The Phosphotyrosine Binding-like Domain of Talin Activates Integrins*

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Cellular regulation of the ligand binding affinity of integrin adhesion receptors (integrin activation) depends on the integrin β cytoplasmic domains (tails). The head domain of talin binds to several integrin β tails and activates integrins. This head domain contains a predicted FERM domain composed of three subdomains (F1, F2, and F3). An integrin-activating talin fragment was predicted to contain the F2 and F3 subdomains. Both isolated subdomains bound specifically to the integrin β3 tail. However, talin F3 bound the β3 tail with a 4-fold higher affinity than talin F2. Furthermore, expression of talin F3 (but not F2) in cells led to activation of integrin α5β3. A molecular model of talin F3 indicated that it resembles a phosphotyrosine-binding (PTB) domain. PTB domains recognize peptide ligands containing β turns, often formed by NPXY motifs. NPXY(Y/F) motifs are highly conserved in integrin β tails, and mutations that disrupt this motif interfere with both integrin activation and talin binding. Thus, integrin binding to talin resembles the interactions of PTB domains with peptide ligands. These resemblances suggest that the activation of integrins requires the presence of a β turn at NPX(Y/F) motifs conserved in integrin β cytoplasmic domains.

Integrin adhesion receptors are essential for the development and survival of multicellular animals. Normal functioning of the >20 human integrins often requires dynamic cellular regulation of integrin ligand binding affinity (integrin activation). Activation of integrins is important in many biological processes, including cell migration, hemostasis, extracellular matrix assembly, tumor metastasis, and the immune response (1, 2). Integrin αβ heterodimers generally possess two short cytoplasmic domains (tails). The integrin β tail plays a central role in the activation process, probably by undergoing regulated interactions with certain cytoplasmic proteins (1, 3).

Talin is an abundant and widely expressed 250-kDa integrin β tail-binding protein implicated in integrin activation (4). Talin is composed of a 50-kDa head and 205-kDa rod domain. The head domain contains a major integrin-binding site (5–7), and expression of a 1071-residue fragment of talin containing the head domain in cells leads to activation of integrin α5β3 (5). The capacity of this fragment to activate integrin α5β3 is lost when the head domain is deleted from it or when the β3 cytoplasmic domain is truncated (5). Talin binding to integrin β tails can be regulated via calpain proteolysis (6) or through the binding of phosphoinositides (8). Furthermore, the phosphorylation of either talin and/or integrin (9, 10) could provide additional mechanisms for regulation of integrin-talin interactions. Thus, the talin head domain is implicated in integrin activation, and modulation of its binding to integrins is likely to contribute to the regulation of integrin activation.

The talin head domain contains a predicted FERM domain (band four-point-one/ermin/radixin/moesin homology domain) (11). FERM domains are found in a number of proteins and often mediate their interactions with the cytoplasmic domain of transmembrane proteins (12, 13). The crystal structures of the FERM domains from moesin, radixin, and band 4.1 (14–17) reveal a very similar overall fold. The FERM domain consists of a trefoil arrangement of three subdomains, each showing similarity to known domains. The first subdomain, F1 (using the nomenclature of Pearson et al. (17)), contains a five-stranded β sheet with an α helix running across it and is similar to ubiquitin. The F2 subdomain is entirely α helical with a short linker region and shows similarity to the acyl-CoA-binding protein. The F3 subdomain is a sandwich of two orthogonal antiparallel β sheets followed by an α helix, this fold is found in a number of structures, including the phosphotyrosine-binding (PTB) and pleckstrin homology domains. In this study, we have localized a principal integrin β tail-binding site of talin to the predicted 96-residue PTB-like subdomain within the talin FERM domain and shown that expression of this PTB-like F3 subdomain leads to integrin activation. Furthermore, like other PTB domain ligands, the capacity of the β3 tail to bind the talin PTB-like domain depends on the integrity of a β turn-forming NPXY motif. These similarities between integrin binding to talin and PTB-domain integrin-ligand interactions suggest that activation of integrins by the talin PTB-like F3 subdomain requires a stable β turn at NPX(Y/F) motifs conserved in many integrin β cytoplasmic domains.

