Dimerization of the Transmembrane Domain of Integron α1b Subunit in Cell Membranes*

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Renhao Li‡‡, Roman Gorelik¶, Vikas Nanda‡, Peter B. Law‡, James D. Lear‡,
William F. DeGrado‡‡, and Joel S. Bennett¶¶

From the ‡Department of Biochemistry and Biophysics and ¶Hematology-Oncology Division, Department of Medicine,
University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Homo- and hetero-oligomeric interactions between the transmembrane (TM) helices of integrin α and β subunits may play an important role in integrin activation and clustering. As a first step to understanding these interactions, we used the TOXCAT assay to measure oligomerization of the wild-type α1b, TM helix and single-site TM domain mutants. TOXCAT measures the oligomerization of a chimeric protein containing a TM helix in the Escherichia coli inner membrane via the transcriptional activation of the gene for chloramphenicol acetyltransferase. We found the amount of chloramphenicol acetyltransferase induced by the wild-type α1b TM helix was approximately half that induced by the strongly dimerizing TM helix of glycophorin A, confirming that the α1b TM domain oligomerizes in biological membranes. Mutating each of the α1b TM domain residues to either Ala, Leu, Ile, or Val revealed that a GXXG motif mediates oligomerization. Further, we found that the residue preceding each glycine contributed to the oligomerization interface, as did the residue at position i + 4 after the second Gly of GXXG. Thus, the sequence XXVGVXGGXXXXXXL is critical for oligomerization of α1b TM helix. These data were used to generate an atomic model of the α1b homodimer, revealing a family of structures with right-handed crossing angles of 40° to 60°, consistent with a 4.0-residue periodicity, and with an interface rotated by 50° relative to glycophorin A. Thus, although the α1b TM helix makes use of the GXXG framework, neighboring residues have evolved to engineer its dimerization interface, enabling it to subserve specific and specialized functions.

By interacting with macromolecular extracellular ligands, integrins mediate essential cell-cell and cell-matrix interactions (1). Further, integrin occupancy transduces information into the cell interior that regulates processes as diverse as cytoskeletal organization, cell migration, cell proliferation, and cellular differentiation, whereas signals initiated within the cell regulate the ability of integrins to interact with ligands. The structural basis for integrin regulation is an area of intense study. For several integrins, there is a correlation between their activation state and the relative positions of the cytoplasmic (CYTO)1 domains of their α and β subunits (2–5). Thus, these integrins are inactive when their CYTO domains are in proximity and are active when the domains are far apart (6). Binding of cytoplasmic proteins such as talin to the cytoplasmic domain of one subunit or the other may influence this equilibrium, providing additional opportunities for regulating the activation state of the integrin (7).

The role of transmembrane (TM) domains in the integrin activation process is currently not well understood. On the basis of in vacuo molecular modeling, Gottschalk et al. (8) proposed a model in which the α and β subunit TM domains interact extensively in both the active and inactive states, with the interaction pattern changing during activation. Other models based on electron microscopy and protein engineering propose that TM domains interact only in the inactive state and move far apart upon activation (4, 6).

The ability of the α and β TM domains to undergo homo-oligomeric interactions potentially adds a second level of regulation to the system. We have found that the α1b TM helix has a greater tendency to form a homodimer than to interact with the β3 TM helix in micelles. Schneider and Engelman (9, 10) reached a similar conclusion using the GALLEX assay. GALLEX is designed to determine the avidity of TM helix association via the suppression of bacterial β-galactosidase synthesis by two separately expressed LexA-TM-containing chimeric proteins. Depending on how it is configured, GALLEX can detect the hetero- or homo-dimerization of chimeric proteins. Using this construct, Schneider and Engelman (10) found that the α1b TM helix had a higher propensity to form homodimers than to dimerize with the β3 TM helix.

The avidity of interaction between the TM helices of α1b is relatively weak compared with that of constitutive homodimers such as glycoporphin A (GpA). This observation is consistent with the hypothesis that α1b TM helix interaction is involved in integrin regulation. Thus, if α/α or α/β TM helix interactions were too strong, the system might be locked into a single activation state. Indeed, mutations that increase the propen-

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‡ Present address: Center for Membrane Biology and Dept. of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, TX 77030

¶ To whom correspondence may be addressed: Dept. of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, 1009 Stellar-Chance Laboratories, 422 Curie Blvd., Philadelphia, PA 19104-6058. Tel.: 215-898-4590; Fax: 215-573-7229; E-mail: wdegrad@ mail.med.upenn.edu.

