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**Interaction Analyses of the Integrin β 2 Cytoplasmic Tail with the F3 FERM
Domain of Talin and 14-3-3 ζ Reveal a Ternary Complex with Phosphorylated
Tail**

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Abstract

Integrins, hetero-dimeric (α and β subunits) signal-transducer proteins, are essential for cell adhesion and migration. Beta cytosolic tails (β -CTs) of integrins interact with a number of cytosolic proteins including talin, Dok1, and 14-3-3 ζ . The formation of multi-protein complexes with β -CTs is involved in the activation and regulation of integrins. The leukocyte-specific $\beta 2$ integrins are essential for leukocyte trafficking, phagocytosis, antigen-presentation and proliferation. In this study, we examined the binding interactions between integrin $\beta 2$ -CT and T758 phosphorylated $\beta 2$ -CT (pT758 $\beta 2$ -CT) with positive regulators talin and 14-3-3 ζ and negative regulator Dok1. Residues of the F3 domain of talin belonging to the C-terminal helix, β -strand 5 and the adjacent loop were found to be involved in binding interactions with $\beta 2$ -CT. The binding affinity between talin F3 and $\beta 2$ -CT was reduced when $\beta 2$ T758 was phosphorylated but this modification promoted 14-3-3 ζ binding. However, we were able to detect stable ternary complex formation of pT758 $\beta 2$ -CT, talin F3 and 14-3-3 ζ that involved the repositioning of talin F3 on $\beta 2$ -CT. We showed that Dok1 binding to $\beta 2$ -CT was reduced when $\beta 2$ T758 was phosphorylated and in the presence of 14-3-3 ζ . Based on these data, we propose a sequential model of $\beta 2$ integrin activation involving these molecules. Our study provides for the first time insights towards $\beta 2$ integrin activation that involves a multi-protein complex.

Keywords: $\beta 2$ integrins, NMR, protein-protein interaction, talin, 14-3-3

Introduction

Integrins are transmembrane receptors that mediate cell-cell and cell-extracellular matrix (ECM) adhesions [1]. Integrins are obligate heterodimers composed of non-covalently linked α and β subunits. Each subunit contains a large extracellular domain, a single-pass transmembrane domain, and a short cytoplasmic tail (CT) [1]. Integrin inside-out activation is a process by which the disruption of the interface between the membrane proximal regions of the α and β subunits and cytoplasmic tails leads to the separation of their transmembrane domains followed by global conformational changes in the extracellular regions [2]. The integrin β -CTs, excluding $\beta 4$ and $\beta 8$, contain two highly conserved NPxY/F motifs. Both motifs are docking sites for cytoplasmic proteins that regulate integrin activation [3].

Talin-1 (henceforth referred to as talin) is a large cytoskeletal protein composed of a 4.1-ezrin-radixin-moesin (FERM)-containing head region and a long rod region [4]. Talin is a well-established positive regulator of integrin activation. It binds directly to the integrin β -CTs and disrupts the membrane proximal interface of the cytoplasmic tails [5]. The phosphotyrosine binding (PTB) fold in the F3 subdomain of talin binds to the membrane proximal NPxY/F motif of the integrin β -CT [6]. It has been reported that the phosphorylation state of the integrin $\beta 3$ membrane proximal NPLY functions as a switch that regulates binding of either talin or Dok1, a cytoplasmic negative regulator of integrin activation [7]. Recently, it has been demonstrated that an alternative phosphorylation switch involving two Ser residues flanking the integrin $\beta 2$ membrane proximal NPLF motif served a similar function [8]. These data exemplify the relevance of integrin β tail post-translational modification(s) as a part

of the mechanism by which fine-tuned regulation of integrin activation could be achieved.

The leukocyte-restricted integrin $\beta 2$ subfamily contains four members that share a common $\beta 2$ subunit, namely $\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$ and $\alpha D\beta 2$ [3]. The $\beta 2$ -CT contains a triplet-Thr motif that lies in between its two NPxF motifs. The phosphorylation status of this triplet-Thr motif modulates the association of the integrin $\beta 2$ -CT with cytoplasm proteins. 14-3-3 family of proteins is known to bind pSer/Thr-containing sequences and they form dimers [9-11]. 14-3-3 ζ binds the pTTT motif in the integrin $\beta 2$ -CT and it serves as a positive regulator of $\beta 2$ integrin ligand-binding [12, 13]. Structural data revealed a 14-3-3 ζ homodimer in complex with two pTTT-containing integrin $\beta 2$ tail peptides, suggesting that 14-3-3 ζ can positively regulate $\beta 2$ integrins functions by promoting receptor clustering [14]. Notably the same study also showed that the phosphorylation of Thr758 (the first residue of the triplet-Thr) in the integrin $\beta 2$ -CT promotes 14-3-3 ζ but inhibits filamin binding [14]. Phosphorylation of the triplet-Thr in the integrin $\beta 7$ -CT has also been shown to reduce filamin binding, a negative regulator of integrin activation, but not talin binding [15]. Further, in $\beta 2$ -CT phosphorylation of residue S756 close to the NPLF754 and T⁷⁵⁸TT motif recruits Dok1, a negative regulator of integrin activation [8]. Taken together, phosphorylation of the triplet-Thr motif and adjacent Ser756 in the integrin $\beta 2$ -CT serves like a switch that regulates the docking of 14-3-3 ζ , filamin A and Dok1 with opposing functions with respect to integrin activation.

Mechanistically the dissociation of filamin A from the integrin β -CT followed by the docking of positive regulators such as 14-3-3 ζ and talin is well rationalized and supported by available data. However, it remains to be clarified if 14-3-3 ζ , talin and

Dok1 with binding sites that are close to each other on integrin β 2-CT, can form ternary complexes or their interactions with the integrin β 2-CT are mutually exclusive. There is also limited information on how phosphorylation of the triplet-Thr in the integrin β 2-CT regulates the binding of 14-3-3 ζ in the presence of talin and *vice versa*. In this work, we set out to understand the interactions between talin F3 domain, 14-3-3 ζ and Dok1 with β 2-CT. Our results showed that talin F3 domain exhibits different affinities for β 2-CT and pT β 2-CT employing residues located at the C-terminal long helix, β -strand 5 and the adjoining loop region. Ternary complex formation of 14-3-3 ζ , talin F3 domain and pT β 2-CT was examined. Based on the docked structure of the ternary complex, the membrane proximal helix of pT β 2-CT interacts with the talin F3 domain and its C-terminal region containing residue pT758 occupies the canonical binding pocket of 14-3-3 ζ . These results provide important insights for multi-protein complex formation between β 2-CT and its interacting partners that are relevant to the activation mechanism of β 2 integrins.

