Structural Basis for the Autoinhibition of Talin in Regulating Integrin Activation

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SUMMARY

Activation of heterodimeric (α/β) integrin transmembrane receptors by the 270 kDa cytoskeletal protein talin is essential for many important cell adhesive and physiological responses. A key step in this process involves interaction of phosphotyrosine-binding (PTB) domain in the N-terminal head of talin (talin-H) with integrin β membrane-proximal cytoplasmic tails (β-MP-CTs). Compared to talin-H, intact talin exhibits low potency in inducing integrin activation. Using NMR spectroscopy, we show that the large C-terminal rod domain of talin (talin-R) interacts with talin-H and allosterically restrains talin in a closed conformation. We further demonstrate that talin-R specifically masks a region in talin-PTB where integrin β-MP-CT binds and competes with it for binding to talin-PTB. The inhibitory interaction is disrupted by a constitutively activating mutation (M319A) or by phosphatidylinositol 4,5-bisphosphate, a known talin activator. These data define a distinct autoinhibition mechanism for talin and suggest how it controls integrin activation and cell adhesion.

INTRODUCTION

Talin is a high molecular weight protein containing an N-terminal head (1–433, talin-H, 50 kDa) and a C-terminal rod domain (434–2541, talin-R, 220 kDa) (Figure 1A) (Rees et al., 1990). Talin-H is globular, containing a FERM domain composed of three lobes, F1, F2, and F3 or phosphotyrosine-binding (PTB) domain (Garciá-Alvarez et al., 2003); talin-R is highly elongated, containing a series of helical bundles separated by linkers (McLachlan et al., 1994; Papagrigoriou et al., 2004; Fillingham et al., 2005; Gingrich et al., 2008). Discovered in high concentrations at regions of cell-substratum contact (Burridge and Connell, 1983), talin has long been known to be a physical linker between integrins and the actin cytoskeleton, and a regulator of a variety of cellular processes such as cell spreading, migration, and prolif-
leads to constitutive activation of full-length talin. Phosphatidylinositol 4,5-bisphosphate (PIP2), a known talin activator (Martel et al., 2001), disrupts the inhibitory talin-PTB/talin-R interaction. These results define a structural mechanism of the talin autoinhibition and suggest how it may serve as a specific cellular brake to control integrin activation.

**RESULTS**

Talin Adopts a Default Low-Activity State for Regulating Integrin Activation

It is well-established that talin-H can activate integrins (Calderwood et al., 1999, 2002; Tadokoro et al., 2003; Kim et al., 2004; Fillingham et al., 2005; Gingras et al., 2008) and it is presumed that this activity is blunted in full-length talin. To directly compare the integrin-activating activity of these two talin forms, we transfected vectors expressing full-length talin or talin-H, all as EGFP constructs, into a CHO cell line stably expressing integrin αIIbβ3-CHO. The activation state of the αIIbβ3 was then measured by the binding of an activation-specific mAb to αIIbβ3 antibody (PAC1) by FACS. To eliminate the influence of varied expression of talin-H and full-length talin, only EGFP-positive cells with similar expression levels were gated. The data in Figure 1B are from three independent experiments and show that, while talin-H induced substantial activation of αIIbβ3 activation, full-length talin was significantly weaker as an activator. Thus, our data provide direct functional evidence for the supposition that integrin activation by full-length talin is dampened and is enhanced by events that change exposure of its head region.

