A Transmembrane Polar Interaction Is Involved in the Functional Regulation of Integrin αLβ2

Ardcharaporn Vararattanavech†, Choon-Peng Chng‡†, Krupakar Parthasarathy†, Xiao-Yan Tang†, Jaume Torres∗ and Suet-Mien Tan∗

1School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore
2Biophysics Team, Large-Scale Complex Systems A*STAR Institute of High Performance Computing, 1 Fusionopolis Way, No. 16-16 Connexis, Singapore 138632, Singapore

Received 23 January 2010; received in revised form 11 March 2010; accepted 15 March 2010
Available online 23 March 2010

Integrins are heterodimeric transmembrane (TM) receptors formed by noncovalent associations of α and β subunits. Each subunit contains a single α-helical TM domain. Inside-out activation of an integrin involves the separation of its cytoplasmic tails, leading to disruption of αβ TM packing. The leukocyte integrin αLβ2 is required for leukocyte adhesion, migration, proliferation, cytotoxic function, and antigen presentation. In this study, we show by mutagenesis experiments that the packing of αLβ2 TMs is consistent with that of the integrin αIIbβ3 TMs. However, molecular dynamics simulations of αLβ2 TMs in lipids predicted a polar interaction involving the side chains of αL Ser1071 and β2 Thr686 in the outer-membrane association clasp (OMC). This is supported by carbonyl vibrational shifts observed in isotope-labeled αLβ2 TM peptides that were incorporated into lipid bilayers. Molecular dynamics studies simulating the separation of αLβ2 tails showed the presence of polar interaction during the initial perturbation of the inner-membrane association clasp. When the TMs underwent further separation, the polar interaction was disrupted. OMC polar interaction is important in regulating the functions of β2 integrins because mutations that disrupt the OMC polar interaction generated constitutively activated αLβ2, αMβ2, and αXβ2 in 293T transfectants. We also show that the expression of mutant β2 Thr686Gly in β2-deficient T cells rescued cell adhesion to intercellular adhesion molecule 1, but the cells showed overt elongated morphologies in response to chemokine stromal-cell-derived factor 1α treatment as compared to wild-type β2-expressing cells. These two TM polar residues are totally conserved in other members of the β2 integrins in humans and across different species. Our results provide an example of the stabilizing effect of polar interactions within the low dielectric environment of the membrane interior and demonstrate its importance in the regulation of αLβ2 function.

© 2010 Elsevier Ltd. All rights reserved.

Keywords: integrins; transmembrane domain; cell adhesion; protein conformation; affinity regulation

Introduction

Integrins are a large family of heterodimeric transmembrane (TM) cell adhesion molecules.1,2 Apart from promoting cell–cell or cell–extracellular matrix attachment required for cell adhesion and migration, integrins play important roles in determining cell fate.3 Each integrin is formed by

*Corresponding authors. E-mail addresses: JTorres@ntu.edu.sg; smtan@ntu.edu.sg.
† A.V. and C.-P.C. contributed equally to this study.
Abbreviations used: TM, transmembrane; OMC, outer-membrane association clasp; JM, juxtamembrane; IMC, inner-membrane association clasp; MD, molecular dynamics; ICAM-1, intercellular adhesion molecule 1; mAb, monoclonal antibody; EI, expression index; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N′-tetraacetic acid; PDB, Protein Data Bank; H/D, hydrogen/deuterium; SDF-1α, stromal-cell-derived factor 1α; BSA, bovine serum albumin; EM, energy minimization; ACN, acetonitrile; TFA, trifluoroacetic acid; ATR, attenuated total reflection.
noncovalent association of an α subunit and a β subunit. The large extracellular domain of each subunit is linked to a short cytoplasmic tail by a single α-helical TM domain, which serves as a transducer of activation signals. The TMs of a nonactivated integrin interact with one another. The structure of the integrin αIIbβ3 TM complex, which includes cytoplasmic juxtamembrane (JM) sequences, has been solved by NMR studies using the peptides αIIb (Ala958-Pro998) and β3 (Pro685-Phe727) in phospholipids. This structure is consistent with computational predictions coupled with experimental restraints. In both studies, Phe992 and Phe993 located in the αIIb JM sequence GFFKR

![Sequence alignments of integrin TMs](image-url)

