Go13 Switch Region 2 Relieves Talin Autoinhibition to activate αIbβ3 Integrin

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Key Points: (1) Go13 relieves talin autoinhibition. (2) Go13-talin interaction represents a novel mechanism for the regulation of integrin activation.
ABSTRACT

Integrins function as bi-directional signaling transducers that regulate cell-cell and cell-matrix signals across the membrane. A key modulator of integrin activation is talin, a large cytoskeletal protein that exists in an autoinhibited state in quiescent cells. Talin is a large 235 kDa protein comprised of an N-terminal 45 kDa FERM domain, also known as the talin head domain (THD), and a series of helical bundles known as the rod domain. The talin head domain consists of 4 distinct lobes designated as F0-F3. Integrin binding and activation is mediated through the F3 region; a critically regulated domain in talin. Regulation of the F3 lobe is accomplished through autoinhibition via anti-parallel dimerization. In the anti-parallel dimerization model, the rod domain region of one talin molecule binds to the F3 lobe on an adjacent talin molecule, thus achieving the state of autoinhibition. Platelet functionality requires integrin activation for adherence and thrombus formation, thus regulation of talin presents a critical node where pharmacological intervention is possible. A major mechanism of integrin activation in platelets is through heterotrimeric G protein signaling regulating hemostasis and thrombosis. Here we provide evidence that Switch Region 2 of the ubiquitously expressed G protein (Gα13) directly interacts with talin, relieves its state of autoinhibition, and triggers integrin activation. Biochemical analysis of Gα13 shows SR2 binds directly to the F3 lobe of talin’s head domain and competes with the rod domain for binding. Intramolecular FRET analysis shows Gα13 can relieve autoinhibition in a cellular milieu. Finally, a myristoylated SR2 peptide shows demonstrable decrease in thrombosis in vivo. Altogether, we present a mechanistic basis for the regulation of talin through Gα13.

INTRODUCTION

Integrins are transmembrane receptors that regulate dynamic cell-cell and cell-matrix interactions (Reviewed (1,2)). Environmental cues regulate a multitude of cellular processes through integrins, such as cell proliferation, shape, adhesion, and migration. Due to the well-established importance of integrin signaling in hemostasis and thrombosis, the mechanisms of integrin regulation remain an area of intense investigation. Upon activation, platelet integrins switch from a low affinity to a high affinity state, thus permitting the binding to multivalent ligands such as vWF and fibrinogen to enable clotting (3-7). The most abundant platelet integrin complex, αIIbβ3, is activated through the cytoskeletal protein talin (1,8-12). Talin is a large 235 kDa protein comprised of an N-terminal 45 kDa FERM domain, also known as the talin head domain (THD). THD is connected to a large rod domain comprised of a series of alpha helical bundles. The talin head domain consists of 4 distinct lobes or regions designated as F0-F3. Integrin binding and activation is mediated through the F3 region; a critically regulated domain in talin. Regulation of the F3 lobe is accomplished through autoinhibition via anti-parallel dimerization, although some evidence suggests an autoinhibited monomer conformation (13-17). In the anti-parallel dimerization model, the rod domain region of one talin molecule binds to the positively charged face of the F3 lobe on an adjacent talin molecule, thus achieving the state of autoinhibition (15).

The primary mechanism for disabling talin autoinhibition is thought to occur through localized distribution of phosphatidylinositol 4,5-bisphosphate (PIP₂) at the plasma membrane surface (11,18,19). This process is mediated by PIP Kinase Iγ via engaging the Cal-DAG-Rap1a/RIAM pathway. PIP₂ can bind to several sites within talin head/ERM domain, and is believed to displace the strong negative interface of the rod domain by attracting the positive charges of the head domain in a so called “push-pull” mechanism (20). RIAM (Rap1–GTP-interacting adapter molecule) effector, on the other hand, has been shown to recruit talin to the membrane as well as specifically binds to the F3 lobe of the FERM domain in an area that relieves rod binding and promoting β3 binding and integrin activation (13). Recently, however, RIAM has been shown to be dispensable for integrin activation in platelets, suggesting the existence of a separate activator that disrupts talin autoinhibition (21,22). In this study, we present a model whereby Gα13-
mediated activation of talin, through the relief of autoinhibition, regulates integrin signaling.

A major mechanism of integrin activation in platelets is through heterotrimeric G protein signaling. In this pathway, platelet agonists bind directly to a variety of G protein-coupled receptors (GPCRs), triggering rapid recruitment and activation of circulating platelets to the site of vascular injury. Heterotrimeric G proteins consist of a complex of α, β, and γ subunits bound to their cognate GPCRs. Subsequent to GPCR activation, the three conformationally-sensitive switch regions of the G protein alpha subunit (SR1-3) permit the exchange of GDP for GTP and dissociation from the β and γ subunits (23,24). Both the α and βγ subunits can interact with downstream molecules resulting in a signaling cascade. While the switch regions are necessary for G protein activation, it is known that these regions also have a secondary function, which enables their specific recognition of the downstream effectors (25-27). Previously, the G protein subunit Go13 has been shown to engage its SR1 to directly bind to p115RhoGEF thus increasing RhoA activity leading to cytoskeletal rearrangements (25). Recently, our group reported that Go13 SR2 directly binds to talin and thereby modulates platelet integrin αIIbβ3 activation (27). This finding provided the first evidence for a Go13-talin interaction, and thus represented a novel regulatory pathway. In the present study, we propose a mechanistic basis for the ability of Go13 SR2 to activate talin; i.e., by relieving talin autoinhibition. This new mechanism for talin activation by Go13 not only has important implications regarding platelet integrin activation and in vivo thrombosis, but also underscores the critical importance of Go13 in numerous developmental and pathological states since Go13 and talin are ubiquitously expressed.

RESULTS

Go13 SR2 binds to F3 Lobe within the FERM domain of Talin: While our recent work reported direct binding of Go13 SR2 to THD (27), the location of Go13 SR2 binding site within the THD domain remained unknown. The FERM domain of talin is comprised of 4 distinct lobes: F0 (aa 1-83), F1 (aa 85-196), F2 (aa 208-306), and F3 (310-400), with flexible linker regions connecting each lobe. To identify the precise region where the SR2 binds to THD, chemical cross linking of biotinylated SR2 (VGGQSERKRWECFDSS, b-Go13SR2_pep) or SR2 scrambled peptide (GCRKFSDRQWFGSRE, b-Go13SR2_randomPeptide) to recombinant THD was employed (Figure 1A). The results shown in Figure 1B demonstrate specific cross linking of b-Go13SR2_pep to THD, while b-Go13SR2_randomPeptide revealed no significant binding. Cyanogen bromide (CNBr) digestion of crosslinked b-Go13SR2_pep and THD was performed, which specifically cleaves the C terminus of methionine residues. As these residues are rare in THD, the predicted cleavage of THD was approximated within 15 distinct regions (Table 1).

Biotinylated fragments of THD were examined by Western blotting (Figure 1C; left panel). For ease of interpretation, the smallest band at ~5 kDa containing b-Go13SR2_pep and CNBr digested THD was analyzed (Figure 1C; arrow #1). Based on the predicted molecular mass, this band could originate from 5 of 15 potential methionine-cleaved peptides present in the THD (Table 1). THD peptides that contain a lysine residue could potentially cross-link to b-Go13SR2_pep via BS3 primary amine cross linking agent, and are thus viable candidates for binding. The predicted molecular mass of b-Go13SR2_pep is ~2.3 kDa, and therefore the sum of the THD CNBr product molecular mass and the 2.3 kDa b-Go13SR2_pep should be ~5 kDa. On this basis all candidate peptides containing lysine residues and molecular weights greater than 2 kDa but less than 4 kDa were considered potential binding sites (Table 1). It can be seen that only THD fragments 2, 6, 11, 13, and 14 fit these criteria for potential binding. To supplement CNBr mapping, an additional thrombin digestion of crosslinked THD and b-Go13SR2_pep was performed. Thrombin cleaves THD at amino acid 328 (see “Thrombin Mapping” Table 2), resulting in two fragments: ~38 kDa and ~7.6 kDa. However, data shown in Figure 1C revealed full size THD as well as a band of ~38 kDa but not a 7.6 kDa band, suggesting that SR2 does not bind to the C terminal side of F3 lobe (Table 2).

To further screen the binding of b-Go13SR2_pep to THD, a dot blot analysis was performed using b-
Ga13SR2<sub>pep</sub> and talin head domain constructs lacking the F2 (ΔF2), F3 (ΔF3), and F2-3 (ΔF2-3) regions. Data shown in Figure 1D, along with the loading controls (Figure 1E), illustrate that b-Ga13SR2<sub>pep</sub> requires a sequence contained within the F2-3 lobes for binding to THD (Left Panel). Furthermore, separate dot blots (Right Panel) demonstrated that F3 is indeed required for SR2 binding. Based on these findings, the CNBr fragments 2, 6, and 11 were ruled out because they do not incorporate F3 (Table 1). Further dot blot analysis also eliminated a portion of F2 and F3 lobes that is represented by the CNBr 13 fragment. Taken together, these data indicate that the candidate amino acid residues reside within the 310-328 region of THD.

This putative binding region within THD was further validated by ELISA measurements by quantifying b-Ga13SR2<sub>pep</sub> binding to recombinant THD carrying F3 mutations at residues M319 and L325. These residues have been examined by others and shown to play a role in maintaining talin autoinhibition as well as talin-mediated clot retraction (10,15,28). As a negative control, amino acid E335 that does not fall within the predicted 310-328 binding region for b-Ga13SR2<sub>pep</sub> was also mutated. In these experiments, b-Ga13SR2<sub>pep</sub> was used to coat the ELISA plate and binding to THD constructs (analyte) was measured using a head domain specific antibody. The results shown in Figure 1F demonstrate that the talin residues 319 and 325, but not 335, are important for Ga13SR2 binding THD.