A Middle Segment of Talin-R, 1654–2344, Interacts with Talin-PTB in Talin-FERM

Since talin-PTB (F3) in talin-H is solely responsible for binding to integrin β3 CTs during integrin activation (Calderwood et al., 2002; Garcia-Alvarez et al., 2003; Tadokoro et al., 2003; Wegener et al., 2007), we reasoned that talin-R might mask the integrin β3 binding site. To test this hypothesis, we used NMR-based 2D $^{1}H$-$^{15}N$ HSQC (heteronuclear single quantum correlation) experiments to examine the interaction between $^{15}N$-labeled talin-PTB and unlabeled talin-R fragments. 2D $^{1}H$-$^{15}N$ HSQC is extremely sensitive for probing protein-target interactions with a wide range of affinities (Bonvin et al., 2005; Vaynberg and Qin, 2006; Takeuchi and Wagner, 2006). The HSQC spectrum of an $^{15}N$-labeled protein contains many peaks; each peak correlates to a proton attached to $^{15}N$ within a particular residue in the protein. Some peaks may be shifted or broadened in the HSQC spectrum if the protein is bound to a target, an excellent indication of the binding interface. The peak broadening or disappearance can be due to the size increase or intermediate rate exchange of the protein complex at the NMR timescale. If any of the unlabeled talin-R fragments bind to $^{15}N$-labeled talin-PTB, some or all $^{1}H$-$^{15}N$ amide signals of talin-PTB should be perturbed and broadened, which in turn provides information on the intramolecular interaction between talin-PTB and talin-R. Based on the available helical bundle structure of talin-R (Papagrigoriou et al., 2004; Fillingham et al., 2005; Gingras et al., 2008) and a secondary structure prediction program (Bryson et al., 2005), we dissected talin-R into nine consecutive fragments (R1, 434–947; R2, 944–1483; R3, 1482–1653; R4, 1654–1848; R5, 1841–1983; R6, 1984–2102; R7, 2103–2229; R8, 2225–2344; and R9, 2338–2541), where the division regions were predicted to be random coil or loop structures so the structural integrities of these fragments should be preserved. A series of HSQC spectra were collected for $^{15}N$-labeled talin F2F3 domain, which contains talin-PTB (F3), in the absence and presence of individual unlabeled talin-R fragments. Starting from the N terminus of talin-R, we found that R1, R2, and R3 had little effect on the HSQC spectrum of talin-F2F3 (see Figures S1A–S1C, available online), whereas R4 (~21 kDa) caused significant line broadening of talin-F2F3 (~22 kDa) (Figure S1D), suggesting that R4 binds to talin-F2F3. From the C terminus, R9 did not bind (Figure S1E), but R8 caused significant line broadening of talin-F3F3, suggesting that it also interacts with talin-F2F3 (Figure S1F). These initial mapping data indicated that talin-R does interact with talin-F2F3 and the binding site involves multiple regions, R4 and R8, but not the N-terminal R1–R3 and C-terminal R9. Based on these initial data, we then prepared another larger expression construct encompassing R4 and R8, i.e., 1654–2344, with a total molecular
weight of ~76 kDa (termed talin-RM). Talin-RM was well-folded, as assessed by its chemical shift dispersion pattern (Figure S2). As predicted, talin-RM also bound to talin F2F3, as indicated by the substantial line broadening and disappearance of talin F2F3 signals in HSQC (MW~100 kDa) (data not shown). By employing a transverse relaxation optimized spectroscopy (TROSY) technique into HSQC, which is tailored for detecting NMR signals of large proteins and protein complexes (Pervushin et al., 1997), we were able to recover the majority of signals, some of which were significantly shifted due to binding (Figure 2A).

To understand the nature of this interaction, we performed backbone signal assignments of talin F2F3 using triple resonance NMR, including HNCACB, CBCACONH, HNCA, HNCO, HC(CO)NH, and C(CO)NH (Bax and Grzesiek, 1993). Chemical shift mapping revealed that only NMR signals of F3 (PTB), but not F2 in talin F2F3, were either significantly shifted or broadened (Figure 2B), thus supporting our hypothesis that talin-PTB binds to talin-RM. To improve the spectral quality and simplify the spectral analysis, we made 15N/2H-labeled talin-PTB and performed its TROSY-HSQC in complex with the unlabeled talin-RM (total complex is ~90 kDa). Both deuteration and TROSY are known to dramatically reduce the line broadening of the proteins, which led to an excellent and well-resolved spectrum of talin-PTB bound to unlabeled talin-RM (Figure S3A). As expected, talin-RM caused significant chemical shift perturbation for talin-PTB (Figure S3A). Surface plasmon resonance (SPR) experiments revealed that the dissociation constant (K_D) between talin-PTB and talin-RM is 577 nM (Table S1 and Figure S3B).

Talin-RM and Integrin Membrane-Proximal β3 CT Compete for an Overlapping Binding Site on Talin-PTB

To precisely map the talin-RM-binding site on talin-PTB, we performed a series of NMR titration experiments. TROSY-HSQC spectra were collected for 15N/2H-labeled talin-PTB in the absence and presence of increasing concentrations of talin-RM to obtain molar ratios of 1:0.0, 1:0.5, 1:1.0, and 1:2. This experimental design allowed us to trace the significantly perturbed signals (Figure S3A). Figure 3A shows the detailed chemical shift perturbation profile. Remarkably, the perturbation pattern was similar to that previously reported for integrin β3 CT or integrin membrane-proximal β3 CT segment fused to PIPKιγ peptide (Wegener et al., 2007) (Figure 3A), suggesting that the talin-RM-binding site on talin-PTB overlaps with that for integrin β3 CT. Interestingly, chemical shift mapping revealed that talin-R4 (1654–1848) also induced a very similar perturbation profile (Figure S4A versus Figure S4B), albeit with slightly reduced chemical shift changes and lower affinity (K_D ~3.6 μM, Table S1) than talin-RM (K_D ~0.58 μM, Table S1). On the other hand, a larger fragment containing C-terminal talin-RM (R6–R8, 1984–2344) induced different and very narrow-range chemical shift changes peaking around 367–375 (Figure S4C) with much lower affinity (K_D ~78.0 μM, Table S1) than talin-RM. Combining these two fragments in a single construct (talin-RM) yields higher affinity (0.58 μM, see Table S1). These data suggest that R4 and R6–R8 bind to different regions in talin-PTB and that R4 plays a more important role in binding to talin-PTB (see more data below). Figure 3B highlights significantly shifted residues in talin-PTB upon binding to talin-RM and integrin β3 CT chimera, respectively, thus providing a more direct view of how the two binding sites might overlap.