**Fig. 1.** Sequence alignments of integrin TMs. (a) Alignment of selected human integrin TM sequences. The GxxxG-like motifs in α and β TMs are shaded. β2 Thr686, which has been previously described to be important in the packing of αLβ2 TMs, is boxed. The integrin αIIbβ3 TM–JM sequences are shown. The two C-terminal TM–cytoplasmic tail boundaries based on recent αIIbβ3 NMR studies of Lau et al. (blue broken line) and Yang et al. (red broken line) are shown. Salt-bridge-forming residues are in orange boxes. (b) Alignment of β2 integrin TM sequences across different species. The residues that could form the H-bond are conserved and shaded. The residue numbers of the human integrins are shown.
insert back into the intracellular membrane leaflet (with the blue broken line in Fig. 1a showing the TM–JM boundary), allowing favorable positioning and interaction of the two salt-bridge-forming residues αLβb1 Arg995 and β3 Asp723. The salt bridge would otherwise not form because of the ~25° crossing at the N-terminal halves of the TMs. However, in another recent NMR study in CD3CN/H2O that used αLβb3 TM and full-length cytoplasmic tail sequences, the helix-to-reverse turn transition of the GFFKR motif in αLγc is reactive towards mAb KIM127, even though the residue is located at the TM interface. 293T cells were transfected with full-length αL mutants and wild-type β2 or, alternatively, wild-type αL and β2 mutants. We then assessed the adhesion properties of these cells to immobilized intercellular adhesion molecule 1 (ICAM-1), which is a αLβ2 ligand (Fig. 2b and c). Activation of αLβ2 (i.e., constitutive adhesion of transfectants to ICAM-1) was detected in mutants αLS1071Fβ2, αL1G1075Fβ2, αLβ2T686F, αLβ2I690F, αLβ2G694F, and αLβ2L697F, albeit with a lower level of binding when compared to cells treated with the β2-integrin-activating monoclonal antibody (mAb) KIM185.13 The addition of the function-blocking mAb IB419 diminished cell adhesion in all cases; therefore, the adhesion observed was specific for αLβ2. The expression levels of the integrins [shown as the expression index (EI)] were comparable in all cases, as determined by flow cytometry.

The above scanning profiles are similar to the Leu-scanning profiles observed previously in αLβb3 TMs. Indeed, the OMC residues of αLβb3 are Gly972 and Gly976 (OMC residues) in the αLβb GxxG motif are important for the packing of αLβb3 TMs. Variations in these OMC residues may affect integrin affinity states. Here, we investigated the TM packing of the integrin αLβ2 (also known as leukocyte-function-associated antigen-1). Integrin αLβ2 is expressed only in leukocytes, and it is required for leukocyte adhesion, migration, cytokotic function, proliferation, and antigen presentation. A notable difference between the TMs of the β2 partner αL, αM, αX, or αD and the TM of the β3 partner αLβb is the presence of a Ser (Ser1071 in αL), instead of a Gly, as the first OMC residue (Fig. 1a). There are a few polar residues in the TMs of TM proteins, and most of these polar residues are Ser and Thr, which may therefore be relevant to the modulation of TM interactions via formation of hydrogen bonds. Indeed, we have previously reported that Thr686 in the β2 GxxG motif has an important role in the packing of αLβ2 TMs, but the way in which this residue contributes to the packing of αLβ2 TMs was not defined. In this study, we hypothesize that the proximity of αL Ser1071 and β2 Thr686 in a lipid environment of low dielectric constant would favor polar interaction of the side chains, and that this interaction is biologically relevant. To test this hypothesis, we performed mutagenesis, molecular dynamics (MD) simulations, and biophysical analyses. We show that H-bond formation between αL Ser1071 and β2 Thr686 is favorable and likely to occur in cells. Furthermore, the OMC H-bond may be important in other members of human β2 integrins and the β2 integrins of other species because of sequence conservation (Fig. 1b).

Results

Examining the interface of αLβ2 TMs

We established the nature of the interface of αLβ2 TMs by using a Phe scan between Ser1071 and Ile1082 in αL, and between Gly685 and Leu697 in β2 (Fig. 2a, box). Phe has a large and apolar side chain and should disrupt TM interactions if the residue is located at the TM interface. 293T cells were transfected with full-length αL mutants and wild-type β2 or, alternatively, wild-type αL and β2 mutants. We then assessed the adhesion properties of these cells to immobilized intercellular adhesion molecule 1 (ICAM-1), which is a αLβ2 ligand (Fig. 2b and c). Activation of αLβ2 (i.e., constitutive adhesion of transfectants to ICAM-1) was detected in mutants αLS1071Fβ2, αL1G1075Fβ2, αLβ2T686F, αLβ2I690F, αLβ2G694F, and αLβ2L697F, albeit with a lower level of binding when compared to cells treated with the β2-integrin-activating monoclonal antibody (mAb) KIM185. Activation of the function-blocking mAb IB419 diminished cell adhesion in all cases; therefore, the adhesion observed was specific for αLβ2. The expression levels of the integrins [shown as the expression index (EI)] were comparable in all cases, as determined by flow cytometry.
MD simulations of wild-type αLβ2 TM packing

