Transmembrane Domain Helix Packing Stabilizes Integrin αIIbβ3 in the Low Affinity State

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SUMMARY

Regulated changes in the affinity of integrin adhesion receptors (“activation”) play an important role in numerous biological functions including hemostasis, the immune response, and cell migration. Physiological integrin activation is the result of conformational changes in the extracellular domain initiated by the binding of cytoplasmic proteins to integrin cytoplasmic domains. The conformational changes in the extracellular domain are likely caused by disruption of intersubunit interactions between the α and β transmembrane (TM) and cytoplasmic domains. Here, we reasoned that mutation of residues contributing to α/β interactions that stabilize the low affinity state should lead to integrin activation. Thus, we subjected the entire intracellular domain of the β3 integrin subunit to unbiased random mutagenesis and selected for activated mutants. Twenty five unique activating mutations were identified in the TM and membrane-proximal cytoplasmic domain. In contrast, no activating mutations were identified in the more distal cytoplasmic tail, suggesting that this region is dispensable for the maintenance of the inactive state. Among the 13 novel TM domain mutations that lead to integrin activation were several informative point mutations that, in combination with computational modeling, suggested the existence of a specific TM helix-helix packing interface that maintains the low affinity state. The interactions predicted by the model were used to identify additional activating mutations in both the α and β TM domains. We therefore propose that helical packing of the α and β TM domains forms a clasp that regulates integrin activation.
**INTRODUCTION**

Integrin heterodimers are essential for the development and functioning of multicellular animals as they mediate cell migration, cell adhesion, and can influence gene expression and cell proliferation(1). All integrin heterodimers are composed of single pass Type I transmembrane (TM) protein subunits, α and β. A central feature of these receptors is their capacity for rapid changes in their adhesive function mediated by changes in their ligand binding affinity, operationally defined here as “activation.” The prototypical integrin, platelet αIIbβ3, is activated through interactions of the cytoplasmic integrin tails (~ 20 and 47 residues for alpha and beta tails, respectively) with intracellular proteins, such as talin (2). These interactions initiate a long-range conformational change in the large extracellular domains (> 700 residues each) resulting in high affinity binding of fibrinogen, von Willebrand factor, and fibronectin and consequently platelet aggregation and adherence to the vessel wall (1).

Initial mutational studies suggested that a salt bridge between αIIbArg\textsuperscript{995} and β3Asp\textsuperscript{723} helps maintain the integrin in the low affinity state by forming part of an interactive face between α and β subunit cytoplasmic domains (3). Protein engineering studies from Springer’s lab have further advanced the idea that specific integrin α/β interactions maintain the low affinity conformation of the receptor. In particular, enforced association of either the C terminal regions of the extracellular domains (4) or of the cytoplasmic domains (5) leads to expression of an inactive integrin. Furthermore, during physiological integrin activation, changes in fluorescence resonance energy transfer between fluorophores joined to the αL and β2 cytoplasmic domains are
consistent with cytoplasmic domain separation(6). Finally, constraining the integrin $\alpha$ and $\beta$ transmembrane domains with inter-subunit disulfide bonds blocks integrin activation from inside the cell. However, this constraint does not prevent activation by divalent cations and antibodies that activate by binding to the extracellular domain(7). Taken together, these data suggest that default low affinity state of integrins is maintained by interactions between integrin $\alpha$ and $\beta$ subunits and that physiological activation occurs when cytoplasmic domain ligands, such as talin, disrupt these interactions.

