Disrupting integrin transmembrane domain heterodimerization increases ligand binding affinity, not valency or clustering

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Residues important in the interaction between the 23-residue transmembrane (TM) domains of the integrin αIIb- and β3-subunits were identified by mutating each non-Leu residue to Leu. Leu substitutions of αIIb at G972, G976, and T981, and of β3 at I693 and G708, increased ligand binding. Substitutions with other amino acids at αIIbG972 and β3G708 could also increase ligand binding. The results are consistent with and extend the helical interface between the integrin α- and β-subunit TM domains previously defined by cysteine scanning and disulfide bond formation. We differentiated between affinity- and valency-based modes of activation by TM domain mutations. The mutant αIIb W967C forms disulfide-linked αIIb-subunits within a (αIIbβ3)2 tetramer. This tetramer behaved as an ideal model for the valency mode of regulation, because it exhibited significantly increased binding to multivalent but not monovalent ligands and basally retained the bent conformation. By contrast, the activating Leu mutants showed increased binding to the monovalent, ligand-mimetic PAC-1 Fab and increased exposure of ligand-induced binding site fides that maintained association of αIIb and β3 TM domains and showed that this prevented activation. Here we use the converse approach of Leu scanning to perturb TM domain association and screen for activating mutations. We show that substitution with Leu of residues located at the heterodimeric interface defined in the previous study activates ligand binding, suggesting that the activating effect of the TM mutations is a consequence of the disruption of the helical interface between the αIIb and β3 TM domains. We demonstrate that activation is a consequence of conformational changes in the extracellular domain and increased affinity for monomeric ligand. Contrasting results are obtained by crossing integrin TM domains to form (αIIbβ3)2 tetramers. Tetramers show no extracellular domain conformational change and increased multimeric, but not monomeric, affinity for ligand.

Materials and Methods

Plasmid Construction and Transient Transfection. Plasmids coding for full-length human αIIb and β3 were subcloned into pEF/V5-HisA or pcDNA3.1/Myc-His(+) as described in ref. 5. Mutants were made by using site-directed mutagenesis with the QuikChange kit (Stratagene), and DNA sequences were confirmed before being transfected into 293T cells by using calcium phosphate precipitation (26).

Soluble Fibrinogen, PAC-1 IgM, and PAC-1 Fab Binding Assay. Ligand-mimetic IgM PAC-1 Fab was from Becton Dickinson, PAC-1 Fab was a generous gift from S. Shattil (27), and the activating anti-αIIb mAb PT25-2 was a generous gift from M. Handa (28). Transiently transfected 293T cells or stably transfected Chinese hamster ovary (CHO) cells in 20 mM Hepes with 150 mM NaCl (HBS) supplemented with 5.5 mM glucose and 1% BSA were

Abbreviations: TM, transmembrane; FITC, fluorescein isothiocyanate; CHO, Chinese hamster ovary; MFI, mean fluorescence intensity; LIBS, ligand-induced binding site.

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incubated with fluorescein-labeled fibrinogen (30 μg/ml), PAC-1 IgM (10 μg/ml), or PAC-1 Fab (10 μg/ml) in the presence of 1 mM EDTA, 5 mM Ca²⁺, or 1 mM Mn²⁺ plus 10 μg/ml mAb PT25-2 at room temperature for 30 min. For fibrinogen binding, cells were coincubated with Cy3 dye-conjugated anti-β₃ mAb AP3 (Cy3-AP3) to a final concentration of 10 μg/ml PT25-2 at room temperature for 30 min. For PAC-1 binding, cells were washed once and suspended with HBS with 1 mM Ca²⁺ and 10 μg/ml FITC-conjugated anti-mouse IgM, and 10 μg/ml Cy3-AP3. For PAC-1 Fab binding, cells were washed once and suspended with HBS with 1 mM Ca²⁺ and 10 μg/ml FITC-conjugated anti-mouse IgG. Cells were incubated at 0°C for 30 min and then subjected to flow cytometry. Binding is presented as specific mean fluorescence intensity (MFI) of FITC-conjugated anti-mouse IgM or fibrinogen expressed as a percentage of the MFI of Cy3-AP3.