** To whom correspondence may be addressed: Hematology-Oncology Division, Dept. of Medicine, University of Pennsylvania School of Medicine, 914 EBB III/III, 421 Curie Blvd., Philadelphia, PA 19104-6058. Tel.: 215-573-3280; Fax: 215-573-7039; E-mail: bennetts@ mail.med.upenn.edu.

1 The abbreviations used are: CYTO, cytoplasmic; TM, transmembrane; CAT, chloramphenicol acetyltransferase; MOPS, 3-(N-morpholino)propanesulfonic acid; GpA, glycoporphin A; MBP, maltose-binding protein; WT, wild-type; ELISA, enzyme-linked immunosorbent assay; MES, 2-(N-morpholino)ethanesulfonic acid.
of the $\beta$-TM helix to form oligomers lead to $\alpha_{TM} \beta$ variants that are constitutively active (11).

The residues that determine the association of the $\alpha_{TM}$ and $\beta$-TM helices have not been thoroughly examined. It is noteworthy that integrin TM domains contain a GXGXG or a similar small residue-XXX-small residue motif. These motifs are a well-established framework for TM helix-helix interaction (12, 13), and Schneider and Engelman (10) have demonstrated that they are involved in mediating homomeric and heteromeric associations of integrin TM helices. Here, we examine the structural basis for $\alpha_{TM}$-TM domain dimerization. Using the TOXCAT system to measure helix-helix interaction in a lipid bilayer (14), our results confirm previous in vitro measurements of the dimerization of the $\alpha_{TM}$ TM helix in micelles.

Furthermore, we have identified the residues that constitute the dimerization interface and used this information to create a model for the structure of the dimer. These data should provide a powerful starting point for future investigations of the role of homo- and hetero-oligomerization of TM helices in integrin activation and clustering.

MATERIALS AND METHODS

Vectors and Strains—The expression vectors pccKAN, pccgpA-wt, and pccgpA-G83I, along with Escherichia coli strain MM39 (14), were kindly provided by Dr. Donald M. Engelman. The EcoRV restriction site between the TM region and the mAb gene in pccKAN was changed to a BamHI site. The vector fragment resulting from NheI-BamHI digestion was purified by gel electrophoresis. The gene encoding the $\alpha_{TM}$ TM domain, amplified from a full-length $\alpha_{TM}$ cDNA and flanked by NheI and BamHI sites, was ligated in-frame with the vector fragment. Subsequent Leu scanning and other point mutants were generated using a QuikChange mutagenesis kit (Stratagene). The sequences of the wild-type and mutant $\alpha_{TM}$ TM regions were confirmed by DNA sequencing. The resulting plasmids were transformed into E. coli MM39 cells for further analysis.

Expression of the Chimeric Protein in MM39—A freshly streaked single colony was inoculated into 5 ml of LB broth containing 100 $\mu$g/ml ampicillin and grown to an $A_{600}$ of 0.6 at 37 °C with vigorous shaking. One ml of the culture was chilled on ice, pelleted, and resuspended in 100 $\mu$l of LDS sample buffer (Invitrogen). After an incubation at 4 °C overnight, 18 $\mu$l of bacterial lysate in LDS buffer, with the addition of 2 $\mu$l of proteinase K (Invitrogen), was vortexed, boiled, and loaded onto a 10% NuPAGE Bis-Tris pre-cast gel in MOPS buffer system (Invitrogen). The separated proteins were transferred to nitrocellulose paper and immunoblotted with an anti-maltose-binding protein (MBP) monoclonal antibody (Sigma).

MalE Complementation Test—The glucose in M9 minimal medium plate was amended by 0.6% maltose as the only carbon source (14). Transformed MM39 cells were streaked onto these plates containing ampicillin and incubated for 2 days at 37 °C.

Protease Digestion of Spheroplasts—Preparation of spheroplasts was carried out following the protocol of Mendrola et al. (15). Proteinase K (Fisher Biotech) was then added to a final concentration of 50 $\mu$g/ml, with or without 1% Nonidet P-40 (Sigma).

