Published in final edited form as:

J Mol Biol. 2010 September 3; 401(5): 882–891. doi:10.1016/j.jmb.2010.06.062.

SPECIFICITY FOR HOMO-VERSUS HETERO-OLIGOMER FORMATION IN INTEGRIN TRANSMEMBRANE HELICES

Hua Zhu^a, Douglas G. Metcalf^b, Craig N. Streu^b, Paul C. Billings^b, William F. DeGrado^b, and Joel S. Bennett^a

^a Hematology-Oncology Division Department of Medicine, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104 USA

^b Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104 USA

Abstract

Transmembrane helices engage in homomeric and heteromeric interactions that play essential roles in folding and assembly of transmembrane proteins. However, features that explain their propensity to interact homomerically or heteromerically and determine the strength of these interactions are poorly understood. Integrins are an ideal model system to address these questions because the transmembrane helices of full-length integrins interact heteromerically when integrins are inactive, but the isolated transmembrane helices are also able to form homo-dimers or homo-oligomers in micelles and bacterial membranes. We sought to determine the features defining specificity for homo versus hetero interactions by conducting a comprehensive comparison of the homomeric and heteromeric interactions of the integrin $\alpha\text{IIb}\beta 3$ transmembrane helices in biological membranes. Using the TOXCAT assay, we found that residues V700, M701, A703, I704, L705, G708, L709, L712, and L713, located on the same face of the $\beta 3$ helix, mediate homodimer formation. We then characterized the $\beta 3$ heterodimer by measuring the ability of $\beta 3$ helix mutations to cause ligand binding to $\alpha\text{IIb}\beta 3$. We found that mutating V696, L697, V700, M701, A703, I704, L705, G708, L712, and L713, but not the small-X₃-small motif, S699-X₃-A703, caused constitutive $\alpha\text{IIb}\beta 3$ activation, as well as persistent $\alpha\text{IIb}\beta 3$ activation-dependent FAK phosphorylation. Because αIIb and $\beta 3$ use the same face of their respective transmembrane helices for homomeric and heteromeric interactions, the interacting surface on each has an intrinsic “stickiness” predisposing towards helix-helix interactions in membranes. The residues responsible for heterodimer formation comprise a network of interdigitated sidechains with considerable geometric complementarity; mutations along this interface invariably destabilize heterodimer formation. By contrast, residues responsible for homomeric interactions are dispersed over a wider surface. While most mutations of these residues are destabilizing, some stabilized homo-oligomer formation. We conclude that the $\alpha\text{IIb}\beta 3$ transmembrane heterodimer shows the hallmark of finely-tuned heterodimeric interaction, while the homomeric interaction is less specific.

Corresponding author: Joel S. Bennett, Hematology-Oncology Division, Department of Medicine, University of Pennsylvania School of Medicine, 914 BRB II/III, 421 Curie Blvd., Philadelphia, PA 19104-6058, Tel: 215-573-3280; Fax: 215-573-7039; bennetts@mail.med.upenn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Integrin; transmembrane domains; oligomerization motifs; TOXCAT; scanning mutagenesis

Introduction

Lateral interactions between transmembrane (TM) helices play essential roles in folding and assembly of multi-span TM proteins as they exit the translocon. They also mediate assembly and signaling from single-span TM proteins in a manner similar to water-soluble modules such as coiled coils, helix-loop-helix motifs, HAMP and other domains that associate/dissociate or rearrange as part of cellular signaling^{1; 2}. The soluble transcription factor Jun is a relevant example. Jun exhibits a substantial propensity to form homooligomers, but an even stronger propensity to interact heteromerically with its physiological partner Fos^{3; 4}. In the case of TM proteins, identifying TM motifs with strong predilection to engage in TM helix associations and dissecting the features that define their preference for homo-versus heterooligomeric assembly will further our understanding of protein recognition and folding.

Integrins provide an ideal system for understanding the propensity for heteromeric versus homomeric helical interactions in membranes. Integrins are heterodimers composed of α and β subunits, each containing a single TM helix⁵. In resting integrins, the α and β TM helices form a tightly interacting heterodimer. However, when the integrins are activated (i.e., competent for clustering, interacting with extracellular ligands, and modulating intracellular signal transduction cascades), the TM domains physically separate^{6; 7}. The extracellular domains of activated integrins remain associated, but undergo global conformational changes in response to dissociation of their TM helices⁶. Thus, the strength of the TM domain heterodimer must be sufficient to maintain integrins in their resting state, but not too strong to prevent TM domain separation, and subsequent integrin activation, in response to cellular stimulation.

As is the case for the oligomerization domains of many water-soluble heterodimeric transcription factors, integrin TM helices, as isolated peptides *in vitro*, are able to interact homomerically as well as heteromerically^{8; 9}. The homomeric association of isolated integrin TM domains has been well documented in micelles and bacterial membranes^{8; 9} and the first inquiries into the role of integrin TM helices focused on their tendency to form homooligomers¹⁰. If homooligomerization occurred only after dissociation of the heterodimeric TM pair, then it would drive the equilibrium towards the activated state and provide a mechanism for clustering. Asn-scanning mutagenesis provided support for this hypothesis; mutations that increased homodimerization of the $\beta 3$ TM helix indeed activated the integrin and electron microscopy of the intact integrin supported this hypothesis¹⁰. However, subsequent studies found that the $\alpha I Ib \beta 3$ activation resulting from $\alpha I Ib$ and $\beta 3$ TM helix mutations might instead result from disruption of $\alpha I Ib \beta 3$ TM helix/helix interaction¹¹ and most^{12; 13; 14}, but not all¹⁵, direct experimental analyses failed to observe TM homooligomerization in integrin activation under physiological conditions. Thus, it appears that homooligomerization is not a dominant or obligatory mechanism in integrin activation and in many cases, it could not be observed in a cellular context. Accordingly, it is interesting to ask what molecular features might discriminate between homo-versus heterooligomerization of integrin TM helices. Such a comparative study gets to the heart of the molecular nature of sequence-specific asymmetric helix-helix interactions in the membrane, for which data is currently almost entirely lacking.

