A Fluorescence Cell Biology Approach to Map the Second Integrin-binding Site of Talin to a 130-Amino Acid Sequence within the Rod Domain*

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The cytoskeletal protein talin, which provides a direct link between integrins and actin filaments, has been shown to contain two distinct binding sites for integrin β subunits. Here, we report the precise delimitation and a first functional analysis of the talin rod domain integrin-binding site. Partially overlapping cDNAs covering the entire human talin gene were transiently expressed as DsRed fusion proteins in Chinese hamster ovary cells expressing α1β3, linked to green fluorescent protein (GFP). Two-color fluorescence analysis of the transfected cells, spread on fibronectin, revealed distinct subcellular staining patterns including focal adhesion, actin filament, and granular labeling for different talin fragments. The rod domain fragment G (residues 1884–2213), devoid of any known actin- or vinculin-binding sites, colocalized with β3-GFP in focal adhesions. Direct in vitro interaction of fragment G with native platelet integrin α1β3, or with the recombinant wild type, but not the Y747A mutant β3 cytoplasmic tail, linked to glutathione S-transferase, was demonstrated by surface plasmon resonance analysis and pull-down assays, respectively. Here, we demonstrate for the first time the in vivo relevance of this interaction by fluorescence resonance energy transfer between β3-GFP and DsRed-talin fragment G. Further in vitro pull-down studies allowed us to map out the integrin-binding site within fragment G to a stretch of 130 residues (fragment 4, residues 1884–2113) that also localized to focal adhesions. Finally, we show by a cell biology approach that this integrin-binding site within the talin rod domain is important for β3-cytoskeletal interactions but does not participate in α1β3 activation.

Integrin-mediated cell adhesion and signaling are crucial events for numerous biological processes such as morphogene-
sis, the immune response, hemostasis, cell growth, and differ-
etiation as well as for cell survival (1). Integrins function as noncovalent αβ heterodimeric transmembrane receptors that link the extracellular matrix to the actin cytoskeleton; they are, however, unable to directly interact with actin filaments. A number of actin-binding proteins, including talin, α-actinin, filamin, myosin, and skelemin, have been identified that act as intermediates in connecting integrins to the actin cytoskeleton (2). Among these, talin was the first intracellular ligand shown to interact directly with integrin β-subunit cytoplasmic tails (3).

Talin is a multifunctional ~270-kDa (2541 amino acids) cytoskeletal protein that forms antiparallel homodimers, which represent the biologically active form of the protein (4). In vitro, talin is found in equilibrium between a membrane-bound and a cytoplasmic form (4, 5), and several studies have emphasized its role in regulating integrin-actin talin-taln complexes (6) and integrin activation (7, 8). Talin colocalizes with integrins at sites of cell-matrix interactions and membrane ruffles of moving cells (9, 10), and it is necessary for the assembly of focal adhesions (FAs).1 The functional role of talin has been elucidated by antibody inhibition studies and genetic approaches. Microinjection of anti-talin antibodies into fibroblasts led to the disruption of FAs and associated stress fibers (11), and down-regulation of talin by antisense mRNA or short hairpin RNAs reduced cell spreading and FA assembly in HeLa cells, integrin processing and transport to the cell surface, and energy-de-
dependent integrin activation (8, 12, 13). Disruption of the Talin1 gene by a knockout approach in mice revealed that undifferentiated Talin1 (−/−) embryonic stem cells exhibited defective cell adhesion and spreading and were unable to assemble FAs or stress fibers, whereas differentiated cells readily formed these adhesion complexes (14). The only partially inhibitory phenotype observed in Talin1 (−/−) mouse fibroblast-like cells may be explained by the presence of a second, recently identi-
fied TALIN2 gene in mammalian cells (15). Indeed, in Drosoph-
illa, disruption of the rhea locus, which corresponds to the unique talin gene, mimics the integrin knockout phenotype, underlining the important role of talin as an obligatory compo-
ent of integrin-mediated adhesion (16).