EXPERIMENTAL PROCEDURES

Antibodies and cDNAs—Anti-talin (8d4; Sigma), anti-hemagglutinin (12CA5; American Type Culture Collection), anti-Tac (7G7B6; American Type Culture Collection), anti-Syk (4D10; Santa Cruz Biotechnology), anti-Myc (Santa Cruz Biotechnology), and anti-GST (B14; Santa Cruz Biotechnology) monoclonal antibodies and anti-Dok polyclonal antibodies were used. Recombinant GST and GST fusion proteins were expressed and purified as described (1, 5, 9). Anti-phosphotyrosine (Santa Cruz Biotechnology) monoclonal antibodies were used to detect phosphorylated proteins. Anti-Phosphotyrosine (Santa Cruz Biotechnology), anti-595 (American Type Culture Collection), anti-Myc (Santa Cruz Biotechnology), and anti-GST (B14; Santa Cruz Biotechnology) monoclonal antibodies were used to detect phosphorylated proteins. Anti-phosphotyrosine (Santa Cruz Biotechnology), anti-595 (American Type Culture Collection), anti-Myc (Santa Cruz Biotechnology), and anti-GST (B14; Santa Cruz Biotechnology) monoclonal antibodies were used to detect phosphorylated proteins.
antibody (Santa Cruz Biotechnology) were obtained commercially. The anti-α5β1, β3 monoclonal antibodies PAC1 and anti-LIBS6 and the α1α2β1-specific antagonist Ro43-5054 have been described previously (3). cDNAs encoding Tac-α5, chicken talin (186–435), mouse talin (1–1071), mouse talin-(434–1071) (residue numbers refer to the sequences in Swiss-Prot Protein Database entry TALI_MOUSE (accession number P29039)), human Syk, Syk-(1–330) (entry K5YK_HUMAN (accession number P43405)), mouse Numb (entry NUMB_MOUSE (accession number Q9ZYS)), mouse Dok (entry TrEMBL (accession number P97465)), and α1α2β1, β3, and β3(Y747A) human integrin tail model proteins have been described previously (5, 18–22). cDNAs encoding mouse talin (–435), talin-(206–305) (F22), and talin-(305–406) (F3) were amplified by PCR and subcloned into the bacterial expression vector pHGEX or the mammalian expression vector pcDNA3.1. The 5′-primer was designed to allow introduction of an N-terminal hemagglutinin tag during expression in mammalian cells. A cDNA for the mouse Dok PTB domain (amino acids 149–256; entry TrEMBL (accession number P97465)) was amplified by PCR and cloned into pCMV-Tag3 (Stratagene) to allow expression of an N-terminally c-Myc-tagged protein.

**Purification of Recombinant Proteins—**Recombinant model proteins of integrin cytoplasmic tails and GST fusion proteins were expressed and purified as previously described (5, 19). Electro spray ionization mass spectrometry revealed that integrin tail model proteins varied by <0.1% from the predicted molecular mass for the non-phosphorylated proteins (19). For surface plasmon resonance (SPR) experiments, GST was removed by addition of thrombin (~1 unit/mg of protein) and mixing overnight at room temperature. p-Aminobenzamidine beads (Sigma) and glutathione-Sepharose 4B beads (Amersham Biosciences) were then added to the mixture to remove the thrombin and GST. Proteins were dialyzed in running buffer (75 mM NaCl, 1.3 mM Na2HPO4, and 2.2 mM NaH2PO4, pH 7.4).

**Affinity Chromatography with Recombinant Integrin Tails—**Affinity chromatography was performed using recombinant integrin tails bound to His-Bind resin (Novagen) as previously described (5, 6, 19). 5 μl of coated beads and 1 μg of purified GST fusion protein were routinely used. Bound proteins were fractionated by SDS-PAGE and analyzed by Western blotting.

**SPR—**SPR analysis was performed as previously described using a Biacore 3000 instrument (6). Briefly, sufficient streptavidin to generate an increase of ~500 resonance units was coupled to activated CM5 sensor chips (6). Recombinant β1 tails were biotinylated on a unique cysteine residue and immobilized on sensor chips via a biotin-avidin linkage (6). Tails were immobilized at a level previously determined to minimize mass transport artifacts. The binding of a range of concentrations of purified recombinant talin subdomains to the β1-coated chip was then analyzed. Analyte was injected using the KINJECT command specifying a 90 s association phase and a 40 s dissociation phase. Following completion of KINJECT, dissociation was followed for at least another 60 s. The use of KINJECT delays removal of the injection needle and so minimizes instrument noise at the start of the dissociation phase; however, a change in resonance units can be detected when the needle is removed following completion of the KINJECT dissociation phase 130 s after the start of the injection. The chip surfaces were regenerated with 2 × NaCl between experiments. All parameters were measured at a flow rate of 20 μl/min in running buffer.

