Calpain Cleavage Promotes Talin Binding to the $\beta_3$ Integrin Cytoplasmic Domain*

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Talin links integrin $\beta$ cytoplasmic domains to the actin cytoskeleton and is involved in the clustering and activation of these receptors. To understand how talin recognizes integrin $\beta$ cytoplasmic domains, we configured surface plasmon resonance methodology to measure the interaction of talin with the $\beta_3$ integrin cytoplasmic domain. Here we report that the N-terminal $-$ 47-kDa talin head domain (talin-H) has a 6-fold higher binding affinity than intact talin for the $\beta_3$ tail. The affinity difference is mainly due to a difference in $K_a$.

Calpain cleavage of intact talin released talin-H and resulted in a 10-fold increase in apparent $K_a$ and a 100-fold increase in apparent $k_{on}$. The increase in talin binding after cleavage was greater than predicted for stoichiometric liberation of free talin-H. This additional increase in binding was due to cooperative binding of talin-H and talin rod domain to the $\beta_3$ tail. Talin resembles ERM (ezrin, radixin, moesin) proteins in possessing an N-terminal FERM (band four-point-one, ezrin, radixin, moesin) domain. These data show that the talin FERM domain, like that in the ERM proteins, is masked in the intact molecule. Furthermore, they suggest that talin cleavage by calpain may contribute to the effects of the protease on the clustering and activation of integrins.

Integrins play important roles in the development and functioning of all multicellular animals (1). Integrins are non-covalent heterodimers of type I transmembrane protein subunits termed $\alpha$ and $\beta$. Each subunit has a large (>700 residue) N-terminal extracellular domain. A single membrane-spanning domain links this extracellular domain to a generally (with the exception of $\beta4$) short (13–70 residues) cytoplasmic domain (2). Integrins bind to insoluble ligands (e.g. collagen fibrils) and link them to the intracellular cytoskeleton. In addition to forming these physical linkages, integrins regulate cell growth, survival, and differentiation (1). These signaling activities and cytoskeletal linkages depend on the integrin cytoplasmic domains (3). Conversely, integrin-mediated adhesion is rapidly and precisely regulated by changes in integrin affinity for ligand (activation) (4, 5) and by affinity-independent mechanisms such as changes in integrin clustering (6). Integrin linkages with the cytoskeleton can affect both integrin activation (7, 8) and affinity-independent (7, 9) regulation of integrin-mediated cell adhesion. Thus, integrin-cytoskeletal linkages play a pivotal role in their regulation and in their signaling properties.

Talin is an actin-binding protein that links integrins to the actin cytoskeleton (10). Talin co-localizes with clustered integrins in all known species that possess these receptors. Furthermore, genetic and cell biological analyses show that talin is required for integrin clustering into focal adhesions (11). Talin-integrin interactions can also regulate integrin activation (12). Talin consists of an N-terminal $-$ 47-kDa globular head domain (talin-H) and a $-$ 190-kDa C-terminal rod (talin-R) domain (13). It is an elongated (60 nm), flexible protein that binds to the cytoplasmic domains of integrins $\beta_1A$ and $\beta_1D$ (but not $\beta_1H$ and $\beta_1P$ (14) $\beta_2$ (15), and $\beta_3$ (16) in vitro and co-immunoprecipitates with $\beta_1$ (17) and $\beta_2$ (15) integrins. Talin also interacts with other focal adhesion proteins such as vinculin (18–20) and actin (20, 21). Talin binding to integrin $\beta$ cytoplasmic domains appears to involve interactions of both the head and the rod domains (12, 22). However, there has been no direct quantitative comparison of the affinities of the two domains of talin for integrin $\beta$ tails. In sum, the binding of talin to integrin $\beta$ cytoplasmic domains is crucial for integrin function and is involved in the regulation of integrin activation and clustering.

