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Talin-driven inside-out activation mechanism of platelet $\alpha\text{IIb}\beta 3$ integrin probed by multimicrosecond, all-atom molecular dynamics simulations

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Abstract

Platelet aggregation is the consequence of the binding of extracellular bivalent ligands such as fibrinogen and von Willebrand factor to the high affinity, active state of integrin $\alpha\text{IIb}\beta 3$. This state is achieved through a so-called “inside-out” mechanism characterized by the membrane-assisted formation of a complex between the F2 and F3 subdomains of intracellular protein talin and the integrin $\beta 3$ tail. Here, we present the results of multi-microsecond, all-atom molecular dynamics simulations carried on the complete transmembrane (TM) and C-terminal (CT) domains of $\alpha\text{IIb}\beta 3$ integrin in an explicit lipid-water environment, and in the presence or absence of the talin-1 F2 and F3 subdomains. These large-scale simulations provide unprecedented molecular-level insights into the talin-driven inside-out activation of $\alpha\text{IIb}\beta 3$ integrin. Specifically, they suggest a preferred conformation of the complete $\alpha\text{IIb}\beta 3$ TM/CT domains in a lipid-water environment, and testable hypotheses of key intermolecular interactions between $\alpha\text{IIb}\beta 3$ integrin and the F2/F3 domains of talin-1. Notably, not only do these simulations give support to a stable left-handed reverse turn conformation of the αIIb juxtamembrane motif rather than a helical turn, but they raise the question as to whether TM helix separation is required for talin-driven integrin activation.

Keywords

Anton; long-scale MD; molecular complex; membrane; CHARMM

INTRODUCTION

Integrin $\alpha\text{IIb}\beta 3$ is a hetero-dimeric receptor complex that mediates platelet aggregation through binding extracellular bivalent ligands such as fibrinogen and von Willebrand factor.¹ Each subunit of this complex features a large extracellular domain (ECD), a single-spanning

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transmembrane (TM) helix, and an intracellular cytoplasmic tail (CT). The heterodimer normally resides in the low-affinity resting state on the cell surface (or bent conformation as shown by X-ray crystallography and electron microscopy²), and can transmit bi-directional signals across the cell membrane via inside-out and outside-in pathways.^{3,4} The former is activated upon interaction of intracellular proteins such as talin and kindlin with residues of the integrin $\beta 3$ CT domain, and results in long-range propagation of conformational changes to the integrin ECD,^{5,6} leading to the receptor adopting a high affinity state for its extracellular ligands.⁷ This high affinity state is believed to feature an extended and “swung-out” (open) conformation of the ECD,^{8,9} as well as separated TMs.^{10–17} However, recent studies of intact $\alpha \text{IIb}\beta 3$ using solution X-ray scattering and electron microscopy¹⁸ have challenged the well-established idea of TM domain separation^{10–17} given that the C-terminal portions of the αIIb and $\beta 3$ subunits and the legs were seen to be close together in all detected conformations of the protein, including bent, extended-closed, or extended-open conformations. Moreover, the addition of talin head domain to purified $\alpha \text{IIb}\beta 3$ embedded in a DMPC/DMPG lipid bilayer nanodisc in the absence of detergent resulted in extension of the receptor and enhanced ligand binding, but not leg separation.⁵ Thus, there is controversy as to whether integrin TM domain separation is necessary for integrin activation under physiologic conditions. Since activation of the $\alpha \text{IIb}\beta 3$ receptor is required to achieve hemostasis, but may lead to harmful thrombus formation and consequent ischemic arterial diseases, understanding how, at the molecular level, “inside-out” signaling propagates from the site of interaction between cellular activators and the receptor CTs to the TM dimer, and ultimately to the ECD, has important clinical implications.

A wealth of recent experimental^{19–24} and computational^{25–28} studies has unveiled important details of the molecular mechanism underlying integrin inside-out activation. However, these insights have mostly been inferred from incomplete or isolated $\alpha \text{IIb}\beta 3$ integrin TM/CT constructs, short all-atom simulations, or serial multiscale approaches combining coarse-grained simulations of membrane association with atomistic refinements. In this study, we carried out all-atom, multimicrosecond molecular dynamics (MD) simulations of the complete $\alpha \text{IIb}\beta 3$ integrin TM/CT domains in an explicit lipid-water environment, and with the αIIb juxtamembrane motif in either a helical or left-handed reverse turn conformations, as observed by NMR in an organic-water mixture²¹ or phospholipid bicelles,²⁹ respectively. The system with a left-handed reverse turn conformation of the αIIb juxtamembrane motif was the only one to remain stable during microsecond-scale MD simulations, and was then also simulated in the presence of the talin-1 F2-F3 domains using the massively parallel special-purpose supercomputer Anton³⁰ for MD simulations. Results of these simulations provide an unprecedented level of molecular detail on the conformational dynamics that promote transition of $\alpha \text{IIb}\beta 3$ to its high affinity state.