Chloramphenicol Acetyltransferase (CAT) Enzyme-linked Immunosorbent Assay (ELISA)—One ml of bacterial culture, freshly grown to an $A_{600}$ of 0.6, was chilled on ice before being pelleted at 4 °C. The pelleted cells were resuspended and washed once using 0.6 ml of Tris-buffered saline buffer (20 mM Tris, pH 8, containing 100 mM NaCl and 2 mM EDTA). Upon resuspension in 0.5 ml of Tris-buffered saline on ice, the cells were treated with lysozyme at a final concentration of 100 $\mu$g/ml. Triton X-100 (to a final concentration of 10 mM), and bacterial protease inhibitor mixture (Sigma) were then added to the mixture. After a 30-min incubation, the mixture was sonicated briefly at 4 °C. On the following day, the supernatant was assayed for CAT concentration using a CAT ELISA kit (Roche Applied Science). Calibration of the assay was carried out using standards provided by the manufacturer. In each experiment, the strongly dimerizing GpA wild type (GpA-WT), the weakly-dimerizing GpA mutant G83I, and $\alpha_{TM}$ 968–989 were included for comparison. CAT ELISA results were expressed as a percentage of CAT induced by GpA-WT in the same experiment. Chimeric protein expression was quantified from immunoblots using a Personal Densiometer SI (Molecular Dynamics) and used to normalize CAT expression by various constructs.

Protein Expression and Purification—Single-site mutations were introduced into the expression vector of wild-type protein using the QuikChange kit (Stratagene) and confirmed by DNA sequencing. The wild-type and mutant TM-CYTO proteins were expressed in E. coli and purified as described previously (16). The purity of each protein was confirmed by reverse phase high pressure liquid chromatography.

Characterization of $\alpha_{TM}$ TM-CYTO Proteins—SDS-PAGE and analytical ultracentrifugation of the $\alpha_{TM}$ TM-CYTO proteins were carried out as described previously (16). For SDS-PAGE, methanol was removed from 5 $\mu$l of protein from a stock solution, and the protein was dissolved in 10 $\mu$l of SDS sample buffer. After vortexing for 1 min, the protein sample was heated to 80 °C for 10 min and loaded onto a 10% NuPAGE Bis-Tris pre-cast gel in MES running buffer (Invitrogen). The sample was electrophoresed at 4 °C for 3 h at a constant current of 27 mA before being stained by Gelcode (Pierce).

For equilibrium sedimentation experiments in a Beckman XL-I analytical ultracentrifuge at 25 °C, D$_2$O was added to the buffer (10 mM dodecylphosphocholine, 20 mM MOPS, 100 mM KCl, 1 mM MgCl$_2$, pH 7.4) to 50.34% to match the density of dodecylphosphocholine. The extinction coefficients were calculated as 16,500 M$^{-1}$cm$^{-1}$ for all the proteins characterized in this report (17). The molecular mass and partial specific volume of wild-type and three mutant TM-CYTO proteins were calculated as described previously (16): $\alpha_{TM}$ WT, 6071 Da and 0.7595 cm$^3$/g; G972L, 6073 Da and 0.7633 cm$^3$/g; and L980A, 5975 Da and 0.7566 cm$^3$/g. The obtained data sets were analyzed as described previously (16).

Perturbation Index Analysis—The perturbation index ($P_i$) for GpA was calculated based on the formalism of Truelein et al. (18) using the exhaustive mutagenesis of the dimer interface in Lemmon et al. (19). A single-site mutation was generated for the $\alpha_{TM}$ TOXCAT data, where the perturbation index reflects the mean fold change in observed activity relative to the 968–989 construct. The fold change was calculated using Equation 1.

$$P_i = 10 \exp \left[ \sum_{j=1}^{n} \log(x_j / x_{wt}) \right]$$

where $n$ is the number of mutations at a given position $i$, and $x_j$ is the TOXCAT activity at position $i$ for mutation $j$. $P_i$ values for GpA and $\alpha_{TM}$ were determined by fitting $P_i$ as a function of position to a continuous periodic function with a period of 4 residue/turn (20).

$\alpha_{TM}$ Structural Model—The atomic model of the $\alpha_{TM}$ dimer was constructed by searching an exhaustive grid of C$_2$-symmetric parallel dimer. The grid is populated with 10$^6$ states representing rigid body transformations of idealized helices. Structural fitness was evaluated using a novel scoring protocol that automatically incorporates mutagenesis data into the energy function. This protocol has been shown to effectively eliminate false positive predictions in other TM proteins. The details of this approach will be described elsewhere. Final predictions from the grid search were minimized in the constant valence force field using the Insight97.0/DISCOVER molecular modeling package (Biosym/MSI, San Diego, CA).