1 The abbreviations used are: FA, focal adhesion; FRET, fluorescence resonance energy transfer; SPR, surface plasmon resonance; CHO, Chinese hamster ovary; GST, glutathione S-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; IMDM, Iscove’s modified Dulbecco’s medium; Pipes, 1,4-piperazinediethanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate; bis-Tris, 2-[bis- (2-hydroxyethyl)amino]-2-hydroxymethyl)propane-1,3-diol.
Characterization of the Talin Rod Integrin-binding Site

In living cells, talin can be cleaved into a globular 47-kDa N-terminal head domain and an elongated 190-kDa C-terminal rod domain by the calcium-dependent protease calpain II (17). The talin head (residues 1–433), which includes a region (residues 86–410) homologous to the N-terminal FERM domain of the band 4.1, ezrin, radixin, moesin family of cytoskeletal proteins (17), contains an actin-binding site (18), three potential membrane-association sites (19, 20), and binding sites for integrins, layilin, focal adhesion kinase, myosin (21), and type 1 γ phosphatidylinositol phosphate kinase (22). The binding affinity of talin for integrins increases upon calpain cleavage or in the presence of phosphatidylinositol 4,5-biphosphate, suggesting that conformational changes within talin unmask cryptic integrin-binding sites (13, 23). Recent biochemical (7, 24), cryo-electron tomographic, and NMR studies (25, 26) have mapped the integrin-binding site in the talin head fragment to a mainly hydrophobic area in the F3 subdomain of the FERM domain. Recombinant F3, which binds integrin tails with a similar affinity as the talin head domain (7), functions as a phosphotyrosine-binding domain (25), recognizing the 744NPXY747 motif in the β3 cytoplasmic tail. Residue Tyr747 appears to be critical for this interaction, since the Y747A mutation abrogates the talin head binding to the β3 subunit (24). Binding of the talin head domain to the cytoplasmic tail of integrin β subunits leads to integrin activation (8), and this was shown by overexpression of the F3 subdomain of the talin FERM domain in CHO α5β3 cells, which results in increased binding of the ligand mimetic anti-α5β3 antibody PAC-1 (7, 24).

The talin rod domain is composed of multiple α-helical ala-
nine-rich repeats (27) and contains a dimerization site (28), an integrin-binding site (3, 29), three vinculin-binding sites (30), a recently identified TES-binding site (31), and two actin-binding sites, the C-terminal one of which is highly conserved among actin-binding proteins (32, 33). Using in vitro solid phase binding assays, the β3 integrin-binding site of the rod domain has recently been shown to be contained within a recombinant fragment encoding the 558 C-terminal residues of talin (29).

The functional significance of the interaction between the talin rod domain and the β-subunit of integrins in integrin-mediated cell adhesion and spreading remains largely unknown. In order to study this specific interaction, we have used a cell biology and a biochemical approach to map the integrin-binding site in the talin rod domain to a poly peptide of 130 amino acids, located between the C-terminal actin- and vinculin-binding sites. We also provide evidence that in FAs this fragment directly interacts with the cytoplasmic tail of β3 but that this interaction does not participate in integrin activation.

EXPERIMENTAL PROCEDURES

Protein Expression and Affinity Purification—Talin cDNA fragments encoding amino acids 1–433 (fragment A), 430–1076 (fragment B), 1984–2541 (fragment F), and 1984–2344 (fragment G) were amplified by reverse transcriptase-PCR using human erythrocyte HEC cell mRNA. DNA fragments encoding amino acids 1984–2344 (fragment H), 1984–2113 (fragment J), and 2093–2344 (fragment K) were amplified by PCR from the talin G construct. The primers used for PCR according to the manufacturer

FRET experiments were performed on a confocal microscope (Bio-Rad MRC1024, krypton-argon laser). DAPI was excited at 488 nm, 568 nm; Nikon Eclipse TE300, 40× oil immersion objective and a LEICA DC 300F camera using Photoshop 6.0 (Adobe Systems).

FRET experiments were performed on a confocal microscope (Bio-Rad MRC1024, krypton-argon laser). DAPI was excited at 488 nm, 568 nm; Nikon Eclipse TE300, 40× oil immersion CFI Plan-Fluar numerical aperture 1.3 objective, using the GFP/DSRed couple as a donor/acceptor pair. A spectral parameter indicating the ability of donor/acceptor fluorescent molecules to exhibit efficient FRET is R0, the Förster distance equal to the donor/acceptor separation at which FRET is 50% efficient. For the GFP/DSRed couple, R0 is 5.7 nm. To determine the Förster radius for this system, the coverslips were washed three times with 5 min in washing buffer. Finally, the coverslips were mounted on microscopy slides in Mowiol/DABCO (Sigma).