Support for the idea that an Arg$^{995}$-Asp$^{723}$ salt bridge is an important constraint for the low affinity state comes from a nuclear magnetic resonance (NMR) spectroscopy study (8). Specifically, in isolated $\alpha$IIb and $\beta$3 cytoplasmic domain peptides the salt-bridge was identified as part of a helical interface between the membrane-proximal regions of $\alpha$ and $\beta$ subunits. Furthermore, this interaction was disrupted by talin, supporting the notion that disruption of this salt-bridge is involved in integrin activation(8). However, other groups have failed to observe intersubunit interactions in the membrane-proximal region suggesting that it is of relatively low affinity(9;10). Therefore, additional intracellular regions of the receptor could contribute $\alpha/\beta$ interactions to “clasp” it into the low affinity state. Indeed, $\textit{in vitro}$ model systems identified heterodimeric interactions between integrin $\alpha$ and $\beta$ transmembrane (TM) domains(11) and such interactions have also been suggested by molecular modeling(12;13) and disulfide cross-linking approaches(7). Mutation of residues contributing to $\alpha/\beta$ interactions that stabilize the low affinity state should lead to integrin activation. Thus, we subjected the entire intracellular domain (cytoplasmic plus TM domain, Fig. 1) of the $\beta$3 integrin subunit to unbiased random mutagenesis and selected
for activated mutants. Through this analysis, we have confirmed the importance of the membrane-proximal domain in maintenance of the low affinity state. In contrast, no activating mutations were identified in the more distal cytoplasmic tail suggesting that this region is dispensable for the maintenance of the inactive state. This approach also identified 13 novel TM domain mutations that lead to integrin activation. Amongst these were several informative point mutations that suggested the existence of a TM helix packing interface that maintains the low affinity state. Computational modeling indicates that these mutations disrupted intersubunit interactions either directly or indirectly by altering helical length/tilt angle. The interactions predicted in the model were used to create additional activating mutations in both the $\alpha$ and $\beta$ TM domains. We therefore propose that $\alpha$ and $\beta$ TM regions interact to form a clasp that constrains integrin activation.
EXPERIMENTAL PROCEDURES

Cell Culture, Cell Lines, and Reagents

Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS), 1% non-essential amino acids (Sigma), 50 units of penicillin/ml, and 50 µg of streptomycin sulfate/ml in a 37°C tissue culture incubator. αIIbC5 cells, an αIIb integrin subunit-expressing CHO cell line, were created by transfecting CHO cells with CDM8 vectors encoding the αIIb integrin subunit along with CD Neo plasmid. Stable colonies were selected with neomycin for two weeks. The pooled stable colonies were subsequently infected with an adenovirus encoding β3 integrin subunit, and sorted for single cells expressing αIIbβ3 integrin. Each individual stable clone was then examined for the absence of β3 subunit to make sure that the adenovirus had not integrated into the genome. The clones that do not express β3 subunit were then transiently transfected with a plasmid that encodes β3 and tested for expression of αIIbβ3. The clones that revealed normal expression of αIIbβ3 after transient expression of β3 subunit were then used for further studies. The anti-αIIbβ3 antibodies D57, PAC1, and Anti-LIBS6, as well as Ro43-5054, an αIIbβ3-specific peptide-mimetic competitive inhibitor, have been described (14-17). The D57 antibody was biotinylated with biotin-N-hydroxy-succinimide (Sigma) (B-D57) according to manufacturer’s instructions.
Construction of Random Mutagenic cDNA Libraries of β3 Integrin Subunit

To facilitate construction of random mutagenic cDNA libraries of transmembrane and cytoplasmic domains of β3 integrin subunit, an Sph I restriction enzyme site was created via a silent mutation at nucleotide 1899 (C->A) of β3 cDNA sequence using site-directed mutagenesis. The Sph I site-containing full-length β3 cDNA was then subcloned into a hygromycin-resistant derivative of CDM8 expression vector (Invitrogen), CDHYG, to create a wide-type β3-expressing vector, CDHYG3A.Sph I. Transiently transfection of this construct in an αIIb-expressing Chinese hamster ovary (CHO) cell line, αIIbC5, or co-transfection with wild-type αIIb cDNA in CHO cells, resulted in a protein product with identical properties to that of wild-type αIIbβ3. Specifically, the mutant species was immunoprecipitated by D-57 (not shown), an antibody against the extracellular domains of αIIbβ3 and was found to be in the low affinity state because it failed to bind PAC1, a monoclonal antibody specific for the high affinity state of αIIbβ3. Furthermore, the Sph I construct gained PAC1-binding ability with treatment of an αIIbβ3-activating antibody, Anti-LIBS6 but failed to bind PAC1 in the presence of Ro43-5054, an αIIbβ3-specific peptide-mimetic competitive inhibitor (not shown). Thus, this silent mutation does not interfere with the normal expression or function of β3 integrin subunit. The spiked mega primer method was chosen to generate random mutagenic cDNA libraries of β3 cytoplasmic and transmembrane domains (18). Specifically, the β3 transmembrane and cytoplasmic domains were divided into four “windows” of 66 nucleotides each with a nine nucleotides overlap between windows (Fig. 1). Spiked oligonucleotide primers corresponding to each window (Fig. 1) were synthesized with a contamination level of
1.5% of incorrect phosphoramidites, i.e., 0.5% of each of the three other bases. 