MATERIAL AND METHODS

System setups

Initial molecular models of the complete $\alpha \text{IIb}\beta 3$ integrin TM/CT domains (Fig. 1), including linkers between the TMs and ECD (specifically: residues αIIb A958-E1008/ $\beta 3$ P685-T762), with the αIIb juxtamembrane segment 991GFFKR995 in either a helical or

left-handed reverse turn conformation were built using as structural templates NMR structures corresponding to PDB codes 2KNC (α IIb G958-E1008/ β 3 A685-T762)²¹ or 2K9J (α IIb A958-P998/ β 3 P685-F727),²⁹ respectively. Specifically, the model with a helical α IIb 991GFFKR995 segment was based entirely on the 2KNC structure, whereas in the model with this segment in a left-handed reverse turn conformation, missing parts of the 2K9J structure (α IIb P998-E1008, β 3 F727-A750, and β 3 T751-T762) were taken from 2KNC, a β 3 model based on the crystal structure of the integrin β 1D CT domain in complex with murine talin-2 F2-F3 (PDB: 3G9W³¹), and the NMR structure of the complete β 3 CT domain (PDB: 2KV9²²), respectively. A complex of this latter complete α IIb β 3 TM/CT model with talin-1 F2-F3 (residues S206-S405) was generated on the basis of the crystal structure corresponding to PDB code 3G9W,³¹ using the integrin β 1D CT domain in complex with the highly homologous murine talin-2 as structural templates for the corresponding β 3 and talin-1 domains, respectively. Residues that had been mutated in the experimental constructs were replaced by corresponding amino acids in normal human α IIb β 3 sequences.

Atomistic MD simulations

The 2KNC-based and 2K9J-based systems without talin and the 2K9J-based model with talin were embedded into pre-equilibrated, TIP3P-hydrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline (POPC) lipid patches of $81 \times 78 \times 129 \text{ \AA}$, taking into consideration the suggested perpendicular orientation of the α IIb TM helix to the plane of the membrane,^{29,32} and the need for a positively charged patch of the talin F2 domain to face the membrane.³¹ The resulting systems in the presence or absence of talin were neutralized by 8 Cl^- or 3 Na^+ counter-ions, and arranged in unit cells of $79 \times 76 \times 124 \text{ \AA}^3$ or $53 \times 81 \times 124 \text{ \AA}^3$ containing $\sim 76,000$ or $\sim 50,000$ atoms, respectively. The CHARMM27 force field topology and parameters³³ with the CMAP backbone energy correction³⁴ were used to describe these systems. Following energy minimization and 50 ns equilibration runs with Desmond 2.4, standard, unrestrained MD production simulations were carried out using the NPT ensemble controlled using the Berendsen thermostat and barostat at 300 K and 1 atm, respectively, as well as reversible reference system propagator algorithms (RESPA) with a timestep of 2.0 fs for both the bonded and the near non-bonded interactions and of 6.0 fs for the far nonbonded interactions. The 2KNC-based and 2K9J-based systems without talin were simulated for ~ 4.0 and $\sim 3.0 \mu\text{s}$, respectively, whereas the 2K9J-based complex with talin was simulated for $\sim 5.0 \mu\text{s}$. Most simulations were carried out on the special-purpose supercomputer Anton.³⁰

Analysis of MD simulations

Simulations were analyzed using various tools, including in-house R scripts to measure crossing and tilting angles of the TM helices, as well as the fraction of intra-molecular HN-NH restraints used to obtain the 2KNC and 2K9J NMR ensembles. Gromacs tools `g_energy` and `g_dist` were used to calculate interaction energies and inter-atomic distances, respectively. Rotation and bending of the TM helices were calculated with the TRAJELIX module³⁵ of the SIMULAID package.³⁶ Briefly, after superposition of the helix axis to the axis of the reference structure, the angle between the vectors projecting the Ca atom on the helix axis in each frame were calculated for each residue, and its increment averaged over

the whole TM region to yield the mean rotation angle. All time-course plots report averages obtained using a moving linear filter with a 100 ns bandwidth, superimposed to raw data sampled with a 1 ns frequency, as well as boxplots indicating the average, the 1st and 3rd quartiles, and 1.5 interquartile range whiskers.