Previous studies have established the tendency of the TM domains of various integrins to interact homomerically, as well as heteromerically, in micelles and bacterial membranes^{8; 9};

¹⁶. The sequence-dependence of heteromeric interactions has been evaluated in bacterial reporter systems that measure the ability of TM helix heterodimers to bring together DNA-binding domains that either induce or repress transcription of specific reporter genes ^{17; 18}. In parallel, sequence-dependent interactions between integrin TM domains have been evaluated by measuring the functional effects of single-site amino acid substitutions in full-length integrins ^{11; 19}. A similar set of residues identified in bacterial reporter system as mediating heteromeric interactions were found to be essential for stabilizing the resting state; in other words, mutations disrupting TM heterodimerization caused constitutive integrin activation.

The interaction of integrin TM helices has been evaluated most extensively for the major platelet integrin $\alpha\text{IIb}\beta 3$ ²⁰. The sequence motifs involved in $\alpha\text{IIb}\beta 3$ TM heterodimerization have been identified, leading to a model for the $\alpha\text{IIb}\beta 3$ TM heterodimer that has been validated by solution NMR and disulfide crosslinking studies ¹⁸. Nonetheless, different results have been obtained for the precise sequence motif in the $\beta 3$ TM helix required to maintain $\alpha\text{IIb}\beta 3$ in its resting state. One group, using transiently transfected mammalian cells repeatedly found only one residue in $\beta 3$, G708, that was functionally important for forming a TM heterodimer with αIIb ^{21; 19}. Others have observed more extended interaction interfaces ^{14; 22}. Further, although the sequence and structural motifs required for homodimerization of the αIIb TM helix have been identified ^{16; 11}, the sequence and structural motifs required for the weaker homooligomerization of the $\beta 3$ TM helix have not yet been determined ¹⁸. We have prepared a series of well-characterized cell lines stably expressing mutants of the $\alpha\text{IIb}\beta 3$ TM helices and evaluated their ability to bind the activation-specific and physiologically-relevant ligand fibrinogen, as well as cluster under activating and non-activating conditions. We found that the faces of the $\beta 3$ TM helix mediating its heteromeric and homomeric interactions are similar and consist of a surface composed largely of hydrophobic residues. Nonetheless, in contrast to the αIIb TM helix, the $\beta 3$ TM helix has a substantially lower intrinsic affinity for association ¹⁸. Thus, it relies on a highly specific interaction with αIIb to stabilize the $\alpha\text{IIb}\beta 3$ TM heterodimer.

Results

Identification of the motif mediating the homomeric association of the $\beta 3$ TM helix

Proteins containing the $\beta 3$ TM helix form homooligomers when dispersed in phospholipid micelles ⁸. To identify the residues mediating this interaction, we scanned the helix with leucine, alanine, valine, and isoleucine mutations and measured the effect of each mutation on homodimer formation using the TOXCAT assay ²³. In TOXCAT, the homomeric association of a fusion protein consisting of an N-terminal ToxR' DNA binding domain and a C-terminal MBP domain in the inner membrane of *E. coli* is driven by the intervening $\beta 3$ TM, resulting in the transcriptional activation of a chloramphenicol acetyl transferase (CAT) reporter gene. The amount of CAT synthesized is a measure of the strength of TM-mediated dimerization ²³.

An important determinant for the tendency of a fusion protein to dimerize in TOXCAT is the length of the inserted TM helix ²⁴. We established the optimal length for the inserted $\beta 3$ TM helix by incrementally deleting one residue from its C-terminal end (Fig. 1A). Importantly, decreasing the length of the $\beta 3$ TM helix had no effect on fusion protein expression in *E. coli* (Fig. 1B). Moreover, the ability of each construct to facilitate growth of an MBP-deficient strain of *E. coli* when maltose was the sole carbon source (MalE complementation) indicated that the MBP domain of each fusion protein was located in the bacterial periplasmic space (Fig. 1C). Nonetheless, despite proper insertion of each fusion protein in the *E. coli* inner membrane, there were substantial differences in CAT synthesis

(Fig. 1D). Because the fusion protein containing $\beta 3$ residues 693–714 generated the greatest CAT signal, this construct was used in subsequent experiments.

Most often, we found that mutations in the $\beta 3$ TM helix perturbing CAT synthesis were disruptive and decreased CAT synthesis (Fig. 2). Occasionally, CAT synthesis was increased, notably by mutations at L697. Immunoblots of bacterial lysates for MBP revealed that none of the mutations had a substantial effect on expression of chimeric proteins (data not shown).

To quantify results shown in Fig. 2, we calculated a Perturbational Index (P_i) as a measure of the overall change in CAT activity at each position in the $\beta 3$ helix due to mutation, regardless whether the mutant increased or decreased activity (Eq. 1). A plot of P_i for a given residue versus its position along the TM helix revealed peaks at approximately every four residues (peaks were observed at V700, M701; A703, I704, L705; G708, L709; L712, L713) (Fig. 3A). We also found the data well described by a sine wave with a repeat unit of 4.0 ± 0.1 residue, significantly greater than the 3.6 residue/turn period of an ideal α helix. A period greater than 3.6 residues in such an analysis typically indicates that residues important for the interaction lie in a stripe at a small angle relative to the helix axis (Fig. 3B). Thus, a second helix would be expected to interact with this surface with a right-handed crossing angle of approximately 40° (Fig. 3C) ²⁵.