Polymerase chain reaction (PCR) was performed using the spiked primer (S.M.P., Fig. 2A) for each window and a 3’ reverse primer that contains an Xba I restriction enzyme site (R.P., Fig. 2A). For second round of PCR reaction (PCR-II, Fig. 2A), the PCR-I product (megaprimer) was used as a reverse primer. PCR reaction was performed with the megaprimer and a forward primer that contains the Sph I site (F.P., Fig. 2A). Products from PCR-II were then subcloned into Sph I-Xba I sites of CDHYG3A.Sph I. The efficiency of random mutagenesis was assessed by cDNA sequencing of 10 randomly-picked transformants from each window. cDNA sequencing indicated that about 70% of inserts in each window contained 1, 2, or 3 point mutations, which is in the reasonable range of efficiency for random mutagenesis. Theoretically, for a window of 66 nucleotides, 150,000 transformants should cover 99% of the possible one-base changes and ~75% of the possible two-base changes. Therefore, a random mutagenic cDNA library containing ~200,000 transformants for each window of transmembrane and cytoplasmic domains of β3 subunit was constructed by large-scale preparation of transformants, and used for subsequent transfection into αIIbC5 cells and identification of activating mutations in the β3 subunit.

**Site Directed Mutagenesis**

Site-directed mutations in both the αIIb and β3 subunits were generated using the QuikChange mutagenesis kit (Strategene) and pCDM8 vectors containing the integrin subunits. Mutants were confirmed by DNA sequencing.
Flow Cytometry

Random mutagenic cDNA library corresponding to each window of β3 transmembrane and cytoplasmic domains was transfected into αIIC5 cells by electroporation. Stable colonies were selected for 2 weeks in the presence of both hygromycin (750 µg/ml) and neomycin (750 µg/ml). About $5 \times 10^6$ cells from pooled stable colonies from each window were then individually sorted on a FACStar Plus (Becton Dickinson) using two-color flow cytometry. The biotinylated monoclonal antibody, B-D57 was used to detect expression of αIIbβ3, while PAC1, an activation specific, monoclonal IgM antibody was used to assess activation state of the αIIbβ3 integrin. For single cell sorting, the pooled stable colonies from each window (~ $5 \times 10^6$ cells/each window) were resuspended by treatment with trypsin and double-stained as described (19;20). Rare cells that exhibited both bright phycoerythrin staining (D57) and fluorescein isothiocyanate (FITC) staining (PAC1) were individually sorted into 96-well plates.

FACS analysis of isolated clonal cell lines was performed on a FACScan using both B-D57 and PAC1 antibodies as described (19;20). PAC1 staining in the presence of Ro43-5054 (2 µM) was used to estimate non-specific binding. In some cases, treatment with Anti-LIBS6 was used to estimate maximal PAC1 binding, since Anti-LIBS directly induces αIIbβ3 binding to PAC1 regardless of the status of cellular activation mechanism (15).

For re-transfection analysis, αIIbC5 cells were transfected with plasmid CDHYG3A. Sph I which encodes wild-type β3 or β3 that contains each identified mutation, using Lipofectamine (Life Technologies) following the manufacturer’s instructions. Forty-eight hours after transfection, the transfected cells were stained with B-D57 and PAC1 in the
presence and absence of Ro43-5054, and FACS analysis was performed as described above.