Results

The F3 domain of talin interacted with β 2-CT using the canonical binding pocket:

Backbone ^{15}N - ^1H correlations of talin F3 were assigned by combined analyses of 3-D HNCACB and CBCA(CO)NH spectra. A series of ^{15}N - ^1H HSQC spectra of F3 were acquired in the presence of different concentrations, molar ratios, 1:0, 1:0.4, 1:0.8, 1:1.2, 1:1.6, 1:2, 1:3, 1:4 and 1:5, of synthetic β 2-CT. Table 1 provides a list of synthetic β 2-CTs used in this study. ^{15}N - ^1H HSQC spectra overlay of talin F3 in free solution (blue contour), and in the presence of β 2-CT at 1:1 (red contour) and 1:3 (brown contour) molar ratios is shown (Fig. 1a). ^{15}N - ^1H HSQC spectra of talin F3

showed chemical shift changes and also broadening of resonances that suggest binding interactions. Residues of talin F3 located at the central segment e.g. E350, W351, L353, T354 and N355 and a continuous stretch of C-terminal region e.g. A³⁸⁹QLIAGY³⁹⁵ exhibited pronounced chemical shift changes in the presence of β 2-CT (Fig. 1b). Additionally, ^{15}N - ^1H cross-peaks of a number of residues, at the central region, of talin F3 were broadened due to its binding to β 2-CT (Fig. 1b). An apparent dissociation constant (K_d) value of 243.8 μM of talin F3/ β 2-CT complex was estimated from the chemical shift changes of talin F3 (Fig. 1c, Table 2). The β 2-CT-induced chemical shift changes (>0.04 ppm) and resonance broadening effect were mapped onto the 3-D structure of talin F3 (Fig. 1d). The 3-D structure of talin F3 represents a canonical PTB fold whereby the C-terminal long helical and the adjacent β -stands including loops may be involved in interactions with β 2-CT (Fig. 1d). ^{15}N - ^1H HSQC titrations were also carried out for ^{15}N -labelled β 2-CT with unlabeled talin F3 protein. ^{15}N - ^1H HSQC spectra of β 2-CT in complex with F3 domain revealed perturbation of most of the residues (Supplemental Fig. S1). Residues of the β 2-CT with $>90\%$ signal attenuation when in complex with talin F3 domain is shown (Fig. 2a). Residues located both in the membrane proximal helix and membrane distal β -strand of β 2-CT were perturbed upon binding to talin F3. Based on chemical shift perturbations, a molecular docked model of talin F3 and β 2-CT was generated (Fig. 2b). In the docked structure, the β 2-CT interacts through its membrane proximal (MP) helical region and residues at the membrane distal (MD) loop with talin F3. The overall topology of the β 2-CT/talin F3 docked structure resembles well with the structures of β 1D-CT/talin 2 F2/F3 complex [16] and β 3 hybrid CT talin F3 complex [17]. Notably, the membrane proximal helix of β 2-CT demonstrated potential ionic

interactions involving residue D731/K316 and non-polar packing of residues Y735/L325, F738/P363 (Fig. 2b). Further, packing interactions with talin F3 can be seen for residues of MD part of β 2-CT e.g. W747/Y377, W747/W359, L753/I396 and L753/L400 (Fig. 2b).

Thr758 phosphorylation of β 2-CT modulated its binding with talin F3:

Phosphorylation of residues in β -CTs modulates its binding affinity with talin and other interacting proteins [7,8,14,18]. Residue Thr758 in the TTT motif of β 2-CT is known to be phosphorylated and it allows binding of 14-3-3 ζ protein to the β 2-CT. However, the consequence of β 2-CT Thr758 phosphorylation on its binding to talin remains unclear. ^{15}N - ^1H HSQC spectra of talin F3 were acquired at F3:pT β 2-CT molar ratios of 1: 0, 1:0.4, 1:0.8, 1:1.2, 1:1.6, 1:2, 1:3, 1:4 and 1:5. Fig. 3a shows chemical shift changes of residues of talin F3 in the presence of pT β 2-CT at 1:3 molar ratio. Residues in the talin F3 central β -sheet and C-terminal helical region showed resonance perturbation in the presence of pT β 2-CT, akin to interactions with the non-phosphorylated β 2-CT. However, most of the residues of talin F3 showed a lower extent of chemical shift changes in the presence of pT β 2-CT compared to the β 2-CT (Fig. 3b). It should also be noted that different residues of talin F3 exhibited broadening of ^{15}N - ^1H HSQC cross-peaks while interacting with β 2-CT and pT β 2-CT, indicating conformational exchange processes (Fig. 3b). Regardless, an apparent K_d value of F3/pT β 2-CT interactions was estimated to be 578 μM from the chemical shift binding curve (Fig. 3c, Table 2). The docked structure of talin F3/ β 2-CT revealed close proximity of residue T758 with negatively charged residues D372 and E335 of talin F3 (Fig. 2b). Therefore, phosphorylation of T758 could cause

charge-charge repulsion, leading to a reduction in binding affinity between pT β 2-CT and talin F3.

Further, we examined interactions of pT β 2-CT with talin F3 employing mutations and peptide fragments of pT β 2-CT. Replacement of critical interfacial aromatic residues W747A and F754A of pT β 2-CT resulted in a significant reduction in binding affinity to talin F3 (Supplemental Fig. S2, Table 2). In order to dissect β 2-CT/F3 binding, two overlapping peptide fragments, KS33 and pTDS20 of β 2-CT were synthesized (Table 1) and examined for binding to talin F3. KS33 peptide fragment interacted with residues in the central β -sheet and the C-terminal helix of talin F3 (Fig. 4a). An apparent K_d , was estimated to be 357 μ M for KS33/talin F3 interactions (Fig. 4b and Table 2). By contrast, talin F3 showed no detectable interactions with pTDS20 peptide fragment (Supplemental Fig. S3). These data demonstrated that β 2-CT and pT β 2-CT interacted with talin F3 domain utilizing similar binding residues, however, with different affinity. The pT β 2-CT/talin interactions were characterized by low affinity complex. Further, analyses of talin binding with non-phosphorylated and phosphorylated β 2 peptide fragments revealed that the non-phosphorylated N-terminal residues (KS33) of β 2-CT contribute primarily to the binding.

Adaptor protein 14-3-3 ζ , talin F3 and pT β 2-CT formed a ternary complex:

NMR transverse relaxation (T_2) is a useful indicator of molecular weight and conformational exchange processes of proteins. We compared backbone ^{15}N T_2 of talin F3 either in free solution or in the presence of pT β 2-CT or a mixture of both pT β 2-CT and 14-3-3 ζ . The ^{15}N T_2 values of most of the residues of talin F3 in free solution showed uniform distribution with an average T_2 value of 50.84 ms

(Supplemental Fig. S4). By contrast, residues of talin F3 exhibited a marked reduction of T_2 values (Supplemental Fig. S4), an estimated average T_2 36 ms, in the presence of 14-3-3 ζ and pT β 2-CT, presumably due to an increase in molecular weight and/or conformational exchange processes involving the ternary complex. Note, ^{15}N T_2 values, an average $T_2 \sim 46$ ms, of talin F3 were not significantly affected in complex with pT β 2-CT (Supplemental Fig. S4).

