NMR Solution Conformations and Interactions of Integrin αLβ2 Cytoplasmic Tails*

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The integrins are bi-directional signal transducers. Devoid of enzymatic activity, the integrin cytoplasmic tail serves as a hub for the recruitment of cytosolic proteins, and many of these are signaling molecules. The leukocyte-restricted integrin αLβ2 is essential for the adhesion, migration, and proliferation of leukocytes. Here we report solution conformations and interactions of the αβ2 cytoplasmic tails by NMR analyses. The αL tail is characterized by three helical segments in the order of helix 1–3 that are connected by two loops with helix 3 having a number of nuclear Overhauser effect contacts with helix 1 and helix 2. The conformation of the β2 tail is less defined with only a helical segment restricted at its N terminus. Acidic residues from the helix 2-loop-helix 3 motif of αL were found to be responsible for its binding to calcium ion. There were detectable interactions between αL and β2 tails, involving helix 1 and helix 3 of the αL tail and the N-terminal helix of the β2 tail. Talin head domain that contains the FERM domain showed binding affinity of $K_d \approx 0.5 \mu M$ with the β2 tail. The binding affinity of αL and β2 tails is $K_d \approx 2.63 \mu M$. These data are in line with the activating property of talin head domain on αLβ2 by which binding of talin head domain to β2 tail disrupts the interface of the αL and β2 tails that constrains αLβ2 in a resting state.

Integrins are cell adhesion molecules that are essential for maintaining the integrity and physiology of metazoans by promoting cell-cell and cell-matrix interactions (1). Integrins are noncovalently associated αβ heterodimeric cell surface glycoproteins. Both the α and β subunits are type I membrane proteins. Generally, each subunit has a large extracellular region, a single transmembrane domain, and a short C-terminal cytoplasmic tail. Integrins are bi-directional signal transducers, and they provide connectivity between the interior of the cell and its external environment. Despite the absence of intrinsic enzymatic activity, the cytoplasmic tails of the integrins provide docking sites for an expanding list of cytosolic proteins, many of which are signaling molecules (2). The cytoplasmic tails also contain phosphorylation sites that impinge on the activity of the integrins (3). The importance of conformational changes in the extracellular regions of the integrins for their functions is well reported (4, 5). The cytoplasmic tails of the integrins may be perceived either as the trigger point or the end receiver of these conformational changes in the context of integrin inside-out or outside-in signaling, respectively (4, 6). Therefore, the interactions between the integrin α and β cytoplasmic tails and the structural changes in these tails when they associate with cytosolic proteins require investigation.

The cytoplasmic tails of the platelet integrin αIIbβ3 are extensively analyzed. Intermolecular interactions of the αIIb and β3 tails have been reported by biochemical (7, 8) and NMR studies despite differences in observations with regards to the conformations of the tails that could be attributed to a difference in the lengths of the tails analyzed (9, 10). The relatively weak interactions between the isolated αIIb and β3 tails, which could present difficulty in detection (11, 12), are proposed to restrain the integrin in a resting state (10). A notable feature in the αIIbβ3 cytoplasmic complex is the potential for αIIb Arg995 and β3 Asp228 to form a salt bridge that was demonstrated functionally by charge-reversal mutations to maintain the resting conformation of αIIbβ3 (13). The association of the N-terminal region of the large cytoskeletal protein talin, referred to as talin head domain, with the integrin β cytoplasmic tails induces integrin conformational changes leading to its activation (14–18). This is attributed primarily to the separation of the cytoplasmic tails by talin head domain binding as evident from fluorescence resonance energy transfer analyses of integrin αLβ2 tails (19). Based on NMR analyses of talin F3 subdomain with the β3 tail, it was proposed that the separation of the integrin cytoplasmic tails is a culmination of events in discrete steps involving talin docking to integrin β tail, stabilization of the membrane proximal region in the β tail, and the electrostatic interactions between a positively charge surface of talin with the acidic head groups of the inner membrane phospholipids (20).

Many integrins share a common β subunit but different α subunits, and it is becoming clear that the sequence variations in the α cytoplasmic tails determine signaling specificity in these integrins. Integrin α2β1 (VLA-2) showed different cell migration on collagen and laminin when the α2 cytoplasmic tail was replaced by that from integrin α5 or α4 (21). Integrin α4β1 (VLA-4) exhibited different adhesion properties to ligand VCAM-1 in shear flow when α4 cytoplasmic tail was exchanged.

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with that from integrin α2 or α5 (22). The different α tails of integrin αLβ2 and αMβ2 confer to these integrins distinct chemokine-induced activation kinetics (23), and confer selective recruitment of the Src kinase Hck to αMβ2 but not αLβ2 (24). Although the membrane proximal regions of the α tails are highly conserved, the membrane distal regions vary in lengths and sequences. The latter allows for structural variations and can also serve as distinct sites for the docking of specific cytosolic molecules. Examples include the specific association of nischarin with α5 (25), paxillin with α4 (26), calcinin integrin-binding protein with α1b (27), and CD45 cytoplasmic domain with αL (28). By contrast, the integrin β cytoplasmic tails, with the exception of β4, share many similarities. A notable feature in these tails is the presence of two NPXY(Y/F) motifs that can serve as internalization signals (29), and each of these motifs serves as separate docking sites for talin and the kindlins, and the latter are co-activators of integrins (14, 30–32).