RESULTS

TM Domain of $\alpha_{TM}$ Subunit Dimerizes in Biological Membranes—Although the $\alpha_{TM}$ TM domain dimerizes in zwitterionic and acidic micelles (16), it has not been established that the domain also dimerizes in membrane bilayers. We used the TOXCAT system to address this question. In TOXCAT, a chimeric protein consisting of an amino-terminal ToxR DNA binding domain, an MBP domain, and an intervening TM domain results in ToxR dimerization, thereby driving transcription activation of a CAT reporter gene, making the E. coli resistant to chloramphenicol. In addition, there is a positive correlation between the level of CAT expression and activity and the extent of TM

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helix-mediated chimera dimerization. For example, inserting the strongly dimerizing TM domain of GpA induces a high level CAT activity, whereas a G83I mutation (GpA-G83I) in the TM domain impairs both dimer formation and CAT activity (12, 19).

The length of the inserted TM helix and the location of the junctions between the helix and MBP and ToxR' are important in defining the degree of CAT expression in the TOXCAT assay. Each defines the positioning of the TM helix in the membrane and influences the orientation of the fused domains with respect to the TM dimer interface. Accordingly, we identified the optimal length for an αIIb TM helix in this assay by incrementally deleting single residues from its amino-terminal end (Fig. 1). As shown by the immunoblot for MBP in whole cell lysates in Fig. 2A, decreasing the length of the TM helix had essentially no effect on the expression of the chimeric protein for six of the seven constructs, whereas the level of expression of the chimeric protein with the shortest TM helix containing residues 969–989 was decreased by ~50% compared with the other chimeras. Two methods were used to confirm that the chimeric proteins were actually inserted into the E. coli inner membrane. First, the ability of each construct to facilitate the growth of an MBP-deficient strain of E. coli when maltose was the sole carbon source (MalE complementation) indicated that the MBP domain of the chimeric protein was located in the bacterial periplasmic space (Fig. 2B). Second, the ability of proteinase K to digest the chimeric proteins in E. coli spheroplasts indicated that the proteins were not sequestered in the bacterial cytoplasm and therefore protected from protease digestion (Fig. 2C).

Whereas the length of the αIIb TM helix had essentially no effect on the extent of chimeric protein expression in E. coli, there were substantial differences in CAT expression as measured by CAT ELISA. As shown in Fig. 3, CAT expression induced by each αIIb TM helix was significantly greater than that induced by the poorly dimerizing GpA mutant G83I with p values ranging from <0.025 to < 0.004 (t test for unpaired samples). Nonetheless, the extent of CAT expression was very sensitive to the length of the αIIb TM insert, varying by more than 2- to 3-fold, depending on the length of the insert. The chimera containing αIIb 968–989 generated the highest ELISA signal and was used for subsequent experiments. Interestingly, this construct placed GXXGX in the same registry as in the originally reported GpA-TOXCAT construct (14). Thus, it is reasonable to compare the ELISA signals from the two TM helices. The signal from the αIIb TM was approximately half of that generated by GpA-WT, indicating that the integrin TM domain was a somewhat weaker dimerization motif.

Identification of a Dimerization Motif in the αIIb TM Helix—

![Fig. 1. αIIb TM domain sequences inserted into the TOXCAT plasmid. The seven αIIb TM domain sequences tested in the TOXCAT assay are aligned and identified by their starting and ending residue numbers as in the mature protein. The inserted TM sequences of GpA WT and GpA-G83I constructs are shown at the top for comparison. Glycine residues in the GXXGX motif are underlined in each sequence.](image)

![Fig. 2. The αIIb TM domain oligomerizes in the E. coli inner membrane.](image)

The experiments described above establish that the αIIb TM helix can dimerize in biological membranes. To identify the sequence motif responsible for dimerization, we performed site-directed mutagenesis, scanning the αIIb TM helix sequentially with Leu, Ala, Val, and Ile residues. The ability of these mutants to complement MBP deficiency (Fig. 4A) indicates that the mutations did not alter the topology of the chimeric proteins in the bacterial inner membrane. Furthermore, randomly selected mutants remained susceptible to protease digestion in spheroplasts, confirming that they were correctly oriented in the bacterial membrane (data not shown). Lastly, as shown in Fig. 4B, the expression levels of the mutants were comparable with that of αIIb 968–989.

