin proteolysis of recombinant His-tagged wild type (WT) and mutant (LI/AA) talin fragment G from bacterial lysate.
Further characterize the different proteolytic fragments, we performed Western blot analysis using a polyclonal anti-talin rod antibody that was raised in-house against a 7-kDa recombinant talin fragment corresponding to helices 50 and 51 of the talin rod domain (aa 2071–2135) (Fig. 6B). This antibody reacted with both wild type and mutant talin G. Following trypsin digestion, the antibody identified the 37-kDa proteolytic fragment of talin G WT but did not react with the proteolytic fragments of mutant talin G LI/AA, providing evidence that the single LI/AA mutation in helix 50 induces a conformational change that can be monitored based on a distinct trypsin proteolytic profile and the loss of epitopes normally exposed by talin helices 50 and 51.

The 2 hydrophobic LI residues in helix 50 are also conserved in other talin rod α helices and correspond to positions 15 and 16 of the consensus 1LXXAXXXVAXXXVXXLXXA19 vinculin recognition sequence (12). To test whether similar mutations in the vinculin consensus sequence would also disrupt the α-helical structure required for talin-vinculin binding, alanine mutations of the 2 hydrophobic residues in helix 4 of vinculin binding site 1 (VBS1-LL/AA) or helix 12 of VBS2 (MV/AA) were introduced into the cDNA encoding DsRed-tagged talin fragment B that comprises VBS1 and VBS2 (aa 430–1076) (1). Interestingly, although wild type fragment B colocalized in focal adhesions, mutant talin fragment B with a single VBS1-LL/AA or VBS2-MV/AA mutation exhibited reduced focal adhesion localization and increased diffuse cytoplasmic staining, whereas fragment B with the double VBS1 and VBS2 mutation appeared as a cytoplasmic precipitate in close proximity to the nucleus, most likely due to improper folding of the recombinant protein (data not shown). Collectively, these results suggest that the LI/AA mutation in helix 50 (IIBS2) and the equivalent mutations in helix 4 (VBS1) or helix 12 (VBS2) affect integrin or vinculin binding by disrupting the α-helical structure of the respective talin contact sites.

The Talin LI/AA Mutation Prevents in Vitro Talin Fragment G-Integrin αIIbβ3 Coprecipitation—To test the effect of the LI/AA mutation on the in vitro interaction of talin fragment G with purified integrin αIIbβ3, we performed a pull-down assay using Mn²⁺ and PAC-1-activated αIIbβ3 integrin. The GST-talin G fragments or GST alone were immobilized on glutathione-Sepharose and used to precipitate Concanavalin A-purified platelet αIIbβ3. As shown in Fig. 7, only talin fragment G WT, but not mutant talin fragment G LI/AA or GST alone, used as a negative control was able to trap αIIbβ3. These data clearly indicate that the LI/AA mutation in talin fragment G prevents its association with integrin αIIbβ3.

Talin IBS2 Is Essential for Linking Integrin β Subunits to the Cytoskeleton—To determine the functional role of talin IBS2 in the integrin-dependent cell adhesion process, we engineered a full-length mouse GFP-talin LI/AA mutant, using wild type mouse talin as a template (29). When expressed in CHO-αIIbβ3-positive cells, fluorescence imaging revealed that both wild type and mutant talin colocalized with the β3 integrin in focal adhesions, demonstrating that recombinant full-length talin was functional and was not hindered by the presence of an N-terminal GFP tag (Fig. 8A). Western blot analysis of the recombinant talin fusion proteins in transfected cells using the TD77 monoclonal antibody that recognizes the extreme C-terminal region of the talin rod (aa 2269–2541) revealed a single band of reduced electrophoretic mobility for both expressed wild type and mutant GFP-talin as compared with endogenous hamster talin, demonstrating that the GFP-talin fusion proteins had the correct molecular mass and did not undergo intracellular proteolytic cleavage (Fig. 8B).
To analyze the effect of the LI/AA mutation on talin function, we expressed GFP-talin WT or GFP-talin LI/AA in a conditional talin1/CreER mouse embryonic cell line in which the floxed talin1 (fl) allele can be inactivated by treatment with 4OHT. As shown in Fig. 9A, prior to 4OHT treatment the cells exhibited a fully spread phenotype, whereas following induction of the Cre recombinase the majority of cells were either round or poorly spread with an irregular morphology, showing short filopodial-like protrusions and very poor and abnormal stress fibers. Fig. 9B shows the changes in cell morphology following GFP-talin WT or GFP-talin LI/AA transfection into 4OHT-treated cells. As expected, GFP-talin WT rescued the abnormal phenotype of 4OHT-treated cells by promoting cell spreading with GFP-talin localizing in focal adhesions. In contrast, the mutant GFP-talin LI/AA was unable to rescue the effect of 4OHT treatment despite strong intracellular expression, as judged by the fluorescence intensity of the transfected cells. These results provide strong evidence that IBS2 in the talin rod is essential to link integrins to the actin cytoskeleton.

