Structures and Interaction Analyses of Integrin αMβ2 Cytoplasmic Tails

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Background: Cytosolic tails of integrins are critical for activation.

Results: Structures of cytosolic tail of αM and phosphorylated αM integrin were determined. The interactions between αM and β2 tails were investigated.

Conclusion: Structures of αM are characterized by N-terminal helix and loop at the C terminus with helix-loop packing. αM tail interacted with β2.

Significance: Structures and interactions provide insights into activation of integrins.

Integrins are heterodimeric (α and β subunits) signal transducer proteins involved in cell adhesions and migrations. The cytosolic tails of integrins are essential for transmitting bidirectional signaling and also implicated in maintaining the resting states of the receptors. In addition, cytosolic tails of integrins often undergo post-translation modifications like phosphorylation. However, the consequences of phosphorylation on the structures and interactions are not clear. The leukocyte-specific integrin αMβ2 is essential for myeloid cell adhesion, phagocytosis, and degranulation. In this work, we determined solution structures of the myristoylated cytosolic tail of αM and a Ser phosphorylated variant in dodecylphosphocholine micelles by NMR spectroscopy. Furthermore, the interactions between non-phosphorylated and phosphorylated αM tails with β2 tail were investigated by NMR and fluorescence resonance energy transfer (FRET). The three-dimensional structures of the 24-residue cytosolic tail of αM or phosphorylated αM are characterized by an N-terminal amphipatic helix and a loop at the C terminus. The residues at the loop are involved in packing interactions with the hydrophobic face of the helix. 15N-1H heteronuclear single quantum coherence experiments identified residues of αM and β2 tails that may be involved in the formation of a tail-tail heterocomplex. We further examined interactions between myristoylated β2 tail in dodecylphosphocholine micelles with dansylated αM tail peptides by FRET. These studies revealed enhanced interactions between αM or phosphorylated αM tails with β2 tail with $K_D$ values $\sim 5.2 \pm 0.6$ and $\sim 4.4 \pm 0.7 \mu M$, respectively. Docked structures of tail-tail complexes delineated that the αM/β2 interface at the cytosolic region could be sustained by a network of polar interactions, ionic interactions, and/or hydrogen bonds.

Integrins are cell surface adhesion molecules formed by specific non-covalent associations between different α and β subunits (1). Each subunit has a large extracellular region that is required for ligand binding, a single transmembrane domain, and a cytoplasmic tail (2, 3). Integrins are bidirectional signaling receptors that allow communication between the interior of a cell and its external microenvironment (1). Integrin occupation and clustering induce cytoplasmic signaling by recruiting cytoplasmic adaptor and cytoskeletal proteins to its cytoplasmic tails, ultimately forming an integrin adhesome (4, 5). In the immune system, integrins mediate many leukocyte functions such as the migration across endothelium, phagocytosis, antigen presentation, cytotoxic cell killing, and selective homing of lymphocytes into specialized tissues (6–8). The four leukocyte-restricted β2 integrins have different α subunits pairing with a common β2 subunit. They are αLβ2 (LFA-1, CD11aCD18), αMβ2 (Mac-1, CD11bCD18), αXβ2 (p150,95, CD11cCD18), and αβ2 (CD11dCD18). The functional importance of the β2 integrins is underscored by the high susceptibility to microbial infections of leukocyte adhesion deficiency I and III patients (9). The adhesive and migratory properties of the leukocytes are compromised in these patients because of disrupting mutations in the β2 subunit (leukocyte adhesion deficiency I) or in the integrin cytoplasmatic regulator kindlin-3 (leukocyte adhesion deficiency III) that regulates the activation of the β2 integrins (10, 11). The integrin αMβ2 is expressed on myeloid, natural killer, and γδ T cells (12–14). It is a promiscuous receptor that binds to many ligands including intercellular adhesion molecules, junctional adhesion molecule-3, iC3b, fibrinogen, microbial saccharides, and denatured proteins (15–19). In addition to its well documented roles as an adhesion molecule, as a phagocytic receptor, and in the regulation of degranulation and apoptosis, integrin αMβ2 has been shown to be involved in the maintenance of tolerance and the control of inflammation (14, 20, 21). Furthermore, αMβ2 has been shown to be involved in the regulation of monocyte differentiation (22–24). Although αM has a short cytoplasmic tail as compared with αL, αX, and αD, it serves important functions in regulating αMβ2 ligand binding and outside-in signaling. It is well established that αMβ2 outside-in signaling regulates neutrophil survival. In particular, αMβ2 with a truncated αM cytoplasmic tail or with...
the αM tail replaced by a βL tail have an impaired ability to protect transfected K562 cells from apoptosis as a result of inactivation of Akt and Erk1/2 (25). Notably, αMβ2, but not the αMβ2 mutant with its αM cytoplasmic tail replaced with that of αL or αX, recruits the Src family kinase Hck (26). Interestingly, a genome-wide association study found an association between systemic lupus erythematosus and a non-synonymous SNP, rs1143678 (C>T), that substitutes amino acid Pro1130 with Ser in the αM tail (27). Phosphorylation of integrin cytoplasmic tails is an important post-translational modification that is necessary for integrin functions (28). In polymorphonuclear leukocytes, the αM cytoplasmic tail is constitutively phosphorylated, whereas the phosphorylation of the β2 tail is dependent on cellular activation (29, 30). There are two phosphorylation sites in the αM cytoplasmic tail, Tyr1121 and Ser1126. Interestingly, it was found that mutation of Ser1126 in the αM cytosolic tail disrupts the ability of αMβ2 to bind intercellular adhesion molecule-1 and intercellular adhesion molecule-2 but not iC3b and denatured BSA (31), suggesting that αM cytosolic tail Ser1126 phosphorylation plays an important role in αMβ2 functional regulation. Indeed, the mutant αMS1126Aβ2 failed to react with the activation reporter mAb KIM127 and bound poorly to intercellular adhesion molecule-1 (32). In addition, cells expressing αMS1126Aβ2, but not wild-type αMβ2, when injected into mice showed reduced extravasation into the spleens and lungs (32). Collectively, these data suggest that the αM cytoplasmic tail is pivotal in the regulation of αMβ2 ligand binding and outside-in signaling. To understand the mechanism of αMβ2 regulation and its cytoplasmic signaling, detailed analyses of its cytoplasmic tails are required. In this study, solution structures of myristoylated αM tail in two forms, phosphorylated (Ser(P)1126) and non-phosphorylated, were determined in solution containing dodecylphosphocholine (DPC)3 micelles. Interactions studies between the αM and β2 tails using NMR and fluorescence resonance energy transfer (FRET) demonstrated a low affinity binding for the non-myristoylated tails in solution. However, FRET experiments carried out in detergent-containing solutions showed high affinity interactions between the tails when one of the tails was myristoylated.