Talin binding to integrin $\beta$ tails is important in governing integrin activation and clustering, events responsible for rapid dynamic changes in integrin function. Consequently, it is likely that physiological mechanisms exist to regulate the binding of talin to integrin $\beta$ tails. Talin contains a FERM domain that is involved in its binding to the $\beta_3$ tail. The talin FERM domain is similar to those present in ezrin, radixin, and moesin (ERM proteins (23)), which mediate the binding of the ERM proteins to the cytoplasmic domains of certain transmembrane receptors (24). In ERM proteins, the membrane protein binding site in the FERM domains are masked in the intact molecule by interactions with the C-terminal tail domain (25). The binding activity of the FERM domain can be unmasked by phosphorylation or the interaction of ERM proteins with polyphosphoinositides (see Ref. 24 and references therein). In the present work, we have made real time measurements of the binding of talin and its two domains to the $\beta_3$ integrin cytoplasmic domain by use of surface plasmon resonance (SPR). Here, we report that talin-H, which contains the FERM domain, has a 6-fold.

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The abbreviations used are: talin-H, talin head domain; talin-R, talin rod domain; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; RU, response unit; ERM, ezrin, radixin, moesin; FERM, four-point-one, ERM; SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid.
higher binding affinity than intact talin for \( \beta_3 \). The affinity difference is mainly due to a difference in the on-rate of binding, indicating that the \( \beta_3 \) cytoplasmic domain binding site(s) is masked in intact talin. Furthermore, calpain cleavage of intact talin resulted in an apparent 16-fold decrease in \( K_d \) and an apparent 100-fold increase in \( k_{on} \). The increase in talin binding after cleavage was due to both the generation of free talin-H and the cooperative binding of talin-R to the talin-H-\( \beta_3 \) tail complex. These data establish that talin resembles ERM proteins in the intramolecular masking of its FERM domain. Thus, physiological modification of the interaction of the talin FERM domain with integrins is likely to be an important control mechanism in cell adhesion. Furthermore, calpain activity is involved in the control of integrin activation and clustering (25). The present data strongly implicate talin as a substrate that could account for some of the effects of calpain on integrin function.

**EXPERIMENTAL PROCEDURES**

**Materials**

Thrombin, p-aminobenzamide-agarose beads, and biotin maleimide were obtained from Sigma. Recombinant calpain II (high purity, 1500 units/mg protein) from rat was purchased from Calbiochem, and calpain inhibitor, E-64, were purchased from Peptide Research Foundation.

**Protein Purification**

Talin was purified from outdated human platelets according to previously published procedures (26, 27) and was at least 90% homogeneous as judged by SDS-PAGE (Fig. 1A). To obtain the rod domain of talin, purified talin in buffer B (50 mM Tris-HCl, pH 8, 0.3 mM EDTA, 0.1 mM dithiothreitol) was digested by thrombin (0.5 units/mg of protein) at room temperature. After a 40-min digestion, benzamidine (final concentration 10 mM) was added, and the cleavage products were separated by gel filtration using a Superdex 200 column (Fig. 1A).

A cDNA encoding amino acids 1–435 of mouse talin was cloned into pET15b vector (Novagen) and purified as described (Fig. 1A). A control model protein containing the N-terminal seven amino acid residues (KLGFFKR) of the \( \alpha_5 \) tail (\( \alpha \)T) was also produced in the same way. Product masses were routinely determined by electrospray ionization mass spectrometry using an API-III quadrupole spectrometer (Sciex) and varied by less than 0.1% from that predicted by the desired sequence.

**Modification of the \( \beta_3 \) Tail Model Protein with Biotin**

Integrin tail model protein (0.8 mg/ml) was solubilized in 0.1 M acetate buffer, pH 4.6. The model protein was then reduced by the addition of 3 mM dithiothreitol at 37 °C for 30 min. After cooling to room temperature, 10 mM biotin-maleimide (100 mM stock in Me2SO) was added into the solution, and the mixture was incubated for 30 min at room temperature. Unreacted biotin-maleimide was removed by extensive dialysis in 0.1 M acetate buffer, pH 4.6, at 4 °C.