Effect of mutations on the heteromeric interactions of the $\beta 3$ TM helix

TM domain separation causes α IIB $\beta 3$ activation which can be detected by measuring fibrinogen binding to the activated integrin. Previously, we have found that mutations perturbing the homomeric interaction of the α IIB TM helix in TOXCAT also caused fibrinogen binding when introduced into the full-length integrin ¹¹. Accordingly, to identify motifs mediating heteromeric interactions of the $\beta 3$ TM helix, we introduced disruptive and non-disruptive $\beta 3$ TM mutations identified in TOXCAT into full-length $\beta 3$ and measured their effect on fibrinogen binding to α IIB $\beta 3$ stably expressed in CHO cells. Disruptive mutations, i.e., those decreasing CAT synthesis in TOXCAT, induced α IIB $\beta 3$ activation (Fig. 2, Fig. 4A). For example, replacing I704 with Leu, Ala, or Val produced a 6.8-fold increase in constitutive fibrinogen binding relative to WT, whereas mutating S699 had little effect on CAT synthesis or fibrinogen binding. Overall, we found that mutating $\beta 3$ residues V696, L697, V700, M701, A703, I704, L705, G708, L712, and L713 caused constitutive α IIB $\beta 3$ activation, but to varying degrees. We then fit the fibrinogen binding results to a continuous sine function. The period of the sine wave (4.0 ± 0.2 residue) was identical to the sine wave fit to the TOXCAT results, as was its phase (Fig. 4B). Thus, $\beta 3$ residues forming the interface of the α IIB $\beta 3$ TM heterodimer are predominantly hydrophobic amino acids with large side chains and lie along the same face of the $\beta 3$ TM helix that mediates its homomeric interactions.

The $\beta 3$ TM mutations causing constitutive fibrinogen binding to α IIB $\beta 3$ are also found to cluster in the interface of the recently described NMR structures of the α IIB $\beta 3$ TM domain heterodimer solved in bicelles ²² or organic solvent ²⁶. The involved residues include V696, L697, V700, M701, A703, I704, L705, G708, and L712. We also found L713 to be important for dimerization, although it is not present in the heterodimer interface in these structures. However, this region of the $\beta 3$ TM helix represents a location at which the two NMR structures differ. Disulfide cross-linking between engineered Cys residues has also been used to probe the geometry of the α IIB $\beta 3$ TM interface ¹⁹. Reasonably strong cross-linking to α IIB residues was observed not only for $\beta 3$ L712, present in the interface of the NMR structures, but also for L713. Specifically, $\beta 3$ L713C and L712C crosslink with similar efficiency to α IIB residues L980C, L983C and V984C, and L913C. These data

support our observation that $\beta 3$ L713 contributes to interactions with αIIb in at least one conformation of resting $\alpha \text{IIb}\beta 3$.

We have also compared the effect of $\beta 3$ mutations on fibrinogen binding to full-length $\alpha \text{IIb}\beta 3$ with the effect of single-site $\beta 3$ and $\beta 1$ TM helix mutations on the heteromeric interaction of the helices on their complementary α subunit TM helices using a dominant negative (DN) bacterial reporter assay¹⁸. As shown in Fig. 4B, the mean P_i profile for the DN assays is very similar to the effect of $\beta 3$ mutations on fibrinogen binding to $\alpha \text{IIb}\beta 3$. The phase and period of the curves are identical within experimental error and highlight the importance of residues spaced at 4-residue intervals, including V696, V700, I704, G708, and L712. Nonetheless, there are significant differences in the effects of neighboring residues: M701A and L705A show large effects on fibrinogen binding, but smaller effects in the DN assay; the opposite is the case for V700A. Interestingly, mutations of $\beta 1$ residue V717, the residue corresponding to M701 in $\beta 3$, have a large effect on the heteromeric interaction of $\beta 1$ with the $\alpha 5$ TM helix, but a smaller effect on its heteromeric interaction with the $\alpha 2$ TM helix. Similarly, mutation of $\beta 3$ residue 703 and the corresponding $\beta 1$ residue had variable effects in the bacterial assay, depending on its binding partner.

It is also instructive to compare the effects of mutations in the homomeric TOXCAT assay and the heteromeric DN assay. The effects of mutations in the homomeric assay are generally smaller than in the heteromeric assay, as determined by the magnitude of the P_i . Although the assays are not identically configured, this finding suggests that the heteromeric interactions are more specific, in the sense that they are much more sensitive to mutation. Moreover, some residues, such as $\beta 3$ A711, that markedly decrease CAT synthesis in TOXCAT had little effect on intact $\alpha \text{IIb}\beta 3$, as assessed by either the DN bacterial or mammalian CHO cell assay. Thus, the heteromeric and homomeric interfaces are similar, but not identical.