Cytometric analysis of the site directed mutations was done by co-transfection of the pCDM8 plasmids containing the αIIb and β3 subunits with Plus reagent and lipofectamine. (Life Technologies). Forty-eight hours after transfection, the transfected cells were stained with B-D57 and PAC1 in the presence and absence of Ro43-5054 and AntiLIBS6, and FACS analysis was performed as described above. Activation index was calculated by using the formula (F-F0)/(Fmax-F0), where F=PAC1 binding, F0= PAC1 binding in the presence of Ro43-5054, and Fmax=PAC1 binding in the presence of antiLIBS 6.

**Reverse Transscriptase-PCR, Subcloning, and Sequencing of β3 Integrin Subunit**

Total cellular RNA from each individual cell line was isolated using TRIzol (GIBCO/BRL). cDNA was synthesized using oligo-dT primer and the cDNA Cycle kit (Invitrogen), and PCR was performed following the manufacturer’s instructions. PCR products were digested with restriction enzymes Sph I and Xba I to create a fragment of ~550 bp. This Sph I-Xba I fragment was then subcloned into the Sph I-Xba I sites of CDHYG3A. Sph I vector and sequenced using primers derived from CDHYG3A. Sph I plasmid but outside of the Sph I-Xba I region. To eliminate possible PCR errors, at least four clones were sequenced for each mutant cell line.
**Computer Simulations**

The modeling procedures for TM helix oligomerization were described elsewhere (21). Briefly, the TMD sequences of integrin αIIb and β3 subunits were built into uniform α-helices having the backbone torsion angles of $\phi = -65^\circ$ and $\psi = -40^\circ$. Their TMD sequences of αIIb and β3 were aligned based on the glycosyl mapping data (22;23). Side chain rotamers were chosen using the backbone-dependent rotamer library program SCWRL (24). Four hundred potential helix packings were first generated using a Monte Carlo search procedure as described (21). The αIIb and β3 TMD dimeric structures were then filtered to remove the structures incompatible with the bilayer constraints. Then, we clustered the remaining structures by Cα RMS distances using NMRCLUSTER(25), which resulted two equally populated clusters: one with crossing point near the N-terminus and the other with a crossing point close to C-terminus. Both models were evaluated for consistency with the experimental results (see below).
RESULTS AND DISCUSSION

Random Mutagenesis Identifies Novel Integrin Activating Mutations in the TM and Membrane-Proximal Cytoplasmic Regions of β3

To generate the β3 random mutants, we used CDHYG3A.Sph I as a template and spiked oligonucleotide primers corresponding to four overlapping windows that cover the entire β3 intracellular domain (Fig. 1). Using the protocol outlined (see Methods and Fig. 2) a cDNA library containing ~200,000 transformants for each window was constructed by large-scale preparation of transformants, and used for subsequent transfection into an αIIb expressing cell line (αIIbC5 cells) and identification of activating mutations in the β3 subunit. We developed a library of stable cell lines and selected activated αIIbβ3 integrin mutants, by their binding to PAC1, an antibody specific for activated αIIbβ3(15) (Fig. 3A, R2). Using this approach we isolated 91 and 192 cell lines bearing activated αIIbβ3 from window I and II, respectively (Table I). In contrast, only 5 cell lines were isolated from either window III or window IV (Table I). Thus, mutations in the membrane-distal segments of the β3 cytoplasmic domain encoded by regions III and IV were less likely to activate αIIbβ3 integrin.

The cell lines expressing activated integrin αIIbβ3 could have arisen as a consequence of mutations within the target window, from adventitious mutations elsewhere in the integrin, or from mutations in genes that indirectly control integrin activation. To identify mutations in the target window that activated αIIbβ3, we sequenced cDNA clones obtained from β3 reverse transcriptase-PCR (RT-PCR) amplicons spanning this region. To confirm that sequenced mutations were responsible
for integrin activation, these amplicons were used to replace this region in wild type β3, the resulting plasmids were transfected into αIIbC5 cells and tested for PAC1 binding in the presence and absence of Ro43-5054. When the transiently expressed mutant integrin was able to bind PAC1, and this PAC1 binding was inhibited by Ro43-5054, we concluded that the mutation was responsible for the activation of αIIbβ3. A total of 25 unique mutations in the transmembrane and membrane-proximal region of cytoplasmic domain of β3 subunit were thus identified, and there were multiple examples of the same mutations present in different clonal cell lines (Fig. 4). Thirteen of them were found amino terminal of Lys716 in the presumptive transmembrane domain and twelve were in the membrane-proximal region of cytoplasmic domain (Fig. 5). No mutation was identified that only affected the region of the cytoplasmic domain C-terminal of Asp723 (Fig 5). The absence of activating mutations C terminal of Asp723 indicates that the C terminus of β3 is not involved in maintenance of the low affinity state of the integrin. The presence of such mutations in the membrane-proximal and transmembrane domains suggests that these sites are important in regulating integrin activation.