**EXPERIMENTAL PROCEDURES**

**Synthesis and Purification of Synthetic Cytoplasmic Tails**—Synthetic cytoplasmic tail peptides of αM and β2 used in this study are given in Table 1. All of the cytoplasmic tail peptides (myristoylated, non-myristoylated, and other derivatives) were purchased from GL Biochem (Shanghai, China) and were further purified using reverse phase HPLC (Waters, Milford, MA) connected to a C18 column (300-Å pore size, 5-μm particle size). A linear gradient of acetonitrile/water with a flow rate of 2 ml/min was used to elute the peptides, and the major peak fractions were lyophilized. The mass of the peptides was confirmed by mass spectrometry.

**Expression Plasmids**—The numbering of the amino acids in the αM and β2 tails is based on the work of Buyon et al. (29). The full-length β2 tail (Lys702–Ser747) was subcloned into the AlwNI site of pET-31b(+) vector (Novagen, EMD, San Diego, CA) containing an N-terminal ketosteroid isomerase (KSI) has been reported previously (33). The cDNA encoding the full-length αM tail (Lys1113–Gln1136) with the terminating stop codon was PCR-amplified from αM-pcDNA3.0 expression plasmid (31) and cloned into the AlwNI site of the pET-31b(+) vector. The forward primer used for the PCR was designed such that formic acid cleavage GGGGSDP sequence (34) was introduced between the KSI and the αM tail sequences. The DP site allows cleavage of the αM tail from the KSI using formic acid in subsequent protein expression and purification procedures. A stop codon was introduced immediately after Gln1136 in the αM tail that disrupts the expression of a C-terminal His6 tag in the pET-31b(+) vector; therefore, we constructed a His6 tag at the N terminus of the KSI to generate the final expression plasmid containing His6-KSI-GGGGSDP-αM tail. This allowed affinity purification of the fusion protein, cleavage of the fusion protein by formic acid, and recovery of full-length αM tail without additional tag sequences. Furthermore, the cytoplasmic tails of αM (Lys1113–Gln1136) and β2 (Lys1126–Ser1126) are numbered as αM (Lys2–Gln4) and β2 (Lys2–Ser4), respectively.

**Protein Expression and Purification**—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 [15N]ammonium chloride/[13C]glucose as described in our previous study (33). For the induction of protein expression, isopropyl β-d-thiogalactopyranoside (0.8 mM) was added, and the bacteria culture was incubated at 25 °C. The harvested cell pellet was resuspended in binding buffer (5

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**TABLE 1**

| Peptide       | Sequence               |
|---------------|------------------------|
| Myr-αM        | Myr–1KLGFKQYKDMMSGEPGPAEQ24 |
| Myr-αMMyr-β2  | Myr–1KLGFKQYKDMMSGEPGPAEQ24 |
| αM            | 1KLGFKQYKDMMSGEPGPAEQ24 |
| αMMyr-β2      | 1KLGFKQYKDMMSGEPGPAEQ24 |
| β2            | 1KALWLDLREYRIFKEKLSQWNNNDPLFKSATTTTINPKFAES26 |
| β2H5W        | 1KALWLDLREYRIFKEKLSQWNNNDPLFKSATTTTINPKFAES26 |
| Myr-β2H5W     | 1KALWLDLREYRIFKEKLSQWNNNDPLFKSATTTTINPKFAES26 |
| Dan-αM        | Dansyl–1KLGFKQYKDMMSGEPGPAEQ24 |
| Dan-αMMyr-β2 | Dansyl–1KLGFKQYKDMMSGEPGPAEQ24 |

3 The abbreviations used are: DPC, dodecylphosphocholine; HSQC, heteronuclear single quantum coherence; dansyl, 5-dimethylaminonaphthalene-1-sulfonfyl; KSI, ketosteroid isomerase; Myr, myristoylated; Myr-P, myristoylated and phosphorylated; Dan, dansylated and phosphorylated; TOCSY, total correlation spectroscopy; PRE, paramagnetic relaxation enhancement; TM, transmembrane domain.
Structure and Interactions of αMβ2 Cytosolic Tails

mm imidazole, 500 mM NaCl, 40 mM Tris, pH 7.9), sonicated on ice, and centrifuged at 20,000 rpm for 20 min at 4 °C. The protein pellet was then resuspended in binding buffer containing 8 M urea, and His6-tagged fusion protein was purified on a nickel-nitritolriacetic acid resin (Qiagen) column. The fusion protein was eluted in elution buffer (300 mM imidazole, 500 mM NaCl, 8 M urea, 40 mM Tris-HCl, pH 7.9) and dialyzed against distilled water overnight at 4 °C. The protein precipitate was pelleted by centrifugation at 4000 × g for 15 min at 4 °C. CNBr in 70% (v/v) formic acid was used for the cleavage of the β2 tail from the KSI fusion partner as described previously (33). For the cleavage of the αM tail from the KSI fusion partner, the fusion protein precipitate was dissolved in deionized water. The KSI protein precipitate was removed by centrifugation at 12,000 × g for 10 min at 4 °C. The cleaved αM or β2 tails were subjected to reverse phase HPLC (Waters) purification on a C18 column (300-Å pore size, 5-μm particle size) by a linear gradient of an acetonitrile/water mixture.