Effect of $\beta 3$ TM helix mutations on FAK phosphorylation

Substantial differences have been reported in the ability of αIIb and $\beta 3$ TM domain mutations to activate $\alpha \text{IIb}\beta 3$ ^{19; 21}. To provide independent confirmation of the results shown in Fig. 4, we tested the ability of various $\beta 3$ TM domain mutations to cause focal adhesion kinase (FAK) phosphorylation. Ligand binding to activated $\alpha \text{IIb}\beta 3$ is followed by the formation of $\alpha \text{IIb}\beta 3$ clusters that initiate downstream signaling events, including phosphorylation of FAK on tyrosine residues²⁷. Previously, we found that causing $\alpha \text{IIb}\beta 3$ activation by mutating the αIIb and $\beta 3$ TM helices was accompanied by persistent FAK phosphorylation, implying that activated $\alpha \text{IIb}\beta 3$ was clustered^{10; 11}. Here, we measured FAK phosphorylation when CHO cells expressing various $\beta 3$ TM domain mutants were adherent to fibrinogen or re-suspended in buffer. As shown in Fig. 5, there was concordance between the ability of $\beta 3$ domain mutations to induce fibrinogen binding to $\alpha \text{IIb}\beta 3$ and cause FAK phosphorylation. Thus, when transfected CHO cells were adherent to fibrinogen, FAK was phosphorylated in cells expressing WT $\alpha \text{IIb}\beta 3$ and various activating and non-activating $\beta 3$ TM helix mutations. However, when CHO cells were re-suspended in buffer, FAK was no longer phosphorylated in cells expressing WT $\alpha \text{IIb}\beta 3$ and the non-activating mutation A711L, phosphorylation was present, but decreased, in cells expressing L705A and L712A, and was present and unchanged in cells expressing A703L, I704L, G708A, and L713A. Further, in results not shown in the Fig. 5, there was no persistent FAK phosphorylation in re-suspended cells when S699 was mutated to Leu and the persistent FAK phosphorylation caused by L697A and L698L was comparable to that of L705A. Because FAK phosphorylation results from clustering of activated integrins, these results provide confirmatory evidence that mutations along the interface of the $\beta 3$ TM helix with αIIb induce $\alpha \text{IIb}\beta 3$ activation.

Visualization of $\beta 3$ TM helix oligomerization interfaces

To visualize the interfaces on the $\beta 3$ TM helix responsible for its homomeric and heteromeric interactions, the functional consequences of the various $\beta 3$ TM helix mutations were divided into equal quartiles corresponding to marked, intermediate, minimal, and no effect and were displayed as such on an α -helix. The residues responsible for homomeric and heteromeric association of the helix, i.e., residues present in the marked and intermediate quartiles, were located on the same face of the helix (Fig. 6). Residues mediating heteromeric association were clustered on the helical face, whereas residues mediating homomeric association were more widely distributed, suggesting less specific interaction and possibly even a dynamic ensemble of associated states in which different conformers have different sensitivity to mutation. We also observed notable differences between the interfaces. For example, mutating G708 had a marked effect on fibrinogen binding, but was only minimally disruptive in TOXCAT. Conversely, mutating residues at the C-terminus of the helix had substantial disruptive effects in TOXCAT, but did not induce constitutive fibrinogen binding. It is also noteworthy that while the $\beta 3$ helix contains a small residue- X_3 -small residue motif (S699- X_3 -A703), this motif is not located in either the hetero- or homooligomer interface, but on the opposite side of the helix where it would be available to possibly mediate other intermolecular interactions.

Discussion

In this paper, we have addressed the features of TM helix sequences that encode for interaction specificity versus affinity. Previous studies of water-soluble coiled-coil domains, such as those of Fos/Jun, found that buried hydrophobic residues at positions “a” and “d” of their heptad repeats provided a strong but non-specific driving force for oligomerization that did not readily distinguish between homomeric and heteromeric association¹. For example, Fos and Jun preferentially associated heteromerically using electrostatic interactions to strongly destabilize homomeric association and weakly stabilize heteromeric association. Similar effects have been observed for a large number of other members of the leucine zipper family of transcriptional factors which use a combination of sidechain packing geometry, hydrogen-bonding, and electrostatics to provide specificity²⁸. Here, we asked whether similar effects might be observed in TM helices, although the nature of the interactions would be different.

The homomeric and heteromeric interactions of the α IIB TM domain have been characterized previously^{11; 16} and the results extended to other integrin α subunits¹⁸. The α IIB TM domain contains a strongly interactive G- X_3 -G motif that mediates homomeric and heteromeric interactions. Nonetheless, α IIB TM-containing heterodimers and α IIB TM homodimers are geometrically distinct and respond differently to mutations. When the α IIB TM domain interacts heteromerically with $\beta 3$, its G- X_3 -G motif packs onto a groove formed by larger residues on the $\beta 3$ helix such that all mutations in α IIB along this interface disrupt the heterodimer¹⁸. The G- X_3 -G motif in α IIB also mediates a glycophorin A-like homomeric interaction¹⁶. But in this case, mutations extend the motif to make it more glycine zipper-like²⁹ and enhance, rather than diminish, its homomeric affinity¹⁶. Thus, the divergent effects of mutations on homomeric and heteromeric interactions indicate that the native α IIB TM sequence is optimized to interact heteromerically with $\beta 3$. However, whether homomeric interactions of α IIB, or $\beta 3$ for that matter, participate in α IIB $\beta 3$ function *in vivo* remains an open question.

Here we provide the first comprehensive comparison of homomeric versus heteromeric interactions of the $\beta 3$ TM helix. Previous studies using several different bacterial reporter systems found that the homomeric and heteromeric interactions of the isolated $\beta 3$ TM helix are substantially weaker than those of the α IIB helix^{17; 18}. Nonetheless, heteromeric

association of $\beta 3$ with αIIb is sufficiently strong to maintain $\alpha \text{IIb}\beta 3$ in its resting conformation until circulating platelets encounter vascular damage. Although like αIIb , the $\beta 3$ TM helix contains a small- X_3 -small motif, S699- X_3 -A703, this motif appears less prone to association than the canonical G- X_3 -G motif. This is born out in our studies showing that S699- X_3 -A703 is not involved in either homomeric or heteromeric $\beta 3$ TM helix interactions. Investigation of heteromeric $\beta 3$ TM helix interactions by mutagenesis of the full-length integrin revealed only activating (disruptive) mutations. By contrast, the TOXCAT assay revealed that mutations can either enhance or disrupt homomeric interactions. Taken together, these observations suggest that the αIIb and $\beta 3$ TM sequences have evolved to be a “matched set” and that mutations to either interaction interface destabilize their association.

