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Structure and Binding Interface of the Cytosolic Tails of $\alpha X\beta 2$ Integrin

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Abstract

Background: Integrins are signal transducer proteins involved in a number of vital physiological processes including cell adhesion, proliferation and migration. Integrin molecules are hetero-dimers composed of two distinct subunits, α and β . In humans, 18 α and 8 β subunits are combined into 24 different integrin molecules. Each of the subunit comprises a large extracellular domain, a single pass transmembrane segment and a cytosolic tail (CT). The CTs of integrins are vital for bidirectional signal transduction and in maintaining the resting state of the receptors. A large number of intracellular proteins have been found to interact with the CTs of integrins linking integrins to the cytoskeleton.

Methodology/Principal Findings: In this work, we have investigated structure and interactions of CTs of the leukocyte specific integrin $\alpha X\beta 2$. We determined the atomic resolution structure of a myristoylated CT of αX in perdeuterated dodecylphosphocholine (DPC) by NMR spectroscopy. Our results reveal that the 35-residue long CT of αX adopts an α -helical conformation for residues F4-N17 at the N-terminal region. The remaining residues located at the C-terminal segment of αX delineate a long loop of irregular conformations. A segment of the loop maintains packing interactions with the helical structure by an extended non-polar surface of the αX CT. Interactions between αX and $\beta 2$ CTs are demonstrated by ^{15}N - ^1H HSQC NMR experiments. We find that residues constituting the polar face of the helical conformation of αX are involved in interactions with the N-terminal residues of $\beta 2$ CT. A docked structure of the CT complex indicates that a network of polar and/or salt-bridge interactions may sustain the heteromeric interactions.

Conclusions/Significance: The current study provides important insights into the conservation of interactions and structures among different CTs of integrins.

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Introduction

Integrins are heterodimeric cell surface receptors that mediate cell attachment and migration, and they modulate cell growth, proliferation and differentiation [1,2]. In humans, there are 24 integrin heterodimers that are categorized into subfamilies based either on the specific-pairing of the α and β subunits or their ligands. Each integrin subunit has a large ectodomain and a transmembrane domain followed by a cytoplasmic tail (CT). Integrin ligand-binding is mediated by its ectodomain while its cytoplasmic tail allows docking of cytosolic proteins, many of which have been shown to regulate integrin ligand-binding via long-ranged allostery or to induce integrin-derived cellular signaling [2]. The $\beta 2$ integrins are expressed exclusively in leukocytes and there are four members in this subfamily, namely $\alpha L\beta 2$ (LFA-1, CD11aCD18), $\alpha M\beta 2$ (Mac-1, CR3, CD11bCD18), $\alpha X\beta 2$ (p150, 95, CR4, CD11cCD18) and $\alpha D\beta 2$ (CD11dCD18) [3]. Integrin $\alpha X\beta 2$ is expressed primarily in myeloid cells, dendritic cells and NK cells. Integrin $\alpha X\beta 2$ has many ligands that overlap with that of integrin $\alpha M\beta 2$, including iC3b, fibrinogen, and denatured proteins [3]. Notably, integrin $\alpha X\beta 2$ has been shown to bind exposed negatively charged residues in

decayed proteins, suggesting that it plays a role in neutrophil migration and pericellular degradation of extracellular matrix [4]. High-fat diet induced less adipose tissue inflammation in integrin $\alpha X^{-/-}$ knockout mice compared with wild-type mice [5]. Further, double-knockout mice (integrin $\alpha X^{-/-}$ and apoE $^{-/-}$), but not apoE $^{-/-}$ mice, showed less accumulation of macrophages in atherosclerotic lesions [6]. These observations are in line with integrin $\alpha X\beta 2$ as a phagocytic receptor and its role in monocyte adhesion to endothelium [7]. Integrin $\alpha X\beta 2$ also serves as a marker to distinguish between two populations of HLA-DR+ human peripheral blood dendritic cells [8]. Although integrins do not possess enzymatic activity, they can trigger intracellular signaling by recruiting cytosolic proteins to their cytoplasmic tails aforementioned. Except for their juxtamembrane regions, the integrin α CTs are divergent in lengths and sequences [3]. There are many lines of evidence that suggest the α CTs in mediating integrin signaling specificity [9–12]. However, structural information of these integrin α CTs is needed to define the underlying mechanisms. Previously, we have reported the solution structures of integrin αL and αM CTs [13,14]. Here we report for the first time the structure of integrin αX CT. Considering that the

structure of the entire integrin α X β 2 ectodomain has been recently solved [15], our data will allow better understanding of integrin α X β 2 function and regulation as a whole.