**MALDI TOP MS, Electrospray Ionization MS, and Liquid Chromatography-MS/MS**

Biotin-modified \( \beta_3 \) Tail Model Protein was desalted on a reverse phase C18 high performance liquid chromatography column (Vydac) and analyzed by electrospray ionization mass spectrometry on an API-III quadrupole spectrometer (Sciex; Toronto, Ontario, Canada). Peptide mass fingerprinting with MALDI-TOP MS was used to identify the biotinylated residue as described previously (28). Briefly, modified proteins were fractionated by SDS-polyacrylamide gel electrophoresis, and the band corresponding to the model proteins was subjected to trypsin in-gel digestion. The digest mixture was analyzed by MALDI TOP MS, as described previously (28) but with the use of a linear detector mode. Liquid chromatography and MALDI-TOF MS analyses were done with an electrospray ionization/torr mass spectrometer (Finnigan-MAT LCQ). Peptide mixtures were separated on 5 μm of C18 MetaChem 150 × 1.0-mm column at a flow rate of 20 μl/min. A gradient of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B) was distributed as follows: 0% B in the first 5 min; 0–30% B in 3–30 min; 30–60% B in 30–100 min; 60–98% B in 100–120 min. Spectra were recorded over the specified range in 2 s and an ion present in the scan and the calculated ion abundance exceeded a specified threshold, then product ion spectra were acquired using the m/z value of the base peak in the last mass spectrum. Using the m/z value and the assumption that the parent is doubly charged, the MALDI TOF MS program calculated the scan range and the collision offset. Product ion spectra were generated usingargon as the collision gas (3–4 milli-torr) and collision energies (laboratory frame) on the order of 10–30 eV. The collision offset varied in a linear manner with the m/z value.

**SPR**

SPR analysis 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. Then streptavidin (5–20 μg/ml in 10 mM acetate, pH 4.5) was injected. Non-reacted activated groups were blocked by injection of 2 M ethanolamine, pH 8.5. Typically, the resonance unit (RU) value increased after this procedure was 200–1500. Biotin-labeled \( \beta_3 \) tail model protein was then bound to the streptavidin-derivatized surface. Free streptavidin was blocked by the addition of 5 mM biotin. The surfaces of the sensor chips were regenerated by injection of 2 M NaCl. The chips were used for about 2–4 weeks in multiple experiments. All of the parameters were measured at a flow rate of 20 μl/min.

**Data Analyses**

**Analysis of the Binding Data in BIAcore**—Sensorgrams for the interaction of talin and integrin tail were analyzed with BIAlogics kinetics evaluation software, as described in the standard mode (29). The kinetics data were interpreted in the context of a first order kinetic model: A + B → AB (30–32). The association rate constants (\( k_{on} \)) were calcu-
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RESULTS

Immobilization of the β₃ Cytoplasmic Domain—Talin-integrin interactions were studied by immobilizing intact cytoplasmic domain model proteins on the sensor chip. To ensure that the β₃ tails were in a unique orientation, we immobilized the tails via a biotin-avidin linkage. Since each model protein has a single cysteine, that cysteine was modified by biotinyl maleimide. To confirm that the appropriate modification had occurred, modified proteins were analyzed. Electrospary ionization MS showed increments of 449 Da, indicating the presence of 1 mol of biotin/mol of tail protein. When tryptic digests of the modified tail were analyzed by MALDI TOF MS, all detected fragments containing the Cys residue (GSMCK) were biotin-modified (Table I). Furthermore, all other detected peptides were at the mass predicted for the unmodified peptide (Table I), indicating that they were not biotinylated. Further analysis of the 1169.6-Da biotinylated fragment, GSMCK, by LC-MS/MS demonstrated that the cysteine residue was modified (Fig. 2). In particular, all expected b ions and y ions derived from the Cys modification were present in the spectrum (Fig. 2). Thus, the β₃ tail model protein was biotinylated on the single Cys residue.