Finally, the putative SR2 binding region within THD was confirmed using the mass spectrometry based mapping of the cross-linked THD complex with biotin-Ga13SR2<sub>pep</sub>. The unique peptides obtained after mass spectrometry with the highest fidelity contained amino acids 307-338. This sequence encompasses completely with the 310-328 region identified using the collective data derived from the CNBr peptide mapping, thrombin peptide mapping, deletion mutagenesis, and point mutagenesis experiments (Table 2). Taken together, these results identify a specific Ga13 SR2 binding site within the THD domain. Importantly, this binding site includes the critical M319 and L325 residues that are present on a flexible region in the F3 lobe that directly engages the rod domain and promotes talin autoinhibition (Figure 1G) (15,16,28-30). Guided by these findings, we elected to examine the mechanism by which Ga13 modulates talin autoinhibition.

**Ga13 SR2 Alters Talin Conformation:** A schematic diagram illustrates critical regions within talin’s full length structure that participate in talin’s dynamic state of autoinhibition (Figure 2A). Specifically, when talin F3 and R9 (light red) bind to one another the crucial integrin binding site present within the F3 lobe is obscured, thereby creating the autoinhibited conformation (5,8,12,30,31). Importantly, the Ga13 SR2-talin binding site (see Figure 1G) overlaps with the talin autoinhibitory binding site created by the interaction of F3 and R9. This juxtaposition of the Ga13 SR2 and F3-R9 binding regions suggests Ga13 may play a role in relieving autoinhibition by inducing a conformational change in talin.

To examine the possibility that Ga13 can relieve talin autoinhibition, a competitive binding assay was performed. GST fusion proteins containing the SR2 sequence (GST- Ga13SR2) or scrambled sequence (GST- Ga13SR2<sub>random</sub>) were incubated with recombinant THD. Recombinant talin rod domain containing the autoinhibitory region (R9-12) or a non-inhibitory protein (BSA) was added to their respective samples. If R9-12 and Ga13SR2 compete for an overlapping binding region on THD, then a decrease in THD binding to GST- Ga13SR2 should be observed (Figure 2B). Alternatively, a decrease in THD binding to GST- Ga13SR2 would also be observed if the binding to GST- Ga13SR2 to THD caused a conformational change in THD such that it could no longer bind R9-12. On the other hand, if R9-12 and Ga13SR2 do not share a binding site on THD, or if Ga13SR2 does not allosterically interfere with THD-R9-12 binding, then a ternary complex would be expected to form between R9-12 and THD. The data shown in Figure 2C (lane 1) demonstrate that GST-Ga13SR2 binds directly to THD, while GST- Ga13SR2<sub>random</sub> did not (lane 2). Recombinant R9-12 was added at either 50 μM, 25 μM, or 5 μM as shown in lanes 3, 4, and 5 (Figure 2C), respectively. A dose-dependent decrease in the THD binding to GST-Ga13SR2 was observed without any detectible R9-12 binding to GST-Ga13SR2. These results demonstrate...
Gα13SR2 significantly reduces the ability of R9-12 to bind to THD, suggesting that Gα13SR2 can interfere with the autoinhibited conformation of talin.

While these data suggest Gα13 may activate talin, few experimental options are available that can test the effect of ligands on the full length talin molecule. To investigate this model further, we devised an assay to evaluate the conformational state of full length talin isolated from human platelets. To examine if Gα13 binding triggers conformational changes in full length talin, a limited proteolysis protection assay was performed. The limited proteolysis approach utilizes a small amount of protease that has little detectible effect on structurally stable proteins. The dimer fraction of platelet talin is reported to exhibit a dumbbell dimer conformation in an autoinhibited state (14,16); thus if Gα13SR2 perturbs this state, talin may become more vulnerable to limited proteolytic degradation. To validate this model, the dimeric fraction of platelet talin was purified and then pre-treated with Gα13SR2Pep or Gα13SR2RandomPep. Next, the complex was exposed to decreasing amount of the protease Endo-AspN, and proteolysis was analyzed by Western blotting (Figure 2D). The results show that with increasing concentration of protease, there is an increase in the level of talin degradation when treated with Gα13SR2Pep but not with Gα13SR2RandomPep (Figure 2D, left panel). This observation suggests that talin is more sensitized to proteolysis after treatment with the Gα13SR2Pep likely due to a change in its conformation.

To further corroborate these findings, we examined a known activator of talin; the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2). PIP2 accumulation in the plasma membrane adjacent to integrin complex is thought to repel the negatively charged autoinhibitory rod domain, and simultaneously pull the positively charged face of talin’s head domain, in a “Push-Pull” mechanism to facilitate talin activation (20). As expected, the addition of monomeric PIP2 at a concentration below the critical micelle concentration was able to increase Gα13SR2-facilitated talin degradation (Figure 2D, center panel). The addition of PIP2 and Gα13SR2Pep had a greater effect on talin degradation than Gα13SR2RandomPep and PIP2 (Figure 2D). Since Gα13SR2RandomPep had no significant effect on talin degradation, all observed proteolysis (Figure 2D, lanes 7 and 8) is presumably attributable to PIP2. These data are consistent with the notion that Gα13SR2 triggers a conformational change in talin, which is significantly more efficient when added in combination with PIP2. This observation suggests a possible cooperative effect whereby the combination of Gα13 and PIP2 may enhance integrin activation.

Direct Binding of Full Length Gα13 to Talin: While Gα13SR2Pep has demonstrable binding to THD, full length Gα13 binding to both THD has yet to be examined outside of the cellular milieu. To further characterize Gα13 interaction with talin, recombinant full length Gα13 purified from SF9 insect cells was used, as previously described (32). Gα13 pre-treated with GDP (inactive) bound significantly less to GST-THD than GTPγS-treated Gα13 (active), consistent with previous findings (Figure 2E) (25). In both cases, no significant binding to GST was observed. An ELISA was used to estimate the affinity between Gα13 and THD, yielding a dissociation constant (Kd) of 13.4 μM. However, the physiological relevance of this affinity will require further characterization of their respective interactions in a milieu more closely resembling the plasma membrane microenvironment.

To further characterize the competition between Gα13 and talin domains, we used a GST-THD fusion construct as bait, and examined its interaction with full length Gα13 in the presence of talin R9-12 module (See model in Figure 2F). To obtain a sufficient amount of purified Gα13 required for this assay, we expressed a chimeric Gα13 subunit containing the N terminal domain of Gα13 (See Methods), as previously described (33). Gα13 in an active GTP bound state interacts with GST-THD (Figure 2G, Lane 1). We then measured Gα13 binding to THD in the presence of talin R9-12 module. Gα13 binding to GST-THD was measured in competition with talin R9-12 at increasing concentrations of 5 μM, 10 μM, 20 μM, 30 μM, and 50 μM of talin R9-12 (Figure 2G, Lanes 2-6). Moreover, biochemical
interaction of inactive $\alpha_{13}$ (30 $\mu$M) bound to GDP was measured with GST-THD in the presence of 30 $\mu$M talin R9-12 (Figure 2G, Lane 7). These results demonstrate that as the binding of talin R9-12 increases, the binding of $\alpha_{13}$ to THD decreases. In contrast, inactive GDP bound $\alpha_{13}$ has little effect on talin R9-12 binding to THD (Figure 2G, lane 7). These data suggest that $\alpha_{13}$ binding to THD occurs at the expense of talin rod binding in a GTP dependent manner. Taken together, these results (Figures 1 and 2) demonstrate a GTP-dependent binding of $\alpha_{13}$SR2 to THD, which competes with the talin rod domain regions responsible for maintaining an autoinhibited state.

**Endogenous $\alpha_{13}$ Changes Talin Conformation in a Cellular Milieu:** While talin activity is often measured indirectly through integrin activation, few options exist that can examine its conformational state directly. Due to the importance of talin in the regulation of integrin activation, we sought to develop a technique that could directly assess gross changes in talin’s conformational state. While it is unclear whether GFP-talin exists as an autoinhibited dimer in CHO cells, it has been shown that GFP-talin causes a low basal level of integrin activation as compared to the GFP-talin head domain (15). Further, a single amino acid mutation of talin’s head domain, M319A, increases integrin activation compared to both wild type full length GFP-talin and GFP-THD (15,31). Additionally, it was shown that the co-transfection of GFP-talin with $\alpha_{13}$, but not SR2 mutants R227A or R232A, increased integrin activation (27). These observations suggest that GFP-talin is partially, if not completely, autoinhibited when overexpressed in this model cell system. To characterize the autoinhibited state of talin in the cellular milieu, we devised a FRET-based assay to assess the effect of $\alpha_{13}$ on integrin activation. Detailed technical aspects of this experimental approach are discussed in the “Methods” section. Briefly, CCPGCC amino acids were engineered into three separate GFP-talin constructs at amino acids 1457, 1655, and 1822 (Figure 3A). In an autoinhibited state, GFP can act as a donor molecule to a cell permeant fluorescent dye known as ReAsH that binds CCPGCC (Figure 3B). The FRET signal should significantly diminish in an activated/elongated state (Figure 3B). The specificity of ReAsH binding was evaluated by immunofluorescence microscopy. Cells transfected with GFP-talin without a CCPGCC motif showed no significant red fluorescence background signal (Figure 3C; Left Panels) as compared to GFP-talin with a CCPGCC motif at amino acid 1822 (GFP-talin-1822) (Figure 3C; Right Panels). These observations demonstrated successful engineering of the CCPGCC motif in talin and highly specific binding of the dye under these conditions.

Next, we elected to measure talin’s conformational state using flow cytometry. This technique was employed due to its sensitivity to even weak fluorescence signals while simultaneously examining large populations of cells to increase the likelihood of detecting FRET positive cells. Flow cytometry utilized only the 488 nm laser to eliminate potential inappropriate activation of ReAsH. The 488 nm laser is sufficient to excite GFP, with only a 2% spectral overlap with the excitation spectrum of ReAsH. The gating of CHO cells, shown in Figure 3D, indicates the distinction whereby the R1 represents the general population of CHO cells, while R2 and R3 show FITC or PE positive cells, respectively. To confirm that no ReAsH signal was detected in the absence of FRET, a tubulin construct that contains the CCPGCC motif, and can therefore bind to the red fluorescence dye, was utilized. ReAsH emission was expected to emit at ~615 nm, which was detected using a Texas red filter (PE). Since only the 488 nm laser was utilized, no red fluorescence signal should occur under these conditions. The results showed no detectable PE signal in cells expressing tubulin containing a CCPGCC motif (Figure 3E; Panel #1). Thus, any signal detected with the GFP-talin constructs was attributed to FRET pairing under these conditions.