From a global perspective, the same face of the $\beta 3$ helix is used for homomeric and heteromeric TM helix interactions, as is evident from the conserved phase of the sine waves in Fig. 3A and 4B. However, there are substantial differences in the essential residues for each type of interaction (Fig. 6). Near the N-terminus of the $\beta 3$ TM helix, the sidechains of L697, M701, and I704 pack against the G- X_3 -G motif of αIIb . Furthermore, G708 forms a geometrically complementary interaction with large residues in the αIIb helix; hence, these $\beta 3$ residues are found to be particularly important for heterodimer formation (Fig. 6). On the other hand, G708 is substantially less important for interaction in the homo-oligomer as assessed by Ala or Leu mutations, although replacement of G708 with Asn stabilizes trimer formation *in vitro*¹⁰. This finding is consistent with the observation that Asn residues can stabilize oligomerization in membranes through hydrogen-bond formation^{30; 31}. Also, residues C-terminal to G708 are more important for homo-oligomerization rather than heterodimer formation.

In conclusion, this work provides a comprehensive mutational analysis of homomeric versus heteromeric TM helix interactions in biological membranes. The αIIb TM sequence drives strong homomeric and heteromeric interactions, mediated by its G- X_3 -G motif^{17; 18}. But whether it interacts homomERICALLY versus heteromERICALLY appears to be defined by the heteromeric pre-association of the $\alpha \text{IIb}\beta 3$ extracellular domains, as well as weaker interactions between its cytoplasmic tails. The $\beta 3$ TM sequence, with a lower intrinsic affinity for association, relies primarily on highly specific interactions with αIIb to stabilize the heteromeric $\alpha \text{IIb}\beta 3$ complex. The use of the less “sticky” $\beta 3$ partner leads to a heterodimer complex that has reduced affinity when compared to idealized homodimers such as that of glycophorin A. It is likely that this feature is essential for switchable systems such as integrins that rely on dynamic TM helix separation as part of their activation mechanism.

Materials and Methods

Vectors and Strains

Expression vectors pccKAN, pccgpA-wt, and pccgpA-G83I and *E. coli* strain MM39²³, were kindly provided by Dr. Donald M. Engelman. The EcoRV restriction site between the ToxR TM region and the malB gene in pccKAN was changed to a BamHI site. Fragments encoding the $\beta 3$ TM domain, flanked by NheI and BamHI sites, were amplified from full-length $\beta 3$ cDNA and ligated in frame into the vector. Point mutations in the $\beta 3$ TM domain were generated using a QuikChange mutagenesis kit (Stratagene). Sequences of the wild-type (WT) and mutant $\beta 3$ TMs were confirmed by DNA sequencing.

Expression of a Chimeric Protein in *E. coli*

E. coli strain MM39 was transformed with plasmids containing cDNA for the WT $\beta 3$ TM domain or various $\beta 3$ TM domain mutants. A single colony was used to inoculate 5 ml of

LB broth containing 100 µg/mL ampicillin and grown to OD₆₀₀ of 0.6 at 37 °C with vigorous shaking. One mL of the culture was chilled on ice, pelleted, and resuspended in 100 µL of LDS sample buffer for analysis by SDS-PAGE (Invitrogen). Eighteen µL of the bacterial lysate in LDS buffer was then loaded onto 10% NuPAGE Bis-Tris pre-cast gels in 3-[N-morpholino]propanesulfonic acid buffer (Invitrogen). The separated proteins were transferred to nitrocellulose and immunoblotted with an anti-MBP monoclonal antibody (Sigma).

MaIE Complementation Test

Glucose in M9 minimal medium plates was replaced by 0.4% maltose as the sole carbon source²³. Transformed MM39 cells were streaked onto these plates containing ampicillin and incubated for two days at 37 °C.

TOXCAT Assay

The TOXCAT assay was performed as reported previously²³. Briefly, one ml of bacterial culture was grown to an OD₆₀₀ of 0.6, chilled on ice, and pelleted at 4 °C. The pelleted cells were resuspended and washed in 0.6 mL TBS buffer (20 mM Tris, pH 8, containing 100 mM NaCl and 2 mM EDTA). Upon resuspension in 0.5 mL TBS on ice, the cells were treated with 100 µg/ml lysozyme for 20 minutes. Triton X-100 (0.4%), 10 mM dithiothreitol, and bacterial protease inhibitor cocktail (Sigma) were then added to the mixture. After a 30 min incubation, the mixture was sonicated briefly and stored overnight at 4 °C. On the following day, supernatants were assayed for chloramphenicol acetyl transferase (CAT) synthesis using a CAT-ELISA kit (Roche). Assay calibration was carried out using standards provided by the manufacturer. In each experiment, the strongly dimerizing TM domain of glycophorin A (GpA)-WT, and the weakly-dimerizing TM domain of the GpA mutant G83I were included as positive and negative controls, respectively. CAT-ELISA results were expressed as a percentage of CAT synthesis induced by GpA-WT in the same experiment. Chimeric protein expression was quantified from immunoblots using a Personal Densitometer SI (Molecular Dynamics) and used to normalize CAT expression by the various constructs.