The biotinylated β₃ tail was immobilized by binding to a streptavidin-coupled sensor chip. To minimize nonspecific binding and mass transfer, we first tested four streptavidin coupling concentrations leading to refractive index changes of ∼200, ∼500, ∼950, and 1500 RU, respectively. The biotinylated β₃ tail model protein was then immobilized at a saturating concentration (16 μM), resulting in an increase in refractive index directly proportional to the initial streptavidin-coupling concentration. Initial experiments at each of these β₃-coating concentrations were conducted with talin-H at concentrations ranging from 10 to 300 nM. Initial estimates of k⁺ using Equation 3 showed a concentration dependence at streptavidin-coupling levels of 950 and 1500 RU (details not shown). At these coupling levels (950 and 1500 RU), k⁺ values decreased as the initial amount of the head domain bound to the sensor chip decreased, increasing mass transfer effects. However, a coupling level of ∼500 RU resulted in clear responses and concentration independence of k⁺ at flow rates ranging from 10 to 30 μl/min (data not shown). Consequently, these conditions were chosen for further analysis.

Talin Head Domain (Talin-II) Binds to the β₃ Cytoplasmic Domain with a Higher Affinity than Intact Talin—Real-time measurements of the talin-H-β₃ interaction revealed a typical association and dissociation phases (Fig. 3A). Binding was specific, since negligible binding of talin-H was seen to a chip coated with αT, a construct that lacks the β₃ cytoplasmic domain sequence (Fig. 3D). At all concentrations of talin-H employed, k⁺ values were nearly constant (4.8 × 10⁻³ s⁻¹) (Table II), as estimated using Equation 3 (Fig. 4A). This indicates negligible rebinding of talin to the β₃ tail during the dissociation phase. k⁻ values were calculated to be 5.3 × 10⁴ M⁻¹ s⁻¹ using

\[ R_t = k_{\text{on}} C \frac{R_{\text{max}}}{1 + e^{-k_{\text{d}} t}} \]  

where \( R_t \) is the response at time \( t \), \( R_{\text{max}} \) is the maximum response, \( C \) is the concentration of analyte on solution, and

\[ k_{\text{d}} = k_{\text{on}} C + k_{\text{off}} \]  

where \( k_{\text{d}} \) is the slope of the dRU/dt versus R plot. The dissociation rate constants \( (k_{\text{d}}) \) values obtained from Equation 2 may not be reliable for low ranges (29). Therefore, k⁻ values were calculated from the dissociation phases of the sensorgrams using Equation 3.

\[ \ln(R/R_0) = -k_{\text{d}}(t - t_0) \]  

where \( R_0 \) and \( R_t \) represent the SPR signal expressed in RU at time \( t \) and the starting time of dissociation, \( t_0 \), respectively. The apparent equilibrium dissociation constant, \( K_d \), was determined from the ratio of these two kinetic constants \( (k_{\text{d}}/k_{\text{on}}) \). Affinities were calculated from rate constants and from analysis of equilibrium binding. By measuring equilibrium resonance units (Rₑ) as a function of ligand concentration, binding data can be analyzed by Scatchard plots using the equation,

\[ R_{\text{off}}/C = R_{\text{on}}/K_d - R_{\text{off}}/K_{\text{on}} \]  

where \( R_{\text{on}} \) is the equilibrium resonance units, \( R_{\text{off}} \) is the resonance signal at saturating concentrations of analyte, and \( C \) is the concentration of free protein.

Affinity Chromatography Experiments with Integrin Cytoplasmic Domain Mimics—500 μg of purified recombinant β₃ cytoplasmic domain proteins was dissolved in a mixture of 5 ml of 20 mM Pipes, 50 mM NaCl, pH 6.8, and 1 ml of 0.1 M sodium acetate, pH 3.5, and bound overnight to 80 μl of Ni²⁺-saturated His-bind resin (Novagen). In control experiments (data not shown) we found that these conditions resulted in saturation of the resin with recombinant protein. Resins were washed twice with 20 mM Pipes, 50 mM NaCl, pH 6.8, and stored at 4 °C in an equal volume of this Pipes buffer containing 0.1% NaN₃ as a preservative.