MD simulations were performed to examine in more detail the packing interface of αLβ2 TMs. We made use of the αIIbβ3 TM-JM NMR structure [Protein Data Bank (PDB) ID 2K9J] reported by Lau et al. as template for the generation of αLβ2 TM–JM (αL Leu1065-Lys1097 and β2 Ile679-Ser723).\textsuperscript{10} αLβ2 TM–JM was inserted into the lipid bilayer and equilibrated, and a simulation run was performed.

**Fig. 2** (legend on next page)
for 30 ns as described in Materials and Methods (Fig. 3a). Interestingly, we observed persistent side-chain H-bond formation between αL Ser1071 and β2 Thr686 in the αLβ2 OMC in all three simulations (Fig. 3b). In these simulations, the packing of αLβ2 TM–JM was stable because the interaction of αL Phe1091-Phe1092 and β2 Tyr701, as illustrated in Fig. 3a, was intact (data not shown). The tilt angle of the αLβ2 TM helices was maintained at ~21° (Fig. 3c), which is close to the ~25° crossing angle of the αlββ3 TM helices.10 These data suggest the presence of a polar interaction in the OMC of αLβ2 TMs.

In another NMR study of αlββ3 TMs with full-length cytoplasmic tail in CD3CN/H2O, the authors suggest that the JM Lys marks the C-terminal boundaries of the αlβ3 TMs (PDB ID 2KNC)11 (Fig. 1a, red broken line). A significant difference between this NMR study and that by Lau et al.11 is the absence of αlβ Phe992 and Phe993 foldback in the IMC, which could be attributed to the different environments used: CD3CN/H2O instead versus lipid bicelles.10 We therefore generated another αLβ2 TM–JM model based on the αlββ3 structure from Yang et al. and performed simulations with the same parameters as described above. We observed distortions in the αLβ2 TM helices located at the top and bottom of the lipid bilayer (Fig. 4a). This accounts for the lack of H-bond formation between αL Ser1071 and β2 Thr686 in the simulations (Fig. 4b). A larger variance in the tilt angle was also observed (Fig. 4c). Collectively, our data suggest that there is a higher propensity of H-bond formation between αL Ser1071 and β2 Thr686 in the first TM packing model (Fig. 3a) as compared to the second model (Fig. 4a).

Infrared spectroscopy analysis of the interaction between αL Ser1071 and β2 Thr686

To verify the predicted polar interaction between αL Ser1071 and β2 Thr686, we performed infrared spectroscopy of peptides αl Lys1062-Gly1090 and β2 Gly676-Leu704. In the β2 peptide, we replaced Ile682 with 13C=18O-labeled Leu to specifically monitor the carbonyl redshift due to H-bond formation. Both αL and β2 peptides were predominantly α-helical when incorporated into lipid bilayers, with an amide I band centered at 1658 cm$^{-1}$ and an amide II band centered at 1546 cm$^{-1}$ (Fig. 5). The linear dichroism of amide I was more than 3 in each experiment, demonstrating a correct incorporation of the peptides into the lipid bilayer, with the long axis of the helix almost perpendicular to the membrane plane. Hydrogen/deuterium (H/D) exchange (see Materials and Methods) was consistent with ~23 residues embedded in the lipid bilayer.

According to harmonic oscillator approximation and numerous experimental evidence in several TM domains embedded in lipids,25–31 absorption for peptide 13C=18O carbonyl in Leu682 is expected to be shifted by ~60 cm$^{-1}$ (redshift) relative to that of 12C=16O (i.e., it should appear at 1590–1600 cm$^{-1}$). Indeed, we have observed previously in β2 TM labeled at Val704 that the band corresponding to this label was centered at 1597 cm$^{-1}$. However, when the β2 peptide labeled at Leu682 was examined, this C=O stretching band appeared at 1586 cm$^{-1}$ (Fig. 5, red trace). The likely explanation for this shift is the presence of an intramolecular H-bond. We have reported a similar effect for the TM domain of the small hydrophobic protein from the human respiratory syncytiotyal virus, which was attributed to the formation of a hydrogen bond between a labeled carbonyl and a Ser residue, four residues away in the same TM.31 In fact, the tendency of serine and threonine residues to form intrahelix hydrogen bonds to carbonyl oxygen at position i–4 has also been reported in water-soluble domains.32 Within the β2 peptide, the candidate residue for interaction with the labeled carbonyl of Leu682 is the side chain of Thr686 that is one turn below Leu682 (Fig. 2a; see β2 Ile682).