We further examined residues of talin F3 that interact with pT β 2-CT in the ternary complex. ^{15}N - ^1H HSQC spectra of talin F3 were found to be invariant upon addition of unlabeled 14-3-3 ζ , suggesting lack of detectable interactions (data not shown). ^{15}N - ^1H HSQC spectra of talin F3, acquired in the presence of equimolar concentration of unlabeled 14-3-3 ζ , showed changes in chemical shifts and intensity in the presence of increasing concentrations of pT β 2-CT. A number of residues of talin F3 showed differences in chemical shifts and line broadening effects between F3/pT β 2-CT binary complex and F3/14-3-3 ζ /pT β 2-CT ternary complex (Fig. 5a). A K_d of 213 μM , between F3/pT β 2-CT in the presence of 14-3-3 ζ , was determined from the chemical shift change (Fig. 5b, Table 2). It was noted that a higher K_d value was obtained for F3/pT β 2-CT in the absence of 14-3-3 ζ (Table 2). Weighted chemical shift changes (>0.04 ppm) and residues that showed peak broadening were mapped onto the 3-D structure of talin F3 (Fig. 5c). Residues located in the $\beta 5$ and $\beta 6$ strands and adjacent loops but with fewer residues in the C-terminal helix were perturbed in the ternary complex (Fig. 5c). The potential involvement of residues W747 and F754 of $\beta 2$ -CT in the talin F3/14-3-3 ζ /pT β 2-CT ternary complex was further examined by individual ^{15}N - ^1H HSQC titrations of ^{15}N -labelled talin F3 and unlabeled 14-3-3 ζ

with $\beta 2$ W747A or F754A variants. In the ternary complex, mutant CTs demonstrated significantly higher K_d values compared to pT $\beta 2$ -CT (Table 2, Supplemental Fig. S5). The binding interactions of the ternary complex of 14-3-3 ζ /F3/pT $\beta 2$ -CT were further determined by ITC, chemical cross linking and size exclusion methods. In addition, ITC data were acquired for binary complex of 14-3-3 ζ and pT $\beta 2$ -CT and phosphorylated peptide fragment pTDS20. Representative ITC thermograms and binding parameters are shown (Fig. 6 and Table 3). Proteins/pT $\beta 2$ -CT interactions were exothermic or enthalpy driven as indicated by negative heat exchange or negative ΔH values (Fig. 6, upper panels, Table 3). Data also suggest that 14-3-3 ζ interacted either with full length pT $\beta 2$ -CT or the shorter CT fragment pTDS20 with similar K_d values of 4.9 μ M or 11 μ M, respectively (Fig 6, panels a and b, Table 3). A recent study, using ITC, also estimated a K_d of 7.9 μ M for the binding of 14-3-3 ζ with pT $\beta 2$ -CT [19]. Further, our results corroborate well with previous reports that the C-terminus or the MD region of phosphorylated $\beta 2$ -CT is largely responsible for the binding with 14-3-3 ζ [14]. The binding affinity of the ternary complex was determined by injecting talin F3 into solutions of pre-formed binary complex of 14-3-3 ζ and pT $\beta 2$ -CT (Fig. 6c). Talin F3 interacted with pT $\beta 2$ -CT/14-3-3 ζ complex with an apparent K_d of 161.8 μ M (Table 3). A comparable K_d value of 213 μ M was estimated from ^{15}N - ^1H HSQC titration experiments of talin F3 (Table 2). Therefore, talin F3 appeared to interact with the complex of pT $\beta 2$ -CT/14-3-3 ζ with relatively higher affinity in comparison to the binary complex of talin F3/pT $\beta 2$ -CT. The determination of binding affinity between pT $\beta 2$ -CT and talin F3 by ITC was unsuccessful presumably due to the weak affinity of interactions that resulted in non-saturable binding thermograms. It has been postulated that interactions of integrin

CTs with effector proteins may be modulated in membrane bilayer [2,16]. Notably, talin binding affinity of $\beta 3$ -CT or CT/CT interactions appeared to be enhanced in model membrane mimic systems [20, 21]. Therefore, we also examined the binding of 14-3-3 ζ with pT $\beta 2$ -CT and Dok1 with di-phosphorylated pSpT $\beta 2$ -CT (*vide infra*) (Table 1) in membrane mimic solutions containing dodecylphosphocholine by ITC (Supplemental Fig. S6). Our results showed that proteins/CTs binding affinity were significantly diminished in membrane mimic solutions. 14-3-3 ζ and Dok1 binds to phosphorylated CTs with K_d values of 649 μ M and 3.5 mM, respectively (Supplemental Fig. S6).

Ternary complex of 14-3-3 ζ , talin F3 and pT $\beta 2$ -CT proteins were further examined by glutaraldehyde mediated chemical crosslinking and size exclusion chromatography. Protein components were mixed in buffer solutions of 2% glutaraldehyde and samples were electrophoresed in SDS-PAGE gel at different time points (Supplemental Fig. S7). Protein band corresponding to the molecular weight of the ternary complex could be detected in the SDS-PAGE gel (Supplemental Fig. S7). In size exclusion chromatography experiments, the ternary complex of 14-3-3 ζ /F3/pT $\beta 2$ -CT can be co-eluted and observed in SDS-PAGE (Supplemental Fig. S8)

Exclusion of Dok1 from $\beta 2$ -CT in the presence of 14-3-3 ζ and talin F3: We probed changes in 31 P NMR signal of phosphorylated $\beta 2$ -CTs i.e. mono phosphorylated pT $\beta 2$ -CT and di-phosphorylated pSpT $\beta 2$ -CT (Table 1) in complex with 14-3-3 ζ , talin F3 and Dok1 proteins. Fig. 7a shows a set of 31 P NMR spectra of pT $\beta 2$ -CT in the absence (lower panel) and in the presence of talin F3, 14-3-3 ζ or 14-3-3 ζ /F3 combined. In free solution pT $\beta 2$ -CT yielded a sharp 31 P NMR signal

characteristic of unstructured protein undergoing fast motion. ^{31}P NMR spectrum of pT β 2-CT in complex with talin F3 showed discernable change in line-width and a slight change in chemical shift compared to the ^{31}P spectrum of free pT β 2-CT, thus infers certain interaction but phosphorylated Thr is not the site of interaction. By contrast, dramatic changes, in terms of chemical shift and line width, in ^{31}P NMR spectrum of pT β 2-CT were observed upon inclusion of 14-3-3 ζ . The downfield shift of ^{31}P signal of pT β 2-CT in the presence of 14-3-3 ζ would indicate direct interactions of the phosphate group of residue pThr758 with binding residues of 14-3-3 ζ . ^{31}P NMR spectrum of pT β 2-CT in the presence of both 14-3-3 ζ and talin F3 revealed further increased line width, with certain change of chemical shift, compared to the binary pT β 2-CT complexes of F3 and 14-3-3 ζ (Fig. 7a). ^{31}P NMR of pT β 2-CT therefore also revealed that 14-3-3 ζ /pT β 2-CT complex can recruit talin F3 in a ternary complex.