To more precisely evaluate how the integrin β3 CT-binding sites on talin-PTB may be involved in binding to talin-RM, we introduced a series of structure-based talin-PTB point mutations, L325R, W359A, S365D, S379R, and Q381V, each of which has been previously shown to impair the talin-mediated integrin activation without affecting the structural integrity of talin-PTB (Wegener et al., 2007). L325R, S365D, S379R, and Q381V disrupt the integrin β membrane-proximal CT binding to talin-PTB, whereas W359A abolishes the integrin CT binding to talin-PTB by removing the bulky interaction of the membrane-distal CT with talin-PTB (Wegener et al., 2007). We also made the M319A mutant. M319 is a surface-exposed hydrophobic residue that is not involved in interacting with either integrin β3 membrane-proximal region (Wegener et al., 2007) or membrane-distal region (García-Alvarez et al., 2003), and thus its mutation to Ala had little effect on the interaction with integrin β3 CT chimera (Figure S5). However, M319 is most significantly perturbed by talin-RM (Figure 3A), suggesting that it may play a crucial role in interacting with talin-RM. Table S1 summarizes the K_D values of talin-RM binding to WT talin-PTB, M319A, L325R, W359A, S365D, S379R, and Q381V by SPR. Compared
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Figure 3. Mapping of the Talin-RM-Binding Site on Talin-PTB
(A) 1H/15N chemical shift changes of talin-PTB upon binding to talin-RM as a function of residue number. As a comparison, the chemical shift changes of talin-PTB upon binding to β3 CT are reproduced using the chemical shift table deposited in the BioMagResBank (accession number 7150) by Wegener et al. (2007). The membrane-distal residues involved in binding to integrin β3 CT chimera are colored in red in the upper panel. Note that W359 is changed dramatically by β3 CT chimera (the change was so big, due to its bulky interaction with talin-PTB [Wegener et al., 2007, that a broken line was used to conserve space].

(B) Significantly perturbed residues on talin-PTB by β3-CT chimera (left) and talin-RM (right) are highlighted using the structure of talin-PTB. The order of dark blue, blue, and light blue indicates the extent of the chemical shift changes, with the dark blue having the most significant changes. The changes have some similarity indicating potential overlapping binding sites for β3 CT and talin-RM, but there are significant differences indicating that the binding sites are not identical.

Figure 3A shows the chemical shift changes of talin-PTB upon binding to talin-RM as a function of residue number. The chemical shift changes of talin-PTB upon binding to β3 CT are also reproduced using the chemical shift table deposited in the BioMagResBank (accession number 7150) by Wegener et al. (2007). The membrane-distal residues involved in binding to integrin β3 CT chimera are colored in red in the upper panel. Note that W359 is changed dramatically by β3 CT chimera (the change was so big, due to its bulky interaction with talin-PTB [Wegener et al., 2007, that a broken line was used to conserve space].

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NMR-based competition experiments also revealed that talin-R3 (Figure S9A), but not talin-R6-R8 (Figure S9B), competed with β3 CT chimera for binding to talin-PTB. Since talin-R4 induced very similar chemical shift perturbations as talin-RM when binding to talin-PTB (Figure S4), this finding suggests that talin-R4 plays a major role in masking the membrane-proximal CT-binding site on talin-PTB. It also further supports our prior chemical shift mapping and affinity-based results indicating that talin-R-M and integrin β3 CT are overlapping but not identical.