RESULTS AND DISCUSSION

The α IIb β 3 TM/CT structure with a helical juxtamembrane segment (2KNC) is unstable in an explicit lipid-water environment

Two experimental model structures have been reported in the literature for the α IIb β 3 TM/CT domain: a) an NMR structure (PDB: 2KNC)²¹ of the complete integrin α IIb β 3 TM and CT domains (α IIb G955-E1008/ β 3 G684-T762) in a CD3CN/H₂O mixture [Fig. 1(A)], and b) an NMR structure (PDB: 2K9J)²⁹ of the same complex with truncated CT domains (α IIb G957-P998/ β 3 P685-F727) in phospholipid bicelles [Fig. 1(B)]. Both model structures are characterized by a right-handed crossing angle between the α IIb and β 3 subunits (-22° , -32° in 2K9J and 2KNC, respectively), and by two main sites of inter-subunit interaction: the so-called outer membrane and inner membrane clasps (or OMC and IMC, respectively). Unlike the OMC [Fig. 1(C,D)], which is characterized by packing interactions between glycines of the α IIb 972GXXXG976 motif and β 3 V700/M701/I704 residues, as well as between α IIb L979/L980 and β 3 L705/G708 residues, the IMC [Fig. 1(E,F)], which includes residues of the highly conserved α IIb juxtamembrane 991GFFKR995 motif, differs significantly between the two NMR structures of the α IIb β 3 TM/CT domains. Specifically, while the α IIb 991GFFKR995 motif exhibits a left-handed reverse turn in lipid bicelles [2K9J²⁹ or Fig. 1(B)], it rather assumes a helical conformation in the CD3CN/H₂O mixture [2KNC²¹ or Fig. 1(A)]. Notably, the left-handed reverse turn was predicted to be the most stable conformation of the α IIb 991GFFKR995 segment by a Rosetta Membrane^{37,38} computational model generated using disulfide-based distance restraints inferred from experiments of intact integrins on the cell surface.³⁹ In both the NMR structure in lipid bicelles (2K9J)²⁹ and the Rosetta model³⁹ this conformation placed both α IIb F992/F993 side chains inside the membrane at the interface between the α IIb and β 3 subunits [Fig. 1(F)], allowing them to form interactions with α IIb M987 and β 3 L712/W715/K716/I719. In contrast, in the NMR structure obtained in the CD3CN/H₂O mixture (2KNC)²¹ the α IIb F992/F993 side chains were not found at the interface between the α IIb and β 3 subunits, and the IMC [Fig. 1(E)] appeared to be stabilized by a weak interaction between α IIb 990VGF992 and β 3 I719.

A common feature of the IMC in the two different NMR structures^{21,29} is a salt bridge between α IIb R995 and β 3 D723 [Fig. 1(E,F)]. Conflicting experimental evidence exists in the literature regarding the ability of this salt-bridge to stabilize an inactive α IIb β 3 conformation. Early experiments in stable CHO transfectants using alanine mutations and “charge-reversal” mutations of the α IIb R995 and β 3 D723 residues suggested a role for each member of this salt bridge in keeping the receptor in a resting state, although mutating D723 to alanine produced more activation than mutating it to arginine.⁴⁰ In addition, NMR experiments in aqueous conditions demonstrated that an α IIb R995D mutation disrupted the interaction between peptides comprising the α IIb tail (K989-E1008) and the β 3 tail (K716-

T762), as did the addition of talin.⁴¹ Later experiments in both 293T transfectants and transient CHO-K1 cells,³⁹ however, showed minimal activation by a $\beta 3$ D723A mutation. Interpreting the results of $\beta 3$ D723 mutations is potentially confounded by the suggestion that $\beta 3$ D723 participates in the activation process through a direct interaction with talin-1 K324.³¹ In view of the above evidence supporting a role for the α IIB R995- $\beta 3$ D723 interaction in maintaining α IIB $\beta 3$ in a low affinity state, it was surprising that the α IIB R995 and $\beta 3$ D723 side chains were within hydrogen-bonding distance in only 16% of the low energy structures identified by Rosetta modeling.³⁹ This study noted that α IIB R995 and $\beta 3$ D723 both sample a large conformational space and that the R995 side chain can also interact with $\beta 3$ D726, creating an alternative salt bridge to maintain the receptor in an inactive conformation.