Results

NMR Analyses of the Myristoylated CT of α X

CTs of integrin, particularly the N-terminal region, are closely localized to the membrane through the transmembrane helical domain. We have prepared an N-terminal myristoylated CT of α X that may act as a probable mimic of transmembrane segment. Such strategy was successfully used for the structure determination of the 20-residue CT of α IIB of platelet integrin α IIB/ β 3 [16] and more recently for the 24-residue CT of α M of integrin α M/ β 2 [14]. It may be noted that fatty acylated CTs of integrins were found to be biologically active under *in vivo* studies [17]. 3-D structures of the myristoylated CTs were obtained in zwitterionic DPC micelles by NMR spectroscopy [14,16]. Despite the longer length of the α X CT in comparison to that of CTs of α IIB and α M well resolved NMR spectra were observed in DPC micelles. Figure 1 shows a section of 2-D ^1H - ^1H NOESY spectrum of the myristoylated α X CT in DPC micelles correlating downfield shifted amide and aromatic proton resonances at 6.6–9.0 ppm with the upfield shifted aliphatic proton resonances 4.5–0.9 ppm. Observation of a large number of NOE correlations among the amide proton resonances (7.8–9.0 ppm) and aliphatic resonances indicates that CT adopts a well defined conformation in DPC micelles. The aromatic ring protons resonating at 6.8–7.4 ppm delineates NOE correlations with the upfield shifted resonances at 0.9–2.5 ppm of aliphatic sidechain protons, indicating a close proximity of these aromatic and alkyl sidechains (Figure 1). The sequence-specific resonance assignments of the CT of α X in DPC were achieved by the combined use of 2-D TCOSY and NOEST spectra.

We carried out paramagnetic relaxation enhancement (PRE) experiments with MnCl_2 to determine the localization of residues of α X CT in DPC micelles. In particular, 2D NOESY spectra of the CT in DPC micelles were acquired in the presence of 1 mM MnCl_2 . It may be noted that paramagnetic Mn^{2+} would enhance the relaxation of resonances of those that are exposed to aqueous environment. Figure 2 shows residual intensity of NH/ $\text{C}\alpha$ H NOESY cross-peaks as a function of amino acid residues of α X

CT. As one would expect, there was a marked diminution of the intensity of NH/ $\text{C}\alpha$ H correlations for most of the residues of α X CT; indicating their close proximity to the paramagnetic Mn^{2+} ions (Figure 2). However, a relatively higher residual intensity of NH/ $\text{C}\alpha$ H cross-peaks can be seen for some of the N-terminal residues namely for G3, F4 and F5 (Figure 2). This data may suggest a probable partial inclusion of these hydrophobic residues within the lipid region of DPC micelles (Figure 2). Taken together, PRE studies established that most of the residues of α X CT are predominantly located in the aqueous milieu.

Chemical Shifts Deviations and Nuclear Overhauser Effects

Secondary structures of amino acids in peptides and proteins can be identified from the deviation of chemical shifts of α H and $^{13}\text{C}\alpha$ nuclei from the random coil values [18]. Figure 3 shows secondary chemical shifts of $^{13}\text{C}\alpha$ (top panel) and α H (bottom panel) of each amino acid of α X CT in DPC micelles. Residues F4-A16 appeared to be experiencing a positive deviation for $^{13}\text{C}\alpha$ and a negative deviation for α H chemical shifts, indicating helical conformations for this segment. By contrast, secondary chemical shift for the residues N17-K35 were not conspicuous, suggesting a loop or random conformations. Helical conformations for F4-A16 were further deduced from the medium range NOE contacts involving diagnostic $\text{C}\alpha\text{H}/\text{HN}$ (i to i+2, i+3 and i+4) and NH/NH (i to i+1 and i+2) resonances. The helical conformation for residues F4-A16 of α X CT was also defined by a number of medium range NOEs of sidechain/sidechain and backbone/sidechain (Figure 4). NOEs could be observed among the aromatic ring protons of F4 and Y9 (Figure 4A) and among the aliphatic sidechain protons of M12 with aromatic sidechain of Y9 (Figure 4B). A few long-range NOEs were detected between residues from the loop with residues of helix (Figure 4 panels D and E). In particular, M12 $\text{C}\gamma\text{H}$ s and A16 $\text{C}\beta\text{H}_3$ showed NOE contacts with amide proton and sidechain protons of I20 and N24. Taken together, secondary chemical shifts and NOE contacts establish that the myristoylated α X CT in DPC micelles assumes a well folded helical conformation for residues F4-A16 at the membrane proximal region followed by less defined secondary conformations for the C-terminus part. However, the C-terminus loop appears to retain a definite orientation through its packing interactions with the helical segment.

Figure 1. Folded conformation of myristoylated α X CT. A section of the two-dimensional ^1H - ^1H NOESY spectrum of α X CT in DPC micelles showing NOE contacts among low-field resonances (6.5 ppm–9.0 ppm) with up-field resonances (0.8 ppm–4.5 ppm). A large number of NOE cross-peak indicates α X CT is well folded in DPC micelles. NOESY spectra were acquired in 200 mM DPC dissolved in 10 mM sodium phosphate buffer, pH 5.6, 308 K.
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Figure 2. Localization of α X CT in DPC micelles by PRE. A bar diagram showing ratio of intensity of NH/ $\text{C}\alpha$ H correlation obtained from ^1H - ^1H TOCSY spectra of α X CT in the absence and in the presence of 1 mM MnCl_2 . As can be seen, most of the residues of α X CT had experienced a marked quenching (low value in intensity ratio) in the presence of paramagnetic Mn^{2+} , suggesting solvent exposure of the CT.
doi:10.1371/journal.pone.0041924.g002

Figure 3. Secondary structure of the α X CT from chemical shift deviations. Bar diagrams representing deviation of $^{13}\text{C}\alpha$ (top panel and $\text{C}\alpha\text{H}$ (bottom panel) chemical shifts from random coil values for amino acid residues of α X CT in DPC micelles.
doi:10.1371/journal.pone.0041924.g003

