The Talin Head Domain Binds to Integrin β Subunit Cytoplasmic Tails and Regulates Integrin Activation*

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The β subunit cytoplasmic domains of integrin adhesion receptors are necessary for the connection of these receptors to the actin cytoskeleton. The cytoplasmic protein, talin, binds to β integrin cytoplasmic tails and actin filaments, hence forming an integrin-cytoskeletal linkage. We used recombinant structural mimics of β3, β,β,D and β, integrin cytoplasmic tails to characterize integrin-binding sites within talin. Here we report that an integrin-binding site is localized within the N-terminal talin head domain. The binding of the talin head domain to integrin β tails is specific in that it is abrogated by a single point mutation that disrupts integrin localization to talin-rich focal adhesions. Integrin-cytoskeletal interactions regulate integrin affinity for ligands (activation). Overexpression of a fragment of talin containing the head domain led to activation of integrin αββ3; activation was dependent on the presence of both the talin head domain and the integrin β3 cytoplasmic tail. The head domain of talin thus binds to integrins to form a link to the actin cytoskeleton and can thus regulate integrin function.

The connection of integrin adhesion receptors to the actin cytoskeleton regulates cell shape, adhesion, and migration (1). Talin, a cytoplasmic protein composed of ~270-kDa subunits, binds to β integrin cytoplasmic tails, vinculin and actin filaments (2–6), and co-localizes with integrins at sites of cell-substratum contact (7). It plays an important role in the establishment and maintenance of integrin-cytoskeleton connections, and loss of talin expression leads to impaired cell adhesion, spreading, and migration (8). Talin consists of an N-terminal ~50-kDa globular head domain and an ~220-kDa C-terminal rod domain (9). The N-terminal talin head domain contains an ~200-residue region similar to a region within the membrane-binding N-terminal ER/MAD association domain (NERMAD) in the ezrin, radixin, and moesin (ERM) family of proteins (9). The NERMAD domain of ERM proteins binds to the cytoplasmic domain of transmembrane receptors (e.g. CD44), and the C-terminal domain binds to actin, linking the receptor to the cytoskeleton (10). In contrast to ERM proteins, previous studies indicate that the C-terminal rod domain of talin contains the integrin-binding site and the vinculin- and actin-binding sites (5, 6, 11). Thus, talin might differ from other ERM proteins in the manner in which it connects membrane proteins to actin filaments.

Talin binds to recombinant structural mimics of dimerized integrin β cytoplasmic tails (2). Here, we examined the interaction of the talin head domain with three of these integrin β cytoplasmic tails and report that either recombinant or proteolytically derived talin head domains bind specifically to all three β tails. Furthermore, overexpression of the talin fragments containing the head domain “activated” integrin αββ3 as judged by increased ligand-binding affinity. Consequently, the talin head domain binds to several integrin β tails and can thus mediate the linkage of these integrins to the actin cytoskeleton and modulate integrin function.

EXPERIMENTAL PROCEDURES

Antibodies and cDNAs—Monoclonal antibodies, anti-talin 84 (Sigma) and TA205 (Srotex), anti-GST B-14 (Santa Cruz Biotechnology) and anti-Tac 7G7B6 (American Tissue Culture Collection, Manassas, VA) were obtained commercially. The anti-αββ3, mAb PAC1 and the αββ3-specific peptide inhibitor Ro43-5054 have been described previously (12). cDNAs encoding Tac-α3, GST-chicken talin-(280–435), -(186–435), and -(1–280) and β3,β,D integrin cytoplasmic tails have been described previously (2, 13, 14). cDNA encoding the β3 cytoplasmic tail was amplified by polymerase chain reaction and cloned into a modified PET15b expression construct (2). Y/A mutations (Fig. 2A) were introduced using the QuikChange™ site-directed mutagenesis kit (Stratagene). A cDNA encoding amino acids 1–435 of mouse talin (9) was cloned into the bacterial expression vector pCGR-2T (Amersham Pharmacia Biotech, Upsala, Sweden). cDNAs for mouse talin encoding amino acids 1–1071 and 434–1071 (9) were cloned into pJ6 R mammalian expression vectors.

Cells and Cell Lysates—Human platelets (obtained as described previously (2)) were lysed by sonication on ice in the presence of either Complete™ protease inhibitor mixture (Roche Molecular Biochemicals), 0.1 mM calpain inhibitor E-64 (Roche Molecular Biochemicals), and 1 mM EDTA, or 1 mM CaCl2, 1 mM MgCl2. Following lysis, Complete™ protease inhibitor mixture, E-64 (0.1 mM final), and EDTA (2 mM final) were added to the inhibitor-free sample. CHO cells stably expressing human integrin αββ3 or αββ3,327A (15) were transfected using lipofectAMINE (Life Technologies, Inc.), and activation of αββ3 was assayed by PAC1 binding as described previously (12).