The effect of single-site mutations on the dimerization of the αIIb TM helix, as measured by CAT ELISA, is summarized in Fig. 5. Mutation of 12 of 21 residues in the αIIb TM helix decreased CAT expression to some extent, indicating that the assay is highly sensitive to the native structure of the helix. In particular, mutation of Gly972 and Gly976 to Ala decreased dimerization, reducing CAT expression to nearly that of non-dimerizing GpA-G83I. These results are consistent with the established role of GXXGX motifs in mediating TM helix interactions (19, 21).
in $\alpha_{IIb}$ to Ala, Leu, or Ile resulted in substantial reductions in CAT activity. By contrast, the succeeding residue, Val\textsuperscript{973}, was less sensitive to the same mutations. Similarly, the position preceding Gly\textsuperscript{976} required a small side chain, either Gly or Ala, for efficient dimerization. Again, mutation of the succeeding residue had no or little effect.

Differences in the interaction of the GpA and $\alpha_{IIb}$ TM helices are further evident when one considers the effect of mutations at a position 4 residues distant from the second glycine of the GXXGX motif. In GpA, this residue is Thr, and mutation of this residue to Ala or other side chains disrupts dimer formation (19). The corresponding residue in the $\alpha_{IIb}$ TM helix is Leu\textsuperscript{980}. Surprisingly, mutating Leu\textsuperscript{980} to Ala resulted in a dramatic 2.5-fold increase in CAT expression. A smaller increase was observed when Leu\textsuperscript{980} was mutated to Val, whose side chain is intermediate in size between Leu and Ala. This finding suggests that Leu\textsuperscript{980} is also located at the dimerization interface, supporting the approximate 4-residue periodicity observed in other GXXXG-bearing TM sequences. In summary, the residues critical for dimerization of the $\alpha_{IIb}$ TM helix are XXVGXXGXXLXX, whereas the critical residues for GpA are XXXGXXGXXLXX.

Quantitative Analysis of the TOXCAT Data—To place the data from TOXCAT on a more quantitative footing, we defined a mean $P_i$, which defines the mean change in CAT activity for the various mutants. Fig. 6A illustrates the variation in $P_i$ versus the position of the mutations in comparison with that observed for the GpA TM helix. Both are qualitatively similar and show a pronounced 4-residue periodicity. The deviation from ideal $\alpha$-helical periodicity (3.6 residues/turn) reflects the $\sim$40° right-handed crossing angle between the two helices (19, 21). Fig. 6, B and C, shows fits of the data to a sine function with 4.0-residue periodicity (Equation 2),

$$P_i = A \sin \left( \frac{2\pi (x + \phi)}{4.0} \right) + B \cdot x + C$$

in which $x$ is the residue number, $\phi$ is the phase (in residues), $A$ is the amplitude, and $B$ and $C$ are the constants describing the linear drift as the chain moves away from the point of closest approach. The quality of fit deteriorated for periodicities greater or less than 4.0. Two features are noteworthy: first, the phase is offset by about 47 $\pm$ 8° for $\alpha_{IIb}$ relative to GpA, suggesting that the helices have been rotated by approximately this factor. Second, the degree of perturbation decreases beyond the first Gly residue in the GXXGX motif in $\alpha_{IIb}$ but not in GpA. This suggests that the region of maximal contact is located closer to the first Gly residue in $\alpha_{IIb}$ than in GpA.

We have also compared the $P_i$ profile for $\alpha_{IIb}$ with the kPROT scale (22), which describes the extent to which a given residue type prefers to engage in helix-helix interactions based on a sequence analysis of single spanning and polytopic membrane proteins (Fig. 6D). There is very good qualitative agreement between these two measures of interaction, particularly in the area surrounding the GXXGX motif.

Characterization of Mutant $\alpha_{IIb}$ TM-CYTO Proteins—In TOXCAT, the orientation of the $\alpha_{IIb}$ TM helix in the bacterial inner membrane is opposite to its orientation in eukaryotic membranes (14). Moreover, MBP and ToxR' are appended to its carboxyl and amino termini, respectively. Thus, to confirm that neither feature affected our results, we directly studied the oligomerization of a protein encompassing the wild-type $\alpha_{IIb}$ TM and CYTO domains and the same protein containing the mutations G972L, G976L, and L980A using SDS-PAGE and analytical ultracentrifugation.