To account for any nonspecific ReAsH signal, a second control, GFP-talin (without a CCPGCC motif) pre-incubated with ReAsH dye was used to gate all GFP+/FRET-cells (Figure 3E, Panel #2). GFP-talin signal (R2) and any signal that appeared on the PE axis (R3) was considered emitting from FRET pairing. Next, each GFP-talin construct containing a CCPGCC motif was tested for FRET signal. Data shown in Figure 3E (panels #3-5) and Figure 3F clearly demonstrate a trend of increasing FRET signal as the CCPGCC motif is
incorporated towards the C terminus of talin. The largest FRET signal was detected when the CCPGCC motif is inserted at amino acid 1822 of talin. This sequence is located immediately after the autoinhibitory R9 domain (Figure 2A), thus supporting the hypothesis that talin is in an autoinhibited state permitting FRET pairing under these conditions. To further investigate the specificity of this interaction, GFP-talin-1822 was mutated at residues M319A and L325K. The M319A mutation has been shown to increase integrin activation nearly threefold as compared to native talin sequence, suggesting that this amino acid is critical for maintaining autoinhibition, and when mutated permits hyperactivation of talin. The results shown in Figure 3G demonstrate a 30% decrease in FRET signal in GFP-talin-1822-M319A construct, while GFP-talin-1822-L325K showed no significant decrease in the FRET signal. Collectively, these data indicate that GFP-talin undergoes FRET pairing when autoinhibited, and can be specifically disrupted by mutating a single amino acid that is critical at the F3/R9 interface.

Next, we investigated talin activation through protein-protein interactions rather than relying only on point mutations. This approach was accomplished by co-transfecting RIAM, a known activator of talin, with GFP-talin-1822. RIAM binding to the F3 lobe of talin FERM domain is sufficient to relieve its autoinhibition, and therefore RIAM was used to validate the assay (13). Co-transfection of RIAM resulted in a 50% reduction of FRET signal (Figure 3H), suggesting a conformational change in talin. Previous studies have shown an increase in integrin activation when RIAM and talin are co-expressed (13,34,35), which is consistent with the observed decrease in the FRET signal.

To examine the effect of Ga13 on talin, GFP-talin-1822 was co-transfected with either wild type Ga13 or Ga13 SR2 mutant R227A (Figure 3I). Similar to RIAM, wild type Ga13 showed a 50% decrease in the FRET signal suggesting a conformational change in talin that suppressed FRET pairing. The specificity of Ga13/talin interaction was further validated through the Ga13 SR2 R227A mutation. In this case, the FRET signal was slightly reduced but was not significantly different from the GFP-talin-1822 control (Figure 3I).

Together, these data suggest the following: 1) GFP-talin exists in an autoinhibited state in the CHOAS cell system, 2) FRET pairing is attained when the red fluorescence acceptor molecule is placed close to the autoinhibitory R9 region, 3) the conformation of talin is altered by single amino acid changes that are known to relieve autoinhibition, and 4) co-expression of talin activating molecules such as RIAM and Ga13 trigger a decrease in FRET signal suggesting a critical role of these molecules in talin regulation. The FRET based methodology, as outlined here, offers a novel experimental tool for detecting gross GFP-talin conformational changes in a cellular milieu, and can be used to screen potential activators of talin. In summary, we conclude that Ga13 plays a significant role in regulating the dynamic conformation of talin.

Endogenous Ga13 Facilitates Talin Activation in CHOAS Cells: Our results thus far suggest that Ga13 plays a functional role in relieving talin autoinhibition. Next, we assessed the role of Ga13 and GFP-talin interaction in cell morphology. Patterned on the FRET assay, the CHOAS cells were used as the model system that most closely approximates integrin activation in platelets. CHOAS cells were transfected with Ga13, Ga13 R227A, GFP-talin, GFP-THD, or a combination of Ga13 and GFP-talin. After 24 hours, the cells were plated on fibrinogen-coated slides for 15 minutes and imaged using immunofluorescence microscopy. Data in Figure 4A (top 3 panels) show that transfection of Ga13 + GFP, Ga13 (R227A) + GFP, and GFP-talin yielded no significant difference in cell spreading. Upon co-expression of Ga13 + GFP-Talin, a significant increase in cell spreading occurred, which was reduced upon co-expression of Ga13 (R227A) + GFP-Talin. These results show a significant increase in spreading only when Ga13 and GFP-Talin are expressed together, and that this cell spreading is SR2 dependent. Next, we examined the effect of GFP-Talin-M319, which earlier showed a decreased FRET signal indicating an active state. As expected, this point mutation alone, Talin-M319, increased cell spreading similar to the level observed with Ga13 + GFP-Talin. To investigate if Ga13 could enhance this phenotype, Ga13 and GFP-Talin-M319A were co-transfected. No increased spreading was observed indicative of talin conformation that is already constitutively active. Lastly, we examined the
effect of GFP-THD on cell spreading. An increase in cell spreading was observed that is similar to that of Gα13 + GFP-Talin as well as GFP-Talin-M319A. Similar to the effect of GFP-Talin-M319A, co-transfection of Gα13 showed no significant impact on cell spreading consistent with the constitutively active state of GFP-THD. Altogether, these data demonstrate that: 1) Gα13 mediated activation of talin leads to increased cell spreading, while the Gα13 SR2 mutant cannot, and 2) talin M319A and THD are constitutively active for cell spreading and do not benefit from the co-expression of Gα13.

To examine the specific localization of the Gα13/talin/integrin complex, immunofluorescence analysis was performed. Gα13 or Gα13 R227A were transfected with GFP-talin into CHOAS5 cells (Figure 4C and 4D). Subsequently, co-localization of β3 integrin and GFP-talin (Figure 4C) or Gα13 and GFP-talin (Figure 4D) was examined. The β3 integrin (Red) and GFP-talin (Green) co-localized (Yellow) in punctate foci throughout the cell when wild type Gα13 was expressed (Figure 4C, top panels). However, upon expression of Gα13 R227A (Figure 4C; middle panels), β3 integrin did not co-localize with GFP-talin as evident by the diffuse non-specific overlapping signal. An enlarged view of the data shown in Figure 4C (Panels A and B) is highlighted in the bottom left and right panels, respectively. These data indicate the functional importance of Gα13 SR2 in determining the intracellular localization of the talin-β3 integrin complex. It can also be seen that GFP-talin (Green) and Gα13 (Red) co-localized (Yellow) when Gα13 was co-transfected (Figure 4D, top panels). When Gα13 R227A was co-transfected (middle panels), there was significantly less overlap between Gα13 R227A and GFP-talin (Figure 4D), indicating a decreased co-localization. An enlarged view of the data shown in Figure 4D (Panels A and B) is highlighted in the bottom left and right panels, respectively. Collectively, these data demonstrate: 1) Gα13 co-localizes with talin directly; 2) Gα13 SR2 mutant R227A prevents talin/Gα13 co-localization; and 3) Gα13 promotes talin localization to integrin αIIbβ3. The striking cooperation between Gα13 and talin led us to evaluate several ex vivo and in vivo models of talin-integrin function to juxtapose in vitro data in the context of physiological significance.

**Gα13 SR2 Modulates ex vivo Platelet Adhesion, Clot Formation, and in vivo Thrombosis:** Platelets serve as a useful model system for assessing the talin activity since aggregation, integrin activation, and clot retraction rely on activated talin molecules. Our previous work demonstrated that myristoylated SR2 peptide (Gα13SR2Pep) inhibits platelet aggregation in response to all agonists tested (27). This global inhibition of platelet aggregation was attributed to inhibition of αIIbβ3 integrin activation (27). Since the biochemical evidence indicates that talin activation can be inhibited by Gα13 SR2, talin-dependent physiological processes are expected to be affected by Gα13 SR2 inhibition as well. To further characterize the effects of Gα13 SR2 on platelet physiology, in vitro adhesion and thrombus formation induced by collagen were assessed. Platelets in whole human blood were pre-treated with Gα13SR2Pep or Gα13SR2RandomPep, perfused over collagen, and analyzed for platelet thrombi formation. A significant reduction in the size and number of platelet thrombi was observed in the Gα13SR2Pep treated blood relative to the Gα13SR2RandomPep treated blood under physiological conditions of shear stress (Figure 5A). These results are consistent with our previous data showing platelet aggregation induced by CRP (collagen related peptide) is inhibited by Gα13SR2Pep (27).

A second talin-dependent physiological process we investigated is clot retraction. Clot retraction requires talin to link the cytoskeleton to integrins in a bidirectional signaling process (28,36). This signaling mechanism provides mechanical response required to reduce the overall size of clots thus restoring normal blood flow. Interestingly, a point mutation in the F3 lobe of THD at amino acid 325 has been shown to cause impaired clot retraction (28). As this critical residue overlaps with the putative binding site of Gα13SR2Pep to THD (Figure 1), it might be expected that Gα13SR2 would play a functional role in regulating clot retraction. To examine this possibility, platelet-rich plasma was pre-treated with Gα13SR2Pep or Gα13SR2RandomPep, and clotting was induced with thrombin (Figure 5B). Thrombus retraction occurred in
an upward direction \textit{in vitro}, and the degree to which it retracted was measured by the volume of liquid beneath. A significant reduction in clot retraction occurred in G\(\alpha_{13}\)SR2\textsubscript{pep} but not in G\(\alpha_{13}\)SR2\textsubscript{RandomPep} treated platelets (Figure 5B). The observed clot retraction defect induced by G\(\alpha_{13}\)SR2\textsubscript{pep} is consistent with previously reported mutations in talin as well as the notion that an unperturbed talin-integrin linkage is necessary to promote effective mechanical stress for regulating the clot retraction. Taken together, G\(\alpha_{13}\)SR2\textsubscript{pep} is able to significantly reduce platelet binding to collagen under physiological shear stress, reduce platelet clot retraction, and is a potent inhibitor of platelet aggregation as previously reported. These \textit{in vitro} data compelled us to examine the functional role of SR2 peptide \textit{in vivo}.