In contrast to the relatively low frequency of α IIB R995- $\beta 3$ D723 interactions identified in the Rosetta conformation sampling, interactions between the $\beta 3$ K716 side chain and the α IIB F992/K994 backbone carbonyl oxygens were recorded in 65% of the structures in the Rosetta conformational sampling, and the functional role of this interaction was supported by demonstrating that mutating $\beta 3$ K716 to any other residue led to the integrin adopting a high-affinity state.³⁹ However, the interactions between $\beta 3$ K716 and α IIB F992/K994 backbone carbonyl oxygens are missing in both NMR structures, although the $\beta 3$ K716 side chain was found in the vicinity of the α IIB M987 backbone carbonyl oxygen in the NMR structure obtained in the CD3CN/H₂O mixture (2KNC²¹).

A multistep inside-out signaling mechanism was proposed²¹ to reconcile the differences between the two existing NMR structures of the α IIB $\beta 3$ TM/CT domains.^{21,29,39} According to this proposal, the α IIB juxtamembrane helical motif of 2KNC corresponds to a native conformation in the aqueous cytoplasm that transitions into an intermediate, transient state represented by the alternative left-handed reverse turn conformation of 2K9J along the integrin activation pathway. To investigate the likelihood of these NMR model structures in an explicit lipid bilayer, we carried out multimicrosecond, unrestrained MD simulations of complete α IIB $\beta 3$ TM/CT models [see Fig. 1(A,B)] built in an explicit, hydrated lipid bilayer following the procedure described in Materials and Methods.

Our extended, 4 μ s all-atom simulations of the 2KNC-based model structure with an α IIB 991GFFKR995 helical segment [Fig. 1(A)] reveal helix unwinding after $\sim 2 \mu$ s in an explicit lipid-water environment, and embedding of both α IIB F992/F993 aromatic rings into the membrane. This observation suggests instability of the 2KNC-based structure in a lipid bilayer as demonstrated by an increased RMSD from the 2KNC experimental structure and a slightly decreased one from the 2K9J structure (see Supporting Information Fig. S1A). Notably, $\sim 70\%$ and $\sim 85\%$ of the NOE distance restraints are satisfied for α IIB and $\beta 3$ respectively during simulation of both systems (Supporting Information Figs. S2A and S2B).

The 2KNC is also notable for the α IIB F992, but not the F993, aromatic ring positioning itself between the α IIB and $\beta 3$ subunits, thus establishing interactions with $\beta 3$ L712/W715 during the simulated timescale. The salt-bridge interaction between α IIB R995 and $\beta 3$ D723 is relatively unstable during the first 2.5 μ s of these simulations, but then stabilizes during

the remainder of the simulation time (see Supporting Information Fig. S3A). Notably, no additional interactions of $\beta 3$ K716 with the α Ib F992/K994 backbone carbonyl oxygens were observed in these simulations (see Supporting Information Fig. S3B and S3C).

Unlike the 2KNC-based structure, the 2K9J-based complete α Ib $\beta 3$ TM/CT model structure is very stable during the achieved 3 μ s MD simulations, maintaining a small RMSD from the initial conformation (Supporting Information Fig. S1B), as well as the main interactions within IMC and OMC. Not only does the left-handed reverse turn of the α Ib 991GFFKR995 segment remain unaltered during these simulations (with an average backbone root mean square deviation of 0.84 Å with respect to the initial conformation), as also inferred from Rosetta modeling,³⁹ but the IMC interactions between the α Ib F992/F993 side chains and α Ib M987 or $\beta 3$ L712/W715/K716/I719 are mostly preserved (see time evolution of these interactions in Supporting Information Fig. S4A) throughout the simulated timescale. This is also the case for the OMC interactions between α Ib G972/G976/L979/L980 and $\beta 3$ V700/M701/I704/L705/G708 (Supporting Information Fig. S4B), given that the right-handed crossing angle between the α Ib and $\beta 3$ TM helices remains mostly confined to a $-33^\circ \pm 8^\circ$ value during simulation [black line in Fig. 2(A)]. This angle was reported to be -32° , -22° , -37° , or -35° in the two structures obtained by NMR,^{21,29} the experimentally driven Rosetta modeling,³⁹ and recent multiscale MD simulations,²⁶ respectively.