When the αL and β2 peptides were reconstituted in the same preparation, the band shifted further to 1583 cm$^{-1}$ (Fig. 5). This 3-cm$^{-1}$ shift was observed in three independent preparations and strongly suggests that the two peptides form heterodimers in these conditions. The additional shift observed is likely to result from a disruption of the β2 H-bond interaction by the αL peptide, where αL Ser1071 would compete with β2 Thr686 for interaction with...
the labeled carbonyl of Leu682. This is consistent with the observed further weakening (redshift) of C=O vibration in Leu682. Furthermore, upon D2O exposure, the band corresponding to the labeled carbonyl did not shift in any of the samples (data not shown), demonstrating that this labeled residue is embedded in the lipid bilayer. These results suggest that polar H-bond formation between αL Ser1071 and β2 Thr686 in a lipid environment is possible. It should be noted that the present infrared spectroscopy analyses used αL and β2 peptide sequences that do not contain the JM sequences. The design of the peptides was based on our previous study showing that the TM-only sequence is sufficient to mediate specific interactions of the integrin TMs.33 Because the OMC and IMC residues serve to maintain complex formation between the integrin TMs, future studies using longer TM peptides that include the JM residues will be needed to assess the possible contribution of JM residues to stabilizing...
αL Ser1071 and β2 Thr686 H-bond interaction in the OMC.

**MD simulations of cytoplasmic tail separation on the αLβ2 TM–JM model**

We also performed steered MD simulations to induce the separation of the αLβ2 cytoplasmic tails of the TM–JM model and examined its effect on OMC polar interaction (Fig. 6). β2 JM was pulled laterally away from αL JM in lipid bilayer to simulate inside-out activation of αLβ2. Pulling was performed on β2 because the integrin β cytoplasmic tail serves as a docking site for the integrin activator talin, which has been reported to induce the separation of cytoplasmic tails.6,34,35 The separating distances of IMC and OMC were monitored during the simulations at three different speeds (Fig. 6a). The simulations were terminated soon after the fraction of residue–residue contacts across the TM interface decayed to zero, signaling the complete separation of the TM helices. An initial lag phase of OMC separation that could be attributed to the need to overcome packing interactions among IMC residues was observed. Despite the presence of the cytoplasmic salt bridge, it did not afford any significant resistance during pulling simulations. The separation of the OMC follows that of the IMC. Overall, the αL Ser1071–β2 Thr686 H-bond was maintained during the initial phase of pulling at different speeds. This was ultimately lost before the complete separation of the TMs (snapshots of the H-
bands are indicated.

The region corresponding to the absorption of the labeled terminal segment of β2 TM. Amide I and amide II regions in the infrared spectrum for labeled integrin TMs incorporated into hydrated lipid bilayer: β2 TM 13C=18O-labeled Leu682 (red), and a mixture (green) of β2-TM 13C=18O-labeled Leu682 and unlabeled αL TM. The region corresponding to the absorption of the labeled carbonyl 13C=18O of Leu682 is indicated by a box and an enlarged view (inset). Frequencies (in cm⁻¹) of the main bands are indicated.

Fig. 5. Infrared detection of intramolecular and intermolecular H-bonds involving main-chain carbonyl at position 682 of β2 TM. Amide I and amide II regions in the infrared spectrum for labeled integrin TMs incorporated into hydrated lipid bilayer: β2 TM Leu704 (blue), β2 TM 13C=18O-labeled Leu682 (red), and a mixture (green) of β2-TM 13C=18O-labeled Leu682 and unlabeled αL TM. The region corresponding to the absorption of the labeled carbonyl 13C=18O of Leu682 is indicated by a box and an enlarged view (inset). Frequencies (in cm⁻¹) of the main bands are indicated.

Discussion

The packing of integrin αLβ2β3 TMs has been the subject of many studies; more recently, its specific interface was resolved by NMR studies and disulfide restraint/Rosetta analyses. Notable features of αLβ2β3 TM packing are as follows: (i) a vertical αLβ2 TM against a slanting β3 TM that has an ~25° crossing angle at the N-terminal halves of the TMs; (ii) reinsertion of the JM residues αLβ2 β992 and Phe993 into the inner leaflet of the lipid bilayer (although not evident in the report by Yang et al.); (iii) definitions of IMC and OMC; and (iv) packing of the OMC deviating from the packing of the GpA dimer.