Fluorescence Microscopy and Fluorescence Resonance Energy Transfer (FRET) Analysis—For fluorescence analysis, GFP- and DSRed-conjugated proteins were visualized in paraffin-embedded fixed cells adherent on fibrinogen-coated glass coverslips. Single images were collected under 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).

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photobleached at 568 nm with 100% laser power until all acceptor (DsRed) was photodestroyed (A2). A second Z-series of the donor was acquired (postbleach; D2). After correction for image acquisition using the Amira software (TGS), maximum intensity projections from the Z-series of GFP images were generated. The FRET-dependent increase in fluorescence of the donor in the photobleached region was visualized by subtracting the prebleach GFP image from the postbleach GFP image (D2 – D1). For each experiment, FRET efficiencies in FA sites (EF<sub>A</sub>) were calculated from the pre- and postbleach donor images according to the following equation: \( EF_A = 1 - (I_{FA, postbleach} / I_{FA, prebleach}) \), where \( I \) is the average intensity measured for the corresponding FA (41). For each cell analyzed, EF<sub>A</sub> values were calculated for 4–8 FA sites in the photobleached region as well as in the control (nonbleached) region, and the results were expressed as means ± S.E.

Pull-down Assays—Platelets were isolated from freshly drawn whole blood as described previously (42) and lysed by sonication on ice in lysis buffer (50 mM NaCl, 150 mM sucrose, 10 mM Pipes, 1% Triton X-100, 0.5% deoxycholate, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 6.8). Lysates were clarified by centrifugation at 4 °C for 30 min at 13,000 rpm, and protein concentration was determined using the Bio-Rad protein assay according to the manufacturer’s instructions.

For direct protein-protein interaction assays, 200 μl of crude bacterial lysates containing the GST fusion proteins talin A, B, F, G, H, J, or K or GST alone were each mixed with 20 μl of glutathione-Sepharose beads for 1 h at 4 °C and subsequently washed four times with PBS plus 0.05% Tween 20. Sepharose beads coated with the different talin fragments were then incubated with 1 μg of platelet lysate for 2 h at 4 °C. Unbound proteins were washed off three times with PBS. Protein complexes were extracted in 25 μl of SDS-PAGE loading buffer (5 min, 100 °C) and separated on a 4–12% NuPAGE Bis-Tris gradient gel (Invitrogen). Western blot analysis of the transferred proteins was performed with a monochlonal anti-GST antibody (Amersham Biosciences) were immobilized on a CM5 sensor chip according to the manufacturer’s instructions (Biacore). Briefly, after activation of the carboxylate groups on the chip by a mixture of N-ethyl-N’-dimethylaminopropyl carbodiimide and N-hydroxysuccinimide (Biacore), a solution of 35 μg/ml goat anti-GST antibody in 10 mM acetate buffer (pH 4.9) was flushed over the four channels of the chip for 7 min at a flow rate of 5 μl/min. The residual activated carboxylate groups were neutralized with 1 mM ethanolamine (pH 8.5); for regeneration, 10 mM HCl was flushed over the channels for 1 min.

For kinetic analysis, two channels were pretreated for 2 min at 5 μl/min with a 400 nM solution of recombinant GST (control channel) or GST–integrin constructs in a solution containing 150 mM NaCl, 10 mM Hepes, and 0.05% NP20 (HBS-N; Biacore). Subsequently, the purified recombinant talin fragment A or G, at a concentration ranging from 0.25 to 2 μM in HBS-N, was allowed to react with the two channels at a flow rate of 20 μl/min for 5 min, followed by a dissociation phase of 7.5 min. After each run, the channels were completely regenerated with 10 mM HCl. The obtained sensorgrams were analyzed with Biacal 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–β<sub>3</sub> from the anti-GST antibodies.

PAC-1 Binding Experiments and Flow Cytometry—Binding of the mouse antibody PAC-1, which is specific for the activated form of α<sub>5</sub>β<sub>3</sub> integrin, was assessed as previously described (36, 43). Briefly, -5 × 10<sup>5</sup> cells in 50 μl of IMDM were preincubated for 20 min at room temperature in the presence or absence of the α<sub>5</sub>β<sub>3</sub>-activating mouse antibody DSGFP (3 μg). For control experiments, nonspecific binding of PAC-1 was assessed by incubating the cells in the presence of 1 μM RGDS peptide. PAC-1 antibody (3.5 μg in 50 μl of IMDM) was then directly added to the suspension, and cells were further incubated for 45 min at room temperature. Cells were washed in cold IMDM and incubated for 30 min on ice with a R-phycocerythrin-conjugated anti-mouse IgM antibody (Jackson ImmunoResearch Laboratories, Inc.), diluted in IMDM. Finally, cells were washed and reuspended in a solution of 137 mM NaCl, 5 mM KCl, 50 mM Hepes, 1 mg/ml glucose, pH 7.4.