Interestingly, W359A, which completely abolished the talin-PTB binding to integrin β3 CT by disrupting the bulky hydrophobic interaction between talin-PTB and membrane-distal β3 CT (Garcia-Alvarez et al., 2003; Wegener et al., 2007), had little effect on the KD of the talin-PTB/talin-RM interaction (Table S1). This observation, together with the above described effects of other mutants, suggests that the binding sites on talin-PTB for talin-RM and integrin β3 CT are overlapping but not identical. To further investigate this possibility, we performed HSQC-based competition experiments. As shown in Figures S6A and S6B, while the majority of signals disappear from 15N-labeled talin-PTB upon binding to the large talin-RM (total MW ~90 kDa, KD ~577nM, see Table S1), these signals return upon addition of equal molar β3 CT chimera (β3 membrane-proximal CT fused to PIPKIγ peptide, MW ~3.5 kDa, KD ~140 nM, see Wegener et al. [2007]), yielding a spectrum identical to that for 15N-labeled talin-PTB bound to the unlabeled β3 CT chimera (Figure S6C). These data demonstrate that the small β3 CT chimera peptide competed with large talin-RM for binding to talin-PTB. In contrast, excess PIPKIγ peptide, which mimics the β3 membrane-distal CT binding and binds tightly to talin-PTB (KD ~270 nM) (de Pereda et al., 2005), did not recover the broadened signals at all (Figure S6D), indicating that PIPKIγ and talin-RM do not have overlapping binding sites on talin-PTB. Since PIPKIγ mimics the β3 membrane-distal CT for binding to talin-PTB (de Pereda et al., 2005; Kong et al., 2006; Wegener et al., 2007), our NMR data are in agreement with our SPR data on W359A mutant (Table S1) indicating that while the integrin membrane-distal CT-binding site is significantly masked by talin-RM, the β3 membrane-distal CT-binding site for talin-PTB is not. Consistently, a F730A mutant in the chimera peptide, which has dramatically reduced membrane-proximal β3 CT binding to talin-PTB (Wegener et al., 2007), did not compete effectively with talin-RM (Figure S7A versus Figure S7B). Note that the β3 CT also binds to talin-RM (Tremuth et al., 2004). However, such binding does not appear to interfere with the talin-PTB/talin-RM interaction, since β3 CT did not affect the talin-RM interaction with talin-PTB W359A (Figure S8). Note that W359A has the same affinity for talin-RM as WT talin-PTB (Table S1) but does not bind to β3 CT (Wegener et al., 2007).

to wild-type talin-PTB, S365D, S379R, and Q381V exhibited markedly reduced binding to talin-RM, whereas L325R had very small effect. As expected, while M319A still binds to integrin β3 CT as WT talin-PTB (Figure S5A versus Figure S5B), it had dramatically weakened interaction (~140-fold) with talin-RM (Table S1).

The effects of S365D, S379R, and Q381V recapitulate those for binding to the integrin membrane-proximal β3 CT and indicate that this site significantly overlaps with that for talin-RM in the talin-PTB domain. To confirm this conclusion, we prepared large quantities of two representative mutants in 15N/2H-labeled form, S365D (reduced binding to talin-RM by ~2.9 × 10^3-fold) and Q381V (reduced binding to talin-RM by ~540-fold) (Table S1) and examined their chemical shift perturbation in the absence and presence of unlabeled talin-RM. Consistent with the SPR data, the extent of the chemical shift changes was decreased for Q381V and much more decreased for S365D as compared to WT talin-PTB (Figure 4).

Interestingly, W359A, which completely abolished the talin-PTB binding to integrin β3 CT by disrupting the bulky hydrophobic interaction between talin-PTB and membrane-distal β3 CT (Garcia-Alvarez et al., 2003; Wegener et al., 2007), had little effect on the KD of the talin-PTB/talin-RM interaction (Table S1). This observation, together with the above described effects of other mutants, suggests that the binding sites on talin-PTB for talin-RM and integrin β3 CT are overlapping but not identical. To further investigate this possibility, we performed HSQC-based competition experiments. As shown in Figures S6A and S6B, while the majority of signals disappear from 15N-labeled talin-PTB upon binding to the large talin-RM (total MW ~90 kDa, KD ~577nM, see Table S1), these signals return upon addition of equal molar β3 CT chimera (β3 membrane-proximal CT fused to PIPKIγ peptide, MW ~3.5 kDa, KD ~140 nM, see Wegener et al. [2007]), yielding a spectrum identical to that for 15N-labeled talin-PTB bound to the unlabeled β3 CT chimera (Figure S6C). These data demonstrate that the small β3 CT chimera peptide competed with large talin-RM for binding to talin-PTB. In contrast, excess PIPKIγ peptide, which mimics the β3 membrane-distal CT binding and binds tightly to talin-PTB (KD ~270 nM) (de Pereda et al., 2005), did not recover the broadened signals at all (Figure S6D), indicating that PIPKIγ and talin-RM do not have overlapping binding sites on talin-PTB. Since PIPKIγ mimics the β3 membrane-distal CT for binding to talin-PTB (de Pereda et al., 2005; Kong et al., 2006; Wegener et al., 2007), our NMR data are in agreement with our SPR data on W359A mutant (Table S1) indicating that while the integrin membrane-distal CT-binding site is significantly masked by talin-RM, the β3 membrane-distal CT-binding site for talin-PTB is not. Consistently, a F730A mutant in the chimera peptide, which has dramatically reduced membrane-proximal β3 CT binding to talin-PTB (Wegener et al., 2007), did not compete effectively with talin-RM (Figure S7A versus Figure S7B). Note that the β3 CT also binds to talin-RM (Tremuth et al., 2004). However, such binding does not appear to interfere with the talin-PTB/talin-RM interaction, since β3 CT did not affect the talin-RM interaction with talin-PTB W359A (Figure S8). Note that W359A has the same affinity for talin-RM as WT talin-PTB (Table S1) but does not bind to β3 CT (Wegener et al., 2007).