Analysis of TOXCAT Results

A perturbation index P_i for $\beta 3$ TM mutations was calculated using the formalism of Treutlein et al.³² and was based on exhaustive mutagenesis of the GpA dimer interface by Lemmon et al.³³. The P_i reflects the mean fold-change in CAT activity measured for each $\beta 3$ TM domain mutation relative to GpA. The fold change is calculated using the equation:

$$P_i = 10 \exp \left[\frac{1}{n} \sum_{j=1}^n |\log(x_j/x_{WT})| \right] \quad (\text{Eq. 1})$$

where n is the number of mutations at a given position i , and x_j is the TOXCAT activity at position i for mutation j . The helical phases for $\beta 3$ TM mutations were then determined by fitting P_i as a function of position to a sine function:

$$(P_i)_x = A \cdot \sin \left(\frac{2\pi(x+\varphi)}{n} \right) + B \quad (\text{Eq. 2})$$

in which x is the residue number, φ is the phase (in residues), A is the amplitude, n is the period in residues, and B is a constant¹⁶.

Stable Expression of α Ib β 3 Mutants in CHO Cells

α Ib and β 3 cDNAs were subcloned into the expression plasmids pcDNA3.1(+)-Neo and pcDNA3.1(+)-Zeo, respectively, prior to co-transfection into CHO cells using FUGENE 6 (Roche). Transfected cells were grown in selection medium containing G418 and Zeocin for three weeks and then sorted twice by fluorescence-activated cell sorting (FACS) for cells expressing high levels of α Ib β 3¹¹.

Fibrinogen Binding to CHO Cells Expressing α Ib β 3

Fibrinogen binding to α Ib β 3 expressed on the CHO cell surface was measured as described previously¹¹. Briefly, CHO cells (2×10^6 cells/ml) were incubated with the β 3-specific monoclonal antibody (mAb) SSA6³⁴ on ice for 30 minutes. Labeled cells were washed and incubated for 30 min at 37 °C with phycoerythrin-conjugated anti-mouse IgG (Molecular Probes), 200 μ g/ml fibrinogen conjugated with Alexa 488 (Molecular Probes), freshly-made 5 mM dithiothreitol (DTT) $\pm$ 5 mM EDTA. The cells were washed, fixed with 0.37% formalin in PBS, and examined by 2-color FACS analysis. Fibrinogen specifically bound to α Ib β 3 was defined as fibrinogen binding inhibited by EDTA¹¹.

Specific fibrinogen binding data were used to calculate a fibrinogen binding index (F_i), using the equation:

$$F_i = (FB_{\text{cmut}}/FB_{\text{DTTmut}})/FB_{\text{cwt}}/FB_{\text{DTTwt}} \quad (\text{Eq. 3})$$

where FB_{cmut} represents constitutive fibrinogen binding to α Ib β 3; FB_{DTTmut} , fibrinogen binding to mutant α Ib β 3 induced by 5 mM DTT; FB_{cwt} , constitutive fibrinogen binding to wild-type α Ib β 3; and FB_{DTTwt} , fibrinogen binding to wild-type α Ib β 3 induced by 5 mM DTT.

Focal adhesion kinase (FAK) phosphorylation

Phosphorylation of FAK in CHO cells stably expressing α Ib β 3 and either adherent to fibrinogen-coated tissue culture plates or placed in suspension in centrifuge tubes was measured as described previously^{9; 16}. Briefly, adherent cells or cells resuspended in 20 mM HEPES buffer, pH 7.4, containing 137 mM NaCl, 2.7 mM $MgCl_2$, 5.6 mM glucose, and 3.3 mM NaH_2PO_4 were lysed with RIPA buffer (10 mM Tris-HCl, pH 7.2, containing 158 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, as well as 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma). FAK was immunoprecipitated from 500 μ g of cellular lysate and 300 μ g of immunoprecipitated protein was subjected to SDS-PAGE in a 3–8% NuPAGE Tris-Acetate gel (Invitrogen). FAK phosphorylation was detected by immunoblotting using the mouse anti-phosphotyrosine mAb 4G10 (Upstate Biotech). The presence of comparable amounts of FAK in each lysate was confirmed by immunoblotting 100 μ g of lysate protein with the anti-FAK monoclonal antibody 4.47 (Millipore).

Acknowledgments

We thank Dr. Don Engelman for kindly providing the TOXCAT plasmid, Roman Gorelik for help in establishing the TOXCAT assay, Dr. Wei Li for her assistance with the CHO cell transfections, and Drs. Bryan Berger, and Gevorg Grigoryan for reviewing the manuscript. This work was supported by grants HL40387, HL81012, GM60610, and GM56423 from the National Institutes of Health.

Abbreviations used

TM	transmembrane
CHO	Chinese hamster ovary
MBP	maltose binding protein
CAT	chloramphenicol acetyl transferase
DTT	dithiothreitol
P_i	perturbation index
F_i	fibrinogen binding index
FAK	focal adhesion kinase