Since the long simulations of the complete α Ib $\beta 3$ TM/CT domain support a stable 2K9J-based model structure in a lipid bilayer, we chose this model to study the system in the presence of talin (Fig. 3).

Whereas the Rosetta modeling indicated that only a small percentage of low energy structures exhibit the IMC salt-bridge interaction between α Ib R995 and $\beta 3$ D723, our simulations of the complete α Ib $\beta 3$ integrin TM/CT domains in an explicit lipid bilayer show a stable α Ib R995- $\beta 3$ D723 interaction with, or without, talin during the simulated timescale [Fig. 4(A)]. While our data are consistent with this salt bridge playing an important role in maintaining the receptor in the resting state, as discussed above, data on the importance of this salt bridge are complex and difficult to reconcile.^{10,21,31,40,41} Similarly, while functional studies of the talin K324D mutant, which was designed on the basis of an overlay of the talin2/ β 1D structure,³¹ suggest that talin disrupts the α Ib $\beta 3$ TM structure by interacting with $\beta 3$ D723 and disrupting the latter's interaction with α Ib R995,²⁹ we did not observe competition of talin-1 with the α Ib R995- $\beta 3$ D723 interaction during the simulated timescale. We cannot, of course, exclude the possibility that we would have observed such competition if we performed longer simulations.

Our simulations confirm the formation of new, mostly stable interactions between the $\beta 3$ K716 side chain and the α Ib F992/K994 backbone carbonyl oxygens in the system without talin [black lines in Fig. 4(B,C), respectively] as seen in 65% of structures sampled by Rosetta.³⁹ These findings further support the proposed functional role of $\beta 3$ K716 based on the observation that its mutation to any other residue leads to the integrin adopting a high-affinity state.³⁹

The α IIB TM helix does not remain perpendicular to the plane of the membrane

Several different experiments have been carried out to infer the location of the α IIB β 3 TM helices in the membrane. Early glycosylation mapping in a cell-free system^{42,43} and NMR structures of the isolated α IIB and β 3 subunits in lipid bicelles^{32,44} suggested that the α IIB TM is perpendicular to the plane of the membrane (with an intracellular border at K994 for the isolated α IIB TM or at G991 in the presence of the β 3 TM helix), whereas β 3 is tilted $\sim 25^\circ$ relative to the normal axis of the membrane, resulting in an intracellular border^{29,39} at D723. Recent experiments using environmentally sensitive fluorophores at the inner or outer membrane-water interfaces of the isolated integrin β 3 TM domain¹⁹ suggested that binding of the FERM (four-point-one, ezrin, radixin, moesin) F3-F2 domains of talin to the β 3 CT domain through the 744NPxY747 motif further enhanced tilting of the β 3 TM domain, thus repositioning β 3 I721 and L694 to a more hydrophobic environment. These findings were supported by multiscale MD simulations of incomplete forms of the integrin α IIB β 3 TM/CT helix dimer with or without talin.^{25,26} Average tilt angles of $\sim 5^\circ$ for the α IIB TM helix and $\sim 30^\circ$ for the β 3 TM helix were monitored during the multiscale simulations of the integrin α IIB β 3 TM/CT helix dimer without talin, although it must be noted that the α IIB TM helix was restrained to be perpendicular to the membrane during the relatively long, coarse-grained runs. Similar simulations carried out in the presence of talin revealed a significant increase in the β 3 TM helix tilt angle of $\sim 15^\circ$ over the course of the simulation, relative to that observed in the multiscale simulations of the α IIB β 3 TM/CT domain without talin.^{25,26}