Dok1 protein is a negative regulator of integrin whereby binding of the PTB domain of Dok1 to the phosphorylated β -CTs displaces talin from integrin [7, 22, 23]. Therefore, we investigated the effect of binding of 14-3-3 ζ and Dok1 proteins to phosphorylated β 2-CT. The PTB domain of Dok1 binds to β 2-CT with high affinity upon phosphorylation of residue Ser756 [8]. We made use of di-phosphorylated pSpT β 2-CT for 14-3-3 ζ and Dok1 interactions. Fig. 7b shows ^{31}P NMR spectra of pSpT β 2-CT in free solution (lower panel), in complex with 14-3-3 ζ (middle panel) and in the presence of 14-3-3 ζ and Dok1 (upper panel). Both of the ^{31}P resonances of pSpT β 2-CT showed large chemical shift changes in the presence of 14-3-3 ζ (Fig. 7b). However, there were no noticeable changes of ^{31}P NMR signals upon further inclusion of equimolar concentration of Dok1 (Fig. 7b). Therefore, ^{31}P NMR studies

of the pSpT β 2-CT demonstrated that Dok1 was unable to interact with the di-phosphorylated pSpT β 2-CT in the presence of 14-3-3 ζ . Taken together, these results suggest that while talin F3 and 14-3-3 ζ could form a ternary complex with β 2-CT, the negative regulator Dok1 was unable to bind to β 2-CT in the presence of 14-3-3 ζ .

Structure model of the ternary complex: At present, we are unable to determine atomic-resolution structure of the entire ternary complex of 14-3-3 ζ /talin F3/pT β 2-CT. However, based on data from existing binary complexes, we modelled the ternary complex of pT β 2-CT, talin F3 and 14-3-3 ζ in order to discern potential inter-molecular contacts (see Materials and Methods). The overall topology of the ternary complex and molecular interactions are shown (Fig. 8a). The ternary complex of 14-3-3 ζ /pT β 2/talin F3 could be modular in nature whereby most of the inter-molecular interactions observed in the binary complexes of 14-3-3 ζ /pT β 2 peptide fragment and talin F3/ β 2-CT are largely conserved. The membrane distal region of pT β 2-CT carrying residue pT758 occupies the binding pocket of 14-3-3 ζ whereas the membrane proximal helix of the CT is engaged in interactions with talin F3 domain. In the docked structure, the phosphate group of pT758 is in close proximity with the guanidinium sidechain of residues R127, R56 and R60 (Fig. 8b), akin to the structure of 14-3-3 ζ /pT β 2-CT complex [14]. The membrane proximal helix of pT β 2-CT maintained its interactions with the β -sheet region of talin F3 through potential salt bridges involving residue D731 of β 2-CT and residues K322, K324 of talin F3 (Fig. 8c). Hydrophobic packing interactions could form between aromatic residues W747 of pT β 2-CT with residues Y377 and W351 of F3 domain (Fig. 8d). The NPLF

motif of pT β 2-CT could serve as a potential hinge between the talin F3 and 14-3-3 ζ with fewer inter-molecular contacts.

Discussion

The interaction between integrin β -CT and the F3 subdomain of talin plays an important role in integrin activation [2, 25-28]. This association brings about the separation of the integrin α and β -CTs that leads to allosteric conformational changes in the integrin ectodomain that are necessary for binding ligands (1-3, 25). Based on x-ray crystallography, the atomic-resolution structure of talin-2 F2F3 domain in complex with integrin β 1D-CT has been reported [16]. Although it is well accepted that talin binds integrin β -CT, there are clear differences in the binding affinities of talin with different integrin β -CTs. NMR interaction studies reported low affinity complexes of talin/integrin β 3-CT, $K_d \sim 273 \mu\text{M}$ and talin/integrin β 1A-CT, $K_d \sim 490 \mu\text{M}$ [29]. However, talin-2/integrin β 1D-CT and talin-1/integrin β 1D-CT complexes showed high affinity interactions with $K_d \sim 36 \mu\text{M}$ and $95 \mu\text{M}$, respectively (16, 29). These data suggest that subtle differences in the sequences of integrin β -CTs could affect their interactions with talin and other binding partners (Supplemental Fig. S9). As compared with other integrin β CTs, β 2-CT contains a few distinct motifs. These include a non phosphorylatable NxxF motif instead of the highly conserved NxxY motif found in other β CTs. It also contains a phosphorylatable TTT motif which may give rise to differential interactions with talin and other effector proteins. Our results showed that talin F3 interacted with integrin β 2-CT with low binding affinity of $K_d \sim 244 \mu\text{M}$ that is comparable to that between talin F3 and integrin CT β 3 or β 1A. However, it is difficult to determine the atomic structure of the talin F3/integrin β 2-

CT complex because of their transient interactions and resonances broadening. Indeed, similar challenges were encountered in the structural characterization of talin F3/integrin β 3-CT complex [17]. Instead, a hybrid peptide or peptide fragment of integrin β 3-CT covalently linked to talin F3/F2 was used for the structure determination [17, 30]. Nevertheless, we were able to map the binding of integrin β 2-CT onto talin F3 that includes the C-terminal long helix, β -strands S5, S4, an adjoining loop and β -strand 7 and a loop between S1 and S2 (Fig. 1d) based on above average chemical shift changes and resonance broadening. These structural elements of talin F3 were also found to be involved in the complex formation with integrin β 3-CT. The docked structure of talin F3 and integrin β 2-CT suggests potential intermolecular interactions within the complex (Fig. 2b). The interactions between talin F3 and the N-terminal MP part of integrin β 2-CT are found to be conserved with that of talin F3/chimeric integrin β 3-CT and talin F3/integrin β 1D-CT complexes [16, 17]. However, comparison of packing interactions involving talin F3 and the MD region of the integrin β -CTs remain challenging due to sequence variations amongst the β CTs. Our ^{15}N - ^1H HSQC binding data showed interactions between talin F3 and the MP region of β 2 peptide fragment (KS33) whereas no detectable binding was observed for the phosphorylated MD peptide (pTDS20) (Table 2). These data suggest that in addition to the MP region, the MD region of the integrin β -CTs may modulate talin/ β -CTs complex formation. Phosphorylation of integrin CTs, both α and β , plays critical roles in regulating their interactions with effector proteins [2, 21, 31, 32]. Phosphorylation of residue Y⁷⁴⁷ in NPxY in integrin β 3-CT promotes binding of Dok1, a negative regulator of integrin activation [7, 22, 23]. Talin-integrin β 3-CT interactions have been shown to be significantly reduced upon phosphorylation of