**Data Analysis—**Sensorgrams were analyzed, and rate constants were calculated as previously described (6). The kinetic data were interpreted in the context of a first-order kinetic model: A + B ↔ AB (23–25). For such a model, the association (k_on) and dissociation (k_off) rate constants are described by Equation 1.

\[
dAB/dt = k_{on}AB - k_{off}AB \tag{Eq. 1}\]

Equation 1 can be expressed with the parameters used in SPR analysis as follows (Equation 2).

\[
dR/dt = k_{on}C \cdot R_{max} - (k_{off}C + k_{off}R)R \tag{Eq. 2}\]

where R is the response in resonance units at time t, R_max is the maximum response at saturating concentrations of analyte, and C is the concentration of the analyte solution. Hence, the gradient of the dR/dt versus R plot, −k_off, is described by Equation 3.

\[
k_{off} = k_{off}C + k_{off} \tag{Eq. 3}\]

The dissociation rate constant value obtained from Equation 3 may not be reliable for low ranges (26); therefore, k_off values were calculated from the dissociation phases of the sensorgrams using Equation 4.

\[
\ln(R/R_0) = - k_{off}(t - t_0) \tag{Eq. 4}\]

where R_0 represents the response in resonance units at the start of dissociation, t_0. The apparent equilibrium dissociation constant, K_off, was then determined from the ratio of these two kinetic constants (k_on/k_off). K_off was also calculated from Scatchard analysis of equilibrium binding using Equation 5.

\[
R_{ex}/C = R_{max}/K_{off} - R_{ex}/K_{off} \tag{Eq. 5}\]

where R_ex is the response in resonance units at equilibrium.

**FACS Analysis of the Activation State of α1α2β1—**Chinese hamster ovary (CHO) cells expressing integrin α1β1 (3) were transiently transfected with cDNAs encoding Tac-α5 (1 μg) and talin fragments (3 μg) using LipofectAMINE Plus (Invitrogen). 24 h later, three-color flow cytometry was performed as described previously (3, 5). Briefly, cells were suspended and stained with PAC1 and then washed and stained with biotinylated anti-Tac monoclonal antibody PAC1 to transfected cells (stained with anti-Tac antibody TGB7B6) was measured, and activation indices were calculated. Results represent mean increase in activation index ± S.E. (n = 6), *p < 0.001 (Student’s t test).
RESULTS

A 250-Amino Acid Fragment of the Talin Head Domain (Talin-(186–435)) Binds the β3 Tail and Activates Integrin αIIbβ3—A talin fragment that contains the head domain (talin-(1–1071)), but not one that lacks the head domain (talin-(434–1071)), can activate αIIbβ3 integrins (5). To determine whether a portion of the talin head domain was sufficient for these functions, we analyzed a fragment of the talin head containing Glu^{186}–Gln^{435} that binds to the integrin β3D tail (5). Talin-(186–435) bound to the β3 (but not αIIb) tail (Fig. 1A), and binding was inhibited by a Tyr-to-Ala mutation in the first NPXY motif, β3(Tyr747Ala) (Fig. 1A). This integrin-binding fragment of the head domain was sufficient to activate αIIbβ3 integrins in CHO cells (Fig. 1B). The magnitude of activation was comparable to that observed for the previously described talin-(1–1071) fragment (5) (mean increase in activation index = 76%; p = 0.001) versus 65% (p = 0.0003). Furthermore, a fragment of talin that lacks this region (talin-(434–1071)) had no effect on the activation state of αIIbβ3 (mean change in activation index = 3%; p > 0.7) (Fig. 1B). The expression of talin-(186–435), talin-(1–1071), and talin-(434–1071) was confirmed in immunoblots of the transfected cells (data not shown). Thus, expression of a 250-amino acid β3 tail-binding fragment of the talin head domain is sufficient to induce αIIbβ3 activation.