Flow cytometry was performed using a Coulter EPICS XL flow cytometer (Coulter, Hialeah, FL). After electronic compensation of the FL1 and FL2 fluorescence channels, PAC-1 binding (FL2) was analyzed on cells, which expressed the relevant GFP fusion proteins (FL1).

**RESULTS**

Subcellular Localization of Recombinant Talin Fragments Covering the Entire Amino Acid Sequence of Human talin1—We have previously shown that fusion of GFP to the cytoplasmic tail of the β<sub>3</sub> integrin subunit allowed surface expression of a fully functional α<sub>5</sub>β<sub>3</sub>-GFP receptor (36). In order to characterize the β<sub>3</sub>-binding site contained within the talin rod domain, we have used a fluorescence cell biology approach to identify DsRed-talin fragments that colocalize with β<sub>3</sub>-GFP when expressed in CHO α<sub>5</sub>β<sub>3</sub>-GFP cells. For this purpose, partially overlapping cDNA fragments encoding the entire amino acid sequence of human talin1 were generated as outlined in Fig. 1 by reverse transcriptase-PCR using human erythroleukemic HEL cell mRNA as a template. Each amplified cDNA fragment was cloned into the pDsRed-N1 vector and verified by automated cDNA sequencing. The constructs were transiently transfected into CHO α<sub>5</sub>β<sub>3</sub>-GFP cells, and the subcellular localization of each fragment in the cells, plated on fibronogen, was analyzed by two-color fluorescence microscopy. Analysis was confined to cells expressing low levels of the tagged protein, because in cells overexpressing DsRed-tagged fusion proteins, the increased cytoplasmic staining obscured specific staining. As shown in Fig. 2, fragment A, which corresponds to the talin head domain and which contains a known integrin-binding site, and fragment B, which carries two vinculin-binding sites (44), both colocalized with β<sub>3</sub>-GFP-stained adhesion structures at the cell periphery. The relatively small size of these structures as compared with larger mature FAs suggests that they represent focal complexes or early FAs. Fragment D exhibited a granular localization, distinct from FA labeling. This granular localization was also observed when talin fragment D was transfected into the human cancer cell line HT-144 (data not shown), suggesting the presence of a potential vesicle-targeting domain in this fragment. Fragment
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Fig. 2. Subcellular localization of recombinant DsRed-talin fragments after transient expression in CHO α₅β₃-GFP cells. CHO cells expressing α₅β₃-GFP were transiently transfected with cDNA encoding talin fragments A, B, D, E, F, and G, fused to DsRed. The transfected cells were detached, plated on fibrinogen-coated coverslips, and fixed with paraformaldehyde prior to observation under a conventional fluorescence microscope. For each talin fragment, β₃-GFP (green) and DsRed-talin (red) images were acquired and merged to visualize protein colocalization. The insets show a magnification of the area boxed. Bar, 10 μm.

F, which has previously been shown to interact with β₃ in vitro (29), colocalized with β₃ in FAs. Additionally, in cells expressing high levels of fragment F, we observed staining of actin filaments, emerging from β₃-GFP-containing FAs, most likely due to the actin-binding site within fragment F. In contrast, fragment E, which corresponds to the C-terminal part of the rod domain, co-localized with cortical actin filaments. We were unsuccessful in expressing fragment C, which corresponds to the central part of the rod domain and which carries the major part of an actin-binding site. Since fragments E and F both contain the conserved actin-binding sequence (I/L)WEQ of the C-terminal part of the rod domain (32), the subcellular localization of a shorter fragment G (amino acids 1984–2344), deleted of sequence corresponding to fragment E, was analyzed. Interestingly, fragment G clearly labeled mature FAs but not actin filaments, suggesting that this fragment could harbor a functional integrin-binding site.