Talin-mediated integrin activation is directly involved in primary thrombus formation and the prevention of blood loss upon vascular damage. Furthermore, point mutation in the talin F3 lobe at amino acid L325 is known to cause impaired integrin activation and increased bleeding times (28). Based on this observation, we examined the effect of G\(\alpha_{13}\)SR2\textsubscript{pep} on bleeding times in mice pre-treated with either vehicle, aspirin, G\(\alpha_{13}\)SR2\textsubscript{pep} or G\(\alpha_{13}\)SR2\textsubscript{RandomPep} (Figure 5C). The results showed a modest but significant increase in bleeding when treated with G\(\alpha_{13}\)SR2\textsubscript{pep} as compared to the vehicle or M-G\(\alpha_{13}\)SR2\textsubscript{RandomPep} (Figure 5C). Interestingly, the G\(\alpha_{13}\)SR2\textsubscript{pep} induced increase in the bleeding time is relatively less than that observed with aspirin alone, even though previous experiments have shown that G\(\alpha_{13}\)SR2\textsubscript{pep} is more effective in blocking platelet aggregation than aspirin (27). This functional divergence in pharmacological activity suggests that the ability to target talin may serve as a more effective therapeutic modality than aspirin, which produces a more severe bleeding phenotype.

Finally, to test the effect of G\(\alpha_{13}\)SR2\textsubscript{pep} or G\(\alpha_{13}\)SR2\textsubscript{RandomPep} on thrombus formation \textit{in vivo}, we employed a well-established laser-induced thrombosis model (37,38). Importantly, the G\(\alpha_{13}\)SR2\textsubscript{RandomPep} treated mice generated larger thrombi than mice treated with G\(\alpha_{13}\)SR2\textsubscript{pep} (Figure 5D and Supplemental Movies 1 and 2). Thrombus formation \textit{in vivo} was analyzed over 140 seconds, and representative pictures of the first 90 seconds are shown (Figure 5D). In summary, these data suggest that G\(\alpha_{13}\)SR2\textsubscript{pep} blocks talin activation resulting in defective platelet adhesion to collagen and clot retraction, increased bleeding time, and decreased \textit{in vivo} thrombus formation.

\textbf{DISCUSSION}

G protein signaling through G\(\alpha_{13}\) has been an area of intense interest recently because of its broad physiological and pathological implications. G\(\alpha_{12}\) and G\(\alpha_{13}\) are often paired together as having similar properties; however, it is quite clear that G\(\alpha_{13}\) plays a far more critical role in numerous developmental and pathological processes. For example, systemic knockout of GNA13 is lethal at the embryonic stage while GNA12 is not (39). Similarly, conditional knockout of G\(\alpha_{13}\) produces hemostatic defects while knockout of G\(\alpha_{12}\) is without such effect (40). Therefore, crucial functional and signaling differences must exist between the two closely related G proteins. In this context, it is known that G\(\alpha_{13}\) plays an important role in the regulation of RhoA activity in a variety of cells, notably through its switch region 1 (SR1). In platelets, G\(\alpha_{13}\)/RhoA activity is primarily modulated through protein-activated receptor (PAR) GPCR signaling (25), but this pathway alone fails to explain the global importance of G\(\alpha_{13}\) in a broad spectrum of biological processes including neurogenesis, embryogenesis, hemostasis, vascular defects and impaired chemokinesis, among others (39,40). Furthermore, unlike most other heterotrimeric G proteins, G\(\alpha_{13}\) is ubiquitously expressed in all cell lines and in all mammalian species. This conservation of G\(\alpha_{13}\), and in particular the conservation of its switch regions, throughout the evolutionary process indicates that G\(\alpha_{13}\) functions in an extremely important capacity in cell development and disease processes. Since the importance of integrin signaling is also of global biological significance, and integrin signaling and G\(\alpha_{13}\) signaling share many biological profiles, we recently investigated whether there is a common mechanistic link between each signaling process. In this regard, we recently reported that G\(\alpha_{13}\) directly binds to another protein that is also embryonically essential and ubiquitously expressed, i.e., talin. Specifically, it was
found that the highly conserved switch region 2 of Gα13 forms a complex with talin’s head domain (THD), also called the FERM domain, and the disruption of this interaction resulted in global inhibition of human platelet aggregation. While these findings provided the first evidence for a novel Gα13-talin signaling pathway, they did not define the underlying mechanism by which Gα13 functions to activate talin-mediated integrin signaling. In this study, we provide a molecular mechanism by which SR2 of Gα13 binds to the talin head domain and disrupts the autoinhibited state of talin.

Our first series of experiments of demonstrated that Gα13SR2rep recognized a single binding site on THD that is critical for talin autoinhibition. Point mutagenesis tests further confirmed these findings. Next, the GST-SR2 fusion proteins were found to sterically interfere with talin’s rod domain R9-12, and similar results were obtained using the full length recombinant Gα13. Finally, we demonstrated that Gα13SR2rep could sensitize full length talin to limited proteolysis, which increased upon the addition of PIP2, suggesting a possible cooperative effect of two modulators. These observations are similar to those attributed to RIAM, which binds and interferes at the F3/Rod autoinhibitory domain on THD, suggesting Gα13 may act in a parallel or perhaps compensatory manner (13,21). If the Gα13/RIAM binding sites on talin overlap, it would be reasonable to suggest that SR2 peptide would not only inhibit Gα13 from binding and activating talin, but prevents RIAM from doing so as well. Future studies into their respective pathways, presumably requiring conditional knockout mouse models, may clarify some of these questions. Interestingly, Gα13 binding to a FERM domain is not without precedent. Gα13 binding to the N terminal half of Radixin, which contains a highly similar sequence to THD identified by CNBr and mass spectrometry data (41). Gα13 binding to Radixin also induces a conformational change; therefore, Gα13 may have a dynamic role in the regulation of FERM domain containing proteins.

In this study, several experimental approaches were used to investigate the functional role of Gα13 in regulating talin conformational changes. In one case, an N terminal GFP tagged full length talin with a calpain-deficient mutation (L432G) was utilized (42). The calpain-deficient mutation was necessary to clarify the open conformation of talin separated by the head and rod domains due to potential proteolysis by calpain. Moreover, a tetracysteine CCPGCC motif capable of binding to an arsenical dye ReASH was introduced into the talin rod domain. ReASH was selected due to the relatively low probability of perturbing talin function with the insertion of small amino acid motifs. Additionally, ReASH was a preferable alternative to adding a second fluorophore at the C terminus of talin often employed by other techniques such as the bimolecular fluorescence complementation (BiFC). Finally, ReASH provides more room for error in the placement of a fluorescence probe than the traditional FRET pairs that display activity at ~10Å, thus increasing the chances of detecting signal without placing the probe in a location which could perturb the functionality of talin. While this assay is designed to examine gross talin conformation, it cannot distinguish between the multitude of inhibited states such as originating from the “donut” model elucidated through small angle x-ray scattering (SAXS) (16), the “dumbbell model” (14), or the “triple domain” comprised of the autoinhibitory rod domains (17). Talin is dynamically regulated by a multitude of proteins, and our FRET assay used in this study demonstrates the importance of Gα13 in these processes, more specifically through switch region 2. Importantly, these data agree with our previous integrin activation studies (27). A decrease in FRET signal was observed after co-transfection of GFP-talin-1822 and Gα13 as compared to the Gα13 R227A mutant (Figure 3I). The reduced FRET signal indicates a switch to an active talin conformation, which agrees with our previous study where Gα13 co-transfection with GFP talin has a greater degree of integrin activation than Gα13 R227A (27).

To demonstrate the effect of full length Gα13 in the cellular milieu, the CHOAS cells were co-transfected with Gα13 and GFP-talin. CHOAS cells expressing both Gα13 and GFP-talin showed relatively larger surface area as compared to cells expressing each construct individually (Figure 4A & B). The effect of Gα13 and GFP-talin co-expression was comparable
to a constitutively active version of talin, GFP-THD. The SR2 mutant, R227A, showed a phenotype similar to either GFP-talin or G\(\alpha_{13}\) alone. These results are consistent with our previous findings where PAC1 binding to CHO A5 cells, reflecting integrin activation, increased after co-transfection of G\(\alpha_{13}\) and GFP-talin but not R227A or R232A SR2 mutants (27). The observed effects of G\(\alpha_{13}\) and talin on cell morphology were also in agreement with their co-localization pattern. Altogether, these data suggest that expression of G\(\alpha_{13}\) can increase cell spreading, but only when full length talin is co-expressed. In platelets, the copy number of talin to G\(\alpha_{13}\) is ~8:1; therefore, this overexpression system does not serve to elucidate precisely what occurs in vivo, but rather to establish that there is an observable co-localization of G\(\alpha_{13}\) with talin that yields increased integrin activation and spreading.