The integrin αLβ2, also referred to as leukocyte function-associated antigen-1, is expressed only in leukocytes, and it serves major roles in leukocyte physiology, including diapedesis, immune synapse formation, and killer cell cytotoxicity (33–35). Ectopic expression of talin head domain induces αLβ2 activation possibly via association of talin head domain with the membrane proximal NPXY motif in the β2 tail (16, 19). Another actin-binding protein α-actinin binds to the membrane proximal sequence His706–Ser723 of the β2 tail of intermediate affinity αLβ2 (36, 37). Interestingly, the binding of filamin to Thr736 in the triplet Thr motif of the β2 tail has an inhibitory effect on αLβ2-mediated T cell adhesion (38). The regulator of adhesion and cell polarization enriched in lymphoid tissues RAPL associates with Rap1-GTP, and the activating effect of this complex on αLβ2 requires the membrane proximal Lys1097 and Lys1099 in the αL tail (39). Collectively, a multifaceted (positive and negative) regulatory network of molecules at the cytoplasmic face of the αLβ2 allows fine-tuning of αLβ2 activity in cells under different conditions and in different regions of a polarized and migrating cell. An impediment toward the understanding of the molecular basis of these regulatory events is a lack of structural information of the complete αLβ2 cytoplasmic tails. In this study, we have characterized solution conformations and interactions between the cytoplasmic tails of αLβ2 integrin. Taken together, the α tails of integrins may have significant conformational diversities that could be important in the regulation and specific activities of integrins in various cell types.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The numbering of the αL and β2 cytoplasmic tails is based on the work of Barclay et al. (40). The cytoplasmic tails of αL (Lys1088–Asp1145) and β2 (Lys702–Ser747) investigated herein are denoted as αL (Lys702–Asp758) and β2 (Lys3–Ser46), respectively (Fig. 1A), for ease of reference in the text and figures. The αL and β2 cytoplasmic tails were PCR-amplified from αL and β2 expression vectors (41) with relevant forward and reverse primers containing the AlwNI site. PCR products were digested with AlwNI and subcloned into the AlwNI site of pET-31b(+) vector (Novagen EMD, San Diego) to generate the ketosteroid isomerase (KSI)4-integrin tail-His6 fusion construct. The AlwNI cut sites employed for the subcloning inherently generates Met sequences in the connecting sequences of KSI-integrin tail and integrin tail-His6. The Met located between integrin tail and His6 was mutated to Gly. In addition, αL Met13 and β2 Met29 were mutated to Ile. These procedures generated KSI-integrin tail-His6 fusion proteins that contain a single Met residue located between the KSI and integrin tail. Instead of removing the His6 tag from the integrin tail, it was retained because we found that the cleavage at Met between the integrin tail and His6 tag was incomplete, which generated two species of integrin tails (with and without His6 tag) that were poorly resolved by reverse-phase HPLC. To prevent complication in downstream analyses, we have therefore retained the His6 tag by substituting the Met between the integrin tail and the tag with Gly as mentioned previously. Amino acid substitutions were made using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing (First Base Sequencing Service, Singapore).

Fusion proteins were expressed in Escherichia coli BL21(DE3) either in LB medium or in M9 minimal medium supplemented with [15N]ammonium chloride and/or [13C]glucose. Protein productions were induced by 0.8 mM isopropyl β-d-thiogalactopyranoside at 28 °C. Harvested cell pellet was resuspended in binding buffer (5 mM imidazole, 500 mM NaCl, 40 mM Tris-HCl, pH 7.9), sonicated on ice, and centrifuged at 20,000 g for 20 min at 4 °C. The pellet was resuspended in binding buffer containing 8 M urea followed by affinity purification on a nickel nitritotriacetic acid resin (Qiagen) column. Fusion protein was eluted in elution buffer (300 mM imidazole, 40 mM Tris-HCl, pH 7.9, 500 mM NaCl, 8 M urea) and dialyzed overnight at 4 °C against distilled water in dialysis tubing (3.5-kDa cutoff) (Pierce). The majority of the protein formed a white precipitate and was pelleted by centrifugation at 4,000 × g for 15 min at 4 °C. The pellet was dissolved in 70% (v/v) formic acid, and cyanogen bromide (CNBr) (37.5 mg per 1 mg of fusion protein) was added and incubated overnight at room temperature. The solution was evaporated to dryness at 28 °C in a rotary evaporator. The remaining protein gel was dissolved in deionized water, and the insoluble KSI protein was removed by centrifugation at 12,000 × g for 10 min at 4 °C. The cleaved tail was subjected to further purification by reverse-phase HPLC (Waters) using a C18 column (300 Å pore size, 5 μm particle size) by a linear gradient of acetonitrile/water mixture. The major peak fractions were collected and lyophilized. The molecular masses of the tails were confirmed by mass spectrometry.

The human talin 1 head domain (Met1–Gln435) was amplified from the expression plasmid pXJ40-HA-talin head domain (16) with relevant forward and reverse primers and cloned into expression vector pET-24a(+) (Novagen) to generate a fusion

4 The abbreviations used are: KSI, ketosteroid isomerase; CNBr, cyanogen bromide; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; TCEP, tris(2-carboxyethyl) phosphine; NOESY, nuclear Overhauser effect spectroscopy; HPLC, high pressure liquid chromatography; ITC, isothermal titration calorimetry.
construct containing a talin head domain with a C-terminal His6 tag. The fusion protein was expressed in *E. coli* BL21(DE3) in LB medium containing 0.8 mM isopropyl β-D-thiogalactopyranoside for 4 h at 30 °C. Harvested cells were resuspended in phosphate buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0), sonicated on ice, and centrifuged at 20,000 × g for 20 min at 4 °C. The fusion protein was recovered from the supernatant by nickel nitrilotriacetic acid affinity purification. Fusion protein was eluted in phosphate elution buffer (50 mM NaH2PO4, 300 mM NaCl, 300 mM imidazole, pH 8.0). The eluted protein was further purified by size exclusion chromatography in 50 mM Tris buffer, pH 7.5, containing 150 mM NaCl on an FPLC system (Amersham Biosciences).

**NMR Spectroscopy**—All NMR experiments were performed at 25 °C on a Bruker DRX 600-MHz instrument equipped with an actively shielded cryoprobe. Triple resonance experiments HNCA, HN(CO)CA, CBCA(CO)NH, HNCACB were carried out to achieve sequence-specific backbone resonance assignments using 15N/13C-labeled 0.8 mM protein samples in 10 mM sodium phosphate buffer, pH 6.2, 150 mM NaCl, and 2 mM tris(2-carboxyethyl) phosphine (TCEP) as reducing agent. The buffer conditions used for the NMR experiments were selected to be close to the cytoplasmic environment. Side chain aliphatic protons were assigned from three-dimensional 1H-15N HSQC-total correlation spectroscopy spectra with mixing time of 70 ms. Three-dimensional 1H-15N NOESY experiments were carried out in the above-mentioned buffer in the absence and in presence of 50 mM calcium chloride. NMR data were processed using the Topspin program suite (Bruker) and analyzed by Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco). All the chemical shifts were indirectly referenced to 2,2-dimethyl-2-silapentanesulfonic acid.