In Vitro Binding of the C-terminal Part of the Talin Rod Domain to the β₃ Integrin Subunit—To elucidate whether fragment G was able to interact with β₃ in vitro, we performed pull-down experiments of α₅β₃ from platelet lysate using recombinant talin fragments. Talin fragments A, B, F, and G were expressed as GST fusion proteins and purified by affinity chromatography on glutathione-Sepharose from crude bacterial lysates. Eluted GST fusion proteins were resolved on a gradient SDS-polyacrylamide gel and stained with Coomassie Blue. The apparent molecular masses corresponded to the calculated masses of 76 kDa (fragment A), 93 kDa (fragment B), 86 kDa (fragment F), and 66 kDa (fragment G). The positions of the molecular weight marker proteins are indicated. B, for β₃ pull-down assays, recombinant GST, used as a control, and GST-talin fragments A, B, F, and G were immobilized on glutathione-Sepharose and subsequently incubated with platelet lysate as described under “Experimental Procedures.” Protein complexes or total platelet lysate (last lane) were resolved by gradient SDS-PAGE and transferred to a nitrocellulose membrane, and β₃ was revealed with the C3a monoclonal antibody directed against the cytoplasmic tail of β₃.

Fig. 3. In vitro binding of the C-terminal part of the talin rod domain to the β₃ integrin subunit. A, talin fragments A, B, F, and G were expressed as GST fusion proteins and purified by affinity chromatography on glutathione-Sepharose from crude bacterial lysates. Eluted GST fusion proteins were resolved on a gradient SDS-polyacrylamide gel and stained with Coomassie Blue. The apparent molecular masses corresponded to the calculated masses of 76 kDa (fragment A), 93 kDa (fragment B), 86 kDa (fragment F), and 66 kDa (fragment G). The positions of the molecular weight marker proteins are indicated. B, for β₃ pull-down assays, recombinant GST, used as a control, and GST-talin fragments A, B, F, and G were immobilized on glutathione-Sepharose and subsequently incubated with platelet lysate as described under “Experimental Procedures.” Protein complexes or total platelet lysate (last lane) were resolved by gradient SDS-PAGE and transferred to a nitrocellulose membrane, and β₃ was revealed with the C3a monoclonal antibody directed against the cytoplasmic tail of β₃.

SPR Analysis of the Direct Protein-Protein Interaction between the Rod Domain Fragment G and the β₃ Cytoplasmic Tail—Since a talin fragment encompassing residues 1541–2541 has recently been shown to contain a binding site for the FA protein TES (31) and to exclude that pull-down of α₅β₃ from platelet lysate. As shown in Fig. 3B, only the talin rod domain fragments F and G, but not B, were able to trap α₅β₃, clearly indicating that fragment G contains the C-terminal β₃-binding site of the rod domain. Experiments with the talin head domain fragment A or GST alone served as positive and negative controls, respectively. These data also provide evidence that the observed FA localization of fragment B, which corresponds to the N-terminal part of the rod domain and contains two vinculin-binding sites, was probably due to interaction with vinculin rather than with integrins.

SPR Analysis of the Direct Protein-Protein Interaction between the Rod Domain Fragment G and the β₃ Cytoplasmic Tail—Since a talin fragment encompassing residues 1541–2541 has recently been shown to contain a binding site for the FA protein TES (31) and to exclude that pull-down of α₅β₃ by fragment G was mediated indirectly through this talin-binding protein TES, we performed SPR analysis using recombinant GST-β₃ cytoplasmic tail fusion proteins as ligands and recombinant talin A and talin G fragments, deleted of the GST moiety, as analytes. Fig. 4A shows the purified recombinant
proteins following SDS-PAGE analysis and demonstrates the absence of cleaved GST in the samples corresponding to the talin fragments A and G.

\[ \text{Calculation of the association rate constants for the talin A- and talin G-} \]