The critical role of G\(\alpha_{13}\) SR2 in the regulation of talin autoinhibition led us to evaluate its translational potential in platelets as a model system. Treatment of whole blood with myristoylated SR2 peptide showed a significant, but not complete, inhibition of platelet adhesion and aggregate size when perfused over collagen as a substrate under physiological conditions of shear stress (Figure 5A). These results provide evidence that platelet adhesion and recruitment on a collagen surface occur through G\(\alpha_{13}\) SR2-mediated events. Similarly, the clot retraction in thrombin-stimulated platelets showed a significant defect when G\(\alpha_{13}\) SR2-talin signaling was blocked with G\(\alpha_{13}\)SR2PeP, which is consistent with the notion that G\(\alpha_{13}\) SR2 plays an important role in this talin-dependent process. Furthermore, in vivo studies in mice demonstrated that infusion of G\(\alpha_{13}\)SR2PeP did, as might be expected, increase bleeding time. However, it is noteworthy that this increase was significantly less than that produced by aspirin infusion, even though ex vivo platelet aggregation studies revealed the reverse profile, i.e., profound inhibition of aggregation with G\(\alpha_{13}\)SR2PeP and only modest inhibition with aspirin (27). This divergence in pharmacological activity suggests that therapeutic targeting of G\(\alpha_{13}\) SR2 may produce an improvement over some prevailing anti-thrombotic methodologies. Finally, the laser-induced thrombosis in mice treated with G\(\alpha_{13}\)SR2PeP showed a significant decrease in both thrombus size and footprint on the vessel wall (Figure 5D). This observation is consistent with our previous in vitro collagen results regarding the critical contributions of G\(\alpha_{13}\) SR2-talin signaling to both platelet adhesion and platelet recruitment.

While reporting our initial G\(\alpha_{13}\) and talin binding studies (27), we demonstrated that G\(\alpha_{13}\) SR2 is linked to talin-mediated integrin activation via the inside-out signaling pathway. Additional results also suggested that G\(\alpha_{13}\) SR2-talin signaling may be involved in the process cellular adhesion (27). The present results are consistent with a role for G\(\alpha_{13}\) SR2 in each of these processes, since they provide evidence that G\(\alpha_{13}\) SR2-talin signaling participates in both platelet adhesion and platelet recruitment to the site of vascular damage. Our model differs from the previous studies (43-45) where the focus was primarily on the inside-in signaling mediated through the direct interaction of G\(\alpha_{13}\) SR1 with \(\beta\)3 integrin. It is to be noted that in the previous study (45), a 6 amino acid peptide (FEEREA) derived from \(\beta\)3 integrin was used as an inhibitor of integrin activation and laser-induced thrombosis in vivo. In fact, only 5-amino acid (EEERA) peptide derived from \(\beta\)3 integrin was sufficient to bind G\(\alpha_{13}\) (45). However, a simple database BLAST search of EEERA sequence reveals hundreds of identical hits, including several in proteins expressed in platelets. In contrast, in the present study, we used a unique 17 amino acid peptide (SR2) derived from G\(\alpha_{13}\) as an inhibitor of multiple platelet functions, including aggregation, adhesion, clot formation, bleeding time, and laser-induced thrombosis in vivo. Future studies will be required to reconcile the functional role of G\(\alpha_{13}\) in inside-out and outside-in signaling pathways, with a particular emphasis on the validity of highly charged and relatively small 5 amino acid peptides of \(\beta\)3 integrin as specific binders of G\(\alpha_{13}\) and their potential utility as anti-thrombotic leads with reduced bleeding diathesis.

In summary, our findings unveil a novel molecular mechanism of G\(\alpha_{13}\) as a regulator of talin activation by relieving its autoinhibition. These studies suggest that the G\(\alpha_{13}\) SR2-talin activation pathway may represent a global integrin signaling process.
Because of the highly conserved nature of G$\alpha_{13}$, talin, and integrins, the broad implications of this regulatory mechanism may thus extend to a multitude of cell types and pathologies.

**MATERIALS AND METHODS**

*Protein Expression and Purification:* GST-G$\alpha_{13}$SR2 or GST-G$\alpha_{13}$SR2$_{\text{Random}}$ fusion proteins were generated as previously described (27). Talin Head Domain (THD) constructs were cloned using pET15b as a backbone containing amino acids 1-400. QuikChange mutagenesis was employed to construct THD mutants M319A, L325K, and E335R. Inverse PCR deletion mutagenesis was used to create THD $\Delta F2$, $\Delta F3$, and $\Delta F2-3$ constructs. For the recombinant talin rod domain constructs, R9-12 (residues 1654-2344) and R13 (residues 2338-2541) were cloned into a pLIC-His vectors as previously described (46,47). Similarly, the GST-THD was cloned into pLIC-GST vector. All THD and rod domain constructs were purified using BL21 strain *E. coli*, 0.2 mM IPTG induction for 3 hours at 37°C, lysis using tip sonication, and purification using a Ni-NTA FastFlow column (GE LifeSciences). Recombinant G$\alpha_{13}$ was purified as previously described using SF9 insect cells (32). Baculovirus constructs containing murine G$\alpha_{13}$, G$\beta$, and 6xhistidinetagged G$\gamma$ constructs were generously provided by Dr. Tohru Kozasa. Briefly, 3 baculovirus containing G$\alpha_{13}$, G$\beta$, and G$\gamma$ were used to co-infect logarithmic phase SF9 cells in SF900II (GE LifeSciences) media for 48 hours. The membrane fractions were isolated as previously described (32), purified using a Ni-NTA FastFlow column, and G$\alpha_{13}$ was eluted using AlF$_3$-GDP. Purification of full length native talin from human platelets was performed as previously described (48), with the following modifications: ion exchange chromatography employed Source15Q resin (GE LifeSciences) for anion exchange, followed by MonoS resin (GE LifeSciences) for cation exchange, followed by S200 resin gel filtration to isolate talin dimer fraction. The S200 column was calibrated using a high molecular weight gel filtration calibration kit, using ferritin (mw = 440 kDa) as a marker for dimeric talin (GE HealthScience).

**THD Cross-Linking and Peptide Mapping:** Recombinant THD at a concentration of 5 mg/ml was cross-linked to biotinylated SR2 peptide (VGGQRSEKRWFECFDS, b-G$\alpha_{13}$SR2$_{\text{pep}}$) or SR2 scrambled peptide (GCRKEVFDQWGSRE, b-G$\alpha_{13}$SR2$_{\text{RandomPep}}$) (27) using BS$_3$ homo-bifunctional amine cross-linker (ThermoFisher Scientific). The reaction was quenched in 50 mM Tris, and resolved with a pre-cast 12% SDS-PAGE (BioRad). Specific cross-linking was determined using a Streptavidin-HRP antibody and resolved with a chemiluminescent substrate (Pierce). Cyanogen bromide cleavage was performed as previously described (49). Briefly, 5 mg/ml THD cross-linked with b-SR2 was added to a solution of 70% formic acid containing 0.1 M cyanogen bromide and incubated for 24 hours at room temperature. After TCA precipitation, fragments were resolved using a pre-cast 4-20% gradient SDS-PAGE. Bands containing the cross-linked peptide were examined using Streptavidin-HRP antibody and resolved with chemiluminescent substrate (Pierce). Additionally, thrombin (0.01 U/ml) was used to cleave THD which is specific for one site in the F3 domain. Samples prepared for mass spectrometry were cross-linked as described above using THD, b-G$\alpha_{13}$SR2$_{\text{pep}}$, and BS$_3$. Biotinylation modifies the primary amine of the SR2 peptide, leaving a single lysine as a substrate for amine cross-linking. The reaction was resolved using a pre-cast 12% SDS-PAGE, and the band that was ~2.0 kDa larger than THD treated with BS$_3$ alone was excised using a sterile scalpel. Samples were examined at the Taplin Mass Spectrometry Core Facility at Harvard Medical School. Unique THD sequences cross-linked to SR2 were identified using a Sequest algorithm, tracking the sequence “BS$_3$-KR.”

**Quantification of THD Mutants by ELISA:** b-G$\alpha_{13}$SR2$_{\text{pep}}$ or b-G$\alpha_{13}$SR2$_{\text{RandomPep}}$ were added to Nunc-Immulon wells (Dynatech) at a concentration of 250 nM in 10 mM NaHCO$_3$/Na$_2$CO$_3$, pH = 9.4, in a volume of 100 µl, and incubated overnight at 4°C. Wells were washed with blocking buffer (PBS, 2% BSA, 0.05% Tween 20). Even distribution of peptide was verified using...
Streptavidin-HRP and TMB Substrate (Pierce) detection. Wells were blocked for 1 hour at room temperature, followed by the addition of 2.5 μM of THD constructs; WT, M319A, L325K, and E335R. Each well was washed, and signal detected using an in-house antibody specific against the head domain of talin (Rb79).

**GST Peptide Binding and Competition Assays:** GST fusion GST-Gα13SR2 or GST-Gα13SR2 Random at a concentration of 2.5 μM was incubated with GSH resin (GE Healthcare Life Sciences) in the binding buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1.0 mM MgCl₂, 1.0 mM DTT, 0.01% TWEEN 20). Subsequently, 5 μM THD was added in the presence of BSA or recombinant talin rod domain R9-12. Samples were washed 4 times in binding buffer, and boiled in SDS sample buffer for 10 min, and resolved using a 10% SDS PAGE. Equal loading of GST fusion proteins was determined with Ponceau staining, THD amount was determined using the Rb79 antibody, while the rod domain was detected using a talin 8d4 antibody (Sigma). Binding of full length Gα13 was accomplished with GST fusion protein expressing talin's head domain (aa1-400) or GST alone. GST-THD (5 μg) or equimolar amounts of GST was incubated with 1 μg of Gα13 in binding buffer (above) with either 30 μM GDP or GTPγS on ice for 1 hour. Each sample was added to 10 μl of GSH resin pre-equilibrated in binding buffer, and bound for 1 hour at 4°C with gentle agitation. Samples were washed 4 times in binding buffer, and boiled in SDS sample buffer. Equal loading was confirmed via Ponceau stain, and Gα13 binding was confirmed via Gα13 mAb (1/500) (Santa Cruz Biotechnology).