Purification of Recombinant Proteins—Recombinant integrin cytoplasmic tails were expressed, purified and characterized as described previously (2). GST-talin fusion proteins were expressed and purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), according to the manufacturer’s instructions.

Affinity Chromatography with Recombinant Integrin Cytoplasmic Tails—Affinity chromatography was performed using recombinant integrin cytoplasmic tails bound to His-Bind® Resin (Novagen). Binding of 200 μl of cell lysate, or 20 μl of purified GST-talin fragments, to 50 μl of coated resin was performed in a total volume of 800 μl as described otherwise.

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1 The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; CHO, Chinese hamster ovary MFI, median fluorescence intensity.
The muscle-specific integrin β₃ Tail—

Talin, a large cytoskeletal actin-binding protein, binds to the integrin cytoplasmic tails in the presence of protease inhibitors (assayed by Coomassie Blue staining of eluted proteins (recombinant integrin were fractionated by SDS-PAGE, and binding of talin fragments was previously (2). Bound proteins were fractionated by SDS-PAGE and analyzed by immunoblotting. Binding of recombinant integrin tails to the resin was verified by Coomassie Blue staining.

RESULTS AND DISCUSSION

Binding of Talin Domains to the Integrin β₃ Tail—Talin, a large cytoskeletal actin-binding protein, binds to the muscle-specific integrin β₃ Tail (Fig. 1A). To determine which domains of talin bind, we permitted endogenous platelet calpain to cleave talin into an ~50-kDa head domain and an ~220-kDa rod domain (Ref. 9; Fig. 1A). In the presence of calpain inhibitors, intact talin resulted in a band with an apparent molecular mass of ~240 kDa that reacted with antibodies specific for the talin head (TA205) and rod (8d4) domains. Platelet lysis in the absence of calpain inhibitors resulted in the complete disappearance of the intact talin and generation of a 50-kDa, TA205-binding, head domain and an apparent 190-kDa, 8d4-reactive, rod domain (Fig. 1B). The discrepancy between the predicted and apparent molecular masses of both intact talin and the talin rod domain is consistent with previous reports (9). β₃ affinity chromatography revealed binding of both head and rod domains (Fig. 1C). Binding of both domains was specific in that it was abrogated by a tyrosine to alanine (Y/A) substitution in the first NPXY motif of β₃ (Figs. 1C and 2A). Mutation of this residue in β₂A or β₂D inhibited binding of full-length talin (data not shown) (2). Thus both domains of talin bind to a β₃ affinity matrix.

Binding of Talin Domains to Other Talin-binding Integrin β Tail Domains—Talin also binds to β₁A and β₂ cytoplasmic tails, (2, 3). However, binding to β₂A is weaker than to β₂D and β₃ (Ref. 2 and data not shown). We therefore tested whether the same talin domains bound to β₁A and β₂D cytoplasmic tails. Both head and rod domains bound to β₁A and β₂ (Fig. 2B). Furthermore, the relative binding of each domain was similar to that of intact talin (data not shown). Substitution of Ala for Tyr in the membrane-proximal NPXY motif inhibited binding of both full-length talin (data not shown), and head and rod domains (Fig. 2B).

Talin forms antiparallel homodimers (6), and previous work (11) implicated the rod domain as the integrin-binding site. Consequently, the head domain could have bound through an association with intact integrin-bound talin. However, the absence of intact talin in the lysates (Fig. 1B) suggests that binding of the isolated domains is not mediated through their dimerization with full-length talin. Furthermore, localization of the dimerization domains to the central region of the rod domain (6) suggests that the head and rod domains do not associate following calpain cleavage. In support of this hypothesis, head and rod domains did not co-immunoprecipitate from calpain-cleaved lysates using either 8d4 or TA205 as precipitating antibodies (data not shown). Consequently, the binding of both head and rod domains to integrins probably represents independent interactions. Independent binding of the head and tail domains would explain the observation that integrin α₃β₃ binds to talin with a stoichiometry of 2:1 (3).

Talin Head Domain Binds Directly to Integrin β Tail Domains—The experiments reported above suggested an interaction between the talin head domain and integrin β tail domains. However, binding of the head domain could occur via a talin-binding intermediary protein present in the platelet lysate. We therefore examined the binding of purified recombinant talin head domain (talin 1A and 1D) to β₁A, β₂D, and β₃ integrin cytoplasmic tails. Talin head domain bound to all three tails. Furthermore, Tyr to Ala mutations in the first NPXY motif inhibited binding (Fig. 3B). In addition, GST failed to bind to any of the cytoplasmic tails, and the head domain failed to bind to the α₃β₃ cytoplasmic tail (data not shown). These results demonstrate that the isolated talin head domain contains an integrin-binding site.