Three-dimensional Structure of the CT of α X in DPC Micelles

NMR structures of α X CT were determined based on 263 NOE-driven distance constraints and 57 backbone dihedral constraints (Φ , Ψ) using CYANA (Table 1). Figure 5A shows a superposition of all backbone atoms ($\text{C}\alpha$, N, and C') of an ensemble of twenty lowest energy structures of α X CT. The RMSD values from the mean structure and the stereo-chemical goodness of the structural ensembles are listed in Table 1. The α X CT is defined by a membrane proximal N-terminal α -helical conformation encompassing residues F4-N17 (Figure 5, panels B and C). The propagation of helical conformation appeared to be terminated at residue G18 that assumes a left-handed helical conformation with a positive value in backbone dihedral angles. The helical structure demonstrates an amphipathic organization of the sidechain of amino acids (Figure 5, panels B and C). In particular, one face of the helix is highly polar/ionic with residues R7, Q8, K10, E11, E14, E15 (Figure 5C). The sidechain of residue Q19, though non-helical, also points towards the hydrophilic side of the helix (Figure 5C). The hydrophobic face of the helix of α X

CT is defined by packing among aromatic and aliphatic sidechain of residues V2, F5, Y9 and M13 (Figure 5B). A part of the C-terminus residues i.e. Q19-T26 displays well defined turn-like conformations as indicated by the close superposition of the structural ensemble for these residues (Figure 5A). There are long-range packing interactions among residues from the turn region with the hydrophobic face of the helix (Figure 5B). In particular, an extended non-polar surface of α X CT can be realized by the mutual packing of residues Y9, M13 of the helix with residues Q27, T28 and P29 in the turn (Figure 5B). By contrast, other C-terminal residues S30-K35 are found to be rather disordered in the NMR structure of α X CT (Figure 5A).

Mapping Binding Residues of α X and β 2 CTs by ^{15}N - ^1H HSQC

Interactions between the CTs of α X and β 2 were probed by obtaining ^{15}N - ^1H HSQC spectra of the ^{15}N -labeled CT samples in the presence unlabelled binding partner in aqueous buffer solutions. The ^{15}N - ^1H HSQC spectrum of α X CT was assigned by the use of stranded triple resonance NMR experiments (see materials and methods). The HSQC spectrum of β 2 CT was previously assigned [13]. Figure 6A shows overlay of the ^{15}N - ^1H HSQC spectra of the α X CT in free (black contour) and after addition of CT of β 2 (gray contour) at a 1:2 molar ratio. Chemical shift changes of amide proton and ^{15}N resonances could be detected for several residues in the HSQC spectra of the α X CT in the presence of β 2 CT, indicating interactions between the CTs. In reverse titrations, chemical shift changes were also observed in the ^{15}N - ^1H HSQC spectra of the β 2 CT upon addition of the cognate CT of α X (Figure 6B). The changes in chemical shifts are summarized in Figures 6C and 6D for the α X CT and for the β 2 CT, respectively. As can be seen, residues Y9, K10, E11, M12, M13, E14/E15, N17 and T28 of the α X CT demonstrated discernable changes in chemical shifts in comparison to other residues, indicating their probable involvement in binding with the β 2 CT (Figure 6C). Notably, most of the residues, K10, E11, M12, E14, E15 and N17, of α X CT displaying binding induced chemical shift perturbation occupy the hydrophilic face of the amphipathic helix. By contrast, residues from the loop region of α X CT delineated limited chemical changes, indicating lack of binding interactions with the β 2 CT (Figure 6C). However, a lone residue T28 from the loop appeared to exhibit chemical shift changes akin to the helical residues (Figure 6C). Interestingly, T28 among the loop residues that has packing interactions with the helical structure (Figure 5B). Perhaps, binding of β 2 CT with the helical region of α X CT might have influenced the packing interactions between the loop and the helix. For the β 2 CT,

Figure 4. Packing interactions in the α X CT in DPC micelles. Selected regions of two-dimensional ^1H - ^1H NOESY spectra of the α X CT showing NOE contacts among; (panel A) aromatic ring proton resonances with the aliphatic sidechain resonances, (panel B) aromatic ring proton resonances of F5 and Y9, (panels C and D) sidechains of M12/I20 and A16/N24.
doi:10.1371/journal.pone.0041924.g004

Table 1. Summary of structural statistics of the twenty lowest energy structures of myristoylated α X CT in DPC micelles.

Distance restraints	Intra-residue ($ i-j =0$)	74
	Sequential ($ i-j =1$)	90
	Medium range ($2 \leq i-j \leq 4$)	96
	Long-range ($ i-j \geq 5$)	3
Dihedral angle constraints (Φ, Ψ)		57
Constraints violations	Average NOE violation ($\text{\AA}$)	0.25
	Maximum NOE violation ($\text{\AA}$)	0.31
^aDeviation from mean structure	Backbone atoms (N, C α , C') ($\text{\AA}$)	1.14 (0.11)
	Heavy atoms ($\text{\AA}$)	1.89 (1.14)
Ramachandran plot analysis	% residues in the most favorable region	82
	% residues additionally allowed region	18
	% residues in the generously allowed region	0
	% residues in the disallowed region	0

^aThe RMSD values for the N-terminal helical region (residue 2–17) are in parentheses.