**GST THD, Gα13, and Rod Domain Binding and Competition Assays:** To obtain sufficient quantities of full length Gα13 required for competition binding assays with talin rod domain, we utilized Gαi13 constructs generously provided by Drs. Kozasa and Kreutz. Briefly, the N terminus of Gα13 is replaced by Gαi1 that permits the generation of soluble Gα subunits without co-expression of cognate β and γ subunits, which in turn dramatically increased the yield (33). The binding assay was performed using GST-THD, Talin R9-12, and Gα13 which were dialyzed overnight in the Binding Buffer (20 mM Tris-HCl, pH 8, 1.0 mM EDTA, 10 mM βME, 300 mM NaCl, 10 mM MgCl₂, 30 μM GDP). Prior to the binding assay, Gα13 was incubated with either 60 μM GTPγS or GDP for one hour on ice.

Gα13 (30 μM), in a total reaction volume of 250 μl, was incubated with 30 μg GST-THD for 2 hours at 4°C. Talin R9-12 was added in increasing concentrations (0 μM, 5 μM, 10 μM, 20 μM, 30 μM, 50 μM). Gα13 (30 μM) bound to GDP was incubated with GST-THD and 30 μM talin R9-12 added subsequently. Each reaction was incubated for one hour at 4°C, followed by incubation with 30 μl GSH resin pre-equilibrated in the Binding Buffer with 0.1% BSA for 2 hours at 4°C. Beads were washed 5 times with the Binding Buffer containing 0.01% Triton X-100. Each sample was boiled in SDS-PAGE sample buffer and resolved using a 4-20% gradient gel, and probed using either Gα13 pAb (Santa Cruz Biotechnology) or anti-rod domain antibody Rb89.

**Limited Proteolysis of Talin Bound to Peptides:** Full length talin (dimer fraction) at a concentration of 5 μM was pre-incubated with 87.5 μM Gα13SR2Pep or Gα13SR2 RandomPep, and/or 45 μM PIP2 (Avanti Polar Lipids) for 30 minutes at room temperature. Subsequently, either Endo LysC or Endo AspN was added at 8.0 ng, 1.6 ng, or 0.32 ng into a total reaction volume of 50 μl in PBS for 30 min at room temperature. Samples were treated with Complete Protease Inhibitor Cocktail (Roche), boiled in SDS Loading buffer, and resolved using 4-20% gradient SDS-PAGE (Bio-Rad Labs). Talin was probed using anti-talin head domain antibody TA205 (Sigma).

**CHO5 Cell Culture and Immunofluorescence:** Cells and reagents were obtained from the following sources: CHO cells stably expressing the platelet integrin αIIbβ3 (CHO5 cells) were kindly provided by Dr. Mark Ginsberg; cells were transfected with GFP-C1-talin (kindly provided by Dr. Jun Qin); GFP-C1, GFP-C1-THD (kindly provided by Dr. Edward Plow); pcDNA4-RIAM was a gift from Vicki Bousios (Addgene plasmid # 32803); and murine pCMV-Sport6-Gα13 or pCMV-Sport6-Gα13(R227A) were originally obtained from the laboratory of Dr. Guy Le Breton. Cell spreading was performed using...
fibrinogen coated slides (100 µg/ml) blocked with 1% BSA for 1 hour at room temperature. CHO-A5 cells transfected with respective plasmids were plated onto fibrinogen coated slides for 15 minutes at 37°C. Subsequently cells were fixed in 4% Paraformaldehyde for 10 minutes and treated with Phalloidin (594) according to manufacturer’s specifications (Molecular Probes) and Hoechst 33342. For protein localization analysis, Gα13 was detected using a Gα13 mouse monoclonal antibody (Santa Cruz Biotechnology). β3 integrin was detected using the goat polyclonal antibody (Santa Cruz Biotechnology). A Nikon Eclipse TE2000-E microscope was used for 60x-100x magnification for Fluorescence Microscopy. MetaMorph series software (Molecular Devices) was used to record images. Green fluorescence detected using FITC conjugated mouse antibody (Molecular Probes) was employed for the detection of Gα13, while AF633 conjugated mouse or goat antibodies were utilized for Gα13 and β3 integrin detection, respectively (Molecular Probes).

**FRET Constructs:** GFP-talin was used as the backbone for all FRET experiments. GFP-talin has been validated by several groups (13,15,27,42), therefore GFP served as the donor molecule. The FRET acceptor molecule, a cell permeant red fluorescence dye known as ReAsH (ThermoFisher Scientific), can bind to a 6 amino acid sequence designated as a tetracysteine motif or “CCPGGC” (50). The tetracysteine motif was engineered into 3 separate constructs of GFP-talin at defined areas of the rod domain (Figure 3A). To avoid perturbation of the structure of talin’s rod domain, this motif was incorporated into flexible linker regions connecting the helical bundles at amino acids 1457, 1655, and 1822. The rationale for selecting these regions was to place FRET acceptor molecules at locations near the autoinhibitory R9 domain. The assumption was that GFP-talin is autoinhibited by an adjacent talin molecule; thus depending on the location of the FRET acceptor, there should be varying levels of FRET pairing. This idea could conceivably apply to an autoinhibited monomer as well. One additional consideration was the cleavage of talin by calpain. Calpain-2 is believed to cleave talin at an unstructured linker region located between the head domain and the rod domain, which can regulate the formation and turnover of focal adhesions (42). Talin proteolytic cleavage would thus reflect a decrease in the FRET signal due to the separation of the rod and head domains rather than a gross conformational change. Therefore, to avoid false positive signals, a calpain-cleavage immune construct designated here as GFP-talin-L432G was utilized for each experiment.

CHO-A5 cells were transfected with each construct individually using the DNA Transfection Reagent (Biotool), and incubated for 24 hours. ReAsH dye was added to a final concentration of 2.0 µM and washed with BAL buffer according to manufacturer’s instructions. A LSRII flow cytometer (BD Biosciences) was used to analyze the FRET signal. Untransfected cells stained with ReAsH dye were used as a negative control to assess any non-specific dye binding. It should be noted that alpha-Tubulin1b (pC2xTC1-alphaTubulin, which was a gift from Amy Palmer [Addgene plasmid # 36326]) was transfected because it possesses a CCPGCC motif and can bind the ReAsH dye, but has no donor molecule (such as GFP). Consequently, it should have no FRET signal and can be used to validate that activation of ReAsH signal does not occur using the 488 nm (FITC) laser alone. Finally, GFP-C1-talin with no CCPGCC motif was incubated with ReAsH dye and used to gate GFP+/FRET negative cells, since no acceptor molecule is present. All PE(-)FITC(+) cells were gated according to the signal emitted by GFP-C1-talin-L432G.

**FRET Analysis and Flow Cytometry:** Each construct (GFP-C1-talin-L432G-1457; GFP-C1-talin-L432G-1655; GFP- C1-talin-L432G-1822) was analyzed using the 488 laser, which should only excite the GFP (donor) molecule. The R0 of ReAsH is ~54Å, thus enabling a wide range for error in the placement of our ReAsH binding motifs (50). However, the overall length of a fully extended talin molecules is roughly 500 Å, thus decreasing the possibility of FRET occurring in an active elongated state. Any signal detected in the PE channel was therefore attributed to ReAsH excitation due to FRET pairing. Fixation of cells greatly diminished the FRET signal in our hands; therefore, equal expression of constructs (Gα13, Gα13 R227A) was verified using Western blotting. It is to be noted that FRET analysis did not use conventional calculation methodology (51,52). Traditional
calculations examine the ratio of median fluorescence intensities of FRET positive and negative populations. We observed that samples with the fewer FRET positive cells had the highest MFI, as only double positive cells expressing the highest levels of GFP-talin-CCPGCC constructs showed FRET to occur. On the other hand, cells with the highest ratio of FRET positive cells showed FRET in cells with lower MFI, suggesting that even low levels of GFP-talin-CCPGCC constructs were capable of emitting higher FRET signals. If MFI is the only metric, it would appear that the samples with the fewest FRET positive cells will show higher efficiency, which is misleading. Thus, our results only examined the ratio of FRET positive cells expressing the GFP-talin-CCPGCC constructs to FRET negative cells, which express the GFP-talin-CCPGCC: PE(+)/FITC(+)/(PE+GFP+)+(PE-GFP+).

Peptide preparations: All peptides were synthesized and HPLC purified (>95% pure) by the Research Resource Center at the University of Illinois, Chicago. Peptides were dissolved in DMSO for all in vitro experiments. For all in vivo assays utilizing myristoylated peptides, a 50 mM stock solution was first prepared by dissolving each peptide in 70% DMSO and 30% PEG300. The stock solution was then diluted with 0.9% saline to bring the final concentration to 3.7 mM, and a 100 μl solution was administered to each mouse, bringing down the total final DMSO concentration to 0.2%.

Collagen Binding and Clot Retraction: Human platelets were provided by Tufts Medical Center Blood Bank and American Red Cross, Dedham, MA. One milliliter of citrated whole blood was treated with 150 μM of Gα13SR2Pep or Gα13SR2RandomPep for 30 minutes at room temperature with gentle agitation. Samples were perfused over Chrono-par Collagen (Chrono-log Corp.) coated slides at a rate of 1000/s using a GlycoTech parallel plate flow chamber (GlycoTech Corp). The chamber was washed with PBS, pH = 7.4, and examined at 10x magnification. Five fields were taken per experiment, and each experiment was performed in triplicate. Clot retraction was performed using platelet rich plasma (PRP) obtained from blood using the sodium citrate (3.2%) anti-coagulant (BD). Platelet count was normalized to 2x10⁷/ml using platelet poor plasma. 100 μl of platelet solution was added to HEPES-Tyrode’s buffer, as well as 2 μl packed RBCs for color contrast, to aid visualization. RBC addition had no effect on clot retraction in parallel experiments. Gα13SR2Pep or Gα13SR2RandomPep were dissolved in DMSO and incubated with each sample for 15 minutes prior to thrombin (1U/ml) addition. The clot-free volume was removed and measured for volume calculation.

Tail Bleeding Analysis: Tail bleeding was performed as previously described (53). Briefly, mice (~25 gram each) were anesthetized with sodium pentobarbital and 100 μl of either vehicle (n=11), 3.7 mM Aspirin (n=9), 3.7 mM SR2, or SR2 Random peptide (n=14) was injected through the tail vein. After 5 minutes, an amputation was performed at 0.5 cm from the tip of the tail. The tail was placed into a pre-warmed saline bath at 37°C and the bleeding times were measured.