It has been hypothesized that the association affinities of integrin α/β TMs are different among integrins because of sequence variations, notably in the OMC. In this study, we have examined the packing of leukocyte integrin αLβ2 TMs. Having established that the packing of αLβ2 TMs is similar overall to that of αLβ2β3 TMs, we went on to show that αLβ2 OMC, unlike αLβ2β3 OMC, is involved in polar interaction. We have previously shown that β2 Thr686 has an important role in the packing of αLβ2 TMs, but the detailed function of this residue was unclear. Our present data addressed this question by showing H-bond formation between side chains of αL Ser1071 and β2 Thr686. This interaction is pivotal to the stability of αLβ2 OMC because site-
directed mutations of αL Ser1071 that disrupt H-bond formation induced αLβ2 activation. Thus, the H-bond between αL Ser1071 and β2 Thr686 provides a polar interaction that packs αLβ2 OMC, as observed in other TM interactions.39–42

The biological importance of this polar interaction in αLβ2 OMC is also supported by the following observations. First, the residue that corresponds to αL Ser1071 in the OMCs of αM, αX, and αD is also Ser, providing support for the generality of the functional importance of the described polar interaction in all β2 integrins. Second, the OMC Ser and Thr that participate in H-bond formation are conserved in the reported β2 integrins across many species, suggesting evolutionary conservation of this interaction. Third, mutation disrupting this polar interaction in transfected SK-β2.7 T cells impeded rear retraction of these cells migrating on

---

**Fig. 6.** Pulling simulations using the αLβ2 TM-JM model based on the αIIbβ3 template (PDB ID 2K9J).10 (a) A lateral pulling force is applied parallel with the plane of the membrane (described in Materials and Methods). Separation between the center-of-mass of Cα atoms of the αL and β2 halves of the OMC and IMC are plotted against simulation time. Residue–residue contacts across the TM interface were computed, and fractional contacts were monitored as an indication of TM separation, shown as filled circles connected by broken lines. Simulation trajectories were resampled at a 100-ps interval for these plots. (b) Snapshots of αLβ2 TM–JM interactions at different time points from the simulation with a pulling speed of 2.5 × 10−4 nm/ps. The H-bond between αL Ser1071 and β2 Thr686, and the electrostatic interaction between αL Arg1094 and β2 Asp709 are circled.
immobilized ICAM-1 in the presence of SDF-1α, suggesting that repacking of these TMs via this polar interaction has a role in αLβ2 deactivation. The repacking of αLβ2 TMs may be more important in the highly migratory leukocytes than, for example, the repacking of αIIbβ3 TMs in platelets because detachment is essential for effective leukocyte locomotion, but less necessary for the function of platelets in blood coagulation.

Our MD simulations data predict that during the initial phase of TM–TM separation, induced by lateral force pulling on the β2 JM cytoplasmic region to mimic inside-out integrin activation, H-bond is maintained. However, the H-bond did not provide significant resistance to the forced separation of the TMs. This is an expected observation for TMs envisioned as signal transducers that allow propagation of activation signals from the cytoplasmic tails to the ectodomains. In this case, the association affinity of the TMs should be sufficiently strong to maintain the packing of the TMs, but also weak enough for their separation in the presence of an unclaspung signal acting on integrin cytoplasmic tails.

In the MD simulations, we also observed that αLβ2 TM helices in lipid bilayers underwent distortions in the αLβ2 TM–JM model generated based on the αIIbβ3 TM–cytoplasmic tail structure of Yang et al., but not in the αLβ2 TM–JM model generated using the αIIbβ3 structural template of Lau et al.10 The distortions of the αLβ2 TMs had a destabilizing effect on the OMC, thus explaining the lack of H-bond between αL Ser1071 and β2 Thr686 in these simulations. Yang et al. have suggested that the two conformations of αIIbβ3 TMs that have different IMC–inner-membrane interactions represent different stages of integrin regulation—one involving a cytoplasmic inhibitor, and the other involving an activator.11 It is an innovative hypothesis, but the αIIbβ3 TMs obtained in CD3CN/H2O11 (a nonphysiological solvent) as opposed to lipid bicelles should be interpreted with caution with regard to its relevance to the regulation of integrin function. Nevertheless, it is important to determine the structures of more integrin TMs with full-length cytoplasmic tails in lipids. In the absence of TM segments and bicelles, the αL cytoplasmic tail has been shown to adopt a trihelical fold, whereas the β2 cytoplasmic tail is similar to that of β3.43 It will therefore be interesting to determine any possible effect of αL Phe1091-Phe1092 reinsertion into lipids on the structure of the αL cytoplasmic tail and the packing of αLβ2 TMs in future NMR studies of the αLβ2 TM–JM complex in lipid bicelles.