NMR Experiments—All NMR spectra were recorded on a Bruker DRX 600-MHz instrument equipped with an actively shielded cryoprobe. NMR data were processed using TOPSPIN 2.1 and analyzed with SPARKY.4 Two-dimensional 1H-1H total correlation spectroscopy (TOCSY) and two-dimensional 1H-15N nuclear Overhauser effect spectroscopy (NOESY) experiments were carried out for myristoylated αM (Myr-αM) and myristoylated and phosphorylated αM (Myr-PαM) at a concentration of 0.7 mM in 10 mM sodium phosphate buffer, pH 5.6, containing 200 mM perdeuterated DPC (DPC-d35) (Cambridge Isotope Laboratories, Andover, MA) at 308 K. NOESY spectra were acquired from myristoylated peptides (200 μM) in 10 mM sodium phosphate buffer, pH 5.6 at 308 K.

TOCSY and NOESY spectra were acquired for non-myristoylated αM tail peptide at a concentration of 0.7 mM in 10 mM sodium phosphate buffer, pH 5.6 at 278 K. The phosphorylated or non-phosphorylated αM tail peptides were also dissolved in 200 mM perdeuterated DPC to record both TOCSY and NOESY spectra. The mixing time for TOCSY and NOESY experiments was fixed to 50 and 200 ms, respectively. Natural abundance 13C-1H heteronuclear single quantum coherence (HSQC) experiments were carried out for Myr-αM and Myr-PαM in DPC solution under similar D2O buffer conditions to obtain 13C chemical shifts. Paramagnetic perturbation studies of the myristoylated or non-myristoylated αM tails with or without 200 mM DPC were carried out in 10 mM sodium phosphate buffer, pH 5.6 by acquiring two-dimensional NOESY and TOCSY spectra with 200- and 500-ms mixing times, respectively, in the absence or presence of 1 mM MnCl2. The changes in the intensity of intraresidue C1H/HN cross-peaks were normalized to the peak intensity of the respective unperturbed spectra. Standard three-dimensional HNCA, HN(CO)CA, HN(CA) CB, and CBCA(CO)NH experiments were performed with 0.5 mM 15N/13C-labeled samples of αM and β2 tails at 298 K to obtain backbone resonance assignments. The interactions between the tails of β2 and αM or PaM were determined by 1H-15N HSQC experiments. HSQC spectra of 15N-labeled β2 or αM tail peptides were acquired in the presence of unlabeled αM or phosphorylated αM or of unlabeled β2 tail peptides, respectively. Samples were prepared by dissolving lyophilized powder of tail peptides in 10 mM sodium phosphate buffer, pH 6.5. The chemical shift changes, after addition of the unlabeled binding partners, of the backbone amide nitrogen and amide proton of each residue were calculated using the following equation: ΔH + Δ15N where ΔH is the absolute value of the change in amide proton chemical shift and Δ15N is the absolute value of the change in backbone amide nitrogen chemical shift.

Circular Dichroism (CD) Studies—Circular dichroic measurements were performed by a Chirascan CD spectrophotometer (Applied Photophysics, Leatherhead, UK) using a cuvette of 0.1-cm path length (Hellma, Müllheim, Germany). CD spectra of 0.2 mM Myr-αM and Myr-PαM in 10 mM sodium phosphate buffer, pH 6.5, containing 60 mM DPC and of 0.3 mM synthetic αM in 10 mM sodium phosphate buffer, pH 6.5 were acquired from wavelengths of 190–240 nm at 0.1-s time steps. CD spectra were averaged over three scans and subtracted from the corresponding background buffer contributions. The final CD spectra were converted to molar ellipticity.

Structure Determination and Docking—Three-dimensional structures of Myr-αM and Myr-PαM were determined using CYANA 2.1 (35). Interproton distance restraints were derived from strong, medium, and weak NOE intensities translated to upper bound distance limits of 2.5, 3.5, and 5.0 Å, respectively. Backbone dihedral angle (φ, ψ) restraints were determined based on 13Cα chemical shifts using PREDICTOR (36). Of the 100 structures, the 20 lowest energy structures were selected for evaluation and analyses. PROCHECK-NMR (37) was used to evaluate the stereochemical quality of the structure ensemble, and structures were visualized using PyMOL, MOLMOL, and Discovery Studio 2.0. RosettaDock (38) was used to generate low energy models of the complexes between the αM tails and β2 tail.

FRET Studies—All fluorescence studies were conducted using a 0.5-cm path length luminescence spectroscopy microcell (PerkinElmer Life Sciences) on a Varian Cary Eclipse spectrophotometer. Peptide samples were prepared in 10 mM sodium phosphate buffer, pH 6.5 containing 1.2 mM DPC. FRET experiments were typically conducted by collecting fluorescence emission spectra of intrinsic Trp residue of Myr-β2 tail mutated (β2H5W) or Myr-β2 at 1 μM concentration in the presence of various concentrations (ranging from 1 to 5 μM) of dansylated and unphosphorylated αM (Dan-αM) and phosphorylated αM (Dan-PαM) tail peptides. Samples were excited at a wavelength of 295 nm, and emission was monitored from 310 to 400 nm. The extent of quenching of Trp emission (resulting from FRET) of Myr-β2H5W at 355 nm was used to determine the dissociation constants (Kd) for the interactions between the β2 and αM tail peptides following a standard nonlinear least square fit. Additional FRET studies were also performed for the non-myristoylated β2H5W in 10 mM sodium phosphate buffer, pH 6.5 and myristoylated β2H5W in 50% acetonitrile, buffer mixture with dansylated αM peptides.
Structure and Interactions of αMβ2 Cytosolic Tails

RESULTS

Effect of Myristoylation on Conformational Stabilization and Localization of αM Cytosolic Tails—We used a myristoylation strategy, i.e. covalently attaching a myristic acid to the N terminus of the αM cytoplasmic tail, to determine three-dimensional structures in the DPC lipid micelles. The long acyl chain (C14) of myristic acid, a probable mimic of the transmembrane domain, may stabilize conformations of peptides (39, 40) in the presence of detergent lipids as demonstrated for the cytosolic tail peptides of integrins in the presence of DPC micelles (39). Furthermore, the acylated cytosolic tails of integrins were also examined by far-UV CD spectroscopy (supplemental Fig. S1). The myristoylated peptides Myr-αM and Myr-PαM in DPC micelles showed negative CD bands at 225 and 208 nm diagnostic of helical structure (43). By contrast, far-UV CD spectra of the non-myristoylated αM peptide delineated an intense CD band of negative ellipticity at ~200 nm, indicating random conformations (43).