The Integrin-binding Fragment of the Talin Head Domain Contains the F2 and F3 Subdomains of the Predicted Talin FERM Domain—As described above, we have localized an integrin-binding and -activating fragment of the talin head to residues 186–435. This fragment contains part of a predicted FERM domain (11). FERM domains are found in a large number of proteins; however, the overall sequence identity is often low (12, 13). BLAST analysis of the talin head domain revealed highly significant similarity to consensus FERM domain sequences (Conserved Domain Database sequences CD: pfam00373 and CD:smart-00295) generated using the Pfam and SMART programs (score 243, E = 2 × 10^{-6}; and score 153, E = 2 × 10^{-38}, respectively). Individual BLAST comparison of the talin head domain with the sequences of crystallized moesin, radixin, and band 4.1 FERM domains demonstrated 25, 25, and 20% identities and 46, 47, and 37% similarities, respectively. These similarities establish significant (E = 4 × 10^{-10}, 4 × 10^{-10}, and 3 × 10^{-6}, respectively) homology of this region of talin to these FERM domains. FERM domains are composed of three subdomains (F1, F2, and F3) (14–17). To identify the boundaries of the subdomains within the talin FERM domain, we aligned the talin head domain sequence with other FERM domains (Fig. 2). Superposition of the moesin secondary and tertiary structural elements (17) allowed us to predict that the mouse talin F2 subdomain extends from Ser^{206} to Leu^{305} and the F3 subdomain from Gly^{309} to Ser^{405} (numbered according to Swiss Protein Database entry TALI_MOUSE (accession number P26039)).
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A.

Talin F2

Talin F3

B.

Talin F2

Talin F3

Fig. 3. Talin F2 and F3 subdomains bind integrin β tails. A, beads coated with recombinant β2, β3(Y747A), or α1ab tail model proteins were incubated overnight with 1 μg of recombinant GST-talin F2 or F3. Bound proteins were fractionated by SDS-PAGE, and recombinant talin proteins were detected by Western blotting with anti-GST antibodies. Loading of the model proteins onto the beads was assessed by Coomassie Blue staining. Starting material represents 5% of the input material. B, the binding of talin subdomains to β3 tails was analyzed by SPR. Sensorgrams of the association and dissociation phases for talin F2 and F3 binding to β3 tails are shown. Talin subdomains at 50, 75, 125 and 200 nM were injected over a surface coated with ~480 resonance units (RU) of biotin-maleimide-modified β3 tail for 90 s at a flow rate of 20 μl/min. Similar results were obtained in three independent experiments.

and F3 subdomains. Therefore, three sequence alignments predicted identical boundaries for the talin F2 and F3 subdomains and indicated that talin-(186–435) contains all of the F2 and F3 subdomains, but only the last predicted β strand of the F1 subdomain. This suggested that the F2 and/or F3 subdomain might be responsible for β tail binding and integrin activation.

The Isolated Talin F3 Subdomain Binds the β3 Cytoplasmic Tail with High Affinity—As described above, a talin fragment containing both predicted F2 and F3 subdomains bound integrin β cytoplasmic domains. We therefore prepared individual F2 (talin-(206–305)) and F3 (talin-(309–405)) subdomains. Affinity chromatography revealed that both F2 and F3 subdomains bound the β3 tail (Fig. 3A). As observed for larger talin fragments, both F2 and F3 exhibited greatly reduced binding to the β3(Y747A) tail and almost undetectable binding to the α1ab tail (Fig. 3A). Both talin F2 and F3 subdomains also bound to the β3A and β3D integrin cytoplasmic tails, and binding was reduced by Tyr-to-Ala mutations in the first NPXY motif of these tails (data not shown). The binding was not due to a nonspecific interaction with the β tail, as purified GST did not bind (data not shown). Furthermore, removal of the GST moiety by thrombin digestion did not impair the ability of either the F2 or F3 subdomain to bind to β3 tails (see below). Thus, both predicted F2 and F3 subdomains of the talin FERM domain bound specifically to the β3 tail.

We examined the real-time binding of each of these subdomains to the β3 tail by SPR. SPR analysis was performed using biotinylated β3 tail model proteins immobilized on a streptavidin-coated sensor chip (coupling level of 500 resonance units), and the data were analyzed in the context of a first-order kinetic model (A + B = AB) (6). These analyses revealed typical association and dissociation phases for both talin F2 and F3 subdomains (Fig. 3B). Under similar conditions, no binding of talin-(1–280) or talin-(1–186) was detected, consistent with their lack of interaction in affinity chromatography experiments (5) (data not shown). Furthermore, as previously observed for the talin head domain (6), neither the talin F2 nor F3 subdomain bound to streptavidin-coated chips lacking immobilized β3 ligand (data not shown). Data were analyzed using Equations 1–5, defined under “Experimental Procedures.” The association rate constant \( k_{on} \) was obtained from Equation 3 (Fig. 4B) using the association phase of the curve, and the dissociation rate constant \( k_{off} \) was obtained from the dissociation phases of the sensorgrams using Equation 4 (23, 25). Using this method, rather than BIAevaluation software, the \( r^2 \) values reported in Fig. 4 provide a measure of the goodness of fit (25).