**LIBS Expression.** The anti-LIBS mAb AP5 was from the Fifth International Leukocyte Workshop (29), LIBS1, LIBS6 (30), and PMI-1 (31) were from M. H. Ginsberg, and D3 was from L. K. Jennings (32). LIBS expression was measured as described in ref. 33. In brief, cells in HBS supplemented with 5.5 mM glucose and 1% BSA were incubated in the presence of 5 mM Ca²⁺ or 1 mM Mn²⁺ plus 100 μM GRRGDSP peptide ligand for 30 min at room temperature and then with LIBS mAbs AP5, LIBS1, LIBS6, D3, and PMI-1 (10 μg/ml) at 0°C for 30 min, followed by staining with FITC-conjugated anti-mouse IgG and flow cytometry. LIBS binding is presented as MFI of FITC-conjugated anti-mouse IgG as a percentage of the MFI of Cy3-AP3.

**Integrin Clustering and Confocal Microscopy.** CHO cells stably expressing wild-type α₃β₃ and mutant α₃β₃ (11) were kindly provided by J. Bennett (University of Pennsylvania, Philadelphia). Cells in culture medium were incubated with Cy3-AP3 (10 μg/ml) at 37°C for 30 min in the absence or presence of rabbit polyclonal anti-mouse IgG (10 μg/ml) and cytochalasin-D (400 nM), followed by fixation with 3.7% formaldehyde. Confocal imaging was performed with a Bio-Rad Radiance 2000 laser-scanning confocal system coupled to an Olympus BX50WI microscope and a ×100 water immersion objective. All image processing was performed with OPENLAB software (Improvision, Lexington, MA). For each condition, the integrin cell-surface distribution patterns of at least 200 cells, from randomly selected fields, were scored as being either “even” (i.e., exhibiting predominantly even cell-surface distribution) or “macroclustered” (i.e., exhibiting significant levels of uneven or patchy cell-surface distribution).

**Results**

**Leu Scanning Shows That Mutations of Several Residues at the Helix-Helix Interface Activate Ligand Binding.** Studies on glycoporphin A TM domains have suggested that Leu substitutions on average disrupt formation of TM homodimers more than substitutions with other hydrophobic residues studied, i.e., Ala, Cys, Val, Ile, and Met (34). We therefore serially mutated all non-Leu residues in the 23 residue integrin αIIb and β₃ TM domains to Leu (Fig. 1A). Mutant αIIB-subunits were coexpressed with wild-type β₃-subunits, mutant β₃-subunits were coexpressed with wild-type αIIB-subunits, and both were screened for enhanced binding to the soluble, multivalent, ligand-mimetic PAC-1 IgM (Fig. 1 B and C). Under activating conditions (in the presence of Mn²⁺ and PT25-2), all mutants bound PAC-1 IgM at levels similar to wild type. In the absence of activation, i.e., in Ca²⁺, wild-type αIIBβ₃ and most mutants exhibited essentially no PAC-1 binding. By contrast, three mutants, αIIBG972L, αIIBG976L, and β₃G708L, bound PAC-1 IgM at nearly maximal levels, and two others, αIIBT981L and β₃I693L, bound partially. We extended...
our previously generated structural model from crosslinking studies (8) to the C termini of the TM domains assuming ideal α-helices and found that all of these activating residues, with the exception of αβ\(_{\text{IIb}}\)T981L, map to the αβ-β3 interface (Fig. 1D). The inconsistency regarding αβ\(_{\text{IIb}}\)T981L may suggest deviation from ideal helices associated with an interacting coiled-coil structure; however, it is noteworthy that mutant αβ\(_{\text{IIb}}\)T981L was only partly activating and, in contrast to all other mutants, was expressed poorly on the cell surface relative to wild type (data not shown).