Materials and Methods

Antibodies and reagents

The hybridomas of mAb KIM185 (specific for integrin β2 and activating),18 mAb KIM127 (specific for integrin β2; reporter mAb for an extended β2 integrin),21,22 and IB4 (specific for the heterodimeric form of β2 integrins)19,44,45
were obtained from the American Type Culture Collection. mAbs were purified from the spent low-IgG fetal-bovine-serum-containing medium of hybridomas using a Protein G Sepharose Fast Flow column (GE Healthcare Life Sciences) in accordance with the manufacturer’s instructions. Recombinant human ICAM-1/Fc was prepared as described previously. All general chemicals were obtained from Sigma-Aldrich, unless stated otherwise.

cDNA expression plasmids

The amino acid numbering of the integrins is based on the mature protein. The full-length integrin αL, αM, αX, and β2 cDNAs in expression plasmid pcDNA3 were used. Site-directed mutations of integrin TMs in the expression plasmids were performed using the QuikChange Site-Directed Mutation kit (Stratagene) with a relevant pair of primers. The plasmids were verified by sequencing (First Base, Singapore). The integrin β2 with a C-terminal mYFP in pcDNA3 has been reported previously. This plasmid was used to generate β2 T686G-mYFP, and mYFP served as a reporter in transfect SK-β2.7 cells.

Cell culture and transfections

Human embryonic kidney 293T and myeloid K562 cells were obtained from the American Type Culture Collection. 293T cells were cultured in complete Dulbecco’s modified Eagle’s medium, and K562 cells were cultured in complete RPMI 1640 medium. All media contained 10% (vol/vol) heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Hyclone). The Polyfect transfection reagent (Qiagen) was used to transfect 293T cells with the integrin expression plasmids (1.5 μg each) following the manufacturer’s instructions. The integrin β2-deficient T-cell line SK-β2.7 was kindly provided by Dr. N. Hogg (Leukocyte Adhesion Laboratory, London Research Institute, UK) and maintained in complete RPMI medium. SK-β2.7 cells (3 × 10⁶ for each reaction) were transfected with either wild-type β2 or β2 mutant plasmids (12 μg each) by electroporation (pulse voltage, 1350 V; pulse number, 2; pulse width, 20 ms) using a pipette-type microporator MP-100 (NanoEn Tek) in accordance with the manufacturer’s instructions.

Flow cytometry

Flow cytometry for integrin expression on transfectants was performed as described previously. Briefly, cells were stained with primary antibody mAb IB4 (20 μg/ml), followed by secondary fluorescein-isothiocyanate-conjugated sheep anti-mouse IgG (Sigma) (1:400 dilution). Stained cells were analyzed on a FACS Calibur flow cytometer with installed CellQuest software (Becton Dickinson). The expression level of integrin was calculated as EI based on percent cells gated positive × geo-mean fluorescence intensity of gated cells.

Cell adhesion assays

Adhesion assays of 293T transfectants to immobilized ICAM-1 or bovine serum albumin (BSA) in microwells were performed as described previously. Cells labeled with 3 mM fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5-(and -6) carboxyfluorescein acetoxymethyl ester (Molecular Probes) were plated onto the immobilized
ligand and incubated for 30 min at 37 °C in a humidified 5% CO₂ incubator. Wells were washed in medium to remove unbound cells. Bound cells were quantified based on fluorescence emitted using a fluorescent plate reader (FL 6000 Bio-Tek Instruments). The mAb KIM127 was performed as described previously.13,17 Cell surface proteins of transfectants were labeled with biotin using the chemical sulfo-N-hydroxysuccinimido-biotin (Pierce). Labeled cells were incubated in complete culture medium containing relevant mAb (2 μg) for 30 min at 37 °C. Excess and unbound mAbs were removed by washing cells at least twice in culture medium. Cells were then lysed, and whole-cell lysate was immunoprecipitated with rabbit anti-mouse IgG (Sigma)-conjugated Protein A Sepharose beads (Amersham Bioscience). Bound proteins were resolved on 7.5% SDS-PAGE under reducing conditions. Biotinylated proteins were immunoprecipitated with rabbit anti-mouse IgG (Sigma)–conjugated Protein A Sepharose beads (Amersham Bioscience). Bound proteins were resolved on 7.5% SDS-PAGE under reducing conditions. Biotinylated proteins were probed with horseradish-peroxidase-conjugated streptavidin, followed by ECL detection (Amersham Bioscience).