To ascertain conformational stabilization of αM tail peptides by myristoylation and micelle incorporation, NOESY spectra were also acquired for the non-myristoylated and myristoylated αM tail peptides either in DPC-containing solutions (supplemental Fig. S2) or in aqueous solutions, respectively (supplemental Fig. S3). Again, the myristoylated αM tail peptides in the presence of DPC micelles yielded a greater number of NOE connectivities in comparison with the non-myristoylated tail peptides in DPC or myristoylated tail peptides in aqueous solutions. The NMR spectra of the myristoylated tail peptides in aqueous solutions appeared to be significantly broadened (supplemental Fig. S3). Such broad resonances may potentially arise as a result of aggregations of the myristoylated peptides in aqueous solutions. In other words, the myristoylated peptides may be improperly folded in the absence of DPC detergent solution. Therefore, the presence of a large number of NOE connectivities for the Myr-αM and Myr-PαM establishes that the cytoplasmic tails of αM, either non-phosphorylated or phosphorylated at residue Ser14, acquire well defined conformations in the presence of DPC micelles.

We further probed whether the myristoylated peptides Myr-αM and Myr-PαM were inserted into the DPC micelles using NMR paramagnetic relaxation enhancement (PRE) experiments. In these studies, two-dimensional 1H-1H NOESY spectra were acquired for the myristoylated peptides in DPC micelles either in the absence or in the presence of 1 mM MnCl₂, and changes in the intensity of NH/C=H cross-peaks were measured (see "Experimental Procedures"). It should be noted that MnCl₂ would exhibit a PRE effect on those amino acid residues that are exposed to the aqueous solution (44). As can be seen, there was a dramatic diminution of the intensity of NH/C=H correlations for most of the residues of Myr-αM and Myr-PαM peptides, indicating their proximity to the paramagnetic Mn²⁺ ions (Fig. 2). However, a relatively restricted diminution of intensity (~40%) of NH/C=H cross-peaks was detected for a few N-terminal residues, Leu²—Phe³, suggesting a limited exposure of these residues to the paramagnetic ions (Fig. 2). The relatively low PRE effect of the N-terminal residues was found to be biologically active (40–42). Fig. 1 shows two-dimensional 1H-1H NOESY spectra correlating low field resonances (6.5–9.0 ppm) with upfield resonances (0.8–4.5 ppm) of the 24-residue synthetic αM tail peptides (Table 1) in Myr-αM (Fig. 1A) and Myr-PαM (Fig. 1B) in DPC micelles at 308 K and non-myristoylated αM in aqueous solution at 278 K (Fig. 1C). As evident from the figure, there are dramatic differences in the NOE connectivities observed for the Myr-αM (Fig. 1A), Myr-PαM (Fig. 1B), and αM (Fig. 1C). There were lesser NOE connectivities in the NOESY spectrum of αM tail in free solution even at 278 K, particularly among the backbone/side chain and side chain/side chain resonances (Fig. 1C). By contrast, the NOESY spectra of the Myr-αM and Myr-PαM demonstrated an overwhelming number of NOE connectivities in solutions containing DPC micelles (Fig. 1, A and B). The paucity of NOE interactions observed for the non-myristoylated αM tail in aqueous solution indicates a lack of significant populations of folded conformations. The structural transitions of the αM cytosolic tail peptides were also examined by far-UV CD spectroscopy (supplemental Fig. S1). The myristoylated peptides Myr-αM and Myr-PαM in DPC micelles showed negative CD bands at 225 and 208 nm diagnostic of helical structure (43). As evident from the figure, there are dramatic differences in the NOE connectivities observed for the non-myristoylated αM tail in aqueous solution (44). As can be seen, there was a dramatic diminution of the intensity of NH/C=H correlations for most of the residues of Myr-αM and Myr-PαM peptides, indicating their proximity to the paramagnetic Mn²⁺ ions (Fig. 2). However, a relatively restricted diminution of intensity (~40%) of NH/C=H cross-peaks was detected for a few N-terminal residues, Leu²—Phe³, suggesting a limited exposure of these residues to the paramagnetic ions (Fig. 2). The relatively low PRE effect of the N-terminal residues...
Structure and Interactions of αMβ2 Cytosolic Tails

may occur as a consequence of a potential insertion of the αM tails into DPC lipid micelles. Note that the N-terminal region of the αM tail contains a stretch of conserved hydrophobic and aromatic residues that may be inserted into the non-polar region of the lipid micelles (Table 1). Therefore, PRE studies were performed for the non-myristoylated tail peptides in lipid-free aqueous solutions and DPC-containing solutions (supplemental Fig. S4). Notably, the N-terminal residues of the non-myristoylated αM tails also demonstrated a lower PRE effect in comparison with the C-terminal residues in lipid-free and DPC-containing solutions (supplemental Fig. S4). These observations indicated that the N-terminal non-polar/aromatic residues of the tail peptides are intrinsically less exposed to the paramagnetic ions, plausibly as a result of structure formation or the presence of bulky aromatic side chains. The overlapping and broad resonances observed for Myr-αM and Myr-αM peptides in aqueous solution precluded residue-specific analyses of PRE studies. Regardless, the marked PRE effect experienced by the majority of the residues of Myr-αM and Myr-αM clearly establishes that these residues are placed into the aqueous milieu. Consequently, the N-terminal myristic acid moiety appears to act as a lipid anchor akin to a transmembrane domain, imparting conformational stabilization and aqueous localization for the cytosolic tail of αM integrin in DPC micelles.