**Activating mutations are predicted to alter the TM helix length**

We had previously established the importance of the membrane-proximal domains of the α and β subunits in regulating integrin activation (20). In consequence, we focused our attention on the numerous mutations in the transmembrane domain. Many of these mutations would be predicted to shorten the β-subunit TM helix (i.e. fewer residues embedded in the membrane) by deletion of one or more residues or by introduction of a charged residue (Table II). Indeed, the majority of the mutations (9/13) would be
expected to disrupt or shorten the TM helix. Previous glycosylation-mapping studies(22;23) indicated that activating mutations in the membrane-proximal domain can shorten the TM helix. The present results extend those findings by showing that such mutations throughout the TM helix activate the integrin.

**Mutagenesis of predicted TM packing residues activates αIIbβ3**

A subset (4/13) of the membrane-embedded activating mutations had no obvious effect on TM helical length. This suggests that the TM segments help stabilize the inactive state through sequence-specific interactions. To investigate this possibility, we used a Monte Carlo simulation method(21) to produce a first approximation of the intersubunit packing of integrin TM domains. We caution that this method assumes idealized rigid α helices and disregards potential changes in membrane insertion. However, several reports indicate that this protocol does yield models that conform well to known the structural and functional data(26-29). Here, the resulting output predicted two alternative structures, one with the TM helices packing near the C-termini (Structure A) and another with the helices packing close to the N-termini (Structure B) (Fig. 6).

The random mutagenesis data focused our attention on Structure A since all of the uncharged activating point mutations clustered in the C-terminal region of the β3 TM domain (G708S, A711T+T720A, I714T) (Fig. 5). Indeed, each of these mutations affected a residue predicted to be a helical contact in Structure A (Fig. 6B). Three additional site directed mutants were employed to test the hypothesis that the interhelical interface predicted in Structure A functioned as a clasp to maintain the integrin in the low affinity state (*vida infra*).
The β3(G708S) mutation was strongly activating. Previous studies (30) showed that introduction of an Asn at this position activated αIIbβ3 and suggested that it did so by forming hydrogen bonds that favor β3 homoligomerization. The Ser substitution could also, in principle, lead to enhanced β3-β3 interactions through such a mechanism. However, the weakly polar nature of the Ser side chain coupled with the observation that insertion of the bulky aliphatic Ile residue at this position (β3(G708I) mutation) was strongly activating suggest that a Gly residue is strictly required at this position for efficient α/β TM packing. (Fig. 7A, B, C).

The packing motif in Structure A allowed us to identify an additional Van der Waals packing residue that helps stabilize the inactive state since the apolar to apolar I704A mutation resulted in an activated integrin (Fig. 7B, C). As well, the Structure A model predicted that αIIb TM residues would pack against the identified β3 residues. Accordingly, we substituted a bulky Ile residue for αIIb Thr981, the residue predicted to pack against β3 Gly708 (Fig. 6B). The observation that this mutant activated αIIbβ3 supports the packing of Thr981 against Gly708 – an interaction that would be stabilized by both Van der Waals packing and a potential Cα/hydroxyl hydrogen bond. Overall, both random mutagenesis and site-directed mutagenesis (Fig. 7C) support the hypothesis that the specific packing of the C-terminal portions of αIIb and β3 transmembrane helices against each other maintains the low affinity state of integrin αIIbβ3.