SDS-PAGE has been used extensively as a simple, direct way to visualize the oligomerization of membrane proteins. In the

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**Fig. 3.** Effect of truncation of the $\alpha_{IIb}$ TM domain on CAT expression. CAT expression induced by the various $\alpha_{IIb}$ TM domains shown in Fig. 1 was determined by CAT ELISA and expressed as the percentage of that induced by GpA-WT. Data shown are the mean ± S.D. of four experiments.

**Fig. 4.** $\alpha_{IIb}$ TM domain mutants are oriented correctly in the E. coli inner membrane. A, MalE complementation assay of representative mutant constructs. pMAL-p2 and pMAL-c2 were included as positive and negative controls, respectively. B, level of expression of chimeric proteins containing selected $\alpha_{IIb}$ TM domain mutations as measured by immunoblotting for MBP.

However, mutations at sites flanking the critical GXXGX motif indicated that the interaction pattern is considerably different from that found in GpA. In GpA, the residues succeeding the critical glycines in GXXGX are important for dimerization, whereas the preceding residues are nonessential (12). Just the opposite was observed in $\alpha_{IIb}$; the residues preceding the critical glycines were important for dimerization, whereas the succeeding residues were found to be substantially more mutable. Thus, mutation of the residue preceding Gly\textsuperscript{972} $\alpha_{IIb}$
**Fig. 5.** Effect of scanning mutagenesis of the αIIb TM domain on CAT expression in the TOXCAT assay. The effect of single-site mutations at consecutive positions in the αIIb TM domain was quantitated by CAT ELISA and compared with the signal from the GpA-WT. Each residue was mutated to either Leu (yellow column), Ala (maroon), Val (green), or Ile (blue). The CAT ELISA results from the L980A and L980V were >GpA-WT. Data presented are the mean ± S.D. of 4–10 independent experiments.

**Fig. 6.** Mutational sensitivity profile of GpA versus αIIb. A, perturbation index ($P_i$) for residues 75–92 of the GpA TM helix (dashed line) and residues 968–988 of the αIIb TM helix (solid line). Profiles are aligned such that relative positions 5 and 9 correspond to the crucial interfacial glycines. The $P_i$ calculation is described under “Materials and Methods.” B and C show the best fit of a sinusoidal equation (Eq. 1) to the data. D compares the $P_i$ (red dashed line) of αIIb with a sequence-derived lipid propensity from the kPROT scale (solid black line). Plotted propensities have been inverted from the original scale (22) such that negative values indicate a preferential interaction with lipid over TM helix bundle interiors for that amino acid type.
**αIIb TM Helix Dimerization in Membranes**

Fig. 7. Study of the oligomerization of wild-type and mutant αIIb TM-CYTO proteins by SDS-PAGE and analytical ultracentrifugation. **A.** SDS-PAGE of the WT αIIb TM-CYTO protein and the G972L, G976L, and L980A mutants. 5 µg of purified protein, dissolved in 10 µl of SDS sample buffer, was applied to each lane. Electrophoresis was carried out for 3 h at a constant current of 27 mA. Molecular mass markers are shown on the right. Differences in the electrophoretic mobilities of the various proteins reflect differences in their molecular masses. **B,** calculated fractions of monomers for WT and mutant proteins as a function of protein/detergent ratio, based on dissociation constants derived from data spanning a peptide/detergent ratio from 0.2/1,000 to 70/1,000. These data show that the G976L mutation substantially decreased αIIb TM helix oligomerization and that the L980A mutation substantially increased αIIb TM helix oligomerization. In contrast to SDS-PAGE, we were also able to observe a significant disruptive effect of the G972L mutation.

**DISCUSSION**

The TOXCAT system is an optimized method for overcoming the technical difficulty of studying the homomeric associations of TM helices in biological membranes (14, 23) and has been used to investigate the homomeric interactions of the GpA TM domain (14), other naturally occurring TM helices such as ErbB4 (15) and VacA (24), and randomized recombinant peptides (12). Previously, we found that the homomeric association of proteins corresponding to the TM and CYTO domains of the integrin αIIbβ3 subunits is consistent with the data shown in Fig. 7A. Fig. 7B displays the calculated fraction of monomers for WT and the mutant proteins as a function of protein/detergent ratio, based on dissociation constants derived from data spanning a peptide/detergent ratio from 0.2/1,000 to 70/1,000. These data show that the G976L mutation substantially decreased αIIb TM helix oligomerization and that the L980A mutation substantially increased αIIb TM helix oligomerization. In contrast to SDS-PAGE, we were also able to observe a significant disruptive effect of the G972L mutation.