In the large-scale, all-atom MD simulations reported here, no restraints were imposed on the orientation of the α IIB TM helix over the course of the simulation, although this helix was initially placed perpendicular to the membrane, with the aforementioned experimentally inferred borders extending into the lipid environment. Our all-atom, 3 μ s MD simulations of the complete 2K9J-based α IIB β 3 TM/CT model structure revealed pronounced tilting of both β 3 and α IIB helices in the lipid bilayer, with or without talin. Specifically, tilt angles increased to $-40^\circ \pm 10^\circ$ for the α IIB TM helix and to $-44^\circ \pm 10^\circ$ for the β 3 helix during simulations without talin [black lines in Fig. 2(B,C), respectively]. During the 5 μ s simulations conducted in the presence of talin, these angles stabilized at $-37^\circ \pm 10^\circ$ and $-35^\circ \pm 10^\circ$ for α IIB and β 3, respectively [red lines in Fig. 2(B,C), respectively], thus positioning α IIB I964-P965 residues into a more hydrophobic environment, and further embedding residues at the intracellular end of α IIB and β 3 into the membrane (see Supporting Information Fig. S5). These results are in agreement with the suggested intracellular borders of the integrin active state at α IIB P998 and β 3 F727 based on the results of a very recent analysis of intact α IIB β 3 in living cells using the substituted cysteine scanning accessibility method (SCSAM).²⁴ This analysis suggests a more pronounced activation-driven tilting of both α IIB and β 3 subunits than that previously inferred. Specifically, the α IIB helix border was shown to shift from the previously suggested K994 in the isolated α IIB³² or G991 in the α IIB β 3 TM construct²⁹ to P998 because of a possible pulling of the extended ECD, and consequent embedding of the α IIB 994KRNP998 stretch in the lipid bilayer. Similarly, the TM border of the β 3 TM domain was found to extend from the previously inferred D723 for the isolated β 3 subunit to E726 in intact α IIB β 3, suggesting a more pronounced activation-driven tilting of β 3 for a more physiologically relevant system.

In search of the molecular determinants that are responsible for the integrin inside-out signaling mechanism

The 5 μ s all-atom MD simulations of the complete 2K9J-based α IIB β 3 TM/CT model structure in the presence of talin-1 presented herein suggest novel, testable hypotheses of stable interactions between the α IIB β 3 TM/CT domain and talin-1 that may be worthy of experimental investigation in search of the molecular determinants that are responsible for the integrin inside-out signaling mechanism. Specifically, these interactions are: α IIB R997-talin E342, α IIB E1002-talin K316, α IIB E1005-talin K364, β 3 T753-talin N355, β 3 F754-talin Y373, and β 3 R760-talin E293 [see interactions in Fig. 3(A,B) and their time evolution in Supporting Information Fig. S6]. Notably, the simulated FERM subdomains of talin, i.e., F2-F3, undergo a major reorientation upon binding to both the β 3 subunit and the membrane. Although the overall folds of the two subdomains remain mostly intact during simulation (RMSD of 2.9 ± 0.42 and 1.4 ± 0.25 Å for F2 and F3 domains, respectively), the F2 domain RMSD changes $\sim 25.67 \pm 1.03$ Å with respect to F3 (see inset in Fig. 3 and Supporting Information Fig. S7). This incidentally allows the F2 domain to establish a stable interaction between a positively charged patch composed of residues K263, K268, K272, K274, and K278 and the membrane (see Fig. 3C and Supporting Information Fig. S8), notwithstanding the lack of anionic lipids in the simplified membrane mimetic used in these simulations. Such anionic lipids have been reported to increase the interaction of talin with membranes.³¹ Notably, the predicted orientation of talin-2 with respect to the membrane in the talin2/ β 1D structure³¹ exhibited equivalent positions for the residues of the talin-2 charged patch. Unlike the ~ 5 μ s all-atom simulations reported herein, recent ~ 1.5 μ s coarse-grained simulations²⁸ failed to see association between talin and the zwitterionic POPC lipid, raising the question as to whether the missed association depends on the choice of the force field or the simulation length. On the other hand, a reorientation of F2 with respect to F3, which is made possible by a very flexible linker between them, was also observed as a result of the serial multiscale simulations.^{26,28} We also note that talin has been reported to activate purified α IIB β 3 in the absence of anionic phospholipids, namely, when embedded in neutral lipid nanodiscs⁵ and when immune-captured to microtiter wells.⁴¹

The simulations reported here also suggest hypotheses of interaction changes between the F2 and F3 subdomains of talin-1 that occur upon F2 domain reorientation prior to anchoring itself to the membrane. Specifically, newly formed interactions during dynamics (Figure 3D) are: F3 E346-F2 K234, F3 E350-F2 K300, F3 E335-F2 R207, and F3 K404-F2 D208. In contrast, the F3 K345-F2 E269 interaction, which is present in the initial structure based on the homology model of the murine talin-2, is lost during simulation. We propose that engineering cysteine mutations at the positions involved in this latter interaction may prevent the positively charged patch of F2 from reaching the membrane, thus interfering with the process of integrin activation. Finally, the simulations reported here suggest stable interactions between talin-1 and the complete β 3 CT, which exhibited a disordered, and hence unresolved, segment in the crystal structure of the β 1D CT with murine talin-2. Specifically, these interactions are: β 3 T753/talin N355, β 3 F754/talin Y373 and β 3 R760/talin E293 (Supporting Information Fig. S6). Notably, the 756NPXY759 motif of the β 3 CT, which has been suggested to interact with kindlin, remains exposed to a possible interaction with this protein.