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\[ \text{values of } 1.5 \pm 0.8 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \text{ and } 4.0 \pm 0.3 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \text{, respectively. These low association rate constants could point to the necessity of conformational changes for the formation of a stable integrin-talin complex or to the overevaluation of the concentration of active talin fragments, since the protein concentration does not always correspond to the concentration of interacting molecules. Nevertheless, the association rate constants for both talin A and talin G were very similar. On the other hand, the dissociation rate constant for talin A (9.7 \pm 1.6 \times 10^{-6} \text{ s}^{-1}) \text{ was much lower than that for talin G (6.3 \pm 6.2 \times 10^{-6} \text{ s}^{-1}), yielding dissociation equilibrium constants of 7.7 and 157 \text{ nm}, respectively. These data demonstrate a direct interaction of talin fragment G with the cytoplasmic tail of } \]

\[ \text{and the } \beta_3 \text{ cytoplasmic tail mutant Y747A at the highest concentration used (2 } \mu\text{M), suggesting that the } \beta_3 \text{ integrin-binding sites for the talin head and the talin rod domain are in close proximity and that both interactions rely on the presence of residue Tyr}^{747}. \text{The observation of a quasi-irreversible dissociation results from two dissociations: the real dissociation rate of the talin fragments from the integrin cytoplasmic tails and the differential dissociation of the control GST and GST-} \]

\[ \text{beta 3 from the anti-GST antibodies} \]
immobilized on the chip. To distinguish the real talin-integrin dissociation rate, a single Langmuir model was chosen, including a drifting base line, which takes the GST-anti-GST dissociation into account.

**FRET Detection of the Molecular Association of β3-GFP with DsRed-talin Fragment G in Focal Adhesions of CHO α3β3-GFP Cells**—To confirm that colocalization of the talin rod domain fragment G with the β3 cytoplasmic tail within the cell is physiologically relevant and corresponds to a true protein-protein interaction, in situ FRET experiments were performed using GFP fused to the β3 integrin cytoplasmic tail as the donor and DsRed fused to different talin fragments (fragments B and G) as the acceptor. CHO α3β3-GFP cells were transiently transfected with DsRed-fused talin fragments B and G and allowed to adhere to fibrinogen, and FRET analysis was performed on a confocal microscope by comparing the images of donor emission (GFP), excited at 488 nm before (D1) and after (D2) acceptor (DsRed) photobleaching. In 15% of the cells transfected with talin fragment G, a decrease in FRET could be detected as a clear increase in donor emission intensity after photobleaching of the acceptor (Fig. 5A, D2-D1). In cells exhibiting this change in FRET, higher amounts of acceptor were expressed so that DsRed-talin fragment G staining was observed not only in FAs but also in the cytoplasm, indicating that the quantity of acceptor is a limiting factor in this approach. However, an increase in donor fluorescence was only detected in FAs (Fig. 5A, arrows), demonstrating that in CHO α3β3-GFP cells, talin fragment G is in very close contact with the cytoplasmic tail of β3. In contrast, photobleaching of DsRed-talin fragment B (Fig. 6A) or DsRed-talin fragment A (data not shown) resulted in no significant increase of donor fluorescence intensity in all cells analyzed. However, it is generally not possible to interpret negative FRET results unless the spatial orientation of the donor and acceptor molecules is controlled (45). Observations made on the difference (D2-D1) images were quantified by calculating FRET efficiencies in FA sites as described under “Experimental Procedures.” A significant difference of FRET efficiency could be observed between the photobleached and the control (nonbleached) region for cells expressing DsRed-talin fragment G (p = 0.0024) but not for cells expressing DsRed-talin fragment B (p = 0.4261) (Fig. 5B).

These results clearly show that the DsRed-talin fragment G is in very close contact with β3-GFP and suggest that the two proteins do interact directly within FAs.

**Delimitation of the β3 Integrin-binding Site to a 120 Residue Sequence within the Talin Rod Domain**—To further map out the integrin-binding site within the amino acid sequence of talin fragment G, five overlapping cDNA fragments (fragments H, I, J, K, and L) were generated (Fig. 1) and expressed as DsRed fusion proteins in CHO α3β3-GFP cells. As shown in Fig. 6A, two overlapping talin fragments, H (287 amino acids) and J (130 amino acids), localized in FAs, whereas the other three fragments (fragments I, K, and L) displayed diffuse cytoplasmic staining, suggesting that the β3 integrin-binding sequence is comprised within residues 1984–2113 of fragment J.

Since DsRed has previously been shown to aggregate and form oligomers (46) and in order to exclude potential nonspecific clustering of DsRed fusion proteins in FA plaques, we performed a control experiment by generating a cDNA construct (Jmyc; Fig. 1), similar to fragment J, but devoid of DsRed and instead fused to a myc-His tag. Indirect immunofluorescence labeling of the transfected CHO α3β3-GFP cells with an anti-myc antibody and a rhodamine-conjugated secondary antibody displayed the same FA staining pattern for talin fragment Jmyc (Fig. 6B) as for DsRed-talin fragment J (Fig. 6A).