As recommended by the manufacturer (BIAcore, Uppsala, Sweden), data collected 10 s after injection start or stop were excluded from the analysis to avoid sample dispersion effects. At the ligand coupling level used, \( k_{on} \) values (calculated from Equation 4) were independent of the concentration of analyte (talin F2 or F3 subdomain) used (Fig. 4A). This indicates negligible rebinding of analyte during the dissociation phase. Plots of \( k_{on} \) versus analyte concentration (Fig. 4B) were also linear.
This is consistent with a simple first-order interaction and facilitates calculation of $k_{on}$ from Equation 3. These analyses allowed us to calculate that talin F3 bound the $\beta_3$ tail with a 4-fold higher affinity than talin F2 ($K_d = 130 \pm 10$ and $540 \pm 40$ nM, respectively), primarily due to a markedly reduced on-rate for talin F2 ($((3.9 \pm 0.2) \times 10^3$ M$^{-1}$ s$^{-1}$) relative to F3 $((3.3 \pm 0.1) \times 10^4$ M$^{-1}$ s$^{-1}$) (Table I). The dissociation constants ($K_d$) calculated using kinetic parameters are in good agreement with those calculated from equilibrium binding Scatchard plots using Equation 5. Kinetic constants represent mean ± S.D.

|        | $K_{on}$ (s$^{-1}$) | $K_{off}$ (s$^{-1}$) | $K_d$ (nM) | $K_d$ (Equilibrium) |
|--------|---------------------|---------------------|------------|---------------------|
| Talin F2 | $(3.9 \pm 0.2) \times 10^2$ | $(2.1 \pm 0.1) \times 10^{-3}$ | 540 ± 40 | 410 |
| Talin F3 | $(3.3 \pm 0.1) \times 10^4$ | $(4.2 \pm 0.2) \times 10^{-3}$ | 130 ± 10 | 100 |

This is consistent with a simple first-order interaction and facilitates calculation of $k_{on}$ from Equation 3. These analyses allowed us to calculate that talin F3 bound the $\beta_3$ tail with a 4-fold higher affinity than talin F2 ($K_d = 130 \pm 10$ and $540 \pm 40$ nM, respectively), primarily due to a markedly reduced on-rate for talin F2 ($((3.9 \pm 0.2) \times 10^3$ M$^{-1}$ s$^{-1}$) relative to F3 $((3.3 \pm 0.1) \times 10^4$ M$^{-1}$ s$^{-1}$) (Table I). The dissociation constants ($K_d$) calculated using kinetic parameters are in good agreement with those calculated from equilibrium binding Scatchard plots using Equation 5 (Fig. 4C and Table I) and support a simple first-order interaction. The presence of the F2 subdomain does not notably enhance or inhibit talin binding to integrin $\beta_3$ tails, as constructs containing the F2 and F3 subdomains retain the ability to bind $\beta_3$ tails (Fig. 1A), and the isolated talin F3 subdomain binds the $\beta_3$ tail with an affinity ($K_d = 130 \pm 10$ nM) that is similar to that of the intact (F2 and F3 subdomain-containing) talin head domain ($K_d = 91$ nM) (6). Although the talin rod domain contains additional integrin $\beta$ tail-binding sites (5, 30), the head domain binds with a 37-fold higher affinity than the rod domain (6), and the F3 subdomain binds with an affinity similar to that of the intact head domain. Therefore, the talin F3 subdomain contains a major integrin $\beta_3$ tail-binding site.

The Talin F3 Subdomain Activates Integrins—As described above, we narrowed an integrin-binding and -activating site in the talin head to a fragment containing the F2 and F3 subdomains and found that both subdomains can bind to the $\beta_3$ tail. We therefore tested the ability of each subdomain to activate this integrin. To do this, we transfected wild-type $\beta_3$-expressing CHO cells with cDNAs encoding hemagglutinin-tagged talin F2 or F3 and measured binding of the activation-specific antibody PAC1 by flow cytometry. Cells transfected with talin F3 (as identified by surface expression of cotransfected Tac-α5) exhibited increased PAC1 binding, resulting in a rightward tilt of the
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Expression of the talin F3 subdomain leads to activation of αIIbβ3. CHO cells expressing αIIbβ3 were transiently transfected with cDNA (1 µg) encoding Tac-α5 along with cDNA (3 µg) encoding talin F2 or F3, talin-(1–1071), talin-(434–1071), or empty vector. The binding of PAC1 (activation-specific anti-αIIbβ3 monoclonal antibody) and 7G7B6 (to identify transfected cells) was then assessed by FACS analysis. A, shown are contour plots of cells stably expressing αIIbβ3 transfected with the indicated cDNAs in the absence or presence of the αIIbβ3 antagonist Ro43-5054. B, the activation indices of transfected cells were calculated as described under "Experimental Procedures," and the percentage change from cells transfected with empty vector is plotted (mean ± S.E., n = 9). *, p < 0.005 (Student’s t test). C, the expression of recombinant talin subdomains in transfected CHO cells was examined by immunoblotting of SDS-PAGE-fractionated cell lysates (7 µg). HA, hemagglutinin.