Induction of in vivo Thrombosis: Laser-induced thrombosis in mice was performed as previously described (37). Briefly, Gα13SR2Pep or Gα13SR2RandomPep (5 μm peptide for every mouse kilogram) were injected through a jugular vein cannulas followed by laser-induced thrombosis of an exposed cremaster muscle artery. Mouse platelets were labeled using a platelet specific β3 integrin antibody conjugated to Alexafluor 647. Laser-induced injuries triggering platelet accumulation at the site of injury, and fluorescent intensity over time was recorded. Three mice were analyzed under each condition with 10-15 observations per mouse. Scale bars (10 μm) and intensities of platelet signal (ranging from 308-7668 AU) are equal between Gα13SR2Pep and Gα13SR2RandomPep Conditions.

Statistics: Statistical significance for all methods with the exception of laser-induced thrombosis, was determined using a Student’s Unpaired t-Test where *P < 0.05 and **P < 0.01. Laser-induced thrombosis significance was determined using the Mann-Whitney test on median fluorescent intensity values of thrombus formation over time.
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Abbreviations: FERM; 4.1, Ezrin, Radixin, and Moesin related proteins. RIAM; Rap1–GTP-interacting adapter molecule. SR2; One of three conformationally sensitive switch regions present in Gα subunits. CNBr; Cyanogen bromide. GTPgS; A non-hydrolyzable form of Guanosine Triphosphate. AlF4-GDP; Tetrahedral aluminum fluoride which forms a complex with GDP bound with Gα subunits which mimics the transition state of GTP hydrolysis. PIP2; Phosphatidylinositol 4,5-bisphosphate. CHOAS; Chinese Hamster Ovary cells constitutively expressing the platelet integrin αIIbβ3. PAC1; Antibody which binds to the active form of platelet integrin αIIbβ3. FRET; Förster (Fluorescence) Resonance Energy Transfer. R0; Förster distance at which half the energy is transferred from a donor to an acceptor molecule. MFI; Median fluorescence intensity. vWF; Von Willebrand factor.

Author Contributions: J.S., A.H.C., A.B., and G.C.L. conceptualized the experiments. Experiments for Figures 1, 2, 3, 4, and 5A&B were performed by J.S. FRET constructs used in experiments for Figure 3 were generated by L.L. Tail bleeding experiment for Figure 5C was performed by J.S.H. and in vivo thrombosis for Figure 5D was performed by G.M. using the resources provided by R.F. The manuscript was written by J.S., A.H.C., and G.C.L., and further improved by the expertise and feedback provided by A.B., G.C.L, and A.H.C.

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FIGURE LEGENDS

Figure 1: G\(\alpha_{13}\)SR2 Binds Talin Head Domain F3.

(A) A Cartoon depiction of THD Cross-linking to biotinylated G\(\alpha_{13}\)SR2\_Pep and subsequent fragmenting using either chemical or enzymatic digestion. Biotinylation detection allowed peptide mapping to elucidate a novel THD binding site. (B) Coomassie blue stain of recombinant THD (Left panel) and streptavidin-HRP detection of cross-linked THD of either G\(\alpha_{13}\)SR2\_Pep or G\(\alpha_{13}\)SR2\_RandomPep. (C) Cyanogen bromide (CNBr) cleavage of THD cross-linked to G\(\alpha_{13}\)SR2\_Pep (Left panel) and thrombin digestion (Right panel). The black arrow on the left panel highlights the smallest detectable fragment for further analysis. The black arrows on the right panel show biotinylated peptide is present on the large THD thrombin fragment, but not a smaller fragment. (D) A dot blot with ligands THD, or THD lacking domains; \(\Delta\)F2, \(\Delta\)F3, or both \(\Delta\)F2-3. Biotinylated G\(\alpha_{13}\)SR2\_Pep analyte was bound differentially to each ligand, as detected by streptavidin-HRP. (E) Loading controls for each dot blot ligand. (F) ELISA of biotinylated G\(\alpha_{13}\)SR2\_Pep ligand bound to THD WT or point mutants. (G) RCSB PDB structure 2KGX depicting THD F3 (Green) binding to R9 (Yellow). Pink color was added to highlight binding site of SR2 elucidated through peptide mapping and mass spectrometry. Blue residues (aa319 and 325) are point mutants which are necessary to bind G\(\alpha_{13}\)SR2\_Pep, while the red residue (335) is not. Statistical significance was determined using a Student’s Unpaired t-Test where *P < 0.05 and **P < 0.01.
Figure 2: Gα13SR2 Sterically Occludes Talin Rod Domain for F3 Binding.
(A) Cartoon depiction of full length talin containing FERM domain THD (F0-3), 13 helical domains, and 1 dimerization domain at the C terminus. Domains F3 and R9 bind to one another to create an autoinhibited state and are shown in light red. R13 cannot bind talin’s FERM domain and is shown in teal. Linker regions between R7-8, R8-9, R9-10 are highlighted in red as reference for future FRET experiments. (B) A cartoon depiction of a competitive binding assay between GST- Gα13SR2 and R9-12 for THD F3 binding. GST- Gα13SR2 binds to THD, and upon the addition of R9-12 the possible binding scenarios are the formation of a ternary complex (non-overlapping binding site), no R9-12 binding due to allosteric changes to talin’s conformation, or competitive binding if R9-12 and SR2 share an overlapping binding site on THD. (C) A binding assay demonstrating GST-Gα13SR2 can bind directly to THD but not GST- Gα13SR2_Random (lanes 1 and 2), but is sterically occluded when increasing amounts of recombinant R9-12 was added at either 50 μM, 25 μM, or 5 μM (lanes 3, 4, and 5). The addition of equal concentrations of BSA does not sterically occlude GST-Gα13SR2/THD interaction (Lanes 6, 7, and 8) demonstrating specificity of R9-12 binding. Finally, no concomitant GST- Gα13SR2/R9-12 complex was formed demonstrating specific competition. (D) Western blot analysis showing the effect of limited proteolysis on full length talin after Gα13SR2_pep or Gα13SR2_RandomPep, or PIP2 combination treatment. Lanes 1-4 show 87.5 μM Gα13SR2_pep (Green) or Gα13SR2_RandomPep (Black) were incubated full length talin and either 8 ng or 0.32 ng of the protease Endo-Asp-N (Red). Lanes 5-8 show the same experiment with the addition of 45 μM of PIP2. Lane 9 is full length talin input. Loss of talin signal is attributed to proteolysis resulting from conformational change. (E) GST pull down assay where GST-THD or GST alone are incubated with Gα13 and GDP or GTPγS. The bottom panel is a Ponceau stain of GST and GST-THD loading controls. The top panel shows the resultant pull down probed with an α-Gα13 mAb. (F) Cartoon depiction of competitive binding assay between Gα13 and talin R9-12 for GST-THD binding. (G) Lanes 1-6 show 30 μM Gα13 bound to GST-THD in the presence of talin R9-12 at increasing concentrations of 0 μM, 5 μM, 10 μM, 20 μM, 30 μM, or 50 μM talin R9-12. Lane 7 shows 30 μM of inactive Gα13 bound to GDP incubated with GST-THD and 30 μM talin R9-12. The top panel is probed with an α-Gα13 pAb, the middle panel was probed with an α-rods domain antibody, and the bottom panel shows a Ponceau stain of GST-THD loading controls.

Figure 3: FRET Based Analysis of Talin Conformation.
(A) Cartoon depicting a GFP-talin dimer containing tetracysteine motifs (CCPGGCC) which can bind ReAsH dye. Each location is at a flexible linker region denoted with a black arrow (highlighted in red in Fig 2a). (B) Cartoon depicting GFP-talin in an autoinhibited state which permits GFP/ReAsH (RED) FRET pairing. Activation relieves autoinhibition and decreases ReAsH FRET signal (gray). (C) GFP-talin containing CCPGCC insert at amino acid 1822 (GFP-talin-1822; right panels) or GFP-talin (left panels). Scale Bars are 20 μm. (D) Flow cytometry based detection of FRET signal. CHOAS cell populations (R1 gate) were evaluated for GFP signal (R2 gate) or ReAsH signal (R3 gate) using the FITC or PE channel respectively, using the 488 nm laser for specific excitation of GFP only. (E) Tubulin-ReAsH construct (#1) demonstrates that no PE signal emitting from ReAsH is visible with the 488 nm laser alone, therefore any signal observed in the PE channel results from GFP-ReAsH FRET pairing. GFP-talin (#2) shows no PE signal. Panels #3-#5 show an increasing level of ReAsH emission in the PE channel, suggesting that moving the location of the CCPGCC motif towards the C-terminus there is an increasing FRET pairing. (F) Quantification of FRET signal observed. (G) Change in FRET signal from GFP-talin-1822 or F3 point mutations M319A or L325K. (H) Percent of FRET+ cells with GFP-talin-1822 co-expressed with vehicle or with RIAM. (I) Percent of FRET+ cells with GFP-talin-1822 co-expressed with vehicle, Gα13, or Gα13 R227A. Statistical significance was determined using a Student’s Unpaired t-Test where *P < 0.05.
Figure 4: Go13 and Talin Co-Localize to Integrins.
(A) Immunofluorescence images of CHOA5 cells plated on fibrinogen coated slides. Phalloidin-594 (Red) was used to highlight the degree of spreading, while Hoechst 33342 (Blue) was used to identify single cells. Scale bars indicate 10 μm. (B) Quantification of relative spreading of each CHOA5 cells. (C) DNA (Blue), β3 Integrin (Red), and GFP-talin (Green) detected in CHOA5 cells in which Go13 (Top Panels) or Go13 R227A (Middle Panels) are co-expressed with GFP-talin. Co-localization between GFP-talin and β3 integrin appears as yellow. Enlarged merged images are shown below. (D) DNA (Blue), Go13 (Red), and GFP-talin (Green) detected in CHOA5 cells in which Go13 (Top Panels) or Go13 R227A (Middle Panels) are co-expressed with GFP-talin. Co-localization between GFP-talin and β3 integrin appears as yellow. Enlarged merged images are shown below. Scale bars for C and D indicate 10 μm. Statistical significance was determined using a Student’s Unpaired t-Test where *P < 0.05 and **P < 0.01.