**Dependence of Activation on the Specific Amino Acid Substitution.**

We selected two residues that were activated when mutated to Leu, αβ\(_{\text{IIb}}\)G972 and β3G708, for additional mutagenesis studies. These two residues were mutated to Ala, Cys, or Asn. At residue αβ\(_{\text{IIb}}\)G972, the substitutions G972A and G972C were not activating, showing that in contrast to Leu, smaller hydrophobic side chains were not activating (Fig. 2A and B). However, the more bulky, polar G972N substitution was activating, although somewhat less than the G972L substitution. At residue β3G708, the G708A and G708N substitutions were not activating, whereas the G708C mutation was activating, although less so than G708L (Fig. 2).

**Disulfide-Bonded αβ\(_{\text{IIb}}\)β3 Tetramer Is an Ideal Model for Studying Valency Effect.**

We previously showed that cysteine mutant αβ\(_{\text{IIb}}\)W967C spontaneously and efficiently formed a homodimeric disulfide bond when cotransfected with the β3-subunit, thereby forming a covalently linked cell-surface integrin tetramer (αβ\(_{\text{IIb}}\)W967C/β3)\(_{\text{IIb}}\) (8) (Fig. 3A). In 293T transfectants, the tetramer could be recognized by all tested mAbs to constitutive αβ\(_{\text{IIb}}\)β3 epitopes, including AP3, 7E3, 10E5, HA5, and AP2 (data not shown), indicating a native fold for this disulfide-linked integrin.

Soluble fibrinogen (Fig. 3B) and PAC-1 IgM (Fig. 3C) bound αβ\(_{\text{IIb}}\)W967C/β3 more efficiently than wild-type αβ\(_{\text{IIb}}\)β3 basally in Ca\(^{2+}\), as well as after activation with Mn\(^{2+}\), PT25-2/Ca\(^{2+}\), or PT25-2/Mn\(^{2+}\). Notably, in Ca\(^{2+}\) the efficiency of αβ\(_{\text{IIb}}\)W967C/β3 tetramer binding to PAC-1 IgM, with potentially 10 binding sites per molecule, was enhanced more than binding to fibrinogen, a divergent ligand. This result suggests that the tetrameric mutant is highly sensitive to the valency of the ligand. Indeed, when binding to the monovalent ligand PAC-1 Fab was assayed under basal conditions in Ca\(^{2+}\), no binding to αβ\(_{\text{IIb}}\)W967C/β3 tetramer was found (Fig. 4).

LIBS mAbs were used to determine the overall conformation of the αβ\(_{\text{IIb}}\)W967C/β3 mutant. All of the LIBS mAbs bound poorly to the tetrameric mutant in Ca\(^{2+}\), similarly to wild type (Fig. 3D), suggesting that the mutant receptor is basally in the bent, resting conformation. This finding, together with the lack of effect on monomeric affinity for PAC-1 Fab, demonstrates that the observed increased binding to PAC-1 IgM and fibrinogen under basal conditions by αβ\(_{\text{IIb}}\)W967C/β3 (Fig. 3B and C) must be attributed to an effect on valency. Thus, this mutant represents an ideal model of valency-regulated ligand binding.

**Monovalent Ligand-Mimetic PAC-1 Fab Binding Shows That the Activating Effect of Leu Mutations of the Integrin TM Domains Is Due to Increased Affinity.**

As described above, the αβ\(_{\text{IIb}}\)W967C mutant did not bind to monomeric PAC-1 Fab in Ca\(^{2+}\) (Fig. 4). By contrast,
the activating Leu mutations, α1hG972L, α1hG976L, and β3G708L, all bound PAC-1 Fab efficiently (Fig. 4). These mutants were activated similarly to the β3N305T mutant (Fig. 4), which introduces an N-glycosylation site into the interface between the β3 I-like and hybrid domains and stabilizes the open, high-affinity conformation of the integrin headpiece (9, 33, 35). High-affinity monomeric binding was also induced by mutation to GAAKR of the GFFKR motif at the junction between the α1b TM and cytoplasmic domains (Fig. 4). Mutation of this motif has long been known to activate ligand binding (36) and has been shown to induce integrin TM domain separation (8) and cytoplasmic domain separation (7). Overall, the data suggest that the activating effect of the α1hG972L, α1hG976L, and β3G708L mutants is due to increased affinity rather than valency. As a further control, the α1hG975L and β3G708N mutants, which did not increase multimeric affinity, also did not detectably increase monomeric affinity in 293T transfectants (Fig. 4).