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pronounced chemical shift and/or intensity changes were observed for the N-terminal residues H5, L6, S7, D8, L9, E11, Y12 and R14. The HSQC cross-peaks of H5 and R14 cannot be detected in the presence of α X CT, presumably as a result of conformational exchange between the free and bound states (Figure 6B).

Molecular Models of the Complex α X/ β 2 CTs

Based on changes of chemical shifts, energy-refined docked structures were generated, using RosettaDock protocol [19], of the complex of α X and β 2 CTs. The N-terminus of the β 2 CT has been determined to assume helical structure in our previous study [13]. A complex formation between the two CTs could be potentially sustained by a number of salt-bridge and polar

interactions along with hydrophobic packing through a parallel orientation (Figure 7). In particular, the docked structure of the hetero-tail complex revealed close proximity between the side-chain of residues R7, K10, E11 of the α X CT with the sidechain of residues of D8, E11 and R10 of β 2 CT, respectively (Figure 7). Further, the sidechain of residues R10 and R14 of β 2 CT may form multiple H-bonds and/or salt bridges with the anionic sidechain of E14 and E15 of α X CT (Figure 7). In addition, polar residues S7 of β 2 CT and Q8 of α X CT are in close proximity in the docked structure. A patch of non-polar packing interactions is probable between the two helices involving aromatic ring of F4 of α X and alkyl chain of L3 and I4 of β 2 CT (Figure 7). Collectively, the tail-tail hetero-complex between α X and β 2 subunits of α X/ β 2 integrin may be stabilized by interactions of membrane proximal

Figure 5. Three-dimensional structure of the α X CT in DPC micelles. (Panel A) Superposition of backbone atoms (N, C α , C') of twenty lowest energy conformers of the α X CT in DPC micelles. (panel B) A space-filling representation of the α X CT structure showing sidechain-sidechain packing interactions between residues from the helix and the loop. (panel C) A representative structure of the α X CT depicting sidechain orientation. Figures were generated by use of PYMOL.

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Figure 6. Interactions between α X CT and β 2 CT by ^{15}N - ^1H HSQC NMR. (panel A) Selected section of ^{15}N - ^1H HSQC spectrum of ^{15}N labeled α X CT in free (in black contour) and in the presence of unlabelled β 2 CT (in grey contour) at a ratio of 1:2 (α X: β 2). (panel B) Selected section of ^{15}N - ^1H HSQC spectrum of ^{15}N labeled β 2 CT in free (in black contour) and in the presence of unlabelled α X CT (in grey contour) at a ratio of 1:2 (β 2: α X). (panel C) A bar diagram showing combined chemical shift changes of ^{15}N and ^1H N resonances (in Hz) of α X CT as a function of amino acid residues. (panel D) A bar diagram showing combined chemical shift changes of ^{15}N and ^1H N resonances (in Hz) of β 2 CT as a function of amino acid residues. doi:10.1371/journal.pone.0041924.g006

helices, whereas the residues at the long loop region of α X or β 2, situated far from the interface, are amenable for binding with other cytosolic proteins.

Discussion

The CTs of integrins are involved in bidirectional signaling by interacting with cytoplasmic proteins [1–3]. Most of the integrin β -subunits have CTs that are well conserved with sequence motifs NPXX(Y/F) binding to talin, kindlin and DOK proteins. By contrast, integrin α -subunits have CTs that are less conserved, except for the membrane proximal region (Figure S1). Notably, different α CTs exhibit specific interactions with cytoplasmic proteins namely α 5 with nischarin [20], α 4 with paxillin [21], α IIB with calcein integrin binding protein [22], and α L with CD45 cytoplasmic domain [23]. Conceivably, interactions between α CTs and the cytosolic binding partners may dictate specific function of integrins. Thus, structural elucidation of various α CTs could be useful not only to gain insights into integrin regulation but also for the development of specific anti-integrin drugs [24,25]. To-date, structures are known for three α CTs namely α IIB/ β 3, α L β 2 and α M β 2 integrins [13,14,16] (Figure 8, top panel). The current study defined the 3-D structure of the myristoylated α X CT in DPC micelles. The α X CT demonstrates a folded structure characterized by the N-terminal amphipathic helix and a distal loop akin to the CTs of α M and α IIB integrins. However, there are

striking differences between the structures of the α X CT and the α IIB CT in terms of the length of the N-terminal helix and molecular contacts of the distal loop with the helix. The NMR structure of the myristoylated integrin α IIB CT which is 20-residue long, is characterized by a short helix (V⁹⁹⁰-R⁹⁹⁷) and an acidic loop (E¹⁰⁰¹EDDEEGE¹⁰⁰⁸) (Figure 8A). The acidic loop binds to metal ions and folds back onto the helix by salt-bridge interactions [16]. By contrast, the N-terminal helix of α X CT is considerably longer involving residues F4-N14, and it is also amphipathic (Figure 5). The hydrophilic or polar face of the amphipathic helix of α X CT is well characterized by an array of possible salt-bridge, (residues R7-E11, K10-E14) and hydrogen bond (residues E14-Q19), interactions. The membrane proximal helix of the α IIB CT is rather hydrophobic with fewer polar interactions. In addition, the acidic loop residues of the α IIB CT acquires a tighter packing with the helix by ionic interactions. On the other hand, the long distal loop, residues I21-K37, of the α X CT experiences a higher degree of conformational variations. Only the first few residues of loop of α X CT is involved in packing interactions with the hydrophobic face of the N-terminal helix. The other residues of the loop of α X CT remains extended. Recently, we have determined NMR structure of the myristoylated α M CT of integrin α M β 2 in DPC micelles [14]. The N-terminal region of the 24-residue long α M CT adopted an amphipathic helix, residue F4-E15, followed by a short, residues G15-Q23, fold back loop [14] (Figure 8B). By contrast, the 3-D structure of 57-residue long α L