35 μl of such a suspension was added to equal molar amounts of talin-H or talin-R or a mixture of the two. After incubation overnight at 4 °C, resins were washed five times with this buffer and finally heated in 20 μl of SDS-polyacrylamide gel electrophoresis sample buffer. Samples were separated on 4–20% SDS-polyacrylamide gels (Novex) and stained with Coomassie Blue.

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\[ k_{\text{on}} = \frac{M}{k_{\text{d}}} \]  

\[ k_{\text{off}} = \frac{M}{k_{\text{d}}} \]  

\[ K_d = \frac{k_{\text{off}}}{k_{\text{on}}} \]  

\[ K_{\text{on}} = \frac{k_{\text{off}}}{k_{\text{on}}} \]  

\[ K_{\text{off}} = \frac{k_{\text{off}}}{k_{\text{on}}} \]  

\[ K_{\text{eq}} = \frac{k_{\text{off}}}{k_{\text{on}}} \]  

where \( M \) is the mass detected.
FIG. 3. Sensorgrams of the association phase and dissociation phase for binding of talin-H (A) and intact talin (B) and its rod domain (C) to the β3 tail. Talin-H (10, 40, 120, 200, 300 nM from the lower trace), intact talin (40, 80, 150, 200, 350 nM from the lower trace), or talin-R (100, 167, 250, 300, 350, and 500 nM from the lower trace) were injected onto the biotin-maleimide-modified β3 tail-coated surface at a flow rate of 20 μl/min, as described under “Experimental Procedures.” Injecting buffer without dissolved protein started the dissociation phase. In D sensorgrams depict the lack of detectable binding of talin-H (300 nM) to a streptavidin-coupled chip containing no bound protein or coated with a truncated model protein, αT.

FIG. 2. LC-MS/MS analysis of biotin-maleimide β3 tail model protein. A tryptic digest of the biotin-maleimide modified β3 tail was injected into an electrospray ionization/ion trap mass spectrometer (Finnigan-MAT LCQ), as described under “Experimental Procedures.” The profile of fragmentation of the biotin-maleimide-linked GSMCK was shown, and each b and y ion was identified as shown in the inset.
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Fig. 4. Estimation of parameters for talin-H binding to the β tail. A, dissociation rate, \( k_{\text{off}} \). Values of \( k_{\text{off}} \) are plotted versus the concentration of the head domain. B, association rate, \( k_{\text{on}} \); \( k_{\text{on}} \) is plotted versus the concentration of free talin-H (C). The slope of the line obtained by linear regression provides the estimate of \( k_{\text{eq}} \); equilibrium association constant, \( K_a \). The fitted values for the response units at equilibrium (Req) were plotted versus the ratio of \( R_{\text{eq}} / C \). The slope of the line, estimated by linear regression, \( K_d \).

Equation 2. \( K_a \) versus concentration plots for 10–300 nM talin-H were linear (Fig. 4B), indicating that the \( k_{\text{on}} \) values are not affected by talin concentration. The dissociation constant, \( K_d \), was then calculated from these kinetic constants to be 9.1 \( \times 10^{-8} \text{ M}^{-1} \). This value is in good agreement with that obtained from equilibrium binding Scatchard plots (1.1 \( \times 10^{-7} \text{ M}^{-1} \)) using Equation 4 (Fig. 4C). Thus talin-H binds to the β3 cytoplasmic domains reversibly, with a \( K_d \) of \( \sim 100 \text{ nM} \).