All-atom, long-scale MD simulations support a mechanism of inside-out signaling that does not require helix separation

Three different mechanisms have been proposed in the literature to explain how talin can trigger α IIB β 3 activation. The first one, which is supported by several *in vitro* studies (see Ref. 4 for a recent review), entails disruptions at both OMC and IMC induced by tilting, pistoning, and/or lateral motions of the β 3 subunit upon its direct interaction with the F3 subdomain of talin. This interaction is sufficient to induce integrin activation,⁴⁵ notwithstanding other potentially important interactions between a positively charged patch of the talin F2 domain and the membrane.³¹ The favorable surface interaction between talin and the phospholipid bilayer has been proposed to dominate the ternary interaction involving talin-1 and the β 3 CT domain.²⁰ The second hypothesis, supported by structural information,³¹ relies on a possible competition between talin K324 and α IIB R995 in its interaction with β 3 D723, thus favoring destabilization of the integrin low-affinity state. Finally, a third hypothesis attributes integrin activation to an interface disruption between the integrin α and β subunits due to talin-induced steric hindrance.⁶ Multiscale MD simulations^{25,26,28} have supported the first of these proposed mechanisms, although inferences were based on all-atom MD simulations of only up to 100 ns following approximate, coarse-grained simulations of α IIB β 3 constructs containing partial CTs. Recent all-atom MD simulations²⁷ of a full-length integrin α IIB β 3 model composed of the α IIB β 3 ECD (PDBid: 3FCS²) and the CD3CN/H₂O NMR structure of the α IIB β 3 TM and CT domains (2KNC²¹) in complex with talin also support the hypothesis that talin's interaction with the membrane-proximal and membrane-distal regions of the α IIB β 3 TM and CT domains significantly loosens the IMC based on conclusions drawn from relatively short, pulling simulations. Experimentally, the role of talin in the mechanism of integrin inside-out activation appears to be further complicated by the presence in the cell of other important integrin regulators such as kindlin, which is known to interact through its F3 domain with the β 3 membrane distal 756NPXY759 motif.^{46,47}

The simultaneous tilting of the α IIB and β 3 TM helices relative to the normal axis of the membrane resulting from our 5 μ s simulations of the restraint-free, all-atom complex formed by the α IIB β 3 TM/CT and F2-F3 talin domains prevents a drastic change in the right-handed crossing angle [$-35^\circ \pm 8^\circ$ in the presence of talin compared with $-33^\circ \pm 8^\circ$ in its absence; see red and black lines, respectively, in Fig. 2(A)] and/or the “scissoring” motion observed in the recent coarse-grained simulations of the α IIB β 3 TM/CT domain where the α IIB TM helix had been restrained to be perpendicular to the membrane plane.²⁶ In contrast to the hypothesis that talin binding induces IMC and OMC disruption within the α IIB β 3 TM/CT system through helix separation,⁴⁸ hinging/scissors,^{26,28,49} and/or piston-like⁴⁹ motions, none of these motions appeared to be significant during the simulations presented herein. This result is in agreement with the proposed lack of TM separation upon integrin activation from recent studies of intact α IIB β 3 using solution X-ray scattering and electron microscopy as well as the studies of talin-induced activation of intact α IIB β 3 in a nanodisc.¹⁸ These latter studies are consistent with our findings that the interaction of the talin F2-F3 domains with the α IIB β 3 TM/CT protein portion did not disrupt the salt-bridge between α IIB R995 and β 3 D723 [red line in Fig. 4(A)] and the failure of our data to support the proposed competition between talin-1 K324 and α IIB R995 for binding to β 3 D723³¹ as a basis for