Figure 7. Docked structure of the α X/ β 2 CTs. Plausible interfacial residues of the complex of CTs of α X/ β 2 integrin. The polar face of the amphipathic helical structure of α X CT (in blue ribbon) appears to be engaged in multiple ionic and/or hydrogen bond interactions with the membrane proximal helix of β 2 (in black ribbon). The probable mutual sidechain-sidechain packing among the non-polar and aromatic residues of α X CT with the β 2 CT at their N-termini is represented by space filling.
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CT was defined by mutual packing of three helices with inter-connecting loops (Figure 8C). The folded conformation of α L CT, sustained by salt bridges and/or hydrogen bonds, display a large negatively charged surface that is involved in binding to metal ions [13]. Recently, NMR structures have been solved for the TM domain of α IIB either with the full-length or C-terminal truncated CT under different solution conditions [26,27]. A helical conformation for the membrane proximal region has been deduced for the full-length CT in the context of the TM domain in a membrane mimetic organic solvent-water mixture [26]. By contrast, NMR structure, in lipid bicelles, of the TM with truncated CT of α IIB revealed a bent or reverse turn conformation for the membrane proximal segment packing with the TM helix [27]. The structural disparity of the membrane proximal region of the α IIB CT noted in these studies may either simply result from the differences in constructs used or an indicative of favored mode of the structural intermediates. Further, NMR derived structure of the full-length TM and CT of α 1 integrin shows helical structure for the membrane proximal region of CT [Lai C et. al. unpublished results, pdb accession number 2L8S]. Aforementioned studies including our current results, therefore, suggest that the membrane proximal region of the full-length α CTs of various integrins assumes a conserved helical structure. However, the C-terminal regions of α CTs appear to show a marked variability in their conformations. It is tempting to speculate that such conformational disparity may allow binding of α CT specific cytosolic proteins resulting in various signaling outcomes.

Integrins undergo complex structural changes from a closed bent conformation to an extended open conformation when activated [1–3]. Mutagenesis and FRET studies in full length

integrins have shown interactions between the CTs of α and β subunits [28–30]. However, analyses of interactions between isolate CTs of α IIB/ β 3 integrin were not unambiguous. NMR structure has been determined for the α IIB and β 3 CTs complex [31], but, initial studies were not able to detect binding between the CTs, probably due to weak interactions [32,33]. A recent study has demonstrated interactions between α IIB and β 3 CTs in DPC micelles whereby a disulfide bond was introduced utilizing a short segment of the TM domains [34]. These results showed conformational stabilization of the β 3 CT in lipid environments influencing its interactions with cognate α IIB CT [34]. Along this line, membrane tethering of the β -CT of α M β 2 integrin has been found to increase the affinity of interactions between the CTs [14]. We have demonstrated that CT of α L and α M interacts with the cognate CT of β 2 by NMR spectroscopy [13,14]. However, the magnitude of chemical shift perturbation was found to be limited indicating weak binding affinity between the free CTs. In this work, we observed interactions between the α X CT and β 2 CTs. ^{15}N - ^1H HSQC results revealed that binding residues are located at the conserved membrane proximal helical segment of α X and β 2 CTs (Figure 6). The docked structure between the CTs of α X and β 2 reveals an interface that is predominantly sustained by a large number of polar and ionic interactions (Figure 7). Previous studies have deduced residues at the interface contact between CTs of α IIB/ β 3, α L/ β 2 and α M/ β 2 integrins (Figure 8, D, E, F). All of these interfaces including α X β 2 have a conserved inter-subunit salt-bridge between R7 of α CT and D8 of β CT at the membrane proximal region (Figure 7 and Figure 8). However, the complex between the CTs of α IIB/ β 3 integrin delineates much shorter interfacial contacts in comparison to that of CTs, including α X β 2,

Figure 8. Structures and interactions of the α CTs of integrins. (top panel) Ribbon representation of the 3-D structures of different α CTs determined in previous studies (panel A) α IIb CT of α IIb/ β 3 integrin, (panel B) α L CT of α L β 2 integrin, (panel C) α M CT of α M β 2 integrin. (bottom panel) Comparison of the complexes of α and β CTs of (panel A) α IIb/ β 3 integrin, (panel B) α L β 2 integrin and (panel C) α M β 2 integrin. doi:10.1371/journal.pone.0041924.g008

of leukocyte integrins (Figure 8D). The docked structure of α X and β 2 CTs suggests a plausible extended interfacial region distal to the membrane proximal helices. It is likely that these interactions between CTs of leukocyte integrins may stabilize the resting state of these integrins. In order to validate the current model of the complex between CTs of leukocyte integrins, we plan to carry out NMR structural studies of the integrin segments that contain the CT and TM.