To further localize the integrin binding site within the talin head domain, we investigated the binding of three overlapping talin head domain fragments to β₂D cytoplasmic tails (Fig. 3, A and C). Western blotting revealed that fusion proteins of the predicted molecular mass were present (Fig. 3C). Only talin-(186–435) bound to the β₂D tails, and this binding was sensitive to Tyr to Ala mutations in the β₂D tail (Fig. 3C). A similar binding pattern was seen for β₂A and β₂D tails (data not shown). The failure of talin-(280–435) and -1 to 280) to bind integrin tails indicates that amino acids within the 186–280 region are necessary, but not sufficient, for tail binding. Further localization of the binding site within amino acids 186–435 was not possible due to the insolubility of all smaller fragments tested.

Sequences within the talin head domain suggest that this region of talin is structurally related to the N-ERMAD of the

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**Fig. 1. The talin head and rod domains both bind to integrin β₃ cytoplasmic tails.** A, a schematic representation of talin. Cleavage by calpain II (after amino acid 433) results in a 50-kDa head domain and a 220-kDa rod domain. The location of the epitopes for the head domain-specific mAb TA205 (amino acids 139–433 in chicken talin (17) and the rod domain-specific mAb 8d4 (amino acids 439–1071 in mouse talin) are indicated. B, talin head and rod domains were generated by lysing platelets (10⁶/ml) in Tris-buffered saline, 0.5% Triton X-100, 0.1% deoxycholate in the presence of protease inhibitors (+), or in 1 mM CaCl₂, (−). Proteins were fractionated by SDS-PAGE under reducing conditions and were transferred to Immobilon-P membranes and probed with monoclonal antibodies TA205 or 8d4. C, lysate prepared in the presence of intact talin and the rod domain-specific mAb 8d4 (amino acids 433–1071 in mouse talin) are indicated.
The Talin Head Domain Binds to Integrin β Tails

**Fig. 2. Integrin βA and βD cytoplasmic tails bind talin head and rod domains.** A shows an alignment of the amino acid sequences of human βA, βD, and βB integrin cytoplasmic tails. The tyrosine residues mutated to alanine in the Y/A mutants are shown as **bold underlined** and correspond to amino acid number 788 in chicken βA (22) and 747 in human βB (23). B, the binding experiments described in the legend to Fig. 1 were repeated using βA, βD(Y/A), βB, and βB(Y/A) coated beads. Bound talin head and rod domain were detected by Western blotting with mAbs TA205 or 8d4, respectively. The lysate shown corresponds to 10% of the starting material in the binding assay.

ERM family of proteins (Ref. 9; Fig. 3B). ERM proteins generally function as membrane-cytoskeleton linker molecules, in which the N-ERMAD domain binds to the cytoplasmic domains of transmembrane receptors, while the C-terminal, C-ERMAD binds filamentous actin (5, 17). Integrin activation by N-terminal fragments of talin Re-expressing recombinant talin head domain fragments. The region of ERM homology (9) is indicated. Beads coated with recombinant βA, βD(Y/A), βB, or βD(Y/A) cytoplasmic tails were incubated with 30 μg of purified GST-talin head domain (talin-(1–435)) (B) or 7 μg of purified GST-talin-(280–435), GST-talin-(186–435), and GST-talin-(1–280) (C). Bound protein was eluted by heating in SDS-sample buffer and samples separated on 4–20% SDS-polyacrylamide gels under reducing conditions. Detection was by Western blotting with an anti-GST mAb. Starting material used for the assays is shown.