IMC destabilization. In contrast, the three residues are involved in a network of stable interactions that are maintained during the entire simulated timescale. Notably, while a stable interaction is formed between the $\beta 3$ K716 side chain and the α IIB F992/K994 backbone carbonyl oxygens [black lines in Fig. 4(B,C), respectively] during the 3 μ s of the α IIB $\beta 3$ TM/CT domain simulated without talin, the latter interaction is lost in the 5 μ s simulation with talin [red line in Fig. 4(C)] as α IIB F992 is displaced from its position at the interface between α and β . This appears to be the result of a slight rotation of one helix with respect to the other, which we propose may contribute to the long-range conformational changes leading to integrin activation. As a rough indication of this motion, we note that the difference between the rotation angles of each TM segment around its axis, $\omega = \omega(\alpha$ IIB) $-\omega(\beta 3)$, is roughly zero for the simulation in the absence of talin (Supporting Information Fig. S9C, black curve), while its average is as big as -25° in the presence of talin (Supporting Information Fig. S9C, red curve).

A destabilization of the IMC in the conformation stabilized by talin binding is evident from interaction energies between residues involved in inter-helical interactions. Specifically, we calculated the total dispersion and electrostatics interaction between residues M987/F992/F993 in α IIB and L712/W715/K716/I719 in $\beta 3$. In the absence of talin, the IMC contributes ~ 12 kJ/mol (i.e., 5kT) to the overall ~ 75 kJ/mol stabilization enthalpy. The time-dependence of these energies is reported with black lines in Supporting Information Figure S10 (plots in panel A and panel C correspond to the IMC and total energies, respectively). When talin is bound, the IMC contribution becomes marginal, thus reducing the overall inter-helix interaction to ~ 59 kJ/mol (red curves in panels A and C of Supporting Information Fig. S10). In contrast, no appreciable difference is observed in the interactions comprising the OMC (defined as residues G972/G976/L979/L980 in α IIB and V700/M701/I704/L705/G708 in $\beta 3$) and the contribution of the OMC to the inter-helix enthalpy is constant throughout the simulation. Although our simulations cannot rule out changes in the overall free-energy of the OMC, the presence of talin does not seem to materially affect its conformation (see Supporting Information Fig. S10B).

Whether the slight IMC destabilization observed upon talin binding in these first large-scale, all-atom MD simulations of the complete α IIB $\beta 3$ TM/CT domain is sufficient to initiate the long-range conformational changes associated with integrin activation or constitutes the first of multiple steps leading to helix separation, is difficult to ascertain unambiguously based on these simulations, and requires further exploration. Indeed we cannot exclude the possibility that the herein reported atomistic simulations are still limited in length, notwithstanding the multimicrosecond scale explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Complete $\alpha_{IIb}\beta_3$ TM/CT models based on: (A) NMR structure of complete integrin $\alpha_{IIb}\beta_3$ TM and CT domains in a CD₃CN/H₂O mixture (PDBid: 2KNC) and (B) NMR structure of the same complex with truncated CT domains in phospholipid bicelles (PDB: 2K9J). Details of the Outer Membrane Clasp (OMC) and the Inner Membrane Clasp (IMC) are shown in panels C, D or E, F for models depicted in A and B, respectively. In all panels, the α_{IIb} and β_3 subunits are shown in blue and red, respectively.

Figure 2.
Time evolution of crossing (A) and tilting angles of $\alpha.IIb$ (B) and $\beta3$ (C) TM helices in the 2K9J-based models simulated in the presence (red line) or absence (black line) of talin-1 F2 and F3 domains.

Figure 3.

Representative snapshot of the 5 μ s MD simulations of the complete α IIb β 3 TM/CT/talin model. Novel interactions observed during simulation (**A**) between residues of the α IIb TM (in blue) and the F3 domain of talin-1 (in dark green), specifically: α IIb R997-talin E342, α IIb E1002-talin K316, α IIb E1005-talin K364, and (**B**) between residues of the β 3 TM (in red) and the F3 domain of talin-1 (β 3 T753-talin N355, β 3 F754-talin Y373, and β 3 R760-talin E293). **C**: Details of the interactions between the positively charged patch of the F2 domain of talin-1 (in light green) and the membrane. The inset shows the rotation of the F2 domain with respect to F3 during the 5 μ s MD simulations.

Figure 4. Selected electrostatic interactions between α IIb and β 3 TM residues located at the IMC. These interactions are: (A) α IIb R995/ β 3 D723 (ionic lock), (B) α IIb F992/ β 3 K716, and (C) α IIb K994/ β 3 K716. The red and black lines indicate the time evolution of these interactions in the 2K9J-based system simulated with or without talin-1 F2 and F3 domains, respectively.