Materials and Methods

Synthesis and purification of myristoylated α X tail

The myristoylated form of the α X CT was purchased in the crude form from GL Biochem (Shanghai, China). The crude peptide was purified by a linear acetonitrile/water gradient, at a flow rate of 2 ml/min, passing through a C18 column (300 Å pore size, 5 μ M particle size) connected to a Waters reverse phase High Performance Liquid chromatography (HPLC) system (Massachusetts, USA). The major peak fraction was collected and lyophilized. The peptide was later reconstituted in the required buffers.

Expression and purification of α X and β 2 cytosolic tails

The CTs of β 2 (residues K724-S769) and α X (residue K1129-K1163) were sub-cloned into the pET-31b(+) vector to generate fusion proteins containing N-terminal ketosteroid isomerase (KSI). Fusion proteins containing KSI were expressed in inclusion body in *E. coli* resisting proteolysis of the target proteins [13,14]. The sub-cloning and expression of the β 2 CT was reported in our previous works [13,14]. In this study, α X CT was sub-cloned into a pET-31b(+) vector (Novagen EMD, San Diego) from α X-pcDNA3.0 expression plasmid. A formic acid-cleavage GGGSDP sequence was introduced between the KSI and the α X CT sequences that enables formic acid digestion at the DP site

of the α X CT from the KSI fusion protein [14]. The pET-31b vector was also modified containing an N-terminal six His residues (his-tag) and a stop codon immediately after the residue K1163 of α X CT to avoid the expression of a C-terminal his-tag from the original vector. Thereby, the α X CT can be purified without any additional tag sequence from the vector. Purification and isotope labeling, ^{15}N and $^{15}\text{N}/^{13}\text{C}$, of the CTs were carried out as described previously [13,14]. Briefly, *E. coli* BL21DE3 cells (New England Biolabs Inc.), containing expressing plasmids, were cultured either in rich LB medium or in minimal media containing ^{15}N , ammonium chloride or ^{15}N ammonium chloride and ^{13}C glucose at 37°C in shaking incubator at 150 rpm. Expression of fusion proteins were induced by isopropyl β -D-1-thiogalactopyranoside (IPTG), 1 mM concentration, at a cell density of 0.7–0.9. The induced cell cultures were kept at 25°C for 18 hours for protein production in an incubator with a shaking speed of 150 rpm. The cells were then collected, via centrifugation at 5000 rpm for 20 mins, and resuspended in 20 mM Tris-Cl, 0.5 M NaCl buffer, pH 8.0. The resuspended cells were then lysed via sonication on ice to release the cellular contents that contain the recombinant fusion proteins. As the KSI fusion proteins were targeted to inclusion bodies, the cell pellets were collected via centrifugation at 14 000 rpm for 30 mins and re-solubilized using a buffer containing 20 mM Tris-Cl, 0.5 M NaCl and 8 M urea, pH 8.0 to release recombinant proteins. The supernatant containing the solubilized KSI fusion protein was affinity purified using Nickel-NitriloTriAcetic (NTA) acid (QIAGEN) beads by making use of the his-tag attached to fusion proteins. The KSI fusion proteins were then eluted with 20 mM Tris-Cl, 0.5 M NaCl and 8 M urea buffer pH 8.0 with 0.5 M imidazole. The eluates were dialyzed against water at 4°C for 2 days to remove urea, causing the formation of KSI-protein precipitates, which were collected by centrifuging at 5000 rpm for 30 mins. KSI- α X fusion

protein was cleaved with 90% formic acid at a ratio of 1 mg KSI- α X to 1 ml of 90% formic acid, purged with N_2 gas to cleave the Asp-Pro connector between KSI and CT of α X for 22 hrs in the dark. The CT of β 2 was obtained by treating fusion protein, dissolved in 70% formic acid, with cyanogen bromide. The reaction was also purged in N_2 gas and left in the dark for 22 hrs. For both reactions, formic acid and/or cyanogen bromide were neutralized with sodium hydroxide using a rotary evaporator, leaving a film of precipitate. The precipitate was dissolved in 10 mM sodium phosphate buffer pH 6.5 and further purified using HPLC. The identities of the cleaved peptides were verified with mass spectrometry.

NMR Experiments

NMR experiments were carried out on a Bruker DRX 600-MHz spectrometer equipped with an actively shielded cryo-probe. 10% deuterium oxide and 2 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were added to all NMR samples. All NMR spectra are referenced to the 1H of DSS. 2-dimensional 1H - 1H Total Correlation Spectroscopy (TOCSY) and Nuclear Overhauser Spectroscopy (NOESY) experiments were carried out at 308 K for 0.7 mM of myristoylated CT of α X dissolved in 10 mM sodium phosphate buffer pH 5.6, containing 200 mM deuterated dodecylphosphocholine (DPC) (CIL, Massachusetts, USA). TOCSY mixing time was fixed at 50 ms and NOESY mixing time was kept at 200 ms. In addition, natural abundance ^{13}C - 1H Heteronuclear Single Quantum Coherence (HSQC) experiment was carried out to obtain ^{13}C chemical shifts for the myristoylated peptide. NMR data was processed using TOPSPIN 2.1 and analyzed with SPARKY [35]. The NOESY spectra were assigned with the aid of the TOCSY spectrum using the sequence specific resonance assignment strategy. Paramagnetic relaxation enhancement experiments were carried out by collecting 2-dimensional TOCSY spectra of myristoylated α X CT dissolved in 10 mM sodium phosphate buffer containing 200 mM deuterated DPC, in the absence and presence of 1 mM $MnCl_2$. The intensity of the intra-residue $C\alpha H/NH$ cross-peaks after the addition of $MnCl_2$ was normalized to the peak intensity of the unperturbed sample. Triple resonance 3-D HNCACB and 3-D CBCA(CO)NH experiments were performed on 0.5 mM of $^{15}N/^{13}C$ α X CT dissolved in 10 mM sodium phosphate buffer pH 6.5 at 298K, to obtain the assignment of backbone resonances. 2-dimensional ^{15}N - 1H HSQC