Activation by mutagenically dissociating the integrin TM helices is consistent with reports that suggest that activation is associated with separation of the cytoplasmic domains(2;6). However, the unbiased random mutagenesis approach identified a preponderance of activating TM domain mutations predicted to shorten the β3 TM
domain indicating that TM helix shortening can also lead to integrin activation. In agreement with this mechanism, previous glycosylation mapping studies suggested that the membrane-proximal domains of the α and β subunits can reside with the membrane bilayers and that certain activating mutations in this region\(^{22;23}\) result in a shortened TM domain.

How might shortening the TM domain lead to disruption of intracellular α/β interactions and consequent integrin activation? In order to avoid hydrophobic mismatch with the fixed width of the membrane bilayer, a shortened TM helix would change its membrane tilt angle and associated register with neighboring helices\(^{31}\). Since helix-helix packing is dependent on specific crossing angles and specific in-register side chain arrays, changes to TM helical length would break the proposed clasp. Previous observations showing that αIIb sequences with a shortened TM segment\(^7\) lost the ability to induce a periodic disulfide crosslinking pattern of the αIIb and β3 transmembrane helices support this notion. In addition, the inactive state intersubunit interactions at membrane-proximal level could cooperate with the TM packing to help maintain the α/β association. Importantly, talin has been shown to bind to the membrane-proximal region\(^{8;32;33}\)– an event that appears to be important for αIIbβ3 activation\(^{33}\). This interaction could contribute to the physiological activation of integrins in two separate but related ways. First, one consequence of talin binding to this region would be to displace the membrane-proximal domain from the bilayer, thereby shortening the TM domain. As noted above this process would likely lead to separation of the intracellular domains. Talin binding could also directly disrupt the cooperative
membrane proximal/TM clasp by breaking the Arg$^{995}$-Asp$^{723}$ salt bridge(8) and associated intersubunit interactions.

Our findings also have implications for integrin-mediated biochemical signals that control cell shape, cell migration, proliferation, and survival. The capacity of integrins to deliver such signals depends on their occupancy with resultant conformational change in the integrin(34) in combination with receptor clustering(35;36). These conformational changes are associated with a dramatic change in the quaternary structure of the integrin, resulting in a switch from a “bent” conformation observed in the crystal structure(37) to an extended one(38) that features a C-terminal separation(39) that would disrupt the TM helical packing proposed here. This disruption could lead to the changes in the intracellular interactions of occupied integrins manifested by focal adhesion targeting and trans-dominant inhibition(34;40). Furthermore, the work of Li et al shows that isolated integrin α and β TM peptides homoligomerize(10)–a process that could contribute to integrin clustering. This suggests a sequential model in which α/β transmembrane separation might then be followed by homoligomerization to favor receptor clustering(10;41). Strikingly, Li et al.’s mutational studies identify β3 Gly$^{708}$ as an important packing residue for β3 homoligomerization and suggest that homoligomerization may occur after TM separation(30). Our findings indicating that Gly$^{708}$ also participates in an α/β interaction that regulates activation lends additional credence to this hypothesis and suggests that the TM helix packing interface proposed here is a nexus for bidirectional transmembrane signaling through integrins.
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FIGURE LEGENDS

Fig. 1. “Windows” for random mutagenic analysis of transmembrane and cytoplasmic domains of the β3 integrin subunit. The coding region for the β3 transmembrane and cytoplasmic domains was divided into four “windows”. Each window contains 66 nucleotides corresponding to the amino acid sequences with a nine nucleotides overlap between windows.

Fig. 2. Polymerase chain reactions for construction of random mutagenic cDNA libraries of the β3 transmembrane and cytoplasmic domain. A. Schematic presentation of the partial β3 cDNA sequence. F.P. and R.P. represent forward primer and reverse primer, respectively, that were derived from the β3 sequence and used in PCR reactions described in B and C. * represents the stop codon of β3 sequence. Newly created Sph I site in the β3 sequence and Xba I site present in the vector sequence are indicated. B. Schematic presentation of two consecutive PCR reactions for construction of random mutagenic cDNA library for each “window” of the β3 transmembrane and cytoplasmic domains. The position of each “window” is indicated. C. PCR product (PCR-I in Fig. 3B) for each “window”. PCR-I reaction was performed as described in “Materials and Methods”. 10 µl of PCR product from each “window” was separated on 1 % agarose gel and stained with ethidium bromide. The sizes of cDNAs are indicated.