Analyses of Secondary Chemical Shifts and NOEs—C"H and 13Cα chemical shift deviations from random coil are an indicator of secondary structures of proteins and peptides (45). Fig. 3 shows the chemical shift deviations of the 13Cα and C"H resonances from their random coil values for Myr-αM (Fig. 3A), Myr-αM (Fig. 3B), and αM (Fig. 3C). The chemical shifts were corrected for the nearest neighbor (i = 2, i - 1, i + 1, and i + 2) effects (46). In particular, the 13Cα atom would experience a downfield shift in helical structure and an upfield shift in β-angle, whereas a reverse trend has been demonstrated for the C"H proton resonances (45). A stretch of at least four or three continuous residues with helical or strand type chemical shift deviations can be assigned as a stable helix or β-strand conformation, respectively (45). The 13Cα and C"H resonances of the Myr-αM and Myr-αM tails were found to experience a positive deviation and a negative deviation, respectively, for the N-terminal residues Phe8–Ser14 (Fig. 3A, C and B). By contrast, the chemical shift deviations for 13Cα and C"H resonances at the C-terminal half, residues Glu15–Gln34, of Myr-αM and Myr-αM were largely limited (Fig. 3A and B). Therefore, the N-terminal segment, residues Phe8–Ser14, of the cytoplasmic tail irrespective of the phosphorylation at residue Ser14 (Ser1127) acquires a stable helical structure when inserted into DPC micelles through myristoylation. On the other hand, the C-terminal region, residues Glu15–Gln34, of the cytoplasmic tail appears to be devoid of regular secondary structures. The chemical shift deviations for the non-myristoylated αM tail in free solution were less pronounced in comparison with the myristoylated tails in DPC micelles, indicating a lack of stable secondary structures (Fig. 3C). However, a population of transient helical structures could be inferred for the N-terminal residues of αM tail in solution because the C"H and 13Cα resonances showed, albeit limited, negative and positive deviations, respectively (Fig. 3C). The myristoylated tail peptides Myr-αM and Myr-αM demonstrated a large number of NOE contacts involving backbone/backbone, backbone/side chain, and side chain/side chain resonances in DPC micelles. Analyses of NOESY spectra revealed medium range C"H/NH NOEs (i to i + 2, i + 3, and i + 4) and NH/NH NOEs (i to i + 1and i + 2) for the N-terminal residues both for Myr-αM (supplemental Fig. S5) and Myr-αM peptides (supplemental Fig. S5), indicating stable helical
conformation for this segment. The cytoplasmic domain of αM in free solution yielded fewer NOE contacts in comparison with myristoylated peptides in DPC micelles (supplemental Fig. S5). However, the N-terminal residues of αM tail showed a number of medium range CαH/NH NOEs evidencing the plausible existence of a population of helical conformations (supplemental Fig. S5). Furthermore, several side chain/side chain NOEs were identified for the Myr-αM and Myr-αMa peptides in DPC micelles. The N-terminal helical regions of Myr-αM and Myr-αMa in DPC micelles were defined by medium range interside chain NOE contacts between aromatic/aromatic and aliphatic/aromatic residues (supplemental Fig. S6). Notably, the aromatic ring protons of Phe5 of Myr-αM (supplemental Fig. S6) and Myr-αMa (supplemental Fig. S6) showed NOE interactions with the ring proton resonances of residue Tyr9. In addition, side chain/side chain NOEs were present for Myr-αM and Myr-αMa peptides between residues Leu2/Phe5 and residues Tyr9/Met12 (supplemental Fig. S6). Interestingly, a number of long range NOEs could be detected among residues Tyr9/Gln24, Met12/Gln24, Met13/Gln24, Met12/Pro18, Ser14/Gln24, and Met12/Glu22 for the Myr-αM peptide (Fig. 4, top panel), implying packing interactions between the C-terminal loop and N-terminal helical region. There were also long range NOEs, e.g. Met12/Gln24, Met13/Gln24, and Ser(P)14/Gln24, observed for the Myr-αMa tail peptide. However, more such long range NOEs could be identified for Myr-αM tail in comparison with Myr-αMa peptide (supplemental Table S1), plausibly indicating diminished interactions between the helix and the loop region.

Solution Structures of Myr-αM and Myr-αMa Cytoplasmic Tail Peptides—Three-dimensional structures of Myr-αM and Myr-αMa were determined by use of NOE-driven distance and backbone dihedral angle (φ, ψ) restraints (Table 2). Fig. 5 shows a superposition of all backbone atoms (Cα, N, and C’) of the 20 lowest energy structures of Myr-αM (Fig. 5A) and Myr-αMa (Fig. 5B). The root mean square deviation values from the mean
structure and the stereochemical goodness of the structural ensembles of Myr-αM and Myr-αPaM are listed in Table 2. The three-dimensional structure of the Myr-αM tail peptide revealed a helical conformation encompassing residues Phe4–Ser14 at the N terminus, whereas residues Glu15–Gln24 at the C terminus acquire a looplike structure (Fig. 5C). The C-terminal loop folds back onto the helical structure, yielding a compact conformation of the Myr-αM tail (Fig. 5D). The N-terminal helical structure of the Myr-αM tail appears to be amphipathic in nature whereby the polar face is determined by the side chains of residues Lys6, Arg7, Lys10, Asp11, and Ser14 (Fig. 5C). There are potential ionic interactions and/or hydrogen bonding between residues Arg7 and Asp11 and residues Lys10 and Ser14 at the polar face of the helix (Fig. 5C). On the other hand, the non-polar face of the N-terminal helix is characterized by the mutual packing interactions among the side chains of aromatic and aliphatic residues, e.g. Phe4, Phe5, Tyr9, Met12, and Met13 (Fig. 5D). The specific positioning of the C-terminal region of the Myr-αM tail in the three-dimensional structure essentially created a non-polar surface involving van der Waals packing interactions among residues Met12 and Met13 from the helix and residues Pro19 and Pro23 and methylene groups of residue Gln24 from the C-terminal loop (Fig. 5D). The N-terminal helical structure, residues Phe4–Ser14, also was demonstrated to be preserved in the Myr-αPaM (Fig. 5E). The disposition of polar and non-polar side chains in the helical structure of Myr-αPaM was found to be amphipathic, akin to Myr-αM (Fig. 5E). In the context of the phosphorylation of Ser14, there could be ionic interactions between the cationic side chain of residue Lys10 and the negatively charged phosphate group of Ser(P)14 in the helical structure of Myr-αPaM (Fig. 5F). However, the tertiary packing interactions between the C-terminal loop and N-terminal helix observed in the Myr-αM tail appear to be largely disrupted in the Myr-αPaM tail (Fig. 5F). We found only a few NOE interactions between the residues at the loop and in the helix for the Myr-αPaM tail, resulting in poorly defined structural organization of the loop.