MD simulations

The αLβ2 TM– JM models were generated based on the recently solved structures of αLIβ3 TMs.10,11 Afforded by the sequence similarity between αLβ2 and αLIβ3 TM–JM regions, residue replacement procedure was performed with PyMOL,2 using the αLIβ3 backbone as template. Side-chain χ₁ and χ₂ angles of β2 Trp701 were adjusted with PyMOL to avoid steric clash with the αL JM Phe1091-Phe1092 side chains. GROMACS MD package49 version 4.0.6 with the modified GROMOS-87 united-atom force field was used to carry out energy minimization (EM) procedures and subsequent MD simulations. In vacuo EM of the αLβ2 TM–JM models was carried out using the steepest descent algorithm to reach a tolerance of 100 kJ/mol/nm, with harmonic positional restraints on protein heavy atoms. Shift functions were used for Coulomb and van der Waals interactions such that forces decay smoothly to zero between 1.0 nm and 1.2 nm. A relative dielectric constant of 6 was used to model the effect of solvent in its absence. The models were placed into a slab of preequilibrated dipalmitoylphosphatidylcholine bilayer of 128 molecules53 that was obtained from the Tieleman group Web site52 following their bilayer inflation/compression procedure.52 In the 2KNC-based model, αL JM Phe1091-Phe1092 side chains were positioned outside the inner membrane. Simple Point Charge52 water molecules were then added to generate a (periodic) simulation box of dimensions 6.7 nm × 6.7 nm × 9.5 nm. The van der Waals radii of selected lipid tail atoms were increased during solvation procedure to avoid adding water molecules to the interior of the membrane. EMs were then performed on αLβ2 TM–JM models in lipid bilayer. The Berger united-atom force-field parameters54 and lipid bilayer topology file were obtained from the Tieleman group. EM was carried out using steepest descent algorithm to reach a tolerance of 1000 kJ/mol/nm, with harmonic positional restraints on the protein heavy atoms. The smooth Particle Mesh Ewald method55 was used to handle long-range electrostatics, with a real-space cutoff of 1.0 nm. Lennard–Jones and electrostatic interactions were evaluated within a cutoff of 1.4 nm, with the neighbor list updated every 10 steps. Equilibration of the system under positional restraints to 310 K was performed using the Velocity–Rescaling thermostat with a coupling constant (τ β) of 0.1 ps.56 System pressure was equilibrated to 1 atm using the Berendsen barostat,57 with a coupling constant τ P of 1.0 ps and a compressibility of 4.4 × 10⁻⁹ bar⁻¹. Semi-isotropic coupling was used in the barostat whereby box dimension changes in x/y directions are coupled, but that in the z direction is independent. After equilibration, positional restraints on protein were released, and the Nose–Hoover thermostat58,59 (τ β = 0.5 ps) and Parrinello–Rahman barostat60 (τ P = 2.0 ps) were employed for the production runs. All bond lengths in protein and lipid molecules were constrained using the LINCS algorithm,61 allowing the use of a 2-fs time step. Water molecule bond lengths were constrained with the SETTLE algorithm.62 Atomic coordinates were saved every 2 ps for subsequent analyses. To investigate the effect of a pulling force that separates β2 from αL, we used the pull code of GROMACS 4.0.5. The 2.5-ns snapshot from run 3 of the αLβ2 TM–JM model (based on the 2K9J template) was used as starting model. The C-terminal section of β2 just within the inner membrane (Ile700-Ala703) was pulled away from the corresponding region on αL (Ile1084-Tyr1087) in a direction along the plane of the membrane. The center of mass of the β2 pull group was restrained to the equilibrium position of a harmonic potential of force constant (500 kJ/mol/Å²) that was moved at a prescribed speed. Three speeds were used: 1 × 10⁻³ nm/ps, 5 × 10⁻⁴ nm/ps, and 2.5 × 10⁻⁴ nm/ps. To prevent the motion of αL, we harmonically restrained the TM Cα atoms with a force constant of 1000 kJ/mol/Å². Temperature and pressure were maintained by the Velocity–Rescaling thermostat and the Berendsen barostat, respectively. All pulling simulations used a time step of 1 fs. GROMACS tools were used for trajectory analysis. VMD55 was used for trajectory viewing and for the alignment of αLβ2 TMs using a script adapted from Orient.tcl (for aligning molecules along their principal axes) from the VMD script library. Residue contacts were computed using a script in Multiscale Modeling Tools for Structural Biology.65