Sensorgrams of intact talin or talin-R binding to β tail revealed dissociable binding. Estimation of kinetic and equilibrium parameters (Table II) indicated that intact talin bound with a 6-fold lower affinity than its head domain (\( K_d \approx 91 \text{ nM} \) for talin-H = and 550 nM for intact talin). This difference was primarily due to a difference in on-rates between the two molecules (\( k_{\text{on}} = 5.3 \times 10^{8} \text{ M}^{-1} \text{ s}^{-1} \) for talin-H and \( 1.3 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1} \) for intact talin). Furthermore, talin-R bound with a 37-fold lower affinity than talin-H (Table II). Thus, talin-H binds β3 with a 6-fold higher affinity than intact talin.

Talin Cleavage Increases Its Affinity for the β3 Cytoplasmic Domain—The difference in affinity of talin versus talin-H could be due to the source of the two proteins, platelets versus a recombinant protein, respectively. Alternatively, it could reflect a constraint on the interaction of talin-H with β3 in intact talin. To test this possibility, we digested talin with calpain, a cleavage known to liberate talin-H from talin-R (33–35). Digestion of talin with calpain (0.1 μg of calpain/mg of protein) resulted in complete cleavage as assessed by SDS-polyacrylamide gel electrophoresis (Fig. 5A, inset). After cleavage there was a dramatic increase in talin binding to the β tail (Fig. 5, A and B).

Kinetic analysis indicated that cleavage resulted in a 100-fold increase in the apparent \( k_{\text{eq}} \), 1.4 \( \times 10^{3} \text{ M}^{-1} \text{ s}^{-1} \), and a 7-fold increase in the apparent \( K_d \), 5.4 \( \times 10^{3} \text{ M}^{-1} \text{ s}^{-1} \), versus 7.2 \( \times 10^{-8} \text{ M}^{-1} \), relative to intact talin. Consequently, the apparent \( K_d \) for this mixture of cleavage products is 3.9 \( \times 10^{-8} \text{ M}^{-1} \), a 16-fold decrease relative to intact talin. Thus, cleavage dramatically enhanced the binding of talin to the β tail. Furthermore, these results confirmed that the capacity of talin-H to bind to the β tail is masked in intact talin.

Cooperative Binding of Talin Fragments to the β Tail—In the foregoing studies, we found that cleavage of talin resulted in markedly increased binding to the β tail. Indeed, the mixture of calpain cleavage fragments appeared to bind to a greater extent than predicted for the sum of independent talin-H and talin-R binding (compare Fig. 3 with Fig. 5). This suggested the possibility of cooperative binding of the two talin fragments to the β tail. To test this possibility, we examined the binding of the individual domains and an equimolar mixture of the two. A mixture of talin-H and talin-R produced a 2-fold enhancement of response relative to the calculated sum of the individual domain binding (Fig. 6A). In SPR, the refractive index increment is approximately proportional to the mass that is bound to the sensor surface (31, 32). Because talin-R (\( M_r = 190,000 \)) is roughly 4 times the mass of talin-H (\( M_r = 50,000 \)), the doubling of the response suggested that in the equimolar mixture of fragments, the molar ratio of bound talin-H to talin-R was 4:1. To test this possibility directly, we performed affinity chromatography with the β3 cytoplasmic domain model protein. Isolated talin-R failed to detectably bind to the affinity matrix, consistent with its low affinity. In contrast, talin-H bound. However, talin-R bound from an equimolar mixture of the two domains (Fig. 6B). Densitometry indicated a ratio of 4 talin-H molecules bound per talin-R molecule. Thus, bound talin-H mediates the binding of talin-R to the β3 integrin cytoplasmic domain.