**MODELING OF αIIb TM DIMER**—The mutagenesis data were then used to construct an atomic model for the αIIb dimer (Fig. 8, A and B), using a novel method that exhaustively searches conformational space and explicitly considers the computed energies of the wild-type, disruptive, and permissive mutations. A family of structures was found that satisfies these mutational restraints. All have right-handed crossing angles, as was observed in GpA, with a value ranging from ~40° to 60°. This finding is consistent with the 4.0-residue periodicity found in the P1 profile. The face of the helix that is buried in the helix-helix interface of the model structures is constant and consistent with the analysis of the P1 profile; examination of Fig. 8C shows that the interface for αIIb is rotated by 50° relative to the GpA dimer interface.

SDS gel shown in Fig. 7A, wild-type αIIb TM-CYTO protein migrated as two discrete bands corresponding to monomer and the dimer species (16). The G972L mutant also migrated as two bands, but it was not apparent using this technique that the mutation affected dimerization. In contrast, the amount of G976L dimer was substantially decreased, and the amount of L980A dimer was substantially increased, as would be predicted from the TOXCAT assay.

Analytical ultracentrifugation provides a quantitative measurement of αIIb TM helix oligomerization. When dispersed into dodecylphosphocholine micelles at pH 7.4, the αIIb TM-CYTO protein is in a monomer-dimer equilibrium (16). The equilibrium radial concentration profiles for mutants G972L and G976L also fit best with a monomer-dimer equilibrium; consistent with the data shown in Fig. 7A, the data for the L980A mutant could be fit equally well to either a single dimeric species or to a monomer-dimer-trimer equilibrium with a very small trimer contribution, consistent with the faint trimer band observed in Fig. 7A. Fig. 7B displays the calculated fraction of monomers for WT and the mutant proteins as a function of protein/detergent ratio, based on dissociation constants derived from data spanning a peptide/detergent ratio from 0.2/1,000 to 70/1,000. These data show that the G976L mutation substantially decreased αIIb TM helix oligomerization and that the L980A mutation substantially increased αIIb TM helix oligomerization. In contrast to SDS-PAGE, we were also able to observe a significant disruptive effect of the G972L mutation.

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**MODELING OF αIIb TM DIMER**—The mutagenesis data were then used to construct an atomic model for the αIIb dimer (Fig. 8, A and B), using a novel method that exhaustively searches conformational space and explicitly considers the computed energies of the wild-type, disruptive, and permissive mutations. A family of structures was found that satisfies these mutational restraints. All have right-handed crossing angles, as was observed in GpA, with a value ranging from ~40° to 60°. This finding is consistent with the 4.0-residue periodicity found in the P1 profile. The face of the helix that is buried in the helix-helix interface of the model structures is constant and consistent with the analysis of the P1 profile; examination of Fig. 8C shows that the interface for αIIb is rotated by 50° relative to the GpA dimer interface.

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eromeric association of TM helices. Consistent with our previous report (16), Schneider and Engelman (10) detected both \( \alpha_{III} \) and \( \beta_3 \) oligomers. The strength of the \( \alpha_{III} \) signal was about one-fourth to one-third that observed for GpA. Given that the optimal location for fusion of the TM domain with the LexA domain was not investigated in this study, we consider this result to be in good agreement with our own finding using the TOXCAT system. Also, consistent with our earlier observations, Schneider and Engelman (10) confirmed that the \( \alpha_{III}/\beta_3 \) interaction is weaker than the corresponding \( \alpha_{III}/\alpha_{III} \) interaction.

We used scanning mutagenesis to identify residues that are present at the \( \alpha_{III} \) TM helix dimer interface. CAT expression was decreased to a variable extent, and the extent of the reduction correlated well with the distance from the predicted helix-helix interface. Thus, residues that were on the opposite face of the helix from the interaction site had little or no effect on dimerization. We found that replacing Gly\(^{972} \) or Gly\(^{976} \), the first and last residues of a potential GXXXG motif, either with a residue having a large hydrophobic side chain or with Ala, markedly disrupted \( \alpha_{III} \) TM dimerization. The GXXXG motif, first recognized as a framework for helix-helix association in studies of the GpA TM helix (19), has been consistently identified as the most overrepresented sequence motif in TM domain databases (13). In the NMR structure of the GpA TM helix dimer, GXXXG permits extensive backbone-backbone contacts at the dimer interface because a groove created by Gly\(^{79} \) and Gly\(^{83} \) of one GpA monomer packs against a ridge created by the side chains of Val\(^{80} \) and Val\(^{84} \) of the other (26).