DISCUSSION

Recent genetic studies in Drosophila have emphasized the importance of the talin rod in linking integrins to the actin cytoskeleton, as the talin head known to activate integrins was unable to fulfill this function (30). The results presented here identify a 23-residue amphipathic α helix (helix 50) of the talin rod as the minimal functional IBS2 structure able to interact with the integrin β3 subunit cytoplasmic tail. We further demonstrate that the IBS2/integrin interaction relies on an intact α helix, because a 2-amino acid point mutation that disrupts this α helix is sufficient to inactivate talin IBS2 function. Finally, we show that talin IBS2 is essential in the rescue of the adherent phenotype of talin1−/− cells.

Interestingly, the IBS2 helix has also been shown to contain a VBS (12). VBSs are defined by a single amphipathic helix of ~25 residues forming six turns, with the hydrophobic residues involved in vinculin binding clustered on one face of the amphipathic helix. Crystallographic and NMR structures have shown that the three major VBSs are contained within helical bundles, with the 6 key hydrophobic residues involved in vinculin binding buried within the hydrophobic core of the α-helical bundles (31–33). Talin binds vinculin with relatively low affinity, confirming that the majority of VBSs in talin are indeed cryptic (34). In native talin, the integrin binding sites are also reportedly cryptic and can be activated by phosphatidylinositol 4,5-biphosphate (35), the Rap1-Riam complex (7), or by calpain cleavage (36). The talin head domain binds with a 6-fold higher affinity to the β3 integrin cytoplasmic tail than native talin (36). However, in contrast to the vinculin binding sites that are still cryptic in the isolated talin rod and need additional activation to become functional, IBS2 appears to be constitutively active, because the isolated talin rod domain purified from platelets as well as recombinant talin rod fragments of various sizes exhibit integrin binding activity, as demonstrated by gel filtration (17), enzyme-linked immunosorbent assay (18), surface plasmon resonance analysis (36), or in vivo protein–protein interactions in focal adhesions (1). In addition, our results provide evidence that, in contrast to the vinculin binding sites (33, 34), the activity of IBS2 does not appear to depend on the stability of the helical bundles that make up the rod domain, as talin fragments of different sizes (fragment F, 557 aa; fragment G, 360 aa; fragment J, 129 aa; and fragment J4, 40 aa) all exhibited integrin binding activity in vivo.

Because IBS2 is constitutively active in the isolated talin rod domain, we speculate that integrin binding relies on the conserved charged residues clustered on the opposite face of the amphipathic helix to the VBS and that IBS2 is most likely exposed on the solvent face of the different talin rod fragments tested. In support of this hypothesis are the structural data provided for the highly conserved F-actin binding THATCH core domain from the human huntingtin-interacting protein-1 related (HIP1R), which shares high sequence homology with related (HIP1R), which shares high sequence homology with
face of the THATCH core, emanating from the third and fourth helices. Mutational analysis of several of these invariant residues revealed that the basic residues Lys and Arg are important for actin binding. The fact that the LI/AA mutation inactivates IBS2 by disrupting the α helix of repeat 50 suggests that this α-helical structure is necessary to provide the right spacing to the highly conserved charged residues clustered on one face of the helix and most likely involved in integrin binding.

Here we have shown for the first time that full-length talin with this 2-amino acid (LI/AA) mutation in the IBS2 site of the rod domain is unable to rescue the adherent phenotype of talin1−/− cells despite the presence of a functional IBS1 site. These results provide direct evidence that IBS2 in the talin rod domain is the site required to link integrins to the cytoskeleton. We and others have previously shown that the integrin binding affinity of the talin rod domain or of talin rod fragments is ~20–30 times weaker than that measured for talin head binding (1, 36, 39), a result difficult to reconcile with the important functional role of IBS2 demonstrated here in linking integrins to the cytoskeleton and the mechanical forces this interaction has to withstand during cell adhesion and migration. However, the recent data by Tanentzapf and Brown (30) suggest that the talin rod may only interact with high affinity ligand-bound integrins, which would explain the weak binding affinities measured in vitro for talin rod interaction with resting αIIbβ3 or recombinant β3 cyttoplasmic tail fragments (1). Thus, our current view is that the talin head activates the integrin leading to ligand binding. Ligand-dependent outside-in signaling leads to a conformational change in the integrin β subunit cytoplasmic tail, exposing de novo a high affinity binding site for talin IBS2. The downstream consequence of integrin engagement with IBS2 would be the induction of a conformational change in the talin rod domain, most likely through a reshuffling of α-helical bundles, resulting in the exposure of vinculin binding sites. Recruitment of vinculin might then stabilize the initial integrin-talin-actin complex by cross-linking talin to F-actin.

Acknowledgments—We thank Dr. Anna Huttenlocher for providing the full-length mouse talin cDNA and Dr. José Gonzalez-Rodriguez for the generous gift of the anti-β3 monoclonal antibody P37. We are also grateful to Dr. J.-C. Faber for providing outdated platelet concentrates.

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