DISCUSSION

Talin links integrin β cytoplasmic domains to the actin cytoskeleton and is involved in both the clustering and activation of these receptors. In the present study, we developed SPR methodology to quantitatively measure the interaction of talin with the β3 cytoplasmic domains. To do this, we covalently modified a β3 cytoplasmic domain model protein to permit its immobilization in a unique orientation on the sensor chip. With this methodology, we found that talin-H displays a 6-fold higher binding affinity than intact talin for the β3 tail. The affinity difference is mainly due to a difference in \( k_{\text{on}} \), suggesting that the capacity of talin-H to interact with the β3 cytoplasmic domain is masked in intact talin. In support of such a constraint, calpain cleavage of intact talin resulted in a 100-fold increase in apparent \( K_d \) and a 100-fold increase in apparent \( k_{\text{on}} \). The increase in talin binding after cleavage was greater than could be accounted for by simple generation of free talin-H. The unexpected increment in talin binding was explained by the cooperative binding of free talin-H and talin-R to the β3 cytoplasmic domain. Our data provide new insight into the structure and function of this important cytoskeletal linker protein. They also provide a plausible biochemical explanation for the effects of calpain on the clustering and activation of integrins.

The present work provides kinetic and equilibrium analysis...
fig. 5. Talin cleavage promotes binding to the $\beta_3$ tail. Purified talin was cleaved with calpain (0.1 $\mu$g/ml) at 22 °C for 1 h. The calpain was inhibited by the addition of 2 $\mu$m calpain inhibitor, E-64. Digestion appeared to yield the talin-H and talin-R quantitatively (panel A, inset). The binding of the starting intact talin (panel A) or the digest (panel B) was assessed by SPR. Both curves depicted the binding of the same input concentrations of intact (A) or digested (B) talin (50, 100, 150, and 200 nM from the lower tracing). Note the dramatic increase in the rate and extent of binding as a consequence of talin cleavage.

Fig. 6. Cooperative binding of talin-H and talin-R to the $\beta_3$ tail. A, SPR analysis. Plotted is the binding of talin-H (circles) or talin-R (squares) versus the input concentration. The binding of an equimolar mixture of the two, each at the indicated concentrations, was also analyzed (diamonds). For comparison, the arithmetic sum of the binding of the two domains (triangles) is also plotted. Note that at each concentration the mixture results in an approximate doubling of response relative to that predicted from the binding of the individual domains. B, affinity chromatography. Talin-H, talin-R, or an equimolar mixture of the two (400 nM) were added to Ni$^{2+}$ beads coated with $\beta_3$ tail model protein. Bound proteins were eluted in SDS sample buffer, fractionated by SDSPolyacrylamide gel electrophoresis, and visualized by Coomassie Blue staining. Scanning densitometry using the input domains as standards indicated that molar ratio of bound talin-H to talin-R was 4:1. C, depicts the loading of the affinity matrix with $\beta_3$ model protein as assessed by Coomassie Blue Staining.

of the binding of talin to the $\beta_3$ integrin cytoplasmic domain. Importantly, the ligand was immobilized via a biotin moiety that was in a unique location in the $\beta_3$ tail model protein. Thus, the $\beta_3$ tail was probably immobilized in a single orientation. Strong support for a single orientation comes from the excellent fit of the binding data to a model of simple bi-molecular interaction with a single class of binding sites. In particular, there was good agreement between the $K_a$ estimated from kinetic and equilibrium analyses. Goldmann (36) uses light scattering to examine the kinetics of talin binding to integrin $\alpha_{1b}\beta_3$ and reported a $K_a$ of 400 nM, in remarkable agreement with our estimated $K_a$ of 550 nM for talin binding to the $\beta_3$ tail (Table II). In contrast, Knezevic et al. (16) report a value of 15 nM for the affinity of talin for integrin $\alpha_{1b}\beta_3$ in an enzyme-linked immunosorbent assay-based assay. It is possible that the immobilization of the ligands or the requirement for multiple washing steps in the enzyme-linked immunosorbent assay account for the discrepancy in the measured affinity of talin for integrin $\alpha_{1b}\beta_3$. The concordance of measured affinities of talin for $\alpha_{1b}\beta_3$ and the $\beta_3$ tail (400 versus 550 nM) in homogeneous real time binding assays supports the idea that the interaction of talin with integrin $\alpha_{1b}\beta_3$ is mediated by its binding to the $\beta_3$ tail (16).