Peptide synthesis and purification

Peptides corresponding to the TM sequences of β2 G8⁻676 PNAALVGVTVQIVLIGYLYLYVLSGILLLVLWKL374 (labeled Leu in boldface) and αL LK1062-QMLYLYVI5GGICGLLL- LLIIVLYVKV1490 were synthesized using 9-fluorenylmethoxycarbonyl solid-phase chemistry (Liberty Microwave Peptide Synthesizer) and Tentagel amide resin (Intavis AG). The β2 TM peptide was isotopically labeled at residue position 682 with [13C]e⁻²⁻⁻¹⁸O-labeled Leu (Cambridge Isotopes, Andover, MA).52,55 The peptides were dissolved in acetonitrile (ACN)/trifluoroacetic acid (TFA) (99:1, vol/vol) and injected into HPLC C18 (β2 TM) or C4 (αL TM) columns. The gradient was based on ACN/water, as described previously.52 Purified peptides were lyophilized overnight after addition of 10 mM HCl to remove TFA adducts. The purity of the peptides was confirmed by mass spectrometry matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using the matrix α-cyano-
4-hydroxycinnamic acid. The matrix was dissolved in ACN/water (50:50, vol/vol) with 0.1% TFA and mixed with a solution containing the peptide (1 mg/ml). Only one peak corresponding to the mass of the peptide was detected (data not shown).

**Infrared spectroscopy**

Infrared spectra were acquired on a Nicolet Nexus spectrometer (Madison) purged with N₂ and equipped with a MCT/A detector cooled with liquid nitrogen. Attenuated total reflection (ATR) spectra were collected with a 25-reflection ATR accessory from Graseby Specac (Kent, UK) and a wire grid polarizer (0.25 μm; Graseby Specac). A total of 200 interferograms collected at a resolution of 4 cm⁻¹ were processed with one-point zero filling and Happ–Genzel apodization.

Peptidomimetics were incorporated into multilamellar liposomes by first dissolving a dry mixture of lipid dimyristoylphosphatidylcholine and lyophilized peptide (50:1 molar ratio) in ethanol. The solution was dried with a stream of N₂, and the resulting peptide–lipid film was dissolved in water, followed by sonication. The liposome solution was deposited on a germanium internal reflection element, dried slowly by evaporation, and examined using ATR. H/D exchange was performed by flowing D₂O-saturated nitrogen into the ATR chamber for a few hours. Amide proton H/D exchange was detected from the reduction in the intensity of amide A and II bands in the nonpolarized spectra, which were obtained from parallel (∥) and perpendicular (⊥) ATR-polarized spectra using the expression 1[∥]/1.44(⊥). The percentage of H/D exchange was calculated as described previously. Dichroic ratios were determined as described previously. The dichroic ratios for amides I and A were calculated from the spectra in H₂O and D₂O, respectively.

**Time-lapse video microscopy**

SK-β2.7 cells transfected with either wild-type β₂ or β₂ mutant plasmids were plated into a coverslip glass-bottom tissue culture dish (MatTek) that was coated with ICAM-1/Fc (1 μg/ml) and SDF-1α (100 ng/ml; Calbiochem) and incubated for 30 min at 37 °C in a humidified 5% CO₂ incubator. Unbound cells (nontransfected or nonviable) were removed by washing in complete medium. Fresh medium supplemented with SDF-1α (100 ng/ml) was added, and the images of bound cells were captured with time on a Zeiss Axiovert 200 M inverted fluorescence microscope (with 20 × objective) housed in a closed system maintained at 37 °C in 5% CO₂ that is equipped with a CoolSnap HQ charge-coupled device camera. Images were analyzed using the software Metamorph® (Molecular Devices Corp.).

**Acknowledgements**

We thank M. Cooray, K. G. Lim, and X. Lin for technical assistance. We thank S. K. Alex Law for sharing the SK-β2.7 cells. We thank N. H. Hiew and other support staff of the Axle Cluster at the A*STAR Computational Resource Center. We have no financial conflict of interest. This work was supported by Singapore A*STAR Biomedical Research Council grant 06/1/22/19/445 (S.-M.T.). A.V. was supported by A*STAR Biomedical Research Council grant 03/1/22/19/238 (J.T.).

**References**