NMR-based competition experiments also revealed that talin-R4 (Figure S9A), but not talin-R6-R8 (Figure S9B), competed with β3 CT chimera for binding to talin-PTB. Since talin-R4 induced very similar chemical shift perturbations as talin-RM when binding to talin-PTB (Figure S4), this finding suggests that talin-R4 plays a major role in masking the membrane-proximal CT-binding site on talin-PTB. It also further supports our prior chemical shift mapping and affinity-based results indicating that talin-R4 and talin-R6-R8 bind to different regions of talin-PTB. Figure 5A summarizes the surface depiction of the talin-PTB structure in which key residues directly involved in binding to talin-RM are highlighted based on the chemical shift mapping, mutagenesis, and competition data. The binding surface is compared to that for the talin-PTB/integrin β3 CT complex (Figure S5B).
(Wegener et al., 2007) and shows that the membrane-proximal β3 CT-binding site significantly overlaps with that for talin-RM, thus providing a view of how talin-R may sterically suppress the β CT binding to talin-PTB in intact talin.

To further evaluate the significance of the talin autoinhibition, we examined the activity of talin M319A in integrin activation. Since this mutant still maintains the integrin binding (Figure S5) but has dramatically reduced the talin-PTB/talin-RM affinity (Table S1), we speculated that full-length talin M319A would activate the integrin. As shown in Figure 5C, M319A indeed dramatically enhanced talin-induced αIIbβ3 activation as compared to WT talin. This enhancement was abolished by RGDS peptide, an inhibitor of ligand binding to β3 integrins (data not shown), indicating specificity. Thus, this observation offers strong support for our hypothesis. Note that the M319A-induced integrin activation is slightly more potent than talin-H. While the precise mechanism for this higher potency remains to be determined, one can envision two possibilities: (1) full-length talin M319A has higher affinity to integrin than talin-H. While the precise mechanism for this higher potency remains to be determined, one can envision two possibilities: (1) full-length talin M319A has higher affinity to integrin than talin-H alone, thus leading to the more potent integrin activation. In addition to talin-H, isolated talin-R also binds to integrin β CT (Xing et al., 2001; Yan et al., 2001; Tremuth et al., 2004) at the membrane-distal region (Tremuth et al., 2004), but not the membrane-proximal site (E.G. and J.Q., unpublished data). Thus, the constitutively open conformation of M319A may have higher affinity for integrin than talin-H alone due to both talin-H (M319A)/integrin and talin-R/integrin interactions. (2) Membrane anchoring of talin is important for integrin activation (Vinogradova et al., 2004; Wegener et al., 2007). It is possible that the open conformation of M319A has stronger capacity to anchor to the membrane than talin-H, resulting in more potent integrin activation.

**Conformational Activation by PIP2**

Given the above findings, an obvious question is how the closed conformation of talin is opened. A well-known talin activator is PIP2, which promotes strong talin binding to integrin β CT (Martel et al., 2001), resulting in the formation of a ternary PIP2/talin/integrin complex in living cells for mediating integrin activation and clustering (Cluzel et al., 2005). Our SPR experiment revealed that talin-H, but not talin-RM, can potently bind to biotinylated PIP2 with high affinity (K_D ~89.2 ± 1.25 nM, Figure S10A). The biotinylated PIP2 was mounted on a biotin sensor chip, and such positioned PIP2 should mimic PIP2 anchored to the membrane. Since talin-H FERM domain and multiple FERM domains bind to PIP2 involving PTB/PH subdomains (Hamada et al., 2000; Bomgard et al., 2003; Cai et al., 2008), we considered if talin-PTB is involved in binding PIP2. Our HSQC-based mapping experiment revealed that PIP2 can indeed interact with talin-PTB with the most significant perturbation around 370–378 (Figure S10B), which overlaps with the talin-RM-binding site (Figure 5D). SPR experiments demonstrated that PIP2 can disrupt the talin-RM/talin-PTB interaction in a concentration-dependent manner (Figure 6A). Such competition was confirmed by HSQC experiments in which soluble C4-PIP2 was able to compete with talin-RM for binding to talin-PTB (Figure 6B). Since PIP2/talin-H interaction has been shown not to interfere with the talin-H/integrin interaction (see Figure 6 in Martel et al. [2001]) and instead it induces conformational change of talin for high-affinity integrin binding (Martel et al., 2001), our findings provide a mechanism by which PIP2 binds to talin-PTB and sterically displaces the inhibitory talin-R to expose the integrin-binding site.