### Interactions between αM and β2 Tails by 15N-1H HSQC

We examined binding interactions between the non-myristoylated tails in aqueous buffer solution. The αM and β2 cytoplasmic tails were overexpressed and isotope (15N and 15N/13C)-labeled for NMR studies (see “Experimental Procedures”). The 15N-1H HSQC spectra of αM and β2 tails were assigned using standard triple resonance NMR experiments (Figs. 6A and 7A). 15N-1H HSQC spectra of either 15N-labeled αM or 15N/13C-labeled β2 were obtained in the presence of various concentrations (at molar ratios 1:1, 1:2, and 1:8) of unlabeled binding partners. There were detectable changes in terms of either chemical shifts or intensity in the 15N-1H HSQC spectra of αM or β2 upon addition of unlabeled tail proteins (Figs. 6A and 7, A and B), indicating interactions between the cytoplasmic tails of αM and β2. However, the binding affinity between the tail peptides appeared to be low as conspicuous spectral changes in 15N-1H HSQC were observed only in the presence of a higher molar concentration of unlabeled tails (supplemental Fig. S7). Similar observations were also made for the NMR interaction studies between tails of αlbb/β3 and αL/β2 integrins (33, 47).

Combined chemical shift changes for 1HN and 15N resonances of αM are shown as a function of residue (Fig. 6B). As can be seen, a number of residues of the αM tail, namely Leu2, Arg7, Lys10, Asp11, Met12, and Ser14, showed higher changes in chemical shifts (≥10 Hz) as compared with the others, indicating their plausible involvement in interactions with the β2 cytoplasmic tail (Fig. 6B). Notably, all of these residues are located in the helical region of the αM tail (Fig. 6C). To map the interacting residues of the β2 tail, 13N-labeled β2 tail was titrated with either unlabeled αM (Fig. 7A) or phosphorylated αM (Fig. 7B) tail peptides. Residues H9, Leu6, Gln11, and Tyr12 of the cytoplasmic tail of β2 demarcated binding-induced chemical shift changes for the αM tail (Fig. 7C) and its phosphorylated variant (Fig. 7D).

### Interactions between β2 and αM Tails by FRET in DPC Micelles

It is likely that membrane anchoring of the cytoplasmic tails by N-terminal myristoylation increases binding affin-
ity between the tails as a result of structural stabilization. We utilized FRET using a dansyl group (as acceptor) and intrinsic Trp (as donor) to detect binding between the tails in the presence of DPC micelles. For these assays, the αM tail and the phosphorylated αM tail peptides were conjugated at their N termini with a dansyl group, whereas a β2 tail analog, termed Myr-β2H5W, was prepared with an N-terminal myristoylation and replacement of two amino acid residues (Table 1). The residues His5 and Trp24 of the native β2 were substituted with Trp and Ala, respectively, in the Myr-β2H5W analog to observe FRET unequivocally. Fig. 8 shows the intrinsic Trp fluorescence of Myr-β2H5W as a function of the concentrations of Dan-αM (A) and Dan-PαM (B) peptides in DPC micelles. As can be seen, additions of increasing concentrations of either Dan-αM or Dan-PαM peptides caused a marked quenching in the fluorescence emission intensity of the Trp residue of the Myr-β2H5W tail, demonstrating a plausible FRET between the Trp and dansyl group of the Dan-αM and Dan-PαM peptides, respectively. In other words, the dansylated αM tail peptides are in close proximity (≤30 Å) with the lipid-anchored β2 tail peptide. Note that to achieve an efficient FRET between the tail peptides the concentrations of DPC were maintained slightly above the critical micelle concentrations (see “Experimental Procedures”). A lower detergent concentration may enhance a close proximity between the donor and acceptor peptides (48).
FRET experiments were also carried out with Dan-αM or Dan-PαM tail peptides and a non-myristoylated analog of β2H5W in detergent-free aqueous solutions. The quenching of Trp fluorescence of the non-myristoylated analog of β2H5W peptide was found to be highly limited upon additions of either Dan-αM or Dan-PαM peptides, indicating a lack of FRET under such conditions (supplemental Fig. S8). This observation could be attributed to a rather low affinity binding between the tails in the absence of lipid tethering. Therefore, the quenching of Trp fluorescence emission intensity observed for the micelle-inserted Myr-αM tail might occur because of an increase in binding affinity between the tails. The changes in Trp fluorescence intensity were used to determine equilibrium dissociation constant ($K_d$) values of the interactions between

![Image](https://example.com/image1.png)

**FIGURE 7. Interactions between [15N-labeled β2 tail with αM and phosphorylated αM tails by [15N-1H HSQC NMR.** A, selected section of the [15N-1H HSQC spectrum of [15N-labeled β2 tail in free solution (in black contour) and in the presence of unlabeled αM tail (in gray contour) at a ratio of 1:8 (β2:αM). B, selected section of the [15N-1H HSQC spectrum of [15N-labeled β2 tail in free solution (in black contour) and in the presence of unlabeled phosphorylated αM tail (in gray contour) at a ratio of 1:8 (β2:phosphorylated αM). C and D, bar diagrams showing combined chemical shift changes of [15N and [1H resonances (in Hz) of the β2 tail as a function of amino acid residues in the presence of αM (C) and in the presence of phosphorylated αM (D). a.u., arbitrary units.

![Image](https://example.com/image2.png)

**FIGURE 8. Interactions between myristoylated β2 tail with dansylated αM tails by FRET in DPC micelles.** A and B, changes in the intensity of the emission spectra of Trp fluorescence of β2 tail upon the additions of dansylated αM (A) and dansylated phosphorylated αM (B) as a result of fluorescence resonance energy transfer from the donor Trp of β2 to the acceptor dansyl group of αM. C and D, determination of binding affinity between myristoylated β2 and αM (C) and myristoylated β2 and phosphorylated αM (D) tails following changes in emission intensity of Trp fluorescence of β2 with various dansylated peptide concentrations.
Myr-β2H5W and dansylated αM tail peptides (Fig. 8, C and D). The \( K_d \) values estimated for Dan-αM and Dan-αM peptides are 5.2 ± 0.6 and 4.4 ± 0.7 \( \mu \text{M} \), respectively. Furthermore, FRET studies between the native Myr-β2 containing Trp24 and dansylated αM demonstrated a lesser quenching of Trp (Trp24) fluorescence of the Myr-β2 upon additions of Dan-αM or Dan-αM peptides, indicating a farness between the Trp24 of Myr-β2 and the dansyl group of αM (supplemental Fig. S8). FRET experiments were also performed for Myr-β2H5W and dansylated αM tails in aqueous solution without DPC micelles. The Myr-β2H5W had a highly limited solubility in aqueous buffer and was soluble only after addition of organic solvents. There was no detectable FRET between Myr-β2H5W and Dan-αM or Dan-αM under such solution conditions, suggesting that myristoylation per se does not favor a tail-tail complex formation (supplemental Fig. S9).