Y⁷⁴⁷ [7, 22, 23]. Given that integrin β 2-CT contains two non-phosphorylatable NPxF motifs instead of the NPxY motifs (Supplemental Fig. S9), the above mechanism of regulation with respect to talin interaction is unlikely. However, integrin β 2-CT contains phosphorylatable Ser and Thr residues that could be involved in integrin activation and regulation [8]. Indeed, we have shown that phosphorylation of integrin β 2-CT residue S756 that resides between the NPxF⁷⁵⁴ and T⁷⁵⁸TT motifs promotes Dok1 binding [8]. Phosphorylation of residue T758 in the TTT motif of integrin β 2-CT and the corresponding residue in integrin β 3 and β 7 CTs were reported to be involved in integrin activation [14]. pT758 of integrin β 2 facilitated binding of 14-3-3 ζ but inhibited the binding of negative regulator filamin to β 2-CT [14, 31-33]. 14-3-3 ζ also binds to phosphorylated α 4 CT of integrin [19], however, its binding to phosphorylated α -CTs of β 2 integrins has yet to be reported. We therefore examined the effect of pT758 on the interactions between integrin β 2-CT and talin F3. Interestingly, talin F3 interacted with pT758 β 2-CT with two-fold lower affinity as compared to β 2-CT (Table 2). Since pT758 β 2-CT recruits 14-3-3 ζ , we performed in depth analyses of the interactions between pT758 β 2-CT, talin F3 and 14-3-3 ζ so as to gain insights into complex formation. We demonstrated that pT758 β 2-CT forms a ternary complex with talin F3 and 14-3-3 ζ . Remarkably, in the ternary complex, pT758 β 2-CT/talin F3 interacted with a lower K_d value. However, the interaction affinity between pT758 β 2-CT/14-3-3 ζ remained invariant as compared to that of the binary complexes (Table 2). Interaction analyses using integrin β 2-CT peptide fragments further mapped the possible interaction sites of talin F3 and 14-3-3 ζ in the ternary complex. It should be noted that in the ternary complex, talin F3 and 14-3-3 ζ did not show any apparent interactions. Talin F3 interacts with the MP region of

integrin β 2-CT whereas 14-3-3 ζ interacts with the pT⁷⁵⁸TT motif and the MD region of integrin β 2-CT (Fig. 8). Interactions of the talin F3 with the MP helix of integrin β -CTs are known to be crucial for talin-mediated activation of integrins [2, 4, 5]. Our study also demonstrated that W747 in the MP region of integrin β 2 is required for talin F3 interaction in both the binary complex and in the ternary complex with 14-3-3 ζ . Residue W747 displays multiple packing interactions with talin F3 that could be important in the complex formation. These data are in line with previous study that showed interactions between talin and integrin β 3, β 1A and β 1D CTs involving this conserved Trp [29].

A recent study demonstrated the possibility of a ternary complex of filamin or domain FLA-IgN21 with integrin α IIb and β 3 CTs such that filamin interacts with both CTs while maintaining their interactions [36]. Notably, the membrane proximal helix of β 3-CT delineated packing with FLA-IgN21 causing a folding back of the β -CT. Further, the Dok1 binding site involving the NPxY motif of β 3 CT in the ternary complex is largely exposed. Based on our findings and that of others [36-38], we propose a sequential model of β 2 integrin inside-out activation followed by outside-in signaling (Fig. 9). In the resting state, the integrin β 2-CT is engaged with the negative regulators Dok1 and filamin (Fig. 9). It should be noted that Dok1 is involved in both inside-out [8] and outside-in [39] signaling of integrins. Upon cellular activation, the following series of inside-out β 2 integrin activation events could occur: (i) kindlin-3 docks onto the distal NPKF motif in the β 2-CT. Kindlin-3 could recruit migfilin that displaces filamin from the β 2-CT [40-42] (ii) β 2-CT pS756 is dephosphorylated and it leads to the release of Dok1 thereby allowing talin head domain to associate with the β 2-CT involving the NPLF motif, (iii) concomitantly, β 2-CT pT758 is

phosphorylated which promotes 14-3-3 ζ binding, (iv) the binding of 14-3-3 ζ to the β 2-CT leads to the repositioning of talin head domain facilitating ternary complex formation. These events lead to the activation of β 2 integrins, allowing them to bind ligands. Given that talin interacts with the actin cytoskeleton and 14-3-3 ζ has the potential to dimerize, such ternary complex formation could have an important role in integrin clustering, which is required for integrin outside-in signaling. In conclusion, our study provides evidence of ternary complex formation of 14-3-3 ζ , pT β 2-CT and talin F3. We have performed co-immunoprecipitation experiment to check for ternary complex using 293T cells transfected with integrin α L β 2, talin head domain and 14-3-3 ζ but was unsuccessful (data not shown). Future cell based experiments using different approaches will be necessary to validate the in vitro data. Notwithstanding, our data provide an important first step toward the understanding of sequential events leading to β 2 integrin inside-out activation and outside-in signaling.

Materials and Methods

Protein expression and purification: N-terminal six His-tag talin F3 domain (G309-S405), Dok1 PTB domain (Q154-G256) and 14-3-3 ζ (M1-S230) were generated by sub-cloning respective synthetic genes with *E. coli* optimized codons into the pET14b vector. Residue Cys 336 of F3 was mutated to Ser to avoid undesirable disulfide bond formation. Transformed *E. coli* BL21(DE3) cells were grown at 37°C either in LB or M9 medium containing [15 N] ammonium chloride and [13 C] D-glucose (Cambridge Isotope Laboratories). Protein expression was induced by adding IPTG (0.5 mM) to the cell culture (OD₆₀₀ ~ 0.6-0.7) followed by incubation for 10-12 hrs at 18°C. Proteins were purified as previously reported [8]. Cells were centrifuged and re-suspended in buffer A (50 mM sodium phosphate buffer, 300 mM NaCl, pH 8.0)

followed by affinity purification on a Nickel-NTA column (GE). His-tagged proteins were eluted in buffer B (50 mM sodium phosphate buffer, 500 mM imidazole, 300 mM NaCl, pH 8.0) using AKTA FPLC UPC-900 system (GE Healthcare UK Ltd., England) and exchanged against buffer C (20 mM sodium phosphate buffer, 50 mM NaCl, pH 6.0). His-tagged proteins were then subjected to size exclusion chromatography in buffer C on a Hiload Superdex 75 16/26 GL preparative column at a flow rate of 0.3 ml/min. Eluted proteins were monitored by absorbance at $\lambda = 276$ nm. The $\beta 2$ -CT (K724-S769) was sub-cloned into the pET-31b(+) vector to generate fusion proteins containing N-terminal ketosteroid isomerase (KSI) as previously described [43]. The same protocol was followed for the purification of $\beta 2$ -CT fusion protein except the buffer A and B contained 8 M urea. The eluted proteins were dialyzed against water to remove urea that resulted in the precipitation of fusion protein. Fusion protein was incubated in 90% formic acid purged with N₂ gas for 22 h in the dark to cleave the Asp-Pro peptide bond between the KSI and $\beta 2$ -CT. Formic acid was neutralized with sodium hydroxide using a rotary evaporator, leaving a film of precipitate. The precipitate was dissolved in water and further purified using HPLC. The identities of the cleaved peptides were verified by mass spectrometry.