was used to determine the interactions between the CTs of α X and β 2. ^{15}N -labelled CT of α X or ^{15}N -labelled CT of β 2 samples were dissolved in 10 mM sodium phosphate buffer, pH 6.5 and titrated with unlabeled cognate CT upto two times the concentration of the labeled sample. The changes in ^{15}N and 1H chemical shift were calculated using the following equation: $\Delta^1H + \Delta^{15}N$, where Δ^1H and $\Delta^{15}N$ refer to the absolute value of the change in chemical shift after addition of the binding partner.

Structure Determination and Docking

An ensemble of conformations of myristoylated α X CT in DPC micelles was obtained by CYANA 2.1 program [36]. The NOESY cross-peaks were qualitatively translated to upper bound distance limits of 2.5 Å, 3.5 Å and 5.0 Å based on the observed signal intensity, with the stronger signal assigned a shorter distance restraint between the two protons. These distances together with the predicted backbone dihedral angles were used to carry out several rounds of structure calculation. Of the 100 structures, twenty lowest energy structures were selected for evaluation and analyses. PROCHECK-NMR [37] was employed to evaluate the stereochemical quality of the structure ensembles and figures were prepared using PYMOL, MOLMOL, and Discovery Studio Visualizer 2.0. The NMR derived structures of CTs of α X and β 2 were placed in close contact to each other based on the residues perturbed in the chemical shift perturbation experiment to form the approximated starting structure for input into the RosettaDock Server [19].

Supporting Information

Figure S1 Comparison of primary structures of representative α and β cytosolic tails of integrins. Alignment of amino acid sequences of α CTs (top panel) and β CTs (lower panel) of integrins α X, α M, α L, α D, α IIB and β 4 and β 2, β 3 and β 1 subunits. (TIF)

Author Contributions

Conceived and designed the experiments: GC XT ATP SMT SB. Performed the experiments: GC XT ATP. Analyzed the data: GC SB. Contributed reagents/materials/analysis tools: GC XT ATP SMT SB. Wrote the paper: GC SB SMT.

References

- Giancotti FG, Ruoslahti E (1999) Integrin signaling. *Science* 285: 1028–1032.
- Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* 110: 673–687.
- Tan SM (2012) The leucocyte β 2 (CD18) integrins: the structure, functional regulation and signalling properties. *Bioscience reports* 32: 241–269.
- Vorup-Jensen T, Carman CV, Shimaoka M, Schuck P, Svitel J, et al. (2005) Exposure of acidic residues as a danger signal for recognition of fibrinogen and other macromolecules by integrin α X β 2. *Proceedings of the National Academy of Sciences of the United States of America* 102: 1614–1619.
- Wu H, Perrard XD, Wang Q, Perrard JL, Polsani VR, et al. (2010) CD11c expression in adipose tissue and blood and its role in diet-induced obesity. *Arteriosclerosis, thrombosis, and vascular biology* 30: 186–192.
- Wu H, Gower RM, Wang H, Perrard XY, Ma R, et al. (2009) Functional role of CD11c+ monocytes in atherosclerosis associated with hypercholesterolemia. *Circulation* 119: 2708–2717.
- Keizer GD, Te Velde AA, Schwarting R, Figdor CG, De Vries JE (1987) Role of p150,95 in adhesion, migration, chemotaxis and phagocytosis of human monocytes. *European journal of immunology* 17: 1317–1322.
- O'Doherty U, Peng M, Gezelter S, Swiggard WJ, Betjes M, et al. (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* 82: 487–493.
- Chan BM, Kassner PD, Schiro JA, Byers HR, Kupper TS, et al. (1992) Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. *Cell* 68: 1051–1060.
- Kassner PD, Hemler ME (1993) Interchangeable α chain cytoplasmic domains play a positive role in control of cell adhesion mediated by VLA-4, a β 1 integrin. *The Journal of experimental medicine* 178: 649–660.
- Weber KS, Klickstein LB, Weber C (1999) Specific activation of leukocyte β 2 integrins lymphocyte function-associated antigen-1 and Mac-1 by chemokines mediated by distinct pathways via the α subunit cytoplasmic domains. *Molecular biology of the cell* 10: 861–873.
- Tang RH, Law SK, Tan SM (2006) Selective recruitment of src family kinase Hck by leukocyte integrin α M β 2 but not α L β 2 or α X β 2. *FEBS letters* 580: 4435–4442.
- Bhunia A, Tang XY, Mohanram H, Tan SM, Bhattacharjya S (2009) NMR solution conformations and interactions of integrin α L β 2 cytoplasmic tails. *The Journal of biological chemistry* 284: 3873–3884.
- Chua GL, Tang XY, Amalraj M, Tan SM, Bhattacharjya S (2011) Structures and interaction analyses of integrin α M β 2 cytoplasmic tails. *The Journal of biological chemistry* 286: 43842–43854.
- Xie C, Zhu J, Chen X, Mi L, Nishida N, et al. (2010) Structure of an integrin with an α I domain, complement receptor type 4. *The EMBO journal* 29: 666–679.
- Vinogradova O, Haas TA, Plow EF, Qin J (2000) A structural basis for integrin activation by the cytoplasmic tail of the α IIB subunit. *Proceedings of the National Academy of Sciences of the United States of America* 97: 1450–1455.
- Liu J, Jackson CW, Gruppo RA, Jennings LK, Gartner TK (2005) The β 3 subunit of the integrin α IIB β 3 regulates α IIB-mediated outside-in signaling. *Blood* 105: 4345–4352.