**NMR-derived Structure Calculations and Modeling of αLβ2 Tail Complex**—Inter-proton distance restraints were obtained from three-dimensional 1H-15N HSQC-NOESY spectra, mixing time of 200 ms, and were categorized as strong, medium, and weak with upper bound distances of 3, 4, and 5 Å, respectively, whereas all the lower limits were set to 2 Å. The pseudoatom corrections were applied to the upper bound distances for methyl and methylene group protons. In addition, backbone dihedral angle (φ and ψ) restraints were derived from TALOS (42). These predicted dihedral angle constraints were used for structure calculation with a variation of ±25° from the average values. An ensemble of solution structures of αL tail was calculated using the program DYANA 1.5 (43). Out of 100 structures calculated, 10 conformers with the lowest energy values were selected to present the NMR ensemble. The stereochemical quality of the structures was determined using the program PROCHECK-NMR (44). A molecular model of αL-β2 tails complex was constructed by iterative docking utilizing chemical shift perturbation data. The salt bridge interactions between

![FIGURE 1. Amino acid sequence alignments and 1H-15N HSQC. A, primary amino acid sequences of αL (top panel) and β2 (bottom panel) cytoplasmic tails showing sequence alignments with homologous integrins using ClustalW. The conserved residues are indicated in bold, and similar residues are bold and italic. Two-dimensional 1H-15N HSQC spectra of αL (B) and β2 (C) tails correlating the single-bond 1H-15N resonances. Both spectra were acquired in 10 mM phosphate buffer, pH 6.2, 150 mM NaCl, and 2 mM TCEP with a Bruker DRX 600-MHz instrument (equipped with cryoprobe) and at 25 °C. Horizontal lines connecting the cross-peaks indicate the side chain amides of Asn/Gln residues. The additional tag residues at the C-terminal of the expressed proteins are labeled as "x". The amino acids are numbered as Lys1–Asp58 for αL tail and Lys1–Ser46 for β2 tail. *, residue Met substituted with Ile.](image-url)
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(A)

(B)

![Diagram and resonance assignments](https://example.com/diagram.png)

**FIGURE 2. Secondary structures of the αL and β2 tails.** Bar diagrams showing sequential and medium range NOEs and the chemical shift deviations from the random coil values for the 13C resonances of the αL (A) and β2 tails (B). The thickness of the bars indicates the intensity of the NOESY peaks, which are assigned as strong, medium, and weak. The overlapping residues are indicated by gray bars. Amino acid sequences of both the tails are shown at the top.

**TABLE 1**

Summary of key inter-helical long range NOEs used to determine the tertiary structure of αL tail

| Amino Acid | NOE Type | Residue |
|------------|----------|---------|
| Lys             | Cα/Cα  | 3       |
| Val             | Cα/Cα  | 18      |
| Lys             | Cα/Cα  | 30      |
| Glu             | Cα/Cα  | 33      |
| Ile             | Cα/Cα  | 40      |
| Asn             | Cα/Cα  | 42      |
| Glu             | Cα/Cα  | 53      |
| Glu             | Cα/Cα  | 57      |

membrane proximal helices, i.e. helix 1 of αL and N-terminal helix of β2, are initially optimized by rotation of side chain dihedral angles. The model structure was further energy-minimized to relieve short inter-atomic contacts using conjugate gradient protocol, force field cvff, Insight II program (Accelrys Inc.).

NMR Interaction Studies by 1H–15N HSQC Experiments—To identify binding between αL and β2 tails, 1H–15N HSQC experiments were carried out using either 15N-labeled αL or β2 tails and unlabeled β2 or αL tails, respectively, at 298 K. In the tail-tail complexes, concentrations of 15N-labeled tails were fixed to 0.3 mM, and concentrations of unlabeled tails were 0.45 mM, resulting in a 1:1.5 molar ratio. 1H–15N HSQC spectra were also obtained after addition of talin head domain (0.5 mM) into a solution of 15N-labeled αL tail and unlabeled β2 tail. The protein samples were prepared in 10 mM sodium phosphate buffer, pH 6.2, 150 mM NaCl, 10 mM CaCl2, and 2 mM TCEP. Under similar buffer conditions, isotope-filtered (15N/14N and 13C/12C) three-dimensional NOESY experiments were recorded using a mixture of 15N/13C-labeled αL tail and unlabeled β2 tail at a 1:1.5 molar ratio. The interactions between β2 tail and talin head domain were studied by acquiring 1H–15N HSQC spectra of 15N-labeled β2 tail as a function of concentrations of tail head domain. Titration were carried out by addition of aliquots of unlabeled talin head domain (1 mM) into 0.3 mM 15N-labeled β2 tail in 10 mM phosphate buffer, pH 6.2, 150 mM NaCl, and 2 mM TCEP at 298 K. The chemical shift changes of backbone 1H and 15N resonances were calculated using the following equation: $\Delta \chi = (\Delta \chi_1 + \Delta \chi_2)$ (10). The calcium binding to αL tail was performed by additions of aliquots of CaCl2 (1 x stock solution) into 15N-labeled αL (0.3 mM) in 10 mM sodium phosphate buffer, pH 6.2, 150 mM NaCl, and 2 mM TCEP. All samples were allowed to equilibrate for 20 min before two-dimensional 1H–15N HSQC spectra were recorded on a Bruker DRX 600-MHz instrument, equipped with cryo-probe and at 298 K.