PAC1 binding could be completely inhibited by the αIIbβ3 antagonist Ro43-5054 (Fig. 5A and Table II). Calculation of the activation index of transfected cells indicated that expression of talin F3 resulted in αIIbβ3 activation (Fig. 5B). However, transfection of talin F2 resulted in PAC1 binding similar to that

contour plot (Fig. 5A). A similar effect was observed for cells transfected with talin-(1–1071) (Fig. 5A). However, cells expressing talin F2 displayed a contour plot similar to that of cells transfected with empty vector or the non-activating talin-(434–1071) fragment (Fig. 5A). In all cases, the increased PAC1 binding could be completely inhibited by the αIIbβ3 antagonist Ro43-5054 (Fig. 5A and Table II). Calculation of the activation index of transfected cells indicated that expression of talin F3 resulted in αIIbβ3 activation (Fig. 5B). However, transfection of talin F2 resulted in PAC1 binding similar to that
observed in cells transfected with empty vector or the non-activating talin-(434–1071) fragment (Fig. 5B and Table II). In all cases, the talin fragments were well expressed (Fig. 5C).

Mutations in an NPXY/F motif that is conserved in most integrin β tails (31) inhibit integrin activation (3, 32). Such a mutation (β3(Y747A)) inhibits the binding of talin F3 (Fig. 3A) to integrin β tails and also inhibits the binding of intact talin (5). We therefore assessed the effect of this mutation on the capacity of talin F3 to activate integrin αIIbβ3. Integrin αIIbβ3 containing this mutation (αIIbβ3(Y747A)) was not activated by expression of talin F3 (Fig. 6 and Table II), although PAC1 binding could be induced by addition of the activating antibody anti-LIBS6 (Fig. 6A). Therefore, expression of the 96-amino acid F3 subdomain of talin (but not the F2 subdomain) activates integrin αIIbβ3, and this activation is inhibited by a mutation that disrupts the NPXY motif in the β3 cytoplasmic tail.

**Talin F3 Is a PTB-like Domain**—The sequence similarity of talin to FERM proteins of known structure (see above) is sufficient to build a reliable three-dimensional homology model of the F3 subdomain of talin. The best model, as judged by the program WHAT IF (29), was obtained using the crystal structure of moesin (15, 17) as template (Fig. 7). The model has good structure-packing quality (Z-score of −2.34) and distribution of hydrophobic residues to the interior/hydrophilic groups to the exterior of the folded domain (root mean square Z-score of 0.96), indicating that the PTB fold is compatible with the sequence of the F3 subdomain of talin. In the predicted structure, Val155 Leu190 Ile192 Val197 Val200 Ile210 Tyr201 Leu203 Ile206 Phe210 Val220 Phe224 Tyr226, and Val228 constitute a hydrophobic core enclosed by the packing of the two orthogonal β sheets, and the C-terminal amphiphilic α helix packs with the apolar side toward the β sandwich. This hydrophobic core is characteristic of the PTB/pleckstrin homology fold and contributes to its stability (33). The predicted PTB domain of talin lacks the long insertion between the β1 and β2 strands observed in the PTB domains of Shc (34), X11 (35), and Numb (36) and structurally is closer to the PTB domain of insulin receptor substrate-1 (IRS-1) (37) (Fig. 2D). C-α atoms of residues in the core of the domain superimpose with a root mean square deviation of 1.1 Å on the IRS-1 PTB domain structure. The PTB domain of IRS-1 binds ligands containing phosphorylated tyrosine in the NPXY motif; interaction with the phosphate group involves basic residues in the β1–β2 and β2–β3 loops. The PTB domain of talin does not contain basic residues in the equivalent phosphate-binding loops. Thus, talin appears to lack the structural requirements to recognize the phosphorylated form of NPXY motifs. Therefore, the talin F3 subdomain binds integrin β tails, activates integrins, and is predicted to be a PTB-like domain.