**DISCUSSION**

A combination of NMR spectroscopy, mutagenesis, and biochemical experiments have revealed how talin-PTB via its PTB subdomain interacts with talin-R to restrain talin in an inactive/autoinhibited conformation. Autoinhibition is a widespread phenomenon in controlling protein functions and occurs in multiple FERM-containing proteins (Pearson et al., 2000; Li et al.,
However, only the talin FERM domain can directly mediate integrin activation (Tadokoro et al., 2003) by binding to the integrin membrane-proximal β CT (Vinogradova et al., 2002; Wegener et al., 2007). The masking of the integrin membrane-proximal β CT-binding site in talin-FERM by talin-R is quite unique as compared to other autoinhibitory FERM-containing proteins, which utilize FERM domains to autoinhibit the functions of other parts of proteins (Pearson et al., 2000; Li et al., 2007; Lietha et al., 2007). For example, focal adhesion kinase (FAK) utilizes its N-terminal FERM F2 domain to mask the C-terminal kinase active site (Lietha et al., 2007), whereas moesin uses its N-terminal FERM F2 and F3 subdomains to mask its C-terminal actin-binding site. Thus, we have unraveled a distinct autoinhibition mechanism for talin in which other parts of the molecule, talin-R, control the function of the FERM domain in integrin activation. Our cell-based experiments provide direct functional evidence to support this mechanism; full-length talin was a poor activator of integrin αIIbβ3, whereas talin-H was substantially more potent (Figure 1). These data are also consistent with the report of Han et al. (2006) showing that talin must be activated to display its integrin-activating activity. Furthermore, talin M319A mutation, which disrupts the talin-PTB/talin-R interaction, but not talin-PTB/integrin interaction, constitutively activated αIIbβ3 (Figure 5C). These data provide strong functional evidence for talin autoinhibition in regulating integrin activation. The autoinhibitory domain was mapped to the middle region (1654–2344) of talin-R, and its affinity for talin-PTB was found to be within the submicromolar range as assessed by SPR using purified components (Table S1). We note that the intramolecular interaction involving the two domains in intact talin is expected to be even stronger by placing the binding surfaces within the same molecule and may provide the tight control of talin activity needed to prevent spontaneous integrin activation. Interestingly, despite its inhibitory conformation that prevents the talin-PTB/integrin membrane-proximal β CT contact, full-length talin can still bind weakly to integrin β CT (the affinity is about 6-fold weaker than that of the talin-H/integrin interaction, see Yan et al. [2001]), suggesting that the integrin-binding site on talin is not completely masked. Our data are consistent with this observation in that the integrin membrane-distal CT-binding site on talin-PTB does not appear to be significantly masked by talin-R. The binding of isolated talin-R to the membrane-distal region of integrin β CT (Xing et al., 2001; Yan et al., 2001; Tremuth et al., 2004) may contribute to the full-length talin/integrin interaction. Based on our observations, we propose a model for how talin controls
inhibin activation (Figure 7). In the closed state, the integrin membrane-proximal β CT-binding site on talin-H is masked by talin-R, although talin can still weakly associate with integrin β CT via its unmasked sites. Upon activation by cellular stimuli, talin undergoes a conformational change so that talin-PTB can access the integrin membrane-proximal β CT, which leads to the integrin α/β CT unclamping and inside-out activation. Since talin also binds to actin via the C-terminal end of talin-R (outside talin-Rm) (Gingras et al., 2008), the enhanced talin/integrin interaction, consequent to the talin conformational change, may alter integrin-actin linkage. In this manner, cells can undergo dynamic cytoskeleton remodeling, in coordination with integrin activation and ligation. A dynamic equilibrium exists between monomeric and dimeric talin, which only shifts to the dimeric state at high talin concentration (>3 μM) (Molony et al., 1987), and the dimeric state may strengthen the integrin-actin linkage. Although the exact dimerization topology for full-length talin is not clear, recent crystallographic studies of the C-terminal actin-binding module (2300–2541) revealed a dimeric structure and suggested that the full-length talin dimer may exist in three possible conformations (Gingras et al., 2008): parallel, tail-to-tail V-shaped, or tail-to-tail antiparallel fashion. Earlier EM studies (Goldmann et al., 1994) suggested that the two globular heads (talin-Hs) are on two opposite ends of the talin dimer and suggested a head-to-tail antiparallel dimer, which raises a possibility that the middle talin-Rm in one subunit interacts with talin-H in the other. However, such head-to-tail antiparallel is incompatible with the crystallographic studies (Gingras et al., 2008), and thus intermolecular autoinhibition is less likely to occur in cells.