Isothermal Titration Calorimetric Studies—Isothermal titration calorimetry (ITC) experiments were performed on a VP-ITC Micro Calorimeter (MicroCal LLC, Northampton, MA). All measurements were made in 10 mM phosphate buffer containing 150 mM NaCl, 2 mM TCEP, and pH 6.2 at 293 K. A fixed concentration of 0.01 mM talin head domain placed in the sample cell was titrated by sequential additions of a 5-μl aliquot of a 0.2 mM β2 tail from the injection syringe, except for the first injection of 2.5 μl. Typically, 40 injections were performed at an interval of 4 min while the reaction cell was constantly stirred at 300 rpm. Similar titrations were carried out using a fixed concentration of 0.01 mM αL tail placed in the sample cell with 25 injections of 5-μl aliquots from 0.2 mM β2 tail placed in the injection syringe. All the reaction conditions were the same as that of the former one. For calcium binding titration, 0.01 mM αL tail placed in the sample cell was titrated by sequential addition of 5-μl aliquots of 100 mM CaCl2 from the injection syringe. Raw data were collected and integrated using MicroCal Origin 5.0 supplied with the instrument. A single set of binding site model, provided with the software, was fitted to the data to obtain association constant, $K_a$, and enthalpy change $ΔH$. Free energy ($ΔG$) and entropy ($ΔS$) changes were calculated using the equations $ΔG = −RT\ln K_a$ and $ΔS = (ΔH − ΔG)/T$, respectively.

RESULTS

Resonance Assignments of the Cytoplasmic Tails of αLβ2 Integrin—The αL and β2 tails were overexpressed into inclusion bodies in E. coli as a fusion protein with KSI. This strategy prevented undesirable proteolytic degradation of the target proteins. The integrin tails were released from KSI by CNBr cleavage and subjected to further purification by HPLC (see under “Experimental Procedures”). A sequence comparison among the α tails from other integrins revealed that the α tails are heterogeneous in length and in amino acid compositions except for the first seven conserved amino acids at the N terminus (Fig. 1A). In all subsequent figures, the amino acids are numbered as Lys1–Asp58 for Lys1088–Asp1145 in the αL tail and Lys1–Ser46 for Lys702–Ser757 in the β2 tail. It is also noteworthy that the αL tail is much longer as compared with other α tails,
including the well investigated 20-residue tail of the \( \alpha L \) integrin (9, 10, 45). By contrast, the \( \beta 2 \) and \( \beta 3 \) tails are similar in length and contain a number of conserved residues.

Assignments for the backbone HN, \( ^{15} \text{N}, ^{13} \text{C} \), and \( ^{13} \text{C} \) resonances of the \( \alpha L \) and \( \beta 2 \) tails were achieved by a combined analysis of triple resonance HNCA, HN(CO)CA, HN(CA)C, and CBCA(CO)NH spectra. The \( ^{1} \text{H}-^{15} \text{N} \) heteronuclear single quantum coherence (HSQC) spectrum of \( \alpha L \) tail with backbone HN and \( ^{15} \text{N} \) correlations of individual residues is shown (Fig. 1B). All the backbone \( ^{1} \text{H}-^{15} \text{N} \) correlations were identified except for four Pro residues. The \( ^{15} \text{N} \) resonances of Gly and Ser/Thr residues are found to be the most upfield-shifted (46). The primary amino acid sequence of \( \alpha L \) contains 10 Gly and 4 Ser residues distributed along the sequence (Fig. 1A). Gly residues resonating at 108–113 ppm at the \( ^{15} \text{N} \) dimension and Ser residues resonating at 115–117 ppm were identified in the HSQC spectrum of the \( \alpha L \) tail (Fig. 1B). \( ^{1} \text{H}-^{15} \text{N} \) HSQC cross-peaks of the 46-residue \( \beta 2 \) tail were also assigned except for residues Phe\(^{15} \), Glu\(^{16} \), Leu\(^{31} \), and Lys\(^{42} \) (Fig. 1C). Interestingly, we found two \( ^{1} \text{H}-^{15} \text{N} \) cross-peaks for residues Ala\(^{34} \), Thr\(^{35} \), Thr\(^{36} \), Phe\(^{43} \), Ala\(^{44} \), Glu\(^{45} \), and Ser\(^{46} \), presumably as a result of cis-trans-isomerization of the Asn\(^{40} \)-Pro\(^{41} \) peptide bond.

Conformational Characteristics of \( \alpha L \) and \( \beta 2 \) Tails—Secondary structures of \( \alpha L \) and \( \beta 2 \) tails were obtained from the deviation of \( ^{13} \text{C} \) chemical shifts from random coil values and short and medium range NOEs (Fig. 2). \( ^{13} \text{C} \) chemical shift deviation is a reliable indicator for identification of helix and sheet secondary structures (47). The \( ^{13} \text{C} \) atom experiences a downfield shift in helical structure and an upfield shift in sheet. A stretch of at least four contiguous residues or a stretch of at least three adjacent residues with helical or strand-type chemical shift deviations can be assigned as a stable helix or sheet conformation, respectively (47). Based on this, three helical segments (helix 1, Lys\(^{6} \)-Ile\(^{13} \); helix 2, Ala\(^{25} \)-Glu\(^{36} \); and helix 3, Gly\(^{38} \)-Leu\(^{43} \)) were derived for the \( \alpha L \) tail (Fig. 2A). Analysis of \( ^{15} \text{N} \)-edited three-dimensional HSQC-NOESY spectra of \( \alpha L \) reveals diagnostic short and medium range NOEs, sequential HN/HN and C\( ^{\alpha} \)/HN (i to i \( +3 \)/\( +4 \), respectively) consistent with the helical structures for these segments. Residues between helix 1 and helix 2 (Glu\(^{14} \)-Pro\(^{29} \)) and residues between helix 2 and helix 3 (Gly\(^{38} \)-Leu\(^{43} \)) are characterized by predominantly sequential NOEs, indicating a lack of regular secondary structures. It is noteworthy that there are a number of Gly and/or Pro residues in these two segments that may be responsible for the disordered or loop-like conformations for these segments. The first five amino acid residues at the N terminus (Lys\(^{1} \)-Phe\(^{5} \)) also appeared to be flexible in the \( \alpha L \) tail. Interestingly, we have identified several NOE connectivities among the three helices of the \( \alpha L \) tail (Table 1). These long range NOEs, e.g. F4C\(^{P} \)/L43HN (Fig. 3A, right panel), N21C\(^{P} \)/G55HN, and I13C\(^{\gamma} \)/E52HN (Fig. 3, B and C) suggest a close proximity (\( \leq 5 \) Å) between helix 3/helix 1 and helix 2/helix 3.
By contrast, the analyses of $^{13}$C chemical shift that deviates from random coil values and NOE of the β2 tail suggest that a large part of the polypeptide chain is devoid of any regular secondary structures (Fig. 2B). Only a short region at the N terminus, residues Leu$^{6}$–Arg$^{14}$, showed a propensity for a helical conformation. However, we could not unambiguously detect any medium range helical NOEs among these residues, suggesting a probable nascent helical conformation (48).