Figure 5: Go13 SR2 Demonstrates Anti-Thrombotic Properties.
(A) Bright field 10x images of platelets bound to collagen coated slides after perfusion of whole blood treated with 150 μM of myristoylated Go13SR2_pep (1) (Left Panel) or Go13SR2_randomP (2) (Right Panel). Quantification was performed by examining the percent of each field covered by platelets. (B) Clot retraction of platelet rich plasma pre-treated with 150 μM of myristoylated Go13SR2_pep (Left Panel) or Go13SR2_randomP (Right Panel) examined after treatment with thrombin for 3 hours. Quantification of clot retraction was performed by measuring the volume non-clot liquid. (C) Tail bleed analysis of mice pre-treated with either Vehicle (DMSO), 3.7 mM myristoylated Go13SR2_randomP, Go13SR2_pep, or Aspirin. Statistical significance was determined using a Student’s Unpaired t-Test where *P < 0.05 and **P < 0.01. (D) Laser induced thrombosis of mice pre-treated with 3.7mM myristoylated Go13SR2_randomP (top panels) or Go13SR2_pep (bottom panels). Thrombosis is shown as a time course from 15 to 90 seconds using representative images of median platelet fluorescence over time. The median fluorescence from n = 3 mice for each condition are shown in the left panel from 0 to 140 seconds. Scale bars indicate 10 μM. Significance was determined using the Mann-Whitney test where *P < 0.05.

Figure 6: Schematic Representation of Go13-Talin-Integrin Activation Pathway.
Go13 directly binds to the F3 domain of talin to relieve its autoinhibited state. Upon activation, talin can bind and activate integrin resulting in conformational changes that convert integrin from a low affinity to high affinity state.

Table 1: Chemical Cleavage of Talin. CNBr cleavage yielded 15 unique peptide fragments from THD. Table contains the predicted sequence, molecular weight, cross-linked molecular weight, lysine residues. The presence of THD segment within each peptide sequence is indicated. Five of the 15 peptides match the criteria for potential binding sites of SR2 to THD.

Table 2: Mapping of Go13SR2 and Talin Binding Interface. Data provide a list of multiple techniques used to map the SR2/THD binding interface and their respective locations. The final row (Green) contains the overlapping region revealed by each technique.
Figure 1

A. Crosslinking of THD and SRII leads to the formation of fragments. The fragments are further analyzed by Peptide Mapping and MS Analysis.

B. Western blot analysis showing the expression of THD and its fragments. THD, ΔF2-3, ΔF3, and ΔF2 are compared.

C. Biotin Detection of the fragments using CNBr and Thrombin.

D. Biotin Detection of THD and its fragments THD, ΔF2-3, ΔF2, and ΔF3.

E. Mass Spectrometry analysis of the fragments showing a peak at 45 kDa.

F. Quantitative analysis of the expression levels of WT, M319A, L325K, and E335R proteins.

G. Structural model of the protein showing the positions of M319, L325, and E335.
Figure 2

Non-overlapping site

Overlapping site

Allosteric Regulation

GST

α-Gα13

GST-THD

GST

GDP

GTPγS

α-Rod

GST-

THD

(GDP)

(GTPγS)

Figure 3

α-Gα13

GST-THD

GST

GDP

GTPγS

α-Rod

GST-

THD

(GDP)

(GTPγS)

Figure 4

α-Gα13

GST-THD

GST

GDP

GTPγS

α-Rod

GST-

THD

(GDP)

(GTPγS)
Figure 3

**A**

```
| 1457 | 1655 | 1822 |
```

**B**

```
FRET (Normalized)  
```

**C**

```
GFP (FITC) | ReAsH (PE) | GFP (FITC) | ReAsH (PE) |
```

**D**

```
FSC-A  
```

**E**

```
FITC-525/50  
```

**F**

```
#1 #2 #3 #4 #5  
```

**G**

```
WT M319A L325K  
```

**H**

```
Vehicle RIAM  
```

**I**

```
GFP-talin-1822  
```
GFP + Go13

Go13 (R227A)

GFP-talin

Go13 + GFP-talin

R227A + GFP-talin

GFP-talin-M319A

Go13 + M319A

GFP-THD

Go13 + GFP-THD

Relative Spreading (AU)

GFP + Go13

Go13 (R227A) + GFP

GFP-talin

Go13 + GFP-talin

GFP-talin-M319A

Go13 + GFP-talin-M319A

GFP-THD

Go13 + GFP-THD

DNA

β3 Integrin

GFP-talin

Merge

Go13

Go13 (R227A)

DNA

Go13

GFP-talin

Merge

Go13

Go13 (R227A)

DNA

Go13

GFP-talin

Merge
Figure 5

Median Sum Intensities

SR2

Aspirin

Vehicle

Bleeding Time (sec)

A

Gα13SR2_{Pep} (150 μM) vs Gα13SR2_{RandomPep} (150 μM)

Field %

#1
#2

B

Gα13SR2_{Pep} (150 μM) vs Gα13SR2_{RandomPep} (150 μM)

Volume (μl)

#1
#2

C

D

Gα13SR2_{Pep} (3.7 mM) vs Gα13SR2_{RandomPep} (3.7 mM)

Bleeding Time (sec)

Vehicle
#2
#1
Aspirin

* indicates statistical significance.
Figure 6

(Low Affinity – Inactive)  (High Affinity – Active)

\[ \begin{align*}
\alpha \text{ Integrin Subunit} & \quad \beta \text{ Integrin Subunit} \\
\text{Extracellular Matrix} & \quad \text{Cytoplasm} \\
\text{Activated Integrin} & \quad \text{Active Talin} \\
\text{Activated Talin} & \quad \text{Inactive Talin} \\
\text{Inside-Out Signaling} & \quad \text{G\alpha13}
\end{align*} \]

- 1: Active (GTP) \( \text{G\alpha13} \) \rightarrow Inactive (GDP) \( \text{G\alpha13} \)
- 2: \text{G\alpha13} \rightarrow \text{Inactive Talin} \rightarrow \text{Active Talin} \rightarrow \text{Activated Integrin} \rightarrow \text{Recycled?} \text{G\alpha13}
- 3: \text{Activated Talin} \rightarrow \text{Activated Integrin}
- 4: \text{Activated Integrin} \rightarrow \text{Extracellular Matrix}
- 5: \text{Recycled?} \text{G\alpha13}
| Fragment | Peptide Sequence | Predicted MW (kDa) | MW with Biotinylated SR2 Peptide (2.3 kDa) | Lysine Present | Talin Head Domain (THD) |
|----------|-----------------|--------------------|------------------------------------------|----------------|------------------------|
| 1        | M               | 0.1492             | 2.4492                                   | No             | F0                     |
| 2        | VALSLKISIGNVVKTM | 1.637              | 3.937                                    | Yes            | F0                     |
| 3        | QFEPSTM         | 0.8389             | 3.1389                                   | No             | F0                     |
| 4        | VYDACRIIREPIREAPAGPPSDFGLFLSDDDPKGIWLEAGKALDDYM | 5.401 | 7.701 | Yes | F0                     |
| 5        | LRNGDTM         | 0.805              | 3.105                                    | No             | F0                     |
| 6        | EYRKQQRPLKIRM   | 1.746              | 4.046                                    | Yes            | F0-1                   |
| 7        | LDGTVKTIM       | 0.9771             | 3.2771                                   | Yes            | F1                     |
| 8        | VDDSKTVTDM      | 1.11               | 3.41                                     | Yes            | F1                     |
| 9        | LM              | 0.2952             | 2.5952                                   | No             | F1                     |
| 10       | TICARIGITNHDEYSVRELMM | 2.434 | 4.734 | No | F1                     |
| 11       | EEEKKEEITGTLRDKDLLRDEKKM | 2.919 | 5.219 | Yes | F1                     |
| 12       | EKLKQKLHTDDELNLWLDHGRTLREQGVEEHEHTLLRLRKFFYSQNVDSRDPVQLNLLYVQARDLNGSHPVSDKACEFAGFQCQIQFGPHNEQHKAGFLDLDKFDLPKEYVKQKGRKIFQAHKNCGQM | 15.441 | 17.741 | Yes | F1-2                   |
| 13       | LARSLKTYGVSSFLVKEKM | 2.217 | 4.517 | Yes | F2-3                   |
| 14       | KGKNNKLVPRLLL GITKEVM | 2.1276 | 4.4276 | Yes | F3                     |
| 15       | RVDEKTKEVIQEWNLTNINKRWAASPKSFTLDFGDYQDGYYSVTTEGEQIAQLIAGYIDIIILKKKS | 7.771 | 10.071 | Yes | F3                     |
| SR2 Binding Residues | Talin Domain Binding Region | Functional Domain | Method of Detection       |
|----------------------|-----------------------------|-------------------|--------------------------|
| aa 1-328 (38 kDa)    | F3                          | Rod Binding Region| Thrombin Mapping (observed) |
| aa 329-400 (7.6 kDa)| F3                          | Rod and Integrin Binding Region| Thrombin Mapping (not observed) |
| aa 289-338 (5 kDa)  | F2-3                        | Rod Binding Region| CNBr Mapping (Observed)   |
| aa 310-402           | F3                          | Rod and Integrin Binding Region| Dot Blot Deletion Mapping (Observed) |
| aa 307-338           | F3                          | Rod Binding Region| Mass Spectrometry        |
| aa 310-328           | F3                          | Rod Binding Region| Overlapping Sites        |
Galpha13 Switch Region 2 Relieves Talin Autoinhibition to activate alphaIIbbeta3 Integrin

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