What is the mechanism to trigger the conformational change of talin to expose its integrin membrane-proximal β CT site in vivo? One mechanism involves PIP2, which promotes strong talin binding to the integrin β1 CT (Martel et al., 2001). Interestingly, the PIP2-producing enzyme, PIPKIγ, is also recruited to integrin adhesion sites by talin (Ling et al., 2002; Di Paolo et al., 2002), providing a mechanism for efficient PIP2/talin interaction. Our NMR and biochemical data have indicated that while the PIPKIγ binding to talin does not affect the autoinhibitory conformation of talin (Figure S6D), the PIPKIγ product, PIP2, does (Figure 6). Thus, upon agonist stimulation, talin may recruit PIPKIγ to locally enrich PIP2, which in turn induces the conformational change of talin to expose its integrin membrane-proximal β CT site, promoting high-affinity talin binding to and activation of integrins. This model is supported by several independent in vivo observations: (1) integrin αβ3/β3 activation is associated with the increased PIPKIγ activity and PIP2 levels in platelets (Hinchliffe et al., 1996). (2) PIP2 level is associated with talin on activated platelets (in which αβ3/β3 is activated), but not on resting platelets (Héraud et al., 1998); and (3) a ternary complex involving PIP2, talin, and integrin αβ3 was formed in the living cells to mediate αβ3 activation and clustering (Cluzel et al., 2005). Another emerging mechanism for the talin activation involves RIAM (Han et al., 2006), which forms a supramolecular complex with talin and Rap1 to activate integrins. It remains to be determined whether RIAM changes the conformation of talin in this supramolecular complex to relieve its autoinhibition. Finally, calpain cleaves talin into a talin-H/talin-R mixture, thereby releasing talin-H for enhanced binding to integrin (Yan et al., 2001). However, activation of integrin αLβ2 by ionomycin did not involve calpain-mediated talin cleavage (Dreolini and Takei, 2007). RIAM-mediated integrin activation also does not involve calpain-dependent talin cleavage (Han et al., 2006). Most data implicate calpain in integrin outside-in signaling (Schoenwaelder et al., 1997; Hayashi et al., 1999; Franco et al., 2004).

In summary, we have demonstrated the structural basis of the autoinhibition for talin in regulating integrin activation. Our findings, together with previous structural data (Vinogradova et al., 2002; García-Alvarez et al., 2003; Wegener et al., 2007), now provide a view of how a series of conformation changes occur for talin-mediated integrin activation (Figure 7).
Structural Basis of Talin Autoinhibition

EXPERIMENTAL PROCEDURES

Sample Preparation for NMR Analyses

A pET15b vector containing a thrombin-cleavable N-terminal His tag GST\(\beta\)SSQVPRGSHM was used to subclone the talin-H fragments talin-F3/PTB (306–405) and talin-F2F3 (206–405). A variant of pET15b, pET30a, was used to clone larger talin-R fragments, talin-R 434–947 (R1), 944–1483 (R2), 1483–1653 (R3), 1654–1848 (R4), 1841–1983 (R5), 1984–2102 (R6), 2103–2229 (R7), 2225–2344 (R8), 2338–2541 (R9), and talin-RM (1654–2344) and a talin R6–R8 construct. The fragments were expressed in E. coli BL21 (DE3), and the cells were harvested and lysed using 10 mg/ml lysozyme. All fragments were purified on nickel columns with 250 mM imidazole in the elution buffer (50 mM phosphate buffer, 100 mM NaCl, 1 mM DTT [pH 7.0]). The eluted proteins were dialyzed against elution buffer, and His tags were cleaved using thrombin. The proteins were then further purified by size exclusion chromatography on Superdex 200 (Amersham Bioscience). Talin-H was prepared as described (Vinogradova et al., 2002). To prepare \(^{15}\text{N}\) and/or \(^{13}\text{C}\)-labeled protein, the cells were grown in M9 minimal media containing \(^{15}\text{NH}_4\text{Cl}\) and/or \(^{13}\text{C}\)-glucose, and all other conditions were the same as for the unlabeled protein. The cells were grown in M9 minimal media containing \(^{15}\text{NH}_4\text{Cl}\) and 90% \(^2\text{H}_2\text{O}\). Point mutants of talin-H were prepared employing the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Inc.) and prepared in the same way as WT proteins. A point mutation (C336S) was also introduced in talin-PTB as reported (de Pereda et al., 2002) to improve protein stability. The \(\beta\)3 CT chimera peptide TIDRKEFKAcEEERAKAIVWYSPLHYSAAR (the sequence in bold is PIPK\(\gamma\)I tail), \(\beta\)3 CT chimera mutant (F730A), and PIPK\(\gamma\)I-(SWVYPLHYSAR) were synthesized by our Biotechnology Core. For improved solubility, the KLLI sequence was not included at the N terminus of the \(\beta\)3 CT, since it is not involved in interacting with talin-PTB (Wegener et al., 2007). The \(\beta\)3 CT chimera binds tightly to talin-PTB (Kd ~140 nM) in slow exchange, which mimicks the \(\beta\)3 CT that has weaker binding to talin-PTB in intermediate exchange (Wegener et al., 2007).