A Compact Conformation of the αL Tail—An ensemble of solution structures of the αL tail was determined based on short/medium and long range NOEs and deduced backbone dihedral ($\phi$, $\psi$) constraints (Fig. 4 and Table 2). The superpositions of backbone atoms (C$^\alpha$, N, and C') of 10 lowest energy structures of the αL tail are shown (Fig. 4, A–D). The individual helical structure of αL appears to show a close superposition with root mean square deviation of 0.14, 0.25, and 0.28 Å for helix 1, helix 2, and helix 3, respectively. The superposition of the backbone atoms of the overall tertiary fold of the αL tail showed relatively higher root mean square deviation values of backbone and all heavy atoms at 0.77 and 1.25 Å, respectively (Fig. 4D and Table 2). The compact structure of αL tail is largely defined as the fold back of helix 3 between helix 1 and helix 2 (Fig. 4E). We also examined plausible side chain-side chain interactions that may be involved in defining the orientations of these helices. There are a number of potential polar and/or ionic interactions between helix 1 and helix 3 and between helix 2 and helix 3 that may impart stabilization to the folded conformation of the αL tail. For example, residues Lys$^{6}$ and Lys$^{10}$ from helix 1 may form salt bridges or hydrogen bonds with residues Glu$^{48}$ and Glu$^{52}$ from helix 3 (Fig. 4F). Furthermore, side chains of residues Lys$^{44}$, His$^{47}$, and Lys$^{57}$ from helix 3 were found to be in close proximity with residues Glu$^{36}$, Ser$^{33}$, and Glu$^{29}$ of helix 2 (Fig. 4G), indicating plausible charge-charge interactions or hydrogen bond formations among these residues. NOE interactions observed among these residues indeed suggest probable occurrence of such ionic and/or hydrogen bond formations in αL structure (Table 1). A hydrophobic packing interaction between residues Phe$^{4}$ and Leu$^{43}$ could also be involved in maintaining the tertiary fold of αL in solution (Table 1). Intra-helical salt bridge interactions can also be present between residues Arg$^{17}$–Glu$^{11}$ in helix 1 and residues Lys$^{43}$–Glu$^{48}$ and Lys$^{49}$–Glu$^{52}$ in helix 3 stabilizing individual helical structures. Amino acid compositions of the integrin tails, including αL and β2, are rich in polar and ionic...
residues (Fig. 1A). Few hydrophobic packing interactions that stabilize well folded structures of proteins were found in the αL conformation.

Binding of Calcium to the αL Tail—Divalent cations like Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ have been shown to interact with the αLbβ3 tail (7). We examined probable Ca$^{2+}$ binding to the αL tail by monitoring changes in 1H-15N HSQC spectra at different concentrations of calcium chloride. Sections of 1H-15N HSQC spectra are shown (Fig. 5, A–C). 1H-15N HSQC cross-peaks of residues Glu26, Gly38, Asp39, Gly41, and Cys42 from the loop between helix 2 and helix 3, and residues His47, Glu48, Lys49, Asp50, Ser51, Glu52, Ser53 from helix 2 of the αL tail showed a change in chemical shift of equivalent level (≥75 Hz). These data suggest that residues from helix 1 and helix 3 of the αL tail are involved in interacting with the β2 tail. Inclusion of talin head domain appeared to disrupt the interactions between the αL and β2 tails because no significant chemical shift perturbations in the 1H-15N HSQC spectra of αL tail were detected in the presence of talin head domain (supplemental Fig. 1).

In the reverse 1H-15N HSQC titration, chemical shifts of the 15N-labeled β2 tail (red contour) were detected in the presence of unlabeled αL tail (black contour) (Fig. 6D). However, the changes in chemical shift were restricted only to the N-terminal residues of the β2 tail, suggesting their involvement in αL tail interaction. Residues His5, Leu6, Asp8, Glu11, and Tyr12 of the β2 tail could be directly involved in the complex formation with αL tail (Fig. 6, E and F). The observations of a downward trend of ITC titration peaks (Fig. 7, top panel) and the resultant negative integrated heats (Fig. 7, bottom panel) suggest that the association of αL and β2 tails is exothermic. The thermodynamic parameters of this association are provided (Table 3).

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were no significant differences in the NOE connectivities between the apo- and holo-forms of protein, indicating a similar helical fold even after Ca$^{2+}$ binding (data not shown).

Interactions between αL and β2 Tails—The 1H-15N HSQC spectra of 15N labeled free αL tail alone (red contour) and in the presence of unlabeled β2 tail (black contour) at 1:1.5 (αL:β2) ratio were obtained (Fig. 6A). Chemical shift changes of HSQC cross-peaks were detected for a number of residues of the αL tail upon addition of the β2 tail, suggesting interactions between the two tails. Chemical shift changes of αL tail as a function of amino acid residues are also shown (Fig. 6B). Chemical shift perturbations, although in different magnitude, were observed for many residues throughout the sequence of the αL tail. However, chemical shift changes were found to be significantly higher (≥75 Hz) only for residues from helix 1 (Lys8, Arg7, Asn8, Lys10, and Arg12) and helix 3 (Lys44, His47, and Lys49) (Fig. 6, B and C). By contrast, only one residue, Glu36, from helix 2 of the αL tail showed a change in chemical shift of equivalent level (≥75 Hz). These data suggest that residues from helix 1 and helix 3 of the αL tail are involved in interacting with the β2 tail. Inclusion of talin head domain appeared to disrupt the interactions between the αL and β2 tails because no significant chemical shift perturbations in the 1H-15N HSQC spectra of αL tail were detected in the presence of talin head domain (supplemental Fig. 1).