Collectively, the FRET experiments delineated that the binding interactions between the cytoplasmic tails might be enhanced while they are inserted into membrane lipids. In addition, FRET studies established that the N-terminal regions of β2 and αM tails are likely to be in close proximity in the tail-tail heterocomplex.

Mode of Interactions of αM and β2 Tails—Molecular models of complexes between unphosphorylated and phosphorylated αM tails and the β2 tail were obtained by iterative docking of αM (A, blue ribbon) or phosphorylated αM (B, dark red ribbon) are likely to be engaged in multiple ionic and/or hydrogen bond interactions with the membrane-proximal helix of β2 (black ribbon). The probable mutual packing interactions among non-polar and aromatic residues of αM and β2 at their N termini are shown in a space-filling representation.

FIGURE 9. Proposed models of αM-β2 tail and phosphorylated αM-β2 tail complexes from RosettaDock. Plausible interfacial contacts between αM-β2 tails (A) and phosphorylated αM-β2 tails (B) of αMβ2 integrin are shown. The polar face of the amphipathic helical structures either of αM (A, blue ribbon) or phosphorylated αM (B, dark red ribbon) are likely to be engaged in multiple ionic and/or hydrogen bond interactions with the membrane-proximal helix of β2 (black ribbon). The probable mutual packing interactions among non-polar and aromatic residues of αM and β2 at their N termini are shown in a space-filling representation.

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Mode of Interactions of αM and β2 Tails—Molecular models of complexes between unphosphorylated and phosphorylated αM tails and the β2 tail were obtained by iterative docking of αM and β2 structures based on \(^{15}\text{N}-^{1}\text{H}\) chemical shift changes. These models were further energetically refined using the RosettaDock protocol (“Experimental Procedures”). The membrane-proximal N-terminal region of β2 or β3 cytoplasmic tails had been shown to adopt helical conformations in previous studies (33, 47, 49, 50). The αM-β2 tail complexes are characterized by intimate interactions between the helical structures, which are orientated in a parallel manner (Fig. 9). A parallel orientation of the two interacting helices is consistent with the FRET studies between Myr-β2H5W and dansylated αM tails that indicated proximity between the N termini of the tails. In the full-length native integrins, the two subunits of the heterodimer assume a parallel orientation (1). In the structures of the model complexes, the residues at the loop of the αM tails are distally placed from the interface and therefore do not
occlude the interactions with the β2 tail (Fig. 9, A and B). As can be seen, the tail-tail complexes may be sustained through a number of side chain-side chain interactions that are predominantly ionic and/or polar in nature (Fig. 9, A and B). In addition, hydrophobic packing interactions are plausible among the conserved N-terminal aromatic residues Pheα and Pheβ of the αM or phosphorylated αM tail and residues Leuβ and Ileβ of the β2 tail (Fig. 9, A and B). At the interfacial regions, the side chain of residue Asp8 of β2 and residues Argγ and Lysγ of αM or phosphorylated αM are found to be in close proximity, implying a potential salt bridge and/or hydrogen bonding interactions between the tails (Fig. 9, A and B). The carboxylate side chain of residue Gluβ of β2 is also in close contact with the cationic side chain of residue Lysα of αM (Fig. 9A) and phosphorylated αM (Fig. 9B). Furthermore, residues Argβ and Argγ of β2 appear to be engaged in a network of multiple ionic and hydrogen bonding interactions with residues Aspα and Gluβ of the αM tail in the β2-αM tail complex (Fig. 9A). For the phosphorylated αM and β2 tail complex, the phosphate moiety of Ser(P)γ is found to be in close proximity with the guanidinium group of residue Argβ of the β2 tail, demarcating ionic and/or hydrogen bond interactions (Fig. 9B). However, the side chains of Argα and Argγ of the β2 tail are only in close contact with the side chains of residues Aspα and Gluβ, respectively (Fig. 9B). It may be noteworthy that the introduction of a bulky phosphate group at the side chain of residue Serγ may render a rearrangement of potential ionic interactions between the phosphorylated αM and β2 tails.

**DISCUSSION**

In humans, there are 24 different integrins formed by specific combinations of 18 α subunits and eight β subunits (1–3). Many integrins share a common β subunit but different α subunits. Conceivably, signaling specificity in these integrins is governed by their α cytoplasmic tails. The membrane-proximal regions of the α tails are well conserved, but the membrane-distal regions significantly vary in lengths and sequences. The latter may permit for structural variations and can serve as distinct docking sites for cytoplasmic molecules. Notably, integrin α2β1 (VLA-2) and integrin α4β1 (VLA-4) exhibited altered functional behaviors when their α tails were replaced by tails from other integrins (51, 52). The α tails of integrin αLβ2 and αMβ2 might be responsible for distinct chemokine-induced activation kinetics and selective interactions of the Src kinase Hck with αMβ2 (26). Different α tails are also known to exhibit specific associations with cytoplasmic proteins, e.g. α5 with nischarin (53), α4 with paxillin (54), αlβ3 with calcinin integrin-binding protein (55), and αL with CD45 cytoplasmic domain (56). By contrast, the β tails with the exception of β4 have high sequence conservation with two NPXY(F/Y) motifs that are docking sites for talins, DOK, and kindlins (supplemental Fig. S10) (57–60).