**Activation of Integrins Is Not a General Property of β3 Tail-binding Proteins or PTB Domains**—Cellular expression of the β3-tail-binding talin F3 subdomain leads to integrin activation. This is not a general effect of β3-tail-binding proteins because talin F2, which also binds the β3 tail, does not result in activation. However, talin F2 binds β3 with a lower affinity than talin F3. Therefore, we tested whether expression of higher affinity β3 tail-binding proteins resulted in activation of αIIbβ3. The non-receptor tyrosine kinase Syk binds to the β3 tail via an N-terminal fragment containing the tandem SH2 (Src homology) domains (Syk-(1–330)). This fragment binds to the β3 tail with an ~5-fold higher affinity (Kv = 24 nM) than the talin F3 subdomain (20). When we transfected cells with either full-length Syk or its N-terminal integrin-binding fragment (Syk-(1–330)), there was no change in integrin activation (Fig. 8A). Expression of the transfected proteins was verified by Western blotting (Fig. 8C). Furthermore, because talin F3 is predicted to adopt a PTB-like fold, we also tested whether expression of other PTB domains resulted in integrin activation. Expression of the PTB domain-containing proteins Numb (22) and Dok (21) and the isolated Dok PTB domain did not significantly alter activation (Fig. 8B). Hence, activation of αIIbβ3 is neither a general property of β3 tail-binding proteins nor of PTB domains.

**DISCUSSION**

Alteration of integrin binding affinity in response to intracellular signals (activation) is an important control mechanism for several integrin functions. Here, we report that a predicted PTB-like 96-amino acid subdomain of the talin FERM domain contains a major integrin-binding site and activates integrin αIIbβ3. PTB domains often recognize peptide sequences containing NPXY motifs (35). Mutations within an NPXY/F sequence that is conserved in most integrin β cytoplasmic domains (31) inhibit talin interaction (5, 19, 39) and integrin activation (3, 32). The conservation of the NPXY motif, its role in talin binding, and its importance for integrin activation suggest that activation by the PTB-like domain of talin is a general property of talin-binding integrins and may require the presence of a β turn formed by the NPXY motif.

The talin head domain is predicted to contain a PTB-like subdomain within a putative FERM domain. FERM domains are found in a wide range of proteins, and the degree of sequence identity between FERM domains is often low (12). However, whereas the band 4.1 FERM domain exhibits only 30% sequence identity and 50% similarity to the moesin and radixin

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**Table II**

| Cells expressing | Transfected with | PAC1 binding (MFI) | % Change in activation index |
|-----------------|-----------------|---------------------|-----------------------------|
| αIIbβ3          | Talin F3               | 14.2 ± 0.7          | 56.3 ± 15.8 (n = 9)          |
| αIIbβ3          | Talin F2               | 8.6 ± 0.3           | −11.0 ± 10.9 (n = 9)        |
| αIIbβ3(Y747A)   | Talin-(4–1071)         | 15.9 ± 0.7          | 57.6 ± 18.9 (n = 9)         |
| αIIbβ3(Y747A)   | Talin-(4–1071)         | 9.3 ± 0.5           | −5.6 ± 10.0 (n = 9)         |
| αIIbβ3          | Empty vector           | 8.6 ± 0.3           | (n = 9)                     |
| αIIbβ3(Y747A)   | Talin F3               | 4.2 ± 0.4           | −4.5 ± 6.2 (n = 6)          |
| αIIbβ3(Y747A)   | Talin F2               | 5.5 ± 0.7           | 8.9 ± 16.7 (n = 6)          |
| αIIbβ3(Y747A)   | Empty vector           | 5.2 ± 0.7           | (n = 6)                     |

* Significantly different from empty vector (Student’s t test, p < 10^-7).  
* Significant change in activation index (Student’s t test, p < 0.005).
FERM domains, it is structurally very similar (root mean square deviation Cα superposition of ~2.0 Å) (15). The presence of a FERM domain within talin has been proposed on the basis of primary sequence alignments performed using several different programs (11–13, 16), and comparison of the talin FERM domain with moesin or radixin reveals only slightly lower levels of identity and similarity (23% identity and 46% similarity, respectively) than seen for the band 4.1 moesin/radixin comparisons. Pearson et al. (17) identified six buried FERM residues that are highly conserved in FERM domains. Five of these six residues are identical in talin, whereas the one non-identical residue is a valine in place of a conserved glycine (Fig. 2). Together, these data suggest that the FERM fold is conserved in talin. FERM domains are composed of three subdomains (F1, F2, and F3), and the F3 subdomains adopt a PTB-like fold (14–17). Modeling the putative talin F3 subdo-