In the reverse 1H-15N HSQC titration, chemical shifts of the 15N-labeled β2 tail (red contour) were detected in the presence of unlabeled αL tail (black contour) (Fig. 6D). However, the changes in chemical shift were restricted only to the N-terminal residues of the β2 tail, suggesting their involvement in αL tail interaction. Residues His5, Leu6, Asp8, Glu11, and Tyr12 of the β2 tail could be directly involved in the complex formation with αL tail (Fig. 6, E and F). The observations of a downward trend of ITC titration peaks (Fig. 7, top panel) and the resultant negative integrated heats (Fig. 7, bottom panel) suggest that the association of αL and β2 tails is exothermic. The thermodynamic parameters of this association are provided (Table 3).

The interaction appears to be driven by a favorable change in enthalpy that may indicate predominant role(s) of ionic and/or hydrogen bonding toward the formation of the αL-β2 tails complex. To determine a plausible orientation of the αL-β2 tails complex, 15N/14N and 13C/12C-filtered NOESY experiments were carried out (see under “Experimental Procedures”). However, we could not detect any intermolecular NOE contacts between the tails. The lack of NOE contacts may result from a fast dissociation of the complex or a low affinity binding between the tails (Table 3). Therefore, the orientation of the αL-β2 tails complex was determined from an iterative docking followed by energy minimization procedure (Fig. 8). The positively charged residue Arg7 from helix 1 of the αL tail (Fig. 8, red) is engaged to form salt bridges with acidic residues Asp8 and Glu11 from the N-terminal helix of the β2 tail (orange). The importance of these salt bridge interactions, in particular Arg7–Asp8, in maintaining inactive states of integrins was determined in αLbβ3 and αLβ2 (13, 16, 49). In the docked structure, potential ionic interactions were also found between acidic residues Glu11, Glu44 in helix 1 of αL tail with residue Arg63 of β2 tail. Interestingly, we also found plausible stabilizing interactions

### Table 2
Summary of structural statistics for the 10 final structures of the αL tail

| Database restraints | intraresidue (i − j = 0) | 69 |
|---------------------|--------------------------|----|
| Sequential (|j − j| = 1) | 90 |
| Medium range (2 < |j − j| < 4) | 81 |
| Long range (|j − j| > 5) | 9 |
| Total | 249 |

| Angular restraints | Φ | 41 |
|-------------------|---|----|
|                      | Ψ | 41 |

| Distance restraints violations | No. of violations | <0.23 Å |
|--------------------------------|------------------|---------|
| Average violation              |                  | <0.48 Å |
| Average target function values |                  | 18.83 (19.71-18.25) |

| Deviation from mean structure | All residues (N, Cx, C") | 0.77 |
|-------------------------------|--------------------------|------|
|                               | Heavy atoms              | 1.27 Å |
|                               | Residues Lysx–Lysx+1     | 0.14 Å |
|                               | Residues Ala→Glu         | 0.25 Å |
|                               | Residues Lysx→Glyx+1     | 0.28 Å |

| Ramachandran plot for the mean structure | Residues in the most favorable region | 88.9% |
|-----------------------------------------|--------------------------------------|------|
|                                        | Residues additionally allowed region | 11.1% |
|                                        | Residues in the generously allowed region | 0% |
|                                        | Residues in the disallowed region | 0% |
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![Image of conformations](image)

**FIGURE 5. Binding of calcium to the αL tail.** Shown are selected regions (A–C) of 1H-15N HSQC spectra of 15N-labeled αL tail in the absence (red) and presence of 40 mM (green), 60 mM (blue), 70 mM (purple), and 80 mM CaCl2 (cyan) in 10 mM phosphate buffer, pH 6.2, 150 mM NaCl, and 2 mM TCEP at 25 °C. **D,** disposition of the side chain of residues of αL tail that showed large changes in chemical shift with increasing concentrations of CaCl2. **E,** electrostatic potential surface diagram of the αL tail showing a negatively charged region. The negative and positive charges are indicated by red and blue, respectively. The neutral residues are presented as white surface. This image was produced with the program PyMOL. **F,** isothermal calorimetric titrations for the interactions of αL tail with calcium. Top, ITC trace obtained at 20 °C by injecting aliquots CaCl2 into sample cell containing αL tail. Bottom, plot of heats of reaction as a function of the molar ratio of CaCl2/αL.

**TABLE 3**

| Thermodynamic parameters for the interactions between β2 tail and talin head domain, αL tail, and β2 tail, and αL tail and CaCl2 | β2/Talin | β2/αL | αL/Ca²⁺ |
|---|---|---|---|
| Kd (μM) | 1.99 | 0.38 | 0.27 |
| ΔH (kcal mol⁻¹) | 8.29 | -7.06 | 172.5 |
| ΔS (kcal mol⁻¹ K) | 16.74 | 0.4 | 179.8 |
| ΔG (kcal mol⁻¹) | -8.45 | -7.47 | -7.28 |
| Kd (μM) | 0.5 | 2.6 | 3.7 |
| n | 1.01 | 1.0 | >1 |

between helix 3 of the αL tail (pink) with the β2 tail (orange). Residues Asp³⁹, Lys⁴⁴, and His⁴³ in helix 3 may be engaged in salt bridges and/or hydrogen bonds with residues His⁵, Asp⁸, and Tyr¹² of the β2 tail. In addition, a cluster of nonpolar amino acids that includes Phe⁵, Pro⁴⁰, and Leu⁴³ of the αL tail were found to be in close proximity with Ile⁸ of the β2 tail.

**Interactions of the β2 Tail with Talin Head Domain—**Talin is a FERM domain-containing and actin-binding protein that connects integrins to the actin cytoskeleton 
(50, 51). The head domain of talin is involved in the activation of several integrins, including αLβ2 (14–16, 18, 19). Here we examined the binding of β2 tail with the talin head domain (Met¹–Gln¹⁸⁷) by 1H-15N HSQC and ITC studies (Fig. 9). The 1H-15N HSQC spectra of β2 tail at various concentrations of unlabeled talin head domain was determined (Fig. 9A). Talin head domain induced marked chemical shift changes or broadening of a number of 1H-15N HSQC cross-peaks of the β2 tail. This suggests talin head domain interacting with the β2 tail. Of note, significant changes in chemical shifts were detected for residues located at the N-terminal membrane proximal and central regions of the β2 tail (Fig. 9B). These suggest a substantial portion of the β2 tail is involved in the interaction with talin head domain. Similar results were reported from 1H-15N HSQC titration of β3 tail with the F3 subdomain of the talin head (52).