To date, the two α tails with known structures are αlβ3 (40) and αL (33). The NMR structure of an N-terminal Myr-αlβ3 tail peptide embedded in DPC micelles was determined previously (40). The αlβ3 tail has a short N-terminal helical segment (Valγ990–Argγ997) followed by a loop in its C-terminal half that packs onto the surface of this helix. This conformation is stabilized by salt bridges formed by residues Lys994 and Arg997 of the N-terminal helix and the C-terminal acidic residues 1001EED-DEEGE1008 (40). Unlike the αlβ3 tail, NMR analyses have revealed that the αL tail in aqueous solution is characterized by a compact structure maintained by mutual interactions among three helical segments (33). Notably, the folded structure of the αL tail contains a large negatively charged surface that can bind divalent metal ions, e.g. calcium, that is similar to the metal ion binding capacity of the negatively charged loop of the αlβ3 tail (33). In this study, we determined NMR structures of the Myr-αM tail in DPC micelles. The Myr-αM tail acquires an amphipathic helical structure for the N-terminal residues Leu1114–Glu1127 (Leu2–Glu15 in the αM tail peptide used herein) and a loop for residues Gly1128–Gln1136 (Gly16–Gln24 in the αM tail peptide used herein) at the C-terminal region (Fig. 5). The C-terminal loop folds back onto the helical structure via non-polar packing. The overall structure of the αM tail is similar to that of αlβ3 despite a lack of sequence homology except for their N-terminal membrane-proximal regions (supplemental Fig. S10). A difference between the two structures is that the C-terminal loop fold-back of the αM tail is mediated by hydrophobic packing, whereas for the αlβ3 tail, helix-loop stabilizations are mediated by electrostatic interactions. In addition, the N-terminal helix of the αM tail is longer (residues Leu1114–Glu1127) than the αlβ3 tail (residues Valγ990–Proγ998).

The Serγ1126 (Serγ14 in the αM tail peptide herein) of the αM tail has been reported to be constitutively phosphorylated, and it is required for inside-out activation of αMβ2 (31). Thus, we also determined the structure of the Myr-αM tail with Ser(P)γ1126 in DPC micelles (Fig. 5). Phosphorylation of Serγ1126 did not induce large conformational changes in the αM tail, but there was lesser packing of the loop region with the N-terminal helix (Fig. 5). Phosphorylation is often found to be a “conformational switch” in proteins and peptides involved in signaling and other cellular functions (61–63). In particular, structural disruptions (either global or local) have been reported for signaling proteins upon phosphorylation of Ser or Thr (64–66). We did not observe significant perturbations in the αM tail with phosphorylated Serγ1126 as compared with the unmodified αM tail, and there was no detectable difference in their binding affinities to the β2 tail. Thus, Serγ1126 phosphorylation is unlikely to modulate directly the conformation of the αM tail or the packing of the αM and β2 tails. However, it may present a docking site for cytoplasmic protein that can perturb either the structure of the αM tail or the interaction of the αM, β2 tails, or both that regulates the activation of αMβ2.

The interaction between the α and β tails of the integrin and the packing of the TMs are required to maintain integrin in a resting state (47, 67–70). Surface plasmon resonance analyses showed that the αlβ3 and β3 tails form a complex with a $K_d$ of 7.7 or 50 μM in the presence or absence of calcium, respectively (71, 72). In the presence of calcium, the NMR-derived structure of the αlβ3 and β3 tails in association revealed ionic/polar interactions involved in the packing of their N-terminal helices (47). However, the interactions between αlβ3 and β3 tails were not detected by others (49, 73). What accounts for this disparity in observations is unclear. In our previous study, a $K_d$ of ~2.6 μM was determined for the complex formation of αL and β2 tails in
the presence of calcium, and a larger surface of the αL trihelical fold contributes to its interactions with the β2 tail (33). Collectively, these studies suggest that the interactions between integrin α and β tails may be dependent on the structure of the α tail, which can be significantly different among the α tails because of length, sequence divergence, and modulation by metal ions.

In this study, non-Myr-αM tail with or without Ser1126 phospho-
phorylation in aqueous buffer solution interacted much more weakly (K_d values greater than mM) with the β2 tail in comparison with the αLβ2 and αIββ3 systems. 15N and 1H chemical shift changes in HSQC spectra could only be seen at higher concentrations of unlabeled binding partners (Figs. 6 and 7). Regardless, these data provided information on the residues of the αM and β2 tails that are perturbed by complex formation. We also examined whether the αM tail can interact with divalent metal ions. However, there was no detectable binding to calcium (data not shown). Interestingly, FRET studies demonstrated that the binding affinity between cytoplasmic tails could be increased significantly when one of the cytoplasmic tails was inserted into lipid micelles. This suggests that the interactions between the α and β tails of an integrin can be stabilized when they are anchored into lipid membranes through the TMs. The energy-minimized molecular models of the αM-β2 and PaM-β2 were obtained by RosettaDock in the absence of lipid micelles. The exact orientations of the tail-tail complexes within DPC micelles are not clear at this moment. However, the docked structures provide molecular insights into integrin activation and regulation of cytosolic tails.

Based on the docked structures of the αM and β2 tails (Fig. 9), ionic and/or van der Waals interactions of the tails are possible. A comparison with the interfacial contacts of the αIββ3 (supplemental Fig. S11) and αLβ2 (supplemental Fig. S11) with the αMβ2 tails demarcated a conserved salt bridge interaction at the membrane-proximal region. However, the docked structure of the αMβ2 tails (and αLβ2 tails) showed more interactions distal to the membrane-proximal region as compared with that of αIββ3 (supplemental Fig. S11). It is likely that the interactions between the TMs of α and β subunits of integrins in lipid membranes may potentially impart stabilizing interactions between the cytosolic tails. An NMR study using peptides of αIββ3 TMs with membrane-proximal tail sequences revealed interactions between the tails (70). We conjecture that in an intact αMβ2 the packing of its TMs and these interactions of its tails together constrain the receptor in a resting state. To better understand the detailed mechanism of αMβ2 inside-out activation, future NMR studies using αM and β2 TM-tail peptides in lipid micelles can be performed. In addition, it will be interesting to identify a cytoplasmic protein(s) that preferentially binds the Ser1126 phosphorylated αM tail.

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Structure and Interactions of αMβ2 Cytosolic Tails

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