**TM Domain Mutations That Increase Monomeric Affinity Partially Increase LIBS Epitope Expression.** The mutant receptors were characterized for binding to two representative LIBS mAbs. Under basal conditions, the activating Leu mutants, α1hG972L, α1hG976L, and β3G708L, as well as the GAAKR mutant, but not the nonactivating α1hG975L mutant, bound LIBS6 mAb to essentially maximal levels, i.e., comparably to binding in the presence of Mn2+ and RGD (Fig. 5A). The same activating Leu mutants and the GAAKR mutant, but not α1hG975L, also elevated binding to mAb D3 under basal conditions, as shown by comparison with wild type. However, in contrast to the LIBS6 epitope, expression of the D3 epitope on all mutants could still be significantly increased by Mn2+ and RGD (Fig. 5B). The same results were obtained in EDTA as in Ca2+, excluding a contribution by ligands in the culture medium to LIBS epitope expression. By contrast to the effect of activating TM domain mutations on D3 epitope exposure, the wedge mutant β3N305T bound D3 and other anti-α1hβ3 LIBS antibodies at maximal levels in Ca2+, i.e., comparably to binding in the presence of Mn2+ and RGD (data not shown) (33). Similarly, the β3 wedge mutant bound anti-β3 LIBS antibodies maximally (35). These results suggest that, unlike the glycan-wedge mutations that strongly stabilize the integrin in the open, extended conformation (33, 35), the activating Leu and GAAKR mutations increase affinity for ligand by shifting the equilibrium toward the extended conformation of the receptor.

**In CHO Transfectants, β3G708N Exhibits Increased Ligand Binding Activity as a Consequence of Increased Affinity Rather Than Increased Valency.** Above, we showed that the β3G708N mutation has no detectable effect on ligand binding by α1hβ3 in 293T cell transfectants. Li et al. (11) have shown that β3G708N mutation in CHO transfectants leads to increased ligand binding compared to wild type. We confirmed that in CHO α1hβ3 transfectants, β3G708N increased binding to the multivalent ligand-mimetic PAC-1 IgM, although to a level <20% of that of the wedge mutant β3N305T (Fig. 6A). The β3G708N mutant also showed increased binding to PAC-1 Fab (Fig. 6B). Binding to multimeric PAC-1 IgM and monomeric PAC-1 Fab by the β3G708N mutant was comparably increased (Fig. 6A and B). In contrast, the tetrameric α1hW967C mutant shows binding to multimeric PAC-1 IgM, but not to PAC-1 Fab (Fig. 4). Therefore, it is concluded that the partially activating effect of β3G708N in CHO cells is due to an effect on affinity rather than valency.

To determine whether the β3G708N mutation induced macroclustering, i.e., areas of the cell surface with increased integrin density that are >200 nm in diameter and therefore detectable by microscopy, we conducted confocal microscopy studies of CHO-cell α1hβ3 transfectants. Under basal conditions in Ca2+, the vast majority of the cells expressing wild-type and β3G708N α1hβ3 demonstrated relatively evenly distributed cell-surface integrin (Fig. 6C). Only 4% and 5% of cells, respectively, showed any substantial macroclustering. We also ranked the cells by expression of α1hβ3 and quantitated clustering in the 10% highest expressing cells in each group. These cells, which expressed about three to five more α1hβ3 than the mean, also showed comparable macroclustering for wild type (9%) and β3G708N (12%). The presence of similar but small subpopulations of cells with macroclustering illustrates the importance of population analysis in addition to showing micrographs of individual cells. After treatment with cytochalasin-D to increase integrin cell-surface diffusivity (37), integrins remained unclus-
Discussion

This investigation has yielded insights into the role that TM domains play in integrin activation. Previously we used cysteine scanning mutagenesis to identify the α-helical interface between the N-terminal regions of the integrin α- and β-subunit TM domains and showed that disulfide bonds introduced into this interface maintained the integrin in the low-affinity state (8). Here, we use the converse approach of Leu scanning mutagenesis to perturb TM domain association. The activating mutations map to the same interface as previously defined by activation-restricting disulfides. This finding strongly suggests that activation is a consequence of disruption of the helical TM interface between the integrin α- and β-subunits. Moreover, we extended previous results by subjecting the entire TM segments to mutagenesis. With the exception of one anomalous mutation that was not well expressed and only partially activating, the results suggest that the previously defined α-helical interface extends all the way across the membrane. We further showed that a variety of the activating mutations both increase monovalent ligand binding and induce conformational changes in the extracellular domain, consistent with our previous finding that association between the TM domains maintains the low-affinity integrin conformation.