A recent NMR structure of a complex between a chimeric peptide, containing N-terminal membrane proximal region of β3 and C-terminal residues of PIPK1γ, with F3 subdomain of talin revealed extended interfacial contacts with residues of membrane proximal and more distal regions (20). Binding of the β2 tail with talin head domain was further investigated by ITC measurements (Fig. 9C and Table 3). The binding of β2 tail to talin head domain was endothermic as suggested by an upward trend of the ITC titration peaks (Fig. 9C, top panel), and the resultant positive integrated heats (Fig. 9C, bottom panel). The ITC results suggest that the talin head domain and β2 tail forms a 1:1 complex with an estimated Kd
localized at specific regions of polarized and migrating T cells (37, 53, 54). To generate a dynamic equilibrium of αLβ2 conformers in these cells, cytosolic signaling and cytoskeleton-bridging molecules play indispensable roles (33, 55, 56). Hence, the αLβ2 cytoplasmic tails that serve as a hub for the recruitment of cytosolic proteins warrant investigations. Despite increasing number of studies examining αLβ2 cytoplasmic tails by biochemical, mutational, and functional analyses, the structures of the αLβ2 cytoplasmic tails remain unknown. This is in contrast to the platelet integrin αIIbβ3 cytoplasmic tails that have been subjected to many structural analyses (9–12, 45, 57). Although the β2 and β3 tails share considerable sequence homology, the αL and αIIb tails are highly divergent in terms of sequence composition and length.

Therefore, we have investigated the solution structures and interactions of the αLβ2 cytoplasmic tails. In solution the isolated αL tail adopts a folded conformation that is characterized by three well defined helical segments. These helices are oriented into a novel compact tertiary structure whereby helix 3 is engaged in interactions with helix 1 and helix 2 (Fig. 4). The specific arrangement of the three helices is presumably strengthened by salt bridges and hydrogen bonds among the helices. The well conserved N-terminal or membrane proximal region of the αL tail forms part of helix 1 (residues Lys6–Ile13). This region was also found to be helical in the structure of the αIIb tail in lipid micelles (45, 57) and when in complex with the β3 tail (9, 10). The packing between helix 1 and helix 3 showed plausible ionic/hydrogen bond interactions between positively charged residues Lys6 and Lys10 with the negatively charged residues Glu48 and Glu52, respectively (Fig. 4F). Interestingly, the folded structure of αIIb tail obtained in DPC micelle was also found to contain salt bridges between conserved residue Lys994 (Lys6 in αL tail) with acidic residues Asp1004 and Glu1005 from the loop (45). However, ionic interactions between helix 2 and helix 3 found here are unique to the αL tail (Fig. 4G).

FIGURE 6. Interactions between the αL and β2 tails. A, expanded region of 1H-15N HSQC spectra of 15N-labeled αL tail in the absence (red contour) and in the presence of unlabeled β2 (black contour) at a ratio of 1:1.5 (αL:β2) in 10 mM phosphate buffer, pH 6.2, 150 mM NaCl, 10 mM CaCl2, and 2 mM TCEP at 298 K. B, bar diagram showing changes in chemical shift (in Hz) as a function of amino acid residue of αL in the presence of β2. The chemical shift changes were calculated based on a sum of changes of absolute values of 1H and 15N chemical shifts in Hz. Filled triangle denotes residue of the αL tail with a reduction in the intensity of HSQC peaks because of line broadening upon binding to β2 tail. Asterisk denotes disappearance of HSQC peak of the αL residue in the presence of β2 tail. Gray bars indicate overlapping residues. C, ribbon representation of the αL tail showing residues, as spheres, affected by interactions with β2 tail. Blue, green, and red represent the changes in 1H and 15N chemical shift at the order of 30–45 Hz and above 45–75 Hz and above 75 Hz, respectively. D, expanded region of 1H-15N HSQC spectra of 15N-labeled β2 in the absence (red contour) and in the presence of unlabeled αL (black contour) at a ratio of 1:1.5 (β2:αL) under the same condition as in A. E, bar diagram showing changes in chemical shift (in Hz) as a function of amino acid residue of β2 tail in the presence of αL tail. The chemical shift changes were calculated as described in B. Asterisk indicates the residue that showed almost complete disappearance of HSQC cross-peaks upon binding to the αL tail. F, ribbon representation of the conformation of β2 tail showing amino acid residues affected by binding with αL tail. Blue and red represent the combined changes in 1H and 15N chemical shift of 15–30 Hz and above 30 Hz, respectively. Residues showing ≤10 Hz chemical shift change are not shown. The images in C and F were produced with the program PyMOL.

−0.5 μM, and the complex formation is driven by a positive change in entropy with a free energy (ΔG) change of −8.45 kcal/mol (Table 3), indicating dominant role of hydrophobic interaction in the complex formation.

**DISCUSSION**

The integrin αLβ2 mediates activation-dependent adhesion and migration of the leukocytes (34). Distinct populations of αLβ2 conformers representing different activation states are...
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The binding of metal ions (Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\)) were characterized for the αLβ2 tail (7), suggesting plausible role of intracellular cations in the regulation of integrin activity. These divalent cations coordinate, at micromolar affinity, with the αLβ2 tail utilizing a stretch of acidic residues, Glu\(^{1001}\)–Glu\(^{1008}\), at the C terminus (Fig. 1A). These acidic residues are largely nonconserved among the amino acid sequences of integrin α tails (Fig. 1A). Here we were able to detect calcium binding of the αL tail. The calcium binding to the αL tail appeared to be stabilized by the acidic residues primarily located in the helix 2-loop-helix 3 region of the αL tail (Fig. 5D). In the folded topology of the αL tail, these residues form a negatively charged surface (Fig. 5E). We also surmise that apart from binding cations, this negatively charged surface may be available for interactions with other positively charged ligands.