We found an increased affinity of talin-H for the $\beta_3$ cytoplasmic domain relative to intact talin. This finding suggests a constraint on the binding activity of talin-H in the intact talin molecule. The presence of such a constraint is supported by the 10-fold lower $k_{on}$ for intact talin relative to talin-H. Its existence is confirmed by the marked increase in binding induced by calpain cleavage of talin. The N terminus of talin contains a FERM domain shared with ERM proteins that link transmembrane receptors with the actin cytoskeleton (37). Within ERM proteins, the capacity of the FERM domain to bind to its transmembrane partners is masked by the C-terminal domain (23). This activity can be unmasked by phosphorylation or binding to certain polyphosphoinositides. Indeed, Martel et al. (38) report that polyphosphoinositides can increase the binding of talin to $\beta_{1A}$ integrins (38). Furthermore, talin-H, which contains the FERM domain, binds to the $\beta_{1A}$ cytoplasmic domain (12) in addition to that of $\beta_3$. Thus, the capacity of talin-H to bind to integrin $\beta_3$ cytoplasmic domains is constrained in intact talin.

Talin-H and talin-R bind cooperatively to the $\beta_3$ tail. This conclusion is based on two observations. First, that an equimolar mixture of the two domains generated approximately twice the response predicted from the arithmetic sum of their individual responses (Fig. 5B). Second, in affinity chromatography experiments, the binding of talin-R was markedly enhanced by the presence of talin-H. In contrast, there was little increase in talin-H binding in the presence of talin-R. This result suggests that talin-H binding to $\beta_3$ alters one or both of the binding partners to promote its binding to talin-R. In addition, from an equimolar mixture, the ratio of bound talin-H to talin-R was 4-1. Limiting quantities and solubility of talin-R made it technically unfeasible to estimate the saturation stoichiometry of talin-R/talin-H. However, mapping of the binding site in talin-R may permit such an analysis in the future. Cooperative binding of talin-R and talin-H explain our previous finding that talin-R bound to a $\beta_3$ affinity matrix from a cell extract containing both talin-R and talin-H (12). In ERM proteins, the C-terminal domain usually directly binds to the FERM domain and blocks its binding function in trans (37). The cooperative
binding of the talin-R and talin-H to β3 suggest a more complex mode of domain interaction in the talin molecule.

The present studies provide a plausible biochemical mechanism for the effects of calpain on integrin clustering and activation. Several reports implicate calpain in the activation of integrins (33, 39). Indeed, μ calpain-deficient platelets exhibit a defect in platelet aggregation, an integrin αIIbβ3 activation-dependent process (33). Expression of talin-H-containing fragments activate integrin αIIbβ3 even when the fragments are present at only a 2-fold excess over endogenous intact talin (12). The present results suggest that the capacity of these talin-H-containing fragments to activate αIIbβ3 may depend on the marked increase in β3 binding affinity resulting from separation of talin-H from the bulk of the molecule. Furthermore, calpain also mediates integrin clustering (25). This clustering could arise from increased ligand-dependent interaction as a consequence of talin-induced increase integrin affinity. Alternately, the integrin-bound head or rod domain could cluster additional integrins through interactions with other integrins, talin molecules, or with other focal adhesion components. Calpain can cleave integrin β cytoplasmic domains (41), a cleavage likely to lead to inhibition of activation. Thus, the balance between calpain cleavage of talin and of the β tail could specify the effect of calpain on integrin activation and clustering. These opposing effects of calpain might also account for the transient nature of certain integrin activation events (40), i.e. talin cleavage activates the integrin; subsequently, integrin β tail cleavage could reverse activation. Thus, the results obtained here provide a plausible biochemical explanation for some of the effects of calpain on integrin functions. They also establish that the binding of talin-H to integrins is regulated by constraints within the talin molecule. A clear understanding of the physiological mechanisms that relieve those constraints will provide valuable insights into the regulation of integrin function and of cytoskeletal organization.

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