We previously identified a mutant, αβW967C, that when cotransfected with wild-type β3 efficiently forms disulfide-linked integrin tetramers on the cell surface (8). We showed here that these tetramers are basally in the bent conformation and exhibit increased binding to multivalent, but not monovalent, ligands. These results are canonical for the valency (or “clustering”) mode of regulation. Thus, αβW967C represents an ideal model for the valency mode of activation. By contrast, Leu and several other amino acid substitutions introduced into the TM helical interface, mutation of the GFFKR motif (αβGAAKR), and introduction of a glycan wedge into the I-like hybrid domain interface (β3N305T) increase monomeric affinity for ligand. Thus, the αβW967C mutation increases valency, whereas all other activating mutations in the TM domains characterized here, including αββ3G708N, act by increasing affinity, but not valency, for ligand.

Our results fail to support a major role for the TM domains in regulating cell-surface oligomerization of integrins, as has recently been suggested by others (11, 25). The mutant β3G708N has been described to stabilize homotrimerization of isolated TM domains in detergent micelles and suggested to enhance ligand binding in CHO cells by driving cell-surface integrin clustering (11). We have confirmed that this mutation partially increased binding for multimeric ligand in CHO cells; however, β3G708N binding to monomeric ligand was enhanced to the same extent. These results suggest that β3G708N activates by the affinity mode of regulation. Moreover, this mutant failed to alter the extent of cell-surface macroclustering compared with wild-type αββ3 under a variety of conditions.

The two most activating mutations we found in the αββ3 subunit, G972L and G976L, are present within a GXXXG motif. GXXXG motifs appear to be generally important in association between TM helices (34), and we have previously demonstrated that this motif in αG976L is in the interface with β3 in the membrane (8). Homomeric association of truncated αββ3 TM domains has been demonstrated in Escherichia coli inner membranes by reporter assays and in detergent micelles by SDS/PAGE and analytical ultracentrifugation (25). The G972L and G976L mutations decrease homomerization in these assays. We find that the same mutations activate binding of soluble monovalent ligand and expression of LIBS epitopes. The results are consistent with a similar interface involving the αββ3 GXXXG motif being involved in heterodimerization with β3 and homomerization with αββ3. Furthermore, the findings that (i) G972L and G976L mutations are activating (here) and (ii) decrease αββ3 homomerization (25) are compatible with activation by separation of αββ3 and β3 TM domains but are incompatible with activation by αββ3 homomerization.

Our results support an important role for separation of the integrin α and β TM domains in regulating the conformation and affinity for ligand of the extracellular domain. Notably, the only example in which we have been able to capture homodimeric integrin TM domain interactions with disulfide bonds is in the resting, low-affinity conformation. The integrin dimer that is formed binds multivalent, but not monovalent, ligands better, demonstrating valency rather than affinity regulation. Despite a large number of α- and β-subunit TM cysteine mutations that were tested within heterodimers containing an activating α-subunit GFFKR mutation, and separation of the α- and β-subunit TM domains in these mutants, none formed disulfide-linked homomers (8). Furthermore, fluorescent res-
onance energy transfer between α-subunits of different integrin heterodimers, or between β-subunits of different integrin heterodimers, shows that integrin activation and separation of α and β TM domains in the plane of the membrane occurs without giving rise to homomeric α TM domain or homomeric β TM domain interactions (23). In the future, it will be interesting to learn whether micro- and macroclustering of integrins on the cell surface, which occurs only after binding to multimeric ligands (23), brings the TM domains of integrins sufficiently close together to drive formation of integrin α- or β-subunit TM domain homomers.

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