Synthetic peptides: Synthetic $\beta 2$ -CT, phosphorylated $\beta 2$ -CT and $\beta 2$ peptide fragments (Table 1) were purchased from GLBioChemTM, Shanghai, China and further purified using HPLC (WatersTM 2489). Molecular weight of the peptides was confirmed by mass spectrometry.

NMR spectroscopy: All NMR experiments were performed on a Bruker DRX 600MHz spectrometer equipped with an actively shielded cryoprobe. ³¹P NMR spectra were recorded at Bruker 400MHz spectrometer. NMR data were processed using NMRPIPE or Topspin 3.0 and analyzed by Sparky (T.D. Goddard and D.G.

Kneller, University of California, San Francisco). The chemical shifts were directly or indirectly referenced to DSS. ^{15}N - ^1H HSQC spectra for ^{15}N -labelled talin F3 and $\beta 2$ -CT were acquired at a concentration of 0.1 mM in buffer C (20 mM sodium phosphate buffer, 50 mM NaCl, pH 6.0). ^{15}N - ^1H HSQC resonance assignments of talin F3 were achieved by 3D experiments (HNCACB, CBCA(CO)NH) of $^{15}\text{N}/^{13}\text{C}$ labelled talin F3, 0.3 mM, samples in buffer C. For NMR titration, ^{15}N - ^1H HSQC spectra of protein samples were recorded after 10 min of equilibration with sequential addition of binding partners. Stock solutions of 3.8 mM, 5.1 mM, 4.2 mM and 3.1 mM of $\beta 2$ -CT, pT $\beta 2$ -CT, KS33, and pTDS20, respectively, were used to titrate against 0.1 mM ^{15}N labelled talin F3 at different molar ratios (1:0.4, 1:0.8, 1:0.12, 1:1.6, 1:2, 1:3, 1:4 and 1:5). ^{15}N - ^1H HSQC titrations were carried out with pT $\beta 2$ -CT and $\beta 2$ peptide fragments in solutions containing equimolar mixture, 0.1 mM, of ^{15}N labelled talin F3 and unlabeled 14-3-3 ζ . ^{15}N - ^1H HSQC spectra of ^{15}N labelled $\beta 2$ -CT were acquired in the presence of unlabeled talin F3 (0.7 mM stock) at different molar ratios (1:0.5, 1:1, 1:1.5, 1:2, 1:2.5 and 1:3). Ligand induced chemical shift changes in ^{15}N and HN resonances were determined using the following equation which is designated as chemical shift perturbation (CSP), $\Delta(\text{H},\text{N}) = [\{(\Delta\text{H})(\text{WH})\}^2 + \{(\Delta\text{N})(\text{WN})\}^2]^{1/2}$ where WH and WN are weighting factors for ^1H and ^{15}N chemical shifts, respectively (WH = 1, WN = 0.154) and ΔH and ΔN are the chemical shift difference for HN and ^{15}N , respectively. The K_d values were calculated from depletion model for ligand binding following the equation; $\Delta(\text{H},\text{N}) = \Delta(\text{H},\text{N})_{\text{max}} [\{ [\text{L}] + [\text{U}] + K_d \} - [\{ [\text{L}] + [\text{U}] + K_d \}^2 - 4[\text{L}][\text{U}] \}^{1/2}] / 2[\text{L}]$, where $\Delta(\text{H},\text{N})$ and $\Delta(\text{H},\text{N})_{\text{max}}$ are the weighted chemical shift and weighted chemical shift at saturation, K_d represents the dissociation constant, and [L] and [U] are the concentrations of protein and unlabelled peptides, respectively. Well resolved ^{15}N - ^1H

HSQC peaks with significant chemical shift changes were used to fit the above equation using OriginPro 9.0 in order to determine K_d values. The dissociation constant is presented as $K_d \pm S.E$ (standard error). The error bar indicates the standard deviation for each titration point.

Relaxation measurement: The transverse relaxation (T_2) rates of ^{15}N nuclei of backbone of talin F3 were determined by collecting a series of ^{15}N - 1H HSQC spectra of 0.1 mM talin F3 alone and talin F3+pT β 2-CT (1:3) talin F3+14-3-3 ζ +pT β 2-CT (1:1:3) with sensitivity enhancement in a Bruker DRX 600 MHz spectrometer at 298 K temperature. T_2 measurements were acquired with delays of 14.4, 28.8, 43.2, 57.6, 72.0, 86.4, 100.8, 115.2, 129.6, 144.0 and 158.4 ms and repeated twice for all the experimental conditions. T_2 values were estimated from by fitting the cross peak intensities to a mono-exponential function as in $I = A \cdot \exp(R \cdot t)$, where I, A, R and t represent signal intensity, constant, relaxation value and time point, respectively.

^{31}P NMR experiments: ^{31}P NMR spectra of pT β 2-CT were recorded on a Bruker DRX 400 spectrometer with dual channel RT probe at 298 K at 0.2 mM peptide concentrations. Data acquisition and processing were performed with the Topspin software (BRUKER) suite. ^{31}P NMR spectra of pT β 2-CT in a buffer containing 20 mM Tris, 50 mM NaCl, pH 6.0 were recorded with talin F3 and 14-3-3 ζ with 0.1 mM of each. Using same buffer condition, ^{31}P NMR spectra of 100 μ M pSApT β 2-CT were acquired with equimolar concentrations of Dok1 PTB domain and 14-3-3 ζ .