Integrin Activation by N-terminal Fragments of Talin Requires the Head Domain—Increases in integrin affinity for ligands (“activation”) can be influenced by cytoskeletal linkages (4, 18–20). We reasoned that if the talin head domain bound to integrin cytoplasmic tails, then overexpression of talin fragments containing this domain might alter integrin affinity. We, therefore, expressed N-terminal fragments of talin (Fig. 4A) and assessed the activation state of αβIβIIa by measuring binding of the activation-specific mAb PAC1 (12). CHO cells expressing recombinant human integrin αβIβIIa were co-transfected with talin-(1–1071) cDNA along with Tac α5 as a marker of transfection. Cells expressing high levels of the transfection marker (FL-2) exhibited increased PAC1 binding (FL-1), resulting in a rightward tilt in the density plot (Fig. 4B). This effect was not seen if the expressed talin fragment lacked the head domain (talin-(434–1071)) or with vector DNA (Fig. 4, A and B). Talin-(1–1071)-induced PAC1 binding was inhibited by the αβIIa antagonist, Ro43-5054, demonstrating the specificity of binding (data not shown). Expression of talin-(1–1071) resulted in a 2.7-fold increase in mean activation index (from 0.21 to 0.56), while a fragment lacking the head domain, talin-(434–1071) (C), had little effect (activation index: 0.18) (Fig. 4D). These fragments had no obvious effect on cell shape (data not shown), consistent with previous results obtained by microinjection of recombinant talin-(102–497) (5). Expression of the talin fragments was confirmed by Western blotting with mAb 8d4, which recognizes endogenous hamster talin and both recombinant mouse talin fragments (Fig. 4C). Densitometry of the blot shown in Fig. 4C revealed that the intensities of signal from talin-(1–1071) and talin-(434–1071) were 0.66 and 1.36 times, respectively, the intensity of the endogenous talin band. However, these fragments are only expressed in the transfected cells. Thus, we calculated that on a per cell basis talin-(1–1071) was 2-fold and talin-(434–1071) was 6-fold overexpressed with respect to endogenous talin, based on transfection efficiencies of 29 and 22%, respectively. We were unable to test whether overexpression of intact talin or isolated head domain results in similar integrin activation as we detected no increase in their expression following transient transfection with cDNAs encoding them in either pJ6 R or pcDNA3.1 expression vectors.
The Talin Head Domain Binds to Integrin \( \beta \) Tails

To determine whether the \( \alpha_{\text{IIb}} \beta_3 \) activation by talin-(1–1071) required the talin-binding \( \beta_3 \) cytoplasmic tail, we tested CHO cells expressing \( \alpha_{\text{IIb}} \beta_3 \Delta 728 \), which lacks the C-terminal 35 amino acids of the \( \beta_3 \) subunit (15). Loss of the \( \beta_3 \) cytoplasmic tail results in an integrin that is deficient in cytoskeletal interactions, will not support cell spreading or initiation of focal adhesions (15). As shown in Fig. 4D expression of talin-(1–1071) did not activate \( \alpha_{\text{IIb}} \beta_3 \Delta 728 \) in CHO cells; however, \( \alpha_{\text{IIb}} \beta_3 \Delta 728 \) could be activated exogenously by addition of the activating mAb anti-LIBS6 (data not shown (12)). Consequently, expression of fragments of talin containing the head domain can increase the ligand-binding affinity of integrin \( \alpha_{\text{IIb}} \beta_3 \) when the integrin contains an intact \( \beta_3 \) cytoplasmic tail.

Following platelet activation talin is redistributed from the cytoplasm to the cytoplasmic face of the plasma membrane (21), raising the possibility that it plays a role in the regulation of adhesive functions of platelets. The data presented above implicate talin in the regulation of \( \alpha_{\text{IIb}} \beta_3 \) ligand binding affinity; however, the molecular mechanism of this regulation remains unclear. Intact talin binding to integrin \( \beta_3 \) tails via its head domain may retain the integrin in an inactive state (4), and the isolated fragment may break these tethers and so activate the integrin (19, 20). Alternatively, talin binding may alter integrin affinity through direct conformational changes or clustering the integrin. Additional studies are under way to distinguish among these potential mechanisms.

In conclusion, we have found (i) that the talin head domain contains a binding site for the cytoplasmic tails of integrins \( \beta \)A, \( \beta \)D, and \( \beta_3 \) and (ii) that expression of a talin fragment containing the head domain activates integrin \( \alpha_{\text{IIb}} \beta_3 \) in a manner dependent on the \( \beta_3 \) cytoplasmic tail. Identification of an integrin-binding site in the talin head domain is consistent with talin’s similarity to the ERM family of proteins. Thus, the talin head domain can form an integrin-binding element in a physical link between integrins and the actin cytoskeleton.

**Note Added in Proof**—The paper by Patil et al. (Patil, S., Jedsadayanmata, A., Wencel-Drake, J. D., Wang, W., Knezevic, I., and Lam, S. C. T. (1999) *J. Biol. Chem.* 274, 28575–28583) describes the interaction of the talin head domain with integrin \( \alpha_{\text{IIb}} \beta_3 \).

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**FIG. 4.** Expression of a talin fragment containing the head domain activates integrin \( \alpha_{\text{IIb}} \beta_3 \). A, a schematic representation of mouse talin. The 8d4 epitope is shown, and bars indicate the sizes of recombinant talin fragments (1–1071 and 434–1071) used in this experiment. B, CHO cells expressing integrin \( \alpha_{\text{IIb}} \beta_3 \) were co-transfected with cDNAs encoding Tac-c5 (1 \( \mu \)g) and talin-(1–1071), talin-(434–1071), or vector control (4 \( \mu \)g), as indicated. Two days after transfection cells were harvested and analyzed by two-color flow cytometry for expression of fragments of talin containing the head domain can form an integrin-binding element in a physical link between integrins and the actin cytoskeleton.