In solution the isolated β2 cytoplasmic tail is largely unstructured except for the N-terminal residues Leu\(^{6}\)–Arg\(^{14}\) similar to the conformation of the free β3 tail (Fig. 2B) (11). We were unable to detect any turn conformations for \(^{25}\)NPLF\(^{31}\) sequence motif in the β2 tail as a result of limited NOEs and spectral overlap. The corresponding sequence motif in β3 tail adopts a β-turn conformation and is involved in the interaction with talin head domain (11). With regard to tails complex formation, the association of αL tail with β2 tail predominantly involved residues located at the membrane proximal helix 1 and helix 3 of the αL tail, and the membrane proximal helix of the β2 tail (Fig. 6). These interactions may form an interface in the αL-β2 tails complex as depicted in a docked model (Fig. 8). The NMR structure of the αLβ2-β3 tails complex revealed an interface stabilized by two membrane proximal helices, each from one tail (10). A notable feature in the interface of the αLβ2-β3 tails complex is the potential salt bridge interactions involving αLβ2 Arg\(^{995}\) and β3 Asp\(^{775}\) and Glu\(^{726}\). Nonpolar packing between αLβ2 Val\(^{990}\) and Phe\(^{992}\) and β3 Leu\(^{718}\) and Ile\(^{719}\) was also determined. These interactions that potentially stabilize the interface of the αLβ2-β3 tails complex are plausible in the docked structure of αL-β2 tails complex. These include potential salt bridges between Arg\(^{7}\) of αL tail with Asp\(^{8}\) and Glu\(^{11}\) of β2 tail and the hydrophobic packing between residues Phe\(^{4}\) of αL and Ile\(^{8}\) of β2 (Fig. 8). Ionic interactions specific to the αL-β2 tails complex because of the close proximities of acidic residues Glu\(^{11}\) and Glu\(^{14}\) (helix 1) of the αL tail with the basic residue Arg\(^{14}\) of the β2 tail are permissible (Fig. 8). All these residues experienced changes in chemical shift as a result of mutual interactions between αL and β2 tails (Fig. 6). The dominant role of ionic and/or polar interactions in the complex formation between αL and β2 tails are also supported by the detection of exothermic heat release in the ITC analyses (Fig. 7).

The cytoskeleton protein talin binds directly with β1, β2, and β3 integrins (2). An in vivo study demonstrated that the head domain of talin interacts with the β2 tail that leads to the activation of αLβ2 by disrupting the αL-β2 tails complex (19). In this study, we detected direct interaction between talin head domain with the β2 tail by NMR and ITC measurement. The binding of talin head domain to the β2 tail induces substantial changes in the chemical shifts of a large number of residues, indicating a considerable region of the β2 tail is engaged in interactions, including the membrane proximal helix. As mentioned previously, the membrane proximal helix of β2 is also involved in the interaction with the
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αL tail. However, binding affinity between αL and β2 tails estimated from ITC studies was found to be significantly lower, \( K_d \approx 2.63 \, \mu M \), as compared with binding affinity, \( K_d \approx 0.5 \, \mu M \), of talin head domain to β2 tail (Table 3). Therefore, it is likely that a high affinity interaction between talin head domain with the β2 tail may disrupt the association of the αL and β2 tails and trigger the activation of αLβ2.

The αLβ2 cytoplasmic tails can undergo phosphorylations that impinge on the activity of αLβ2 (58). In the αL tail, Ser\(^{1140} \) (Ser\(^{53} \) herein) was found to be abundantly phosphorylated in resting T cells, and mutation of Ser\(^{1140} \) to Ala abrogated αLβ2 affinity up-regulation by the small GTPase Rap1 (59). Of note, the substitution of Ser\(^{1140} \) with Ala did not affect heterodimerization of αL and β2 (59), and in this study, Ser\(^{1140} \) is not perturbed when the αL tail interacts with the β2 tail (Fig. 6B). This suggests that Ser\(^{1140} \) phosphorylation may serve to modulate interactions of cytosolic factors with the αL tail. In this regard, it is interesting to note that Ser\(^{1140} \) in helix 3 is located at the negatively charged surface of the αL tail, and its phosphorylation can enhance the negative charge of this surface (Fig. 5, D and E). In the β2 tail, phosphorylation of Thr\(^{236} \) (Thr\(^{35} \) herein) but not Ser\(^{734} \) (Ser\(^{53} \) herein) allows the docking of 14-3-3 proteins, thereby modulating the activity of αLβ2 (59–61). Thr\(^{236} \) is also one of the threonine triplets in the β2 tail that was reported to undergo phosphorylation, two threonines at a time, in T cells stimulated with phorbol ester (62).

In a series of studies that examined the interaction of αLβ and β3 tails, differences in observations were made. These are possibly attributed to different approaches employed that included coiled-coil fusion proteins, tails with transmembrane domains, and fragments of tails other than full-length tails of the αLβ and β3 subunits (9–12). In vivo, the orientation of the tails and their juxtaposition when in complex may be affected by the positions of the transmembrane domains. Recent NMR studies suggest that the first charged residue Lys on the intracellular side of αLβ and β3 does not necessarily demarcate the C-terminal end of the transmembrane domains because of the tilting of transmembrane domains in the lipid bilayer (63, 64). Interestingly, the αLβ cytoplasmic tail residues Phe\(^{992} \) and Phe\(^{993} \) after the Lys\(^{989} \) insert back into the membrane. These Phe residues are conserved in the αL tail (Phe\(^{6} \) and Phe\(^{5} \) herein, see Fig. 1A). Whether the insertion of these residues into the membrane as in αLβ may change the conformation of the αL tail and its mode of interaction with the β2 tail is not known at present. Furthermore, the αLβ tail adopts a “closed” conformation with its C-terminal region folded back and interacting with its N-terminal helix (45). It was shown that membrane-permeable myristoylated wild-type αLβ tail peptide inhibited fibrinogen binding to platelets, whereas a mutant peptide, with P998A/P999A mutations that disrupt the turn between the N-terminal helix and C-terminal region, had no inhibitory effect (45). This demonstrates the relevance and importance of the αLβ tail conformation in αLββ3 function. In the αL tail conformation, a turn is observed between helix 1 and helix 2 and another between helix 2 and helix 3. Hence, it will be interesting in future studies to determine the physiological significance of the αL tail conformation in αLβ2 functional assays using, for example, a T cell-based system and a series of myristoylated wild-type and fold-disrupting mutant αL tail peptides.

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