Finally, we performed pull-down experiments of α3β3 using purified GST-fused talin fragments G (66 kDa), H (60 kDa), J (37 kDa), and K (50 kDa) (Fig. 6C). As shown in Fig. 6D, talin...
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Fig. 6. Delimitation of the β₃ integrin-binding site within fragment G of talin. A, five overlapping DsRed-talin fragments (fragments H, I, J, K, and L), covering the sequence of talin G, were transiently expressed in CHO α₁mβ₃-GFP cells. The transfected cells were detached, plated on fibronectin-coated coverslips, and fixed prior to observation under a conventional fluorescence microscope. Bar, 10 μm. B, the subcellular localization of the myc-His-tagged talin fragment J (Jmyc), was visualized with an anti-myc monoclonal mouse antibody and rhodamine-conjugated goat anti-mouse IgG antibody. Bar, 10 μm. C, talin fragments G, H, J, and K were expressed as GST fusion proteins and analyzed as described in the legend to Fig. 3A. The apparent molecular masses corresponded to the calculated weights of 66 kDa (G), 60 kDa (H), 37 kDa (J), and 50 kDa (K). D, pull-down of β₃ from platelet lysate using GST-talin fragments G, H, J, and K was performed as outlined in the legend to Fig. 3B.

Discussion

Talin is a multifunctional cytosolic actin-binding protein that plays a major role in linking integrins to the actin cytoskeleton. It is therefore not surprising that talin contains, in addition to its three actin-binding sites (18), multiple redundant functional domains, notably three binding sites for acidic phospholipids (47), three vinculin-binding sites (30), a binding site for the recently identified focal adhesion protein TES (31), and two integrin-binding sites (3, 24, 29). In the present study, we have delimited the second integrin-binding site of talin to a sequence of 130 residues in the rod domain and have demonstrated the in vivo relevance of this interaction using a cell biology approach and fluorescence energy transfer.

Overlapping DsRed-tagged fragments covering the entire amino acid sequence of talin were expressed in CHO α₁mβ₃-GFP cells to study subcellular localization patterns of the different recombinant proteins. This way, we have mapped out the second integrin-binding site of talin to residues 1984–2113 in the C-terminal part of the rod domain, located between the highly conserved (IL/WEQ actin-binding domain (residues 2345–2541) and the third vinculin-binding site (residues 1944–1969). The distinct subcellular staining of the transiently expressed DsRed-talin fragments in CHO α₁mβ₃-GFP cells of either FAs, actin filaments, or granular structures underscores the reliability of the approach and excludes artificial or non-specific clustering of the recombinant fusion proteins due to the presence of the DsRed tag (46). Also, the specificity of the subcellular localization of fragment J was further confirmed with the expression of a similar fragment (Jmyc), fused to a myc–His tag and devoid of the DsRed moiety.

A result of interest was the subcellular localization of the fragments that contained, in addition to the β₃ integrin-binding site, a second functional domain such as the C-terminal actin-binding site (fragment F) or a potential new vesicle targeting domain (fragment D). Fragment F was localized in FAs when expressed at low levels and localized along actin filaments, suggesting a lower affinity of the actin-binding motif as compared with the integrin-binding site of the talin rod domain. Fragment D labeled granular structures, which may imply an involvement of talin in vesicle transport processes. Although the significance of this localization remains to be investigated, these data are reminiscent of findings showing that down-regulation of talin either by an antisense (12) or by a gene targeting approach (48) compromised integrin trafficking and...
surface expression and led to a marked change in the actin cytoarchitecture.