Fig. 3. Isolation and characterization of clonal cell lines that express activated αIIbβ3 integrin mutants. A. Pooled stable colonies transfected with each random
mutagenic cDNA library of the β3 transmembrane and cytoplasmic domains were stained with antibodies BD-57 and PAC1 as described in “Materials and Methods”. The cells that revealed both BD-57 and PAC1 staining (gate R2) were single cell-sorted into 96-well plates filled with selection medium. B. FACS analysis of A5 cells and a representative clonal cell line that expresses activating αIIbβ3 integrin mutant.

Fig. 4. Identified activating mutations in the β3 transmembrane and cytoplasmic domains. The activating mutations identified in the β3 transmembrane and cytoplasmic domains are listed separately. Number of events for each mutation is indicated in brackets. & represents different mutations identified in the same clone.

Fig. 5. Frequency of activating mutations in the β3 transmembrane and cytoplasmic domains. Frequency of activating mutations is plotted against each amino acid residue in the β3 transmembrane and cytoplasmic domains. Amino acid sequence of the β3 transmembrane and cytoplasmic domains is illustrated.

Fig. 6. Dimer models of integrin αII and β3 subunits’ TMD. A. Structure A) Calculated model shows a left-handed helix-crossing angle of 30° with crossing point near C-terminus. Packing residues are colored on the dimer model and indicated on the TMD sequence. From the packing residues, G708 mediates the most-close packing. Alternative packing Structure B) Model structure shows a left-handed helix-crossing angle of 40° with crossing point near N-terminus. B. Interhelical residues involved in Structure A. αIIb residues are highlighted in green, β3 residues are highlighted in red.
**Fig. 7. Site direct mutagenesis supports simulation Structure A as the inactive state conformation.** A. CHO cells were transfected with plasmid coding for wt and mutant versions of αIIb and β3. Shown here a representative dot plots of FACS analysis for cells transfected with wildtype and G708I mutant integrin. Harvested cells were stained for integrin expression (D-57) and activated αIIbβ3 (PAC1). B. Activation indices for wildtype and site-directed mutants β3(I704A), β3(G708I), αIIb(T981I) were calculated by measuring PAC1 staining in the presence or absence of Anti-LIBS6 and Ro43-5054 (see Methods). C. Random and site-directed activating mutants map to interhelical residues outlined in computer simulation Structure A (underlined amino acids). Sites of activating random and site-directed mutations are shown with rectangles and circles respectively.
| Window | Cell Lines Isolated | Activation Re-Confirmed | Unique Mutations Identified |
|--------|---------------------|-------------------------|-----------------------------|
| I      | 288                 | 91                      | 7                           |
| II     | 468                 | 192                     | 22                          |
| III    | 65                  | 5                       | 0                           |
| IV     | 30                  | 0                       | 0                           |
### Table II. Activating β3 Transmembrane Mutations

Mutations that directly shorten the TM helix  
(Loop-out and truncation mutations)

\[\Delta(\text{Val}^{695}\text{Val}^{696})\]  
\[\text{Leu}^{697}\rightarrow\text{Pro} \& \Delta(\text{Leu}^{698}\text{Ser}^{699}\text{Val}^{700})\]  
\[\Delta(\text{Ser}^{699}\text{Val}^{700})\]  
\[\text{Leu}^{709}\rightarrow\text{His} \& \Delta(\text{Leu}^{697}\text{Leu}^{698})\]  
\[\text{Trp}^{715}\rightarrow\text{Stop}\]

Mutations that are predicted to shorten the TM helix  
(Apolar to charged mutations)

\[\text{Leu}^{697}\rightarrow\text{Arg}\]
\[\text{Leu}^{712}\rightarrow\text{Arg}\]
\[\text{Leu}^{713}\rightarrow\text{His}\]
\[\text{Trp}^{715}\rightarrow\text{Arg}\]