18. Wishart DS, Sykes BD (1994) The ^{13}C chemical-shift index: a simple method for the identification of protein secondary structure using ^{13}C chemical-shift data. *Journal of biomolecular NMR* 4: 171–180.
19. Lyskov S, Gray JJ (2008) The RosettaDock server for local protein-protein docking. *Nucleic acids research* 36: W233–238.
20. Alahari SK, Reddig PJ, Juliano RL (2004) The integrin-binding protein Nischarin regulates cell migration by inhibiting PAK. *The EMBO journal* 23: 2777–2788.
21. Liu S, Thomas SM, Woodside DG, Rose DM, Kiosses WB, et al. (1999) Binding of paxillin to α 4 integrins modifies integrin-dependent biological responses. *Nature* 402: 676–681.
22. Naik UP, Patel PM, Parise LV (1997) Identification of a novel calcium-binding protein that interacts with the integrin α Ib cytoplasmic domain. *The Journal of biological chemistry* 272: 4651–4654.
23. Geng X, Tang RH, Law SK, Tan SM (2005) Integrin CD11a cytoplasmic tail interacts with the CD45 membrane-proximal protein tyrosine phosphatase domain 1. *Immunology* 115: 347–357.
24. Yin H, Slusky JS, Berger BW, Walters RS, Vilaire G, et al. (2007) Computational design of peptides that target transmembrane helices. *Science* 315: 1817–1822.
25. Bernard E, Parthasarathi L, Cho MK, Aylward K, Raab M, et al. (2009) Ligand switching in cell-permeable peptides: manipulation of the α -integrin signature motif. *ACS chemical biology* 4: 457–471.
26. Yang J, Ma Y-Q, Page RC, Misra S, Plow EF, et al. (2009) Structure of an integrin α Ib β 3 transmembrane-cytoplasmic heterocomplex provides insight into integrin activation. *Proceedings of the National Academy of Sciences of the United States of America* 106: 17729–17734.
27. Lau T-L, Kim C, Ginsberg MH, Ulmer TS (2009) The structure of the integrin α Ib β 3 transmembrane complex explains integrin transmembrane signalling. *EMBO J* 28: 1351–1361.
28. Kim M, Carman CV, Springer TA (2003) Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* 301: 1720–1725.
29. Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, et al. (1996) Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. *The Journal of biological chemistry* 271: 6571–6574.
30. Vallar L, Melchior C, Plancon S, Drobecq H, Lippens G, et al. (1999) Divalent cations differentially regulate integrin α Ib cytoplasmic tail binding to β 3 and to calcium- and integrin-binding protein. *The Journal of biological chemistry* 274: 17257–17266.
31. Vinogradova O, Velyvis A, Velyviene A, Hu B, Haas T, et al. (2002) A structural mechanism of integrin α Ib β 3 “inside-out” activation as regulated by its cytoplasmic face. *Cell* 110: 587–597.
32. Ulmer TS, Yaspan B, Ginsberg MH, Campbell ID (2001) NMR analysis of structure and dynamics of the cytosolic tails of integrin α Ib β 3 in aqueous solution. *Biochemistry* 40: 7498–7508.
33. Li R, Babu CR, Lear JD, Wand AJ, Bennett JS, et al. (2001) Oligomerization of the integrin α Ib β 3: roles of the transmembrane and cytoplasmic domains. *Proceedings of the National Academy of Sciences of the United States of America* 98: 12462–12467.
34. Metcalf DG, Moore DT, Wu Y, Kielec JM, Molnar K et al (2010) NMR analysis of the α Ib β 3 cytoplasmic interactions suggest a mechanism for integrin regulation. *Proceedings of the National Academy of Sciences of the United States of America* 107: 22481–22486.
35. Goddard TD, Kneller DG SPARKY. Graphical NMR Assignment and Integration program. University of California, San Francisco.
36. Guntert P, Mumenthaler C, Wuthrich K (1997) Torsion angle dynamics for NMR structure calculation with the new program DYANA. *Journal of molecular biology* 273: 283–298.
37. Laskowski RA, MacArthur MW, Moss DS, Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Cryst* 26: 283–291.