The talin rod domain was initially identified as the integrin-binding domain (3), and subsequent *in vitro* binding studies localized this binding site to the 558 C-terminal amino acids of talin (residues 1984–2541) (29). Our data precisely delimit this integrin-binding site to a small fragment of 130 amino acids (residues 1984–2113 of talin fragment J). In the absence of any notable sequence similarity of fragment J with the FERM domain of the talin head, the binding mechanism, the binding site, and the affinity of the rod domain for the β3 tail are likely to be different from that recently reported for the talin head domain (25). We used real time SPR to demonstrate the *in vitro* interaction of the recombinant talin fragments (A and G) with the β3 cytoplasmic tail and determined the respective binding affinities corresponding to a $K_d$ of 7.7 nM for the head domain fragment A and 157 nM for the rod domain fragment G. Even if we consider the possibility that not all recombinant talin molecules are interacting molecules, the very low association rate constants for both fragment A and fragment G with the β3 integrin cytoplasmic tail suggest that the formation of a stable complex requires conformational changes in one or both of the interacting partners. The difference in β3 integrin affinity for the talin head and rod domain, which has already been observed by Yan et al. (23), is thus essentially due to a difference in the dissociation rate constant, which is 65 times lower for fragment A as compared with fragment G. The 10-fold difference in the affinity of the talin head domain for β3 reported here as compared with the data reported by Yan et al. (23) is most likely due to differences in the experimental setting, notably the species origin of the talin head domain (human versus mouse) and the β3 cytoplasmic tail construct (GST-β3 fusion protein versus a biotin-maleimide-labeled model protein containing a four-heptad repeat motif and a spacer of 4 glycines linked to the N-terminal part of the β3 cytoplasmic tail). Since the β3 Y747A mutation abolished the interaction of the mutant β3 subunit cytoplasmic tail with both fragment A and fragment G, we conclude that the talin head and rod domains recognize either one or two closely proximal sites in β3, suggesting a mutually exclusive interaction of the two domains with the β3 cytoplasmic tail rather than a cooperative binding (23).

Taking advantage of the GFP/DsRed pair of autofluorescent tags, we have shown fluorescence resonance energy transfer between β3-GFP and DsRed-fused talin fragment G, which emphasizes the *in vivo* relevance of the talin rod domain interaction with β3. FRET measurements were possible, since the FRET pair GFP/DsRed exhibits good wavelength separation of donor and acceptor emission spectra. Moreover, the spectral characteristics of GFP allow the use of the 568-nm line of the krypton/argon laser to excite DsRed with no excitation of GFP, a condition required when using the acceptor photobleaching method to assess FRET. However, measured FRET efficiency was low, and FRET could only be detected in ~15% of the cells expressing talin fragment G. This can be explained by the fact that in CHO αIIbβ3-GFP cells, the transfected DsRed-talin fragments compete with endogenous talin for interacting with the different binding partners. Since the analyzed cells were attached and spread, it can be assumed that a major part of the β3-GFP cytoplasmic tails was still bound to endogenous talin, thus decreasing the ratio between the acceptor (DsRed-talin fragment) and the donor (β3-GFP cytoplasmic tail) in FAs so that FRET efficiency was diminished. Other factors that influence FRET efficiency are the slow chromophore maturation of DsRed of typically 48 h to reach 90% of maximum fluorescence (49) as well as the orientation and the total amount of fluorescent proteins within the cell (45). Therefore, it is difficult to interpret negative FRET results as in the case of talin fragments A and B.

Several studies have conclusively established the functional role of the talin head domain in inside-out signaling as such that it mediates spatial separation of the α and β cytoplasmic domains, which subsequently leads to integrin activation (7, 8, 24–26, 50). Using the activation-specific PAC-1 antibody, we demonstrated that, unlike the talin head domain, the rod do-

![Figure 7](image-url)
main fragment G by itself is unable to activate α5β3. Although the talin rod domain has recently been shown to activate vinculin (51, 52), which suggests a regulatory role in FA assembly, its exact function in binding to integrins remains to be investigated further. However, a direct role in outside-in signaling appears likely, where interaction of the rod domain with numerous cytoskeletal proteins such as vinculin, actin, and the newly identified TES protein may account for the clustering of integrins by strengthening the link between integrins and the actin cytoskeleton in mature FAs. This hypothesis is supported by the observation that the transfected talin head domain was localized in relatively small structures on the cell periphery that resembled focal complexes or early FAs rather than mature FAs, whereas talin fragments containing the rod domain integrin-binding site (fragments F, G, H, J, and Jres) labeled larger mature FAs. The Tyr747 of the β3 cytoplasmic tail seems to be required for both talin head and rod domain binding, and this suggests a mutually exclusive interaction. It can be speculated that α5β3 will be required for both talin head and rod domain binding, and this may account for the clustering of integrins by strengthening the link between integrins and the actin cytoskeleton in mature FAs.

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