Isothermal Titration Calorimetry (ITC): All samples were prepared in 20 mM sodium phosphate and 50 mM NaCl (pH 6.5). ITCs were performed in a VP-ITC200 instrument at 298 K. For 14-3-3 ζ /phosphorylated CTs binding, fixed amounts of either pT β 2-CT or pTDS20 peptide were injected into cell, 200 μ l, containing 14-3-3 ζ . For ternary complex, fixed concentrations of F3 were injected into cell, 200 μ l,

containing equimolar mixture of 14-3-3 ζ and pT β 2-CT. Interactions of 14-3-3 ζ and Dok1 with pT β 2-CT and pSpT β 2-CT, respectively, were carried out in 20 mM sodium phosphate and 50 mM NaCl (pH 6.5) containing 10 mM dodecylphosphocholine. ITC experiments in dodecylphosphocholine were performed either with 25 μ M 14-3-3 ζ (in cell) or 20 μ M Dok1(in cell). pT β 2-CT (690 μ M) and pSpT β 2-CT (290 μ M) were kept in syringe. Typically, a first injection of 0.2 μ l was followed by 19 injections of 2 μ l, with 150 s time delay between two injections and stirred at a speed of 900 rpm. Taking care of heat of dilution, a blank titration was performed by injecting proteins into buffer. All raw data were processed using MicroCal Origin software.

Chemical cross-linking: Glutaraldehyde mediated protein cross linking experiments were carried out with reaction mixtures containing 14-3-3 ζ :talins F3:pT β 2-CT at 1:1:3 molar ratios and 14-3-3 ζ :pT β 2-CT at 1:3 in 20 mM sodium phosphate and 50 mM NaCl (pH 8.0) with a total volume of 100 μ l, in the presence of 2 μ l of 2% freshly prepared solution of glutaraldehyde at 37°C. Samples were collected at different time intervals and reactions were quenched by addition of 1.5 M Tris-HCl, pH 8.0. Cross-linking was observed by conducting electrophoresis in 16% SDS-polyacrylamide gel.

Size exclusion chromatography: A Hiload Superdex 75 16/26 GL preparative column using AKTA FPLC UPC-900 system (GE Healthcare UK Ltd., England) was employed for size exclusion chromatography. A solution of 500 μ l of 14-3-3 ζ :talins F3:pT β 2-CT in 1:1:3 ratio injected and eluted with 20 mM sodium phosphate buffer, 50 mM NaCl, pH 6.0 at a flow rate of 0.30ml/min. Formation of ternary complex was determined by 16% SDS-polyacrylamide gel.

Docking of talin F3 with pT β 2-CT and 14-3-3 ζ : Structural models of β 2-CT and pT β 2 were constructed based on the 3-D structures of β 1D/talin 2 [14] and pT β 2

peptide/14-3-3 ζ [12] complexes, using InsightII (Accelrys Inc.). Docked models of binary β 2-CT/talin F3 (pdb:2h7d) and ternary pT β 2-CT/talin F3/14-3-3 ζ (pdb:2v7d) complexes were derived using ZDOCK program (<http://zdock.umassmed.edu>) based on chemical shift changes and cross-peak boarding. The model structures were validated using ADIT Validation Server from the Protein Data Bank (PDB).

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FIGURE LEGENDS

Figure 1. Mapping talin F3 interactions with β 2-CT. (a) Overlay of ^{15}N - ^1H HSQC spectra of talin F3 in the absence of β 2-CT (blue contour), and in the presence of increasing concentrations of β 2-CT at 1:1 (red contour) and 1:3 (brown contour) ratios. Chemical shift changes of some representative residues T354, G371 and Q390 are highlighted. (b) Bar diagram showing combined chemical shift perturbation for ^{15}N and HN resonances of each residue of talin F3 upon binding to β 2-CT at F3: β 2-CT 1:3 ratio. The dotted line marked average chemical shift perturbation. (c) Normalized chemical shift differences are plotted against the concentrations of β 2-CT for talin F3 to determine equilibrium dissociation constant (K_d) value. (d) A ribbon representation of the three-dimensional structure of talin F3. Residues that exhibited above average chemical shift perturbations and resonance broadening in the presence of β 2-CT are shown as blue and red, respectively.

Figure 2. Interactions of β 2-CT with talin F3 and F3/ β 2-CT docked. (a) A bar diagram showing change of intensity in ^{15}N - ^1H HSQC spectra of β 2-CT upon addition of talin F3 at 1:3 ratio. (b) A ribbon representation of the docked structure of talin F3 (in cyan) and β 2-CT (light red) highlighting some of the residues at the interface of the complex.

Figure 3. Mapping talin F3 interactions with pT β 2-CT. (a) Bar diagram showing combined chemical shift perturbation for ^{15}N and HN resonances of each residue of talin F3 upon binding to β 2-CT at F3: β 2-CT 1:3 ratio. The dotted line marked average

chemical shift perturbation. (b) Bar diagram showing differences in weighted chemical shift of residues of F3 talin in the presence of β 2-CT and pT β 2-CT. ^{15}N - ^1H HSQC cross-peaks of some residues of F3 delineated resonance broadening upon binding interactions either with β 2-CT or pT β 2-CT are marked as down arrows and up arrows, respectively. Residues of F3 showing resonance broadening upon binding interactions with β 2-CT and pT β 2-CT are indicated as cross. (c) Normalized chemical shift differences are plotted against the concentrations of pT β 2-CT for talin F3 to determine equilibrium dissociation constant (K_d) value.

Figure 4. Mapping talin F3 interactions with membrane proximal β 2-CT peptide

KS34. (a) Bar diagram showing combined chemical shift perturbation for ^{15}N and HN resonances of each residue of talin F3 upon binding to KS34 at F3:KS34 1:3 ratio. The dotted line marked average chemical shift perturbation. (b) Normalized chemical shift differences are plotted against the concentrations of KS34 for talin F3 to determine equilibrium dissociation constant (K_d) value.

Figure 5. Mapping talin F3 interactions with pT β 2-CT and 14-3-3 ζ .

(a) Overlay of sections of ^{15}N - ^1H HSQC spectra of representative residues of talin F3 alone (in blue contour), in the presence of 3 fold excess of pT β 2-CT in green contour and in the presence of both pT β 2 and 14-3-3 ζ (in red contour) (F3:14-3-3 ζ :pT β 2 1:1:3). (b) Normalized chemical shift differences are plotted against the concentrations of pT β 2-CT for talin F3 to determine equilibrium dissociation constant (K_d) value. (c) A ribbon representation of the three-dimensional structure of talin F3. Residues that exhibited above average chemical shift perturbations and resonance broadening in the presence of β 2-CT and 14-3-3 ζ are shown as blue and red, respectively.

Figure 6. Thermodynamic characterization of interactions between pT β 2-CT, talin F3 and 14-3-3 ζ in ternary complex by ITC. (a) ITC titrations of binding of full-length pT β 2-CT with 14-3-3 ζ . (b) ITC titrations of binding of Thr phosphorylated peptide fragment pTDS20 of pT β 2-CT (Table 1) with 14-3-3 ζ . In both titrations, aliquots of fixed amount of phosphorylated β 2-CT peptides were injected into sample cells containing 14-3-3 ζ . (c) ITC titrations of binding of talin F3 with the pre-formed complex of full-length pT β 2-CT and 14-3-3 ζ . Aliquots of fixed amount of F3 samples were injected into sample cells containing equimolar concentrations of pT β 2-CT and 14-3-3 ζ . The top panels of the ITC thermograms show heat exchange of binding whereas the heat of reaction as function of molar ratios is shown at the bottom panels.