References

1. Grigoryan G, Keating AE. Structural specificity in coiled-coil interactions. *Curr Opin Struct Biol*. 2008; 18:477–83. [PubMed: 18555680]
2. Swain KE, Falke JJ. Structure of the conserved HAMP domain in an intact, membrane-bound chemoreceptor: a disulfide mapping study. *Biochemistry*. 2007; 46:13684–95. [PubMed: 17994770]
3. Newman JR, Keating AE. Comprehensive identification of human bZIP interactions with coiled-coil arrays. *Science*. 2003; 300:2097–101. [PubMed: 12805554]
4. Vinson C, Acharya A, Taparowsky EJ. Deciphering B-ZIP transcription factor interactions in vitro and in vivo. *Biochim Biophys Acta*. 2006; 1759:4–12. [PubMed: 16580748]
5. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell*. 2002; 110:673–87. [PubMed: 12297042]
6. Takagi J, Petre B, Walz T, Springer T. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell*. 2002; 110:599–611. [PubMed: 12230977]
7. Litvinov RI, Nagaswami C, Vilaire G, Shuman H, Bennett JS, Weisel JW. Functional and structural correlations of individual α IIb β 3 molecules. *Blood*. 2004 in press.
8. Li R, Babu CR, Lear JD, Wand AJ, Bennett JS, DeGrado WF. Oligomerization of the integrin α IIb β 3: roles of the transmembrane and cytoplasmic domains. *Proc Natl Acad Sci U S A*. 2001; 98:12462–7. [PubMed: 11606749]
9. Schneider D, Engelman DM. Involvement of transmembrane domain interactions in signal transduction by α/β integrins. *J Biol Chem*. 2004; 279:9840–9846. [PubMed: 14681217]
10. Li R, Mitra N, Gratkowski H, Vilaire G, Litvinov R, Nagasami C, Weisel JW, Lear JD, DeGrado WF, Bennett JS. Activation of integrin α IIb β 3 by modulation of transmembrane helix associations. *Science*. 2003; 300:795–8. [PubMed: 12730600]
11. Li W, Metcalf DG, Gorelik R, Li R, Mitra N, Nanda V, Law PB, Lear JD, Degrado WF, Bennett JS. A push-pull mechanism for regulating integrin function. *Proc Natl Acad Sci U S A*. 2005; 102:1424–9. [PubMed: 15671157]
12. Kim M, Carman CV, Yang W, Salas A, Springer TA. The primacy of affinity over clustering in regulation of adhesiveness of the integrin { α }L{ β }2. *J Cell Biol*. 2004; 167:1241–1253. [PubMed: 15611342]
13. Luo BH, Springer TA, Takagi J. A specific interface between integrin transmembrane helices and affinity for ligand. *PLoS Biol*. 2004; 2:776–786.
14. Partridge AW, Liu S, Kim S, Bowie JU, Ginsberg MH. Transmembrane domain helix packing stabilizes integrin α IIb β 3 in the low affinity state. *J Biol Chem*. 2005; 280:7294–300. [PubMed: 15591321]
15. Vararattanavech A, Lin X, Torres J, Tan SM. Disruption of the integrin α L β 2 transmembrane domain interface by β 2 Thr-686 mutation activates α L β 2 and promotes micro-clustering of the α L subunits. *J Biol Chem*. 2009; 284:3239–49. [PubMed: 19029120]

16. Li R, Gorelik R, Nanda V, Law PB, Lear JD, DeGrado WF, Bennett JS. Dimerization of the transmembrane domain of Integrin α IIb subunit in cell membranes. *J Biol Chem.* 2004; 279:26666–73. [PubMed: 15067009]
17. Schneider D, Engelman DM. GALLEX, a measurement of heterologous association of transmembrane helices in a biological membrane. *J Biol Chem.* 2003; 278:3105–11. [PubMed: 12446730]
18. Berger BW, Kulp DW, Span LM, DeGrado JL, Billings PC, Senes A, Bennett JS, DeGrado WF. Consensus motif for integrin transmembrane helix association. *Proc Natl Acad Sci U S A.* 2010; 107:703–8. [PubMed: 20080739]
19. Zhu J, Luo BH, Barth P, Schonbrun J, Baker D, Springer TA. The structure of a receptor with two associating transmembrane domains on the cell surface: integrin α IIb β 3. *Mol Cell.* 2009; 34:234–49. [PubMed: 19394300]
20. Bennett JS. Structure and function of the platelet integrin α IIb β 3. *J Clin Invest.* 2005; 115:3363–9. [PubMed: 16322781]
21. Luo BH, Carman CV, Takagi J, Springer TA. Disrupting integrin transmembrane domain heterodimerization increases ligand binding affinity, not valency or clustering. *Proc Natl Acad Sci U S A.* 2005; 102:3679–84. [PubMed: 15738420]
22. Lau TL, Kim C, Ginsberg MH, Ulmer TS. The structure of the integrin α IIb β 3 transmembrane complex explains integrin transmembrane signalling. *Embo J.* 2009; 113:4747–4753.
23. Russ WP, Engelman DM. TOXCAT: a measure of transmembrane helix association in a biological membrane. *Proc Natl Acad Sci U S A.* 1999; 96:863–8. [PubMed: 9927659]
24. Langosch D, Brosig B, Kolmar H, Fritz HJ. Dimerisation of the glycophorin A transmembrane segment in membranes probed with the ToxR transcription activator. *J Mol Biol.* 1996; 263:525–30. [PubMed: 8918935]
25. Moore DT, Berger BW, DeGrado WF. Protein-protein interactions in the membrane: sequence, structural, and biological motifs. *Structure.* 2008; 16:991–1001. [PubMed: 18611372]
26. Yang J, Ma YQ, Page RC, Misra S, Plow EF, Qin J. Structure of an integrin α IIb β 3 transmembrane-cytoplasmic heterocomplex provides insight into integrin activation. *Proc Natl Acad Sci U S A.* 2009; 106:17729–34. [PubMed: 19805198]
27. Parsons JT. Focal adhesion kinase: the first ten years. *J Cell Sci.* 2003; 116:1409–16. [PubMed: 12640026]
28. Grigoryan G, Keating AE. Structure-based prediction of bZIP partnering specificity. *J Mol Biol.* 2006; 355:1125–42. [PubMed: 16359704]
29. Kim S, Jeon TJ, Oberai A, Yang D, Schmidt JJ, Bowie JU. Transmembrane glycine zippers: physiological and pathological roles in membrane proteins. *Proc Natl Acad Sci U S A.* 2005; 102:14278–83. [PubMed: 16179394]
30. Choma C, Gratkowski H, Lear JD, DeGrado WF. Asparagine-mediated self-association of a model transmembrane helix. *Nat Struct Biol.* 2000; 7:161–6. [PubMed: 10655620]
31. Zhou FX, Cocco MJ, Russ WP, Brunger AT, Engelman DM. Interhelical hydrogen bonding drives strong interactions in membrane proteins. *Nat Struct Biol.* 2000; 7:154–60. [PubMed: 10655619]
32. Treutlein HR, Lemmon MA, Engelman DM, Brunger AT. The glycophorin A transmembrane domain dimer: sequence-specific propensity for a right-handed supercoil of helices. *Biochemistry.* 1992; 31:12726–32. [PubMed: 1463744]
33. Lemmon MA, Flanagan JM, Treutlein HR, Zhang J, Engelman DM. Sequence specificity in the dimerization of transmembrane α -helices. *Biochemistry.* 1992; 31:12719–12725. [PubMed: 1463743]
34. Weisel JW, Nagaswami C, Vilaire G, Bennett JS. Examination of the platelet membrane glycoprotein IIb/IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. *J Biol Chem.* 1992; 267:16637–16643. [PubMed: 1644841]
35. Lau TL, Partridge AW, Ginsberg MH, Ulmer TS. Structure of the integrin β 3 transmembrane segment in phospholipid bicelles and detergent micelles. *Biochemistry.* 2008; 47:4008–16. [PubMed: 18321071]