Fig. 6. Mutation of the β3 NPXY motif blocks αIβ3 activation. CHO cells expressing αIβ3(Y747A) were transiently transfected with cDNA (1 μg) encoding Tac-α5 along with cDNA (3 μg) encoding talin F2 or F3 or empty vector and analyzed as described in the legend to Fig. 5. A, shown are contour plots of cells transfected with the indicated cDNAs in the presence or absence of the αIβ3-activating antibody anti-LIBS6. B, the activation indices of transfected cells were calculated, and the percentage change from cells transfected with empty vector is plotted (mean ± S.E., n = 6). C, the expression of recombinant talin subdomains in transfected CHO cells was examined by immunoblotting of SDS-PAGE-fractionated cell lysates (7 μg). HA, hemagglutinin.
main by homology to the moesin structure indicates that the resulting fold has good structure packing quality and is similar to the IRS-1 PTB domain.

Loss-of-function mutations in the F3 module of known FERM domains are predicted to be within the hydrophobic core of the protein and are therefore likely to disrupt folding (15). Furthermore, known mutations that disrupt PTB domain-ligand interactions do not fall within residues conserved in talin (35–37). Thus, the identification of surface-exposed residues in talin that are involved in binding to integrins will require a structural analysis of the complex of the talin PTB domain with an integrin β tail. In this study, we have identified a 96-residue module of talin that binds and activates integrin αIIbβ3, whereas the previous minimal integrin-activating fragment of talin was a 1071-amino acid fragment of undefined structure. The structure and dynamics of the β3 cytoplasmic domain have been analyzed by NMR spectroscopy of model protein mimics of the β3 tail (40). The identification of the F3 subdomain as the fragment responsible for activation will enable an NMR analysis of a protein-protein interaction involved in integrin activation.

Our data suggest that the interaction of integrin β cytoplasmic domains with talin resembles the binding of a PTB domain to a peptide ligand in several ways. PTB domain-binding sequences, including NPXY motifs, form β turns when bound to a PTB domain (38). Consistent with such an interaction, the integrin β3 NPXY motif has the propensity to form a β turn (40), and this propensity is lost as a result of mutations of the NPXY motif that block both talin binding and integrin activation. Additional PTB domain-ligand interactions are provided by the amino acid sequence N-terminal to the β turn of the recognition motif (38). Consistent with such an interaction, minimal talin-binding peptides contain the conserved first NPXY motif of integrin β3A along with the preceding five amino acids (10, 39), providing additional similarities between the peptide binding properties of talin and authentic PTB domains. The integrin β3 tail does not adopt a stable β turn conformation in solution (40). However, its propensity to form a turn and its binding to the PTB-like F3 subdomain of talin suggest that a stable turn will be present when talin binds to integrins. We report here that the talin PTB-like F3 subdomain activates integrins, whereas certain other β tail-binding proteins do not. Furthermore, mutations that disfavor formation of a stable β turn block activation (3, 40). Hence, the integrin β tail-talin interaction bears many of the characteristics of PTB domain-ligand interactions, and the ability of the talin PTB-like F3 subdomain to activate integrins may require a stable β turn at the conserved NPXY/F motif.

This study demonstrates NPXY-dependent ligand binding to the PTB-like FERM subdomain. Previous structural analyses have suggested that NPXY-dependent interactions of FERM domains could occur (17); however, this study represents the first experimental documentation of this principle (16). There are now 261 proteins in the non-redundant data base predicted to contain FERM domains (13). Thus, our studies may suggest
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A more general model for other FERM domain interactions. They may also suggest a facile means for identification of potential binding partners for FERM domains by the presence of β turn-forming NPXY and related (38) sequences.

PTB domains were initially characterized as domains that bind to phosphorylated tyrosines in the context of NPXY motifs (38). However, their binding is often independent of phosphorylation of the phosphorylated NPXY(36, 41, 42). The binding of talin or its PTB-like F3 domain to phosphorylated tyrosines may inhibit talin binding (10). Furthermore, in our model of the talin PTB subdomain, basic donor groups that would coordinate the phosphate moiety are absent, suggesting that binding of the phosphorylated NPXY motif is energetically disfavored. Integrins are closely associated with talin at focal adhesions, stable attachments formed in relatively non-migratory cells. Tyrosine phosphorylation of the β tail leads to displacement of the integrin from these talin-rich structures (43).

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