Mutations that are not predicted to shorten the TM helix

\[\text{Gly}^{708}\rightarrow\text{Ser}\]
\[\text{Ala}^{711}\rightarrow\text{Thr} \& \text{Thr}^{720}\rightarrow\text{Ala}\]
\[\text{Ile}^{714}\rightarrow\text{Thr}\]
\[\text{Ile}^{714}\rightarrow\text{Thr} \& \Delta(\text{Ile}^{721})\]
Figure 1

Transmembrane Domain

Cytoplasmic Domain

693 700 710 720 730 740 750 760

GPDILVLLSVMGAILLIGLALLIWKLLIITHDRKEAFKFEERARAKWDANNPLYKEATSTFTNITYRG

I II III IV

Windows
Figure 2A

Partial β3 cDNA

```
5'  F.P.  Sph I  Xba I  3'  
/   ↓    ↓     ↓       
1899  *  2370    R.P.
```

Figure 2B

PCRs

```
5'  Sph I  S.M.P.  Window  Xba I  3'  
\   ↓     ↓    I     II    III    IV    ↓   

PCR I:  

PCR II:  Sph I

W-I
W-II
W-III
W-IV
```
Figure 2C
Figure 3

A.

B.

A5 Cell

Mutant Cell
Figure 4

| Transmembrane Domain | Cytoplasmic Domain |
|----------------------|--------------------|
| Membrane Proximal    | Membrane Distal    |

| Position | Mutation Description | Number |
|----------|----------------------|--------|
| 693      | ΔVal<sup>69</sup>Val<sup>69</sup> | 1      |
| 700      | Leu<sup>697</sup>→Arg | 2      |
| 710      | Δ(Leu<sup>699</sup>Ser<sup>699</sup>Val<sup>700</sup>) | 1      |
| 720      | ΔSer<sup>699</sup>Val<sup>700</sup> | 1      |
| 730      | Gly<sup>700</sup>→Ser | 2      |
| 740      | Leu<sup>709</sup>→His & Δ(Leu<sup>697</sup>Leu<sup>699</sup>) | 1      |
| 750      | Ala<sup>711</sup>→Thr & Thr<sup>720</sup>→Ala | 2      |
| 760      | Leu<sup>712</sup>→Arg | 1      |
|          | Leu<sup>712</sup>→His | 1      |
|          | Ile<sup>714</sup>→Thr | 1      |
|          | Ile<sup>714</sup>→Thr & Δ(Ile<sup>714</sup>) | 1      |
|          | Trp<sup>714</sup>→Arg | 7      |
|          | Trp<sup>714</sup>→Stop | 20     |
|          | Lys<sup>716</sup>→Asn | 1      |
|          | Leu<sup>717</sup>→Arg | 1      |
|          | Leu<sup>717</sup>→His | 1      |
|          | Ile<sup>718</sup>→Leu | 2      |
|          | Ile<sup>719</sup>→Asn & Asp<sup>723</sup>→Asn | 1      |
|          | ΔIle<sup>720</sup>Thr<sup>720</sup>Ile<sup>721</sup> | 3      |
|          | Thr<sup>720</sup>→Pro | 1      |
|          | Ile<sup>721</sup>→Thr | 2      |
|          | ΔIle<sup>721</sup> | 1      |
|          | Leu<sup>713</sup>→Ala & Ile<sup>721</sup>→Ser | 1      |
|          | Leu<sup>712</sup>→Met & Asp<sup>723</sup>→Glu | 1      |
|          | Asp<sup>714</sup>→His | 1      |

&: different mutations in the same clone
(): Number of events
Figure 5
Figure 6

A

Structure A

Structure B

B

Packing Motif of Structure A

\[
\begin{align*}
\alpha_{IIb} & : \text{WWVVLGVGILG}^{967}\text{IILITILV}^{989}\text{LAMWK} \\
\beta_3 & : \text{LVVLLSVMGA}^{694}\text{IILLIGLA}^{716}\text{ALLIWK}
\end{align*}
\]
Figure 7C

\[ \alpha_{\text{IIIb}} WWVLVGVLGGLLLLILVLMAMWK \]
\[ \beta_3 \text{ LVVLLSVMGAALLLLALLWK} \]