Fig. 1.

β 3 TM domain expression in MalE-deficient MM39 *E. coli*. (a) The β 3 TM domain sequences tested in the TOXCAT assay are aligned and identified by their starting and ending residues in the mature protein. TM sequences of WT-GpA and GpA-G83I, positive and negative controls for the TOXCAT assay, are shown at the top. (b) Anti-MBP immunoblots of proteins from *E. coli* transformed with plasmids containing cDNA for the various length β 3 TM domains shown in A. (c) Growth of MalE-deficient MM39 *E. coli* transformed with plasmids containing β 3 TM domain cDNA on M9 minimum medium where maltose is the only carbon source. Only cells expressing MBP in the periplasm survive. *E. coli* transformed with pMAL-p2 and pMAL-c2 vectors expressing MBP in the periplasm or cytoplasm were included as positive and negative controls, respectively. (d) CAT expression induced by the various length β 3 TM domains shown in A, expressed as a percent of the CAT induced by WT GpA (100%). Data shown are the mean and S.D. of triplicate measurements.

Fig. 2. Effect of scanning mutagenesis of the $\beta 3$ TM domain on CAT expression in TOXCAT. Single-site Leu (red), Ala (pink), Val (gray), or Ile (blue) mutations were made at consecutive positions in the $\beta 3$ TM domain. Their effect was quantified by CAT-ELISA and compared to the signal from GpA-WT. The horizontal line at 40% corresponds to CAT synthesis by WT $\beta 3$. Data presented are the mean and S.D. of 4–10 independent experiments

Fig. 3.

A. Perturbation index (P_i) for $\beta 3$ residues 697–714. TOXCAT results shown in Fig. 2 were used to calculate a P_i as described in Materials and Methods. The red line is the best fit of the data to a sinusoidal function as described in Materials and Methods. **B.** Ribbon diagram showing that the arrangement of sidechains at positions i , $i+4$, $i+8$... gives rise to a ridge of sidechains spiraling in a right-handed manner along an α -helix. **C.** A second helix (shown in gray) will have a right-handed crossing angle when docked against the first helix.

Fig. 4. Fibrinogen binding to CHO cells expressing $\alpha\text{IIb}\beta 3$ containing $\beta 3$ TM domain mutations. **A.** Alexa 488-fibrinogen binding to $\alpha\text{IIb}\beta 3$ was measured by flow cytometry in the absence (constitutive binding) or presence (stimulated binding) of 5 mM DTT. Fibrinogen binding measurements were used to calculate a binding index for each mutation as described in Materials and Methods. Data shown are the mean and SE of 3–10 measurements. **B.** Comparison of the fibrinogen binding data shown in **A** (solid lines) and a mean perturbation index calculated from mutational analyses of $\beta 3$ TM helix binding to αIIb and $\beta 1$ TM helix binding to $\alpha 2$ and $\alpha 5$ reported in reference ¹⁸ (dashed lines) with a sinusoidal function with a 4.0 residue periodicity fit to the fibrinogen binding data (red line).

Fig. 5.

FAK phosphorylation in CHO cells expressing WT α IIb β 3 and α IIb β 3 containing β 3 TM mutations. Phosphotyrosine (pTyr) immunoblots of FAK from lysates of CHO cells maintained in suspension (*top row*) or adherent to immobilized fibrinogen (*third row*). Total FAK in the immunoprecipitates, detected by an anti-FAK antibody, is shown in the *second and fourth rows*.

Fig. 6.

Models of the $\beta 3$ TM helix showing the location of residues participating in homomeric and heteromeric interactions. Models were constructed using PyMol (DeLano Scientific) and are based on the NMR structure reported by Lau et al.³⁵. Residues along the $\beta 3$ helix are depicted as colored spheres whose size reflects the magnitude of the disruptive effect of mutations on either homomeric and heteromeric $\beta 3$ helix interactions. Red, yellow, green, and blue spheres indicate marked, intermediate, minimal, and no effect, respectively. Gray spheres indicate positions in the helix that were not studied because mutants at these position expressed poorly in CHO cells.