Figure 7. Interactions of pT β 2-CT and pSpT β 2-CT with 14-3-3 ζ , Dok1 and talin F3. (a) ^{31}P NMR spectra of pT β 2-CT in free solution, in the presence of talin F3 (pT β 2:F3, 1:0.5), 14-3-3 ζ (pT β 2:14-3-3 ζ , 1:0.5) and talin F3, 14-3-3 ζ (pT β 2:F3:14-3-3 ζ 1:0.5:0.5). (b) ^{31}P NMR spectra of pSpT β 2-CT in free solution, in the presence of 14-3-3 ζ (SpT β 2:14-3-3 ζ , 1:1) and with Dok1, 14-3-3 ζ (pT β 2:Dok1:14-3-3 ζ , 1:1:1).

Figure 8. Docked structure of the ternary complex of talin F3, 14-3-3 ζ and pT β 2-CT. (a) A ribbon representation of the topology of the ternary complex dimeric 14-3-3 ζ in green, talin F3 in cyan and pT β 2-CT in light red. (b) Ionic interactions of residue pT758 of pT β 2-CT with cationic residues R126, R56 and R61 of 14-3-3 ζ at the canonical phosphorylated peptide binding pocket. (c-d) Potential ionic and non-

polar packing interactions of pT β 2 with talin F3, residue D731 of the membrane proximal helix and residue D749 of β 2-CT are in close proximity of cationic residues of talin F3 (panel c), potential aromatic packing interactions may involve residue W748 of pT β 2-CT with residues Y376 and W351 of talin F3 (panel d).

Figure 9. A proposed model of β 2 integrin activation that involves complex formation with talin and 14-3-3 ζ . In its resting state, β 2 integrin adopts a bent conformation with clasped TMs and CTs. This is maintained by filamin that binds to two regions within β 2-CT and the membrane proximal region in the α -CT. In the resting β 2 integrin, Ser756 could be phosphorylated which promotes Dok1 binding. Upon cellular activation, kindlin-3 is recruited to the NPKF motif of the β 2-CT. Kindlin-3 binds to migfilin that could displace filamin from its β 2-CT binding sites but filamin could still retain its interaction with the α -CT. Concomitantly, pSer756 could be dephosphorylated by yet unknown mechanism. This leads to the dissociation of Dok1 from its β 2-CT binding site. The release of both filamin and Dok1 from the β 2-CT allows talin binding. This disrupts the salt-bridge interaction between the β 2 integrin CTs, leading to the separation of the CTs and TMs. As a consequence, the β 2 integrin undergoes global conformational changes from a bent to an extended conformation, which is a hallmark of integrin inside-out activation. At this stage, Thr758 in the β 2-CT could be phosphorylated and it promotes 14-3-3 ζ binding. 14-3-3 family of proteins is known to form homodimers. Hence, a 14-3-3 ζ monomer on one β 2-CT could homodimerize with another 14-3-3 ζ monomer on another β 2-CT, leading to β 2 integrin clustering. Given that talin rod region binds actin, the β 2 integrin cluster is further stabilized by talin-filamentous actin interactions. The

clustering of $\beta 2$ integrins and their linkages to the actin cytoskeleton not only increase the overall avidity of ligand binding, they also induce outside-in signaling.

Table 1. Primary structures of synthetic β 2-CT and β 2-CT peptide fragments used in this study*.

Name	Residue	Sequence
β 2-CT	724-769	K-A-L-I-H-L-S-D-L-R-E-Y-R-R-F-E-K-E-K-L-K-S-Q- W-N-N-D-N-P-L-F-K-S-A-T-T-T-V-M-N-P-K-F-A-E-S
pT β 2-CT	724-769	K-A-L-I-H-L-S-D-L-R-E-Y-R-R-F-E-K-E-K-L-K-S-Q- W-N-N-D-N-P-L-F-K-S-A- pT -T-T-V-M-N-P-K-F-A-E- S
pTDS20	751-769	D-N-P-L-F-K-S-A- pT -T-T-V-M-N-P-K-F-A-E-S
KS33	724-757	K-A-L-I-H-L-S-D-L-R-E-Y-R-R-F-E-K-E-K-L-K-S-Q- W-N-N-D-N-P-L-F-K-S
pT β 2WA	724-769	K-A-L-I-H-L-S-D-L-R-E-Y-R-R-F-E-K-E-K-L-K-S-Q- A-N-N-D-N-P-L-F-K-S-A- pT -T-T-V-M-N-P-K-F-A-E-S
pT β 2FA	724-769	K-A-L-I-H-L-S-D-L-R-E-Y-R-R-F-E-K-E-K-L-K-S-Q- W-N-N-D-N-P-L-A-K-S-A- pT -T-T-V-M-N-P-K-F-A-E- S
pSpT β 2-CT	724-769	K-A-L-I-H-L-S-D-L-R-E-Y-R-R-F-E-K-E-K-L-K-S-Q- W-N-N-D-N-P-L-A-K- pS -A- pT -T-T-V-M-N-P-K-F-A- E-S

*phosphorylated residues and Ala replacements are bold faced.

Table 2. Determination of K_d values of β 2-CT, pT β 2-CT, talin F3 and 14-3-3 ζ interactions by ^{15}N - ^1H HSQC titrations

Complex	K_d (μM)
^{15}N -F3/ β 2-CT	244 ± 14
^{15}N -F3/pT β 2-CT	578 ± 74
^{15}N -F3/pT β 2-WA	1361 ± 248
^{15}N -F3/pT β 2-FA	668 ± 75
^{15}N -F3/KS33	357 ± 31
^{15}N -F3/14-3-3 ζ /pT β 2-CT	213 ± 25
^{15}N -F3/14-3-3 ζ /pT β 2 FA	493 ± 61
^{15}N -F3/14-3-3 ζ /pT β 2 WA	564 ± 62

Table 3: ITC interaction analyses of binding of pT β 2-CT, 14-3-3 ζ and talin F3

Cell Conc. (μ M)		Syringe Conc. (μ M)		K_d	ΔH (Kcal/mol)	$T\Delta S$ (Kcal/mol)	ΔG (Kcal/mol)
14-3-3 ζ	25	pT β 2	580	4.9 $\pm$ 1.2	-13.22	-5.97	-7.25
14-3-3 ζ	10	pT β 2 MD	220	11.03 $\pm$ 1.2	-10.29	-11.7	6.54
pT β 2:14-3-3 ζ	25	F3	800	161.8 $\pm$ 31.2	-17.94	-12.75	-5.18

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9