The sequence arrangement GXXXG with Val, Ile, or Leu at positions \( i \pm 1 \) is also overrepresented in TM domain databases (13) and is a frequent packing motif in high affinity homomeric association selected from a randomized GpA TM helix sequence library (12). In the \( \alpha_{III} \) TM helix, Gly\(^{972} \) is preceded by an immutable Val, whereas Gly\(^{976} \) is preceded by Gly, which can be replaced by Ala, but not Val or Leu, without affecting \( \alpha_{III} \) TM helix dimerization. Thus, although GXXXG is a framework for helix-helix association, the diversity of residues neighboring GXXXG, exemplified by the \( \alpha_{III} \) and GpA helices, likely imposes specificity on this interaction.

Although the structural model shown in Fig. 8 explains the most perturbing mutations, it does not account for some aspects of the mutagenesis data shown in Fig. 5. For example, residues 981–984 are sensitive to mutations, even though the helices have diverged and are no longer in contact at this position. This may reflect a limitation of our method that uses straight helices in the modeling process. It is possible that gradual bending or a kink downstream from the second Gly in the GXXXG motif may extend the interfacial region toward the carboxyl terminus of the TM helix.

In addition, mutation of Leu\(^{980} \) to Ala increased TOXCAT activity severalfold, an effect detected by SDS-PAGE and analytical ultracentrifugation as well. Mutation of Leu\(^{980} \) to Val also increased TOXCAT activity, but to a lesser extent. It is noteworthy that Val is present in the corresponding position in the TM helix of \( \alpha_7 \), which is known to have a very high tendency to form homodimers (9). The analogous residue in the GpA TM helix is Thr\(^{87} \), and mutation of this Thr severely disrupts GpA TM dimerization (19). It is difficult to explain these observations by considering the ensemble of structures alone. L980A failed to significantly improve the computed energy of interaction between the helices. We considered the possibility that this mutation induces an alternate conformation. Indeed, calculations showed that the minimum energy conformation for this mutation was much more similar to the GpA structure. Thus, the nature of the residue at position 980 may be important for defining the rotation of the helices.

Previously, we reported that the reversible homomeric association of the \( \beta_3 \) TM domain may play a role in the regulation of both the \( \alpha_{III}/\beta_3 \) activation state and \( \alpha_{III}/\beta_3 \) clustering (11). Because the \( \alpha_{III} \) TM domain undergoes reversible dimerization, it is also possible that the homomeric association of this domain could regulate \( \alpha_{III}/\beta_3 \) function. Vinogradova et al. (5) have proposed that a clasp between the membrane-proximal regions of the \( \alpha_{III} \) and \( \beta_3 \) CYTO domains maintains \( \alpha_{III}/\beta_3 \) in an inactive state and that disruption of the clasp enables \( \alpha_{III}/\beta_3 \)

![Image](https://via.placeholder.com/150)
activation. Our previously reported data (11, 16) raise the possibility that disruption of the clasp is accompanied by the homomeric interaction of the αIIb and β3 TM domains, thereby providing a physiologic context for movement of the αIIb and β3 stalks (6). Consistent with this hypothesis, we have found that the affinity of the αIIb TM helix for dimerization is modest (16), making it amenable to regulation. In this study, we have provided a structural basis for this decreased affinity. Thus, although the αIIb TM helix makes use of the common GXXXG framework for helix-helix interaction, neighboring residues have evolved to re-engineer its dimerization interface, enabling it to subserve a specific and specialized function.

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Note Added in Proof—While this paper was undergoing review, two papers were published confirming that the length and phase of an inserted IM helix is important for optimal reporter gene expression in assays like TOXCAT (Ruan, W., Becker, V., Klingsmuller, U., and Langosch, D. (2004) J. Biol. Chem. 279, 3273–3279; Ruan, W., Lindner, E., and Langosch, D. (2004) Protein Sci. 13, 555–559).

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