NMR Spectroscopy

All heteronuclear NMR experiments were performed as described by Bax and Grzesiek (1993). All triple resonance NMR experiments for 1 mM talin-F2F3 were performed on a Varian 600 MHz instrument equipped with a triple resonance probe and shielded 2-gradient unit. These experiments were performed at 25°C (pH 7.0) in 150 mM NaCl, 50 mM phosphate buffer, and 1 mM DTT. All HSQC experiments were performed on Bruker Avance 600 with 900 MHz spectrometers using the cryogenic triple resonance probes. \(^{15}\text{N}\)-labeled and/or \(^{13}\text{C}^{15}\text{N}/90\%^{2}\text{H}\)-labeled talin-PTB or talin-F2F3 (0.2 mM) were used with and without the talin-R fragments at 1:2 ratio in all HSQC experiments. For chemical shift mapping, TROSY-based HSQC (Pervushin et al., 1997) for talin-PTB or talin-F2F3 in complex with talin-R\(_b\) was performed on a Bruker 900 MHz spectrometer. Weighted chemical shift changes were calculated using the equation \(\Delta\delta_{\text{obs}} = [\Delta\delta_{\text{res}}W_{\text{res}} + \Delta\delta_{\text{NMR}}W_{\text{NMR}}]^{1/2}\), where \(W_{\text{res}} = 1\) and \(W_{\text{NMR}} = 0.154\) are weighting factors based on the gyromagnetic ratios of \(^1\text{H}\) and \(^{15}\text{N}\). All the spectra were processed with nmrPipe (Delaglio et al., 1995) and visualized with PIPP (Garrett et al., 1991).

Surface Plasmon Resonance Measurements

SPR was performed using a BIA Core 3000 instrument (Amersham Pharmacia Biotech). CM5 sensor chips were activated using the amine coupling kit from Amersham Pharmacia Biotech. Talin-R\(_b\) was immobilized to the activated surface face, and talin-PTB and its mutants were injected at 0.1–5 \(\mu\)M and flow rates of 20 \(\mu\)l/min. For PIP2/talin-H interaction, biotinylated C\(_b\)-PIP2 (C-45B6a, Echelon Biosciences, Inc.) was immobilized on a biotin chip and talin-H was injected at 20 \(\mu\)l/min. Experiments were performed at 37°C in 10 mM HEPES (pH 7.4). Binding affinities were calculated by using the BiAcore 3000 evaluation software (Biacore AB, Uppsala, Sweden).

Plasmids and Transfections

Plasmid DNA encoding full-length mouse talin was kindly provided by Richard Hynes, Massachusetts Institute of Technology, and it was cloned into pEGFP-N1 vector (Clontech Lab, Inc) encoding a red-shifted variant of WT GFP using EcoRI and AgeI restriction sites. The final DNA had a C-terminal GFP fusion and was confirmed by sequencing. Full-length talin M319A DNA mutant was prepared employing the QuickChange Site-Directed Mutagenesis Kit (Stratagene) using the cloned GFP-fusion talin DNA as a template. The resulting mutant DNA was confirmed by sequencing.

Integrin Activation

The effects of full-length talin, full-length talin M319A, and talin-H on integrin activation were analyzed using \(\text{PIP}_3\)-\(\text{CHO}\) and an activation-specific mAb (PAC1) as described previously (Ma et al., 2008). Briefly, \(\text{PIP}_3\)-\(\text{CHO}\) cells were transfected with EGFP-talin, EGFP-talin-H, or EGFP vector alone; 24 hr after transfection, the cells were harvested and stained with PAC1 followed by Alexa Fluor 633 goat anti-mouse IgM conjugate. After washing, the cells were fixed and analyzed on a FACScalibur instrument (BD Scientific, Franklin Lakes, NJ). PAC1 binding was analyzed only on a gated subset of cells positive for EGFP expression. The mean fluorescence intensities of PAC1 bound to the control EGFP expressing cells. Three independent experiments were performed, and the Student’s t test was used for statistical analyses.

ACCESSION NUMBERS

The Biological Magnetic Resonance Data Bank accession number for the chemical shift assignments reported in this paper is 15792.
SUPPLEMENTAL DATA
The Supplemental Data include one table and ten figures and can be found with this article online at http://www.molecule.org/cgi/content/full/31/1/124/DC1/.

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REFERENCES
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