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

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Contributed by Timothy A. Springer, December 20, 2004

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 an (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 (LIBS) epitopes, suggesting that they partially adopt an extended conformation. Furthermore, the previously described β3G708N mutation in Chinese hamster ovary cells enhanced ligand binding affinity, not valency, and did not alter cell-surface clustering as defined by confocal microscopy. Our studies provide evidence that disrupting the integrin heterodimeric TM helix–helix interface activates ligand binding mainly by increasing the monomeric affinity for ligand, but not the receptor valency, i.e., clustering.

Integrins are noncovalently associated heterodimeric cell-adhesion molecules that transmit signals bidirectionally across the plasma membrane and regulate many biological functions including wound healing, cell differentiation, and cell migration. Integrins bind to ligands in the extracellular matrix and on cell surfaces. The “avidity,” or overall strength, of cellular adhesive interactions results from both the affinity of individual receptor–ligand bonds and the valency, or the total number of bonds formed. The affinity of integrins for ligands is conformationally regulated (1–4). On physiological cell surfaces, integrins can assume multiple conformations, each with a distinct affinity. Various studies have indicated that close apposition of the membrane-proximal regions of each subunit and the overall bent structure of the extracellular domain are hallmarks for the low-affinity state of integrins, whereas the extended conformation, with separated legs, represents the high-affinity state (5–9). On the other hand, the valency of integrins for ligands is regulated by changes in their cell-surface distribution. Although a variety of studies suggest that integrin clustering serves to enhance the propensity to form initial adhesions with multivalent substrates (10–14), others have shown that valency regulation does not precede ligand binding, but instead functions in adhesion strengthening after binding to multivalent ligands (15–23).

Although considerable progress has been made, the role of integrin transmembrane (TM) domains in integrin signaling still remains controversial. We previously used disulfide crosslinking studies to determine the helical interface between integrin α- and β-subunit TM domains and showed that restraining this specific heterodimeric helical interface with disulfide bonds maintains integrins in the low-affinity state (8). By contrast, Li et al. (11, 24, 25) have shown that in detergent micelles, integrin TM domains have the potential to form homooligomers rather than heterodimers. Based on two mutations, with most experiments on the mutation β3G708N, they hypothesized that the homomeric associations between TM segments provide a driving force for integrin cell-surface oligomerization (i.e., clustering), thereby enhancing binding to multivalent ligands (11). However, homomeric interactions were only demonstrated with model TM peptides in detergent micelles, and not with intact integrins on cells. The basis for enhanced ligand binding by the β3G708N mutation characterized in this study thus remained unclear.

Here, we use full-length integrin αIIbβ3 expressed on the surface of mammalian cells to study the effects of mutations of the TM domains. Previously we used cysteine scanning to introduce disulfides 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 crosslinking of αIIb TM domains to form (αIIbβ3)2 tetramers. Tetramers show no extracellular domain conformational change and increased multicentric, 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/VS-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 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...
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 (filled bars). Binding was measured with two-color immunofluorescence (see Materials and Methods) and is expressed as the MFI of PAC-1 staining as a percentage of MFI of staining with AP3 mAb. Data are representative of two independent experiments. (D) Relative orientation between the αIIb and β₃ TM domain α-helices as determined by disulfide scanning of residues shown between dashed lines (B). Activating residues are shown as black circles, partially activating residues are shown as gray circles, and others are shown as white circles.

**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 GRGDSP 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 αIbβ₃ and mutant αIbβ₃ (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 glycoprophin 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 αIbβ₃ TM domains to Leu (Fig. 1A). Mutant αIbβ₃-subunits were coexpressed with wild-type β₃-subunits, mutant β₃-subunits were coexpressed with wild-type αIbβ₃-subunits, and both were screened for enhanced binding to the soluble, multivalent, ligand-mimetic PAC-1 IgM (Fig. 1B 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 αIbβ₃ and most mutants exhibited essentially no PAC-1 binding. By contrast, three mutants, αIbG972L, αIbG976L, and β₃G708L, bound PAC-1 IgM at nearly maximal levels, and two others, αIbT981L and β₃I693L, bound partially.
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 αIIbT981L, map to the αIIb-β3 interface (Fig. 1D). The inconsistency regarding αIIbT981L may suggest deviation from ideal helices associated with an interacting coiled-coil structure; however, it is noteworthy that mutant αIIbT981L 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, αIIbG972 and β3G708, for additional mutagenesis studies. These two residues were mutated to Ala, Cys, or Asn. At residue αIIbG972, the substitutions G972A and G972C 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 αIIbβ3 Tetramer Is an Ideal Model for Studying Valency Effect. We previously showed that cysteine mutant αIIbW967C spontaneously and efficiently formed a homodimeric disulfide bond when cotransfected with the β3-subunit, thereby forming a covalently linked cell-surface integrin tetramer (αIIbW967C/β3)2 (8) (Fig. 3A). In 293T transfectants, the tetramer could be recognized by all tested mAbs to constitutive αIIbβ3 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 αIIbW967C/β3 more efficiently than wild-type αIIbβ3 basally in Ca2+, as well as after activation with Mn2+, PT25-2/Ca2+, or PT25-2/Mn2+. Notably, in Ca2+ the efficiency of αIIbW967C/β3 tetramer binding to PAC-1 IgM, with potentially 10 binding sites per molecule, was enhanced more than binding to fibrinogen, a divalent 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 Ca2+, no binding to αIIbW967C/β3 tetramer was found (Fig. 4).

LIBS mAbs were used to determine the overall conformation of the αIIbW967C/β3 mutant. All of the LIBS mAbs bound poorly to the tetrameric mutant in Ca2+, 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 αIIbW967C/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 αIIbW967C mutant did not bind to monomeric PAC-1 Fab in Ca2+ (Fig. 4). By contrast,
the activating Leu mutations, α\textsubscript{IIb}G972L, α\textsubscript{IIb}G976L, and β\textsubscript{3}G708L, all bound PAC-1 Fab efficiently (Fig. 4). These mutants were activated similarly to the β\textsubscript{3}N305T mutant (Fig. 4), which introduces an N-glycosylation site into the interface between the β\textsubscript{3} 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 α\textsubscript{IIb} 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 α\textsubscript{IIb}G972L, α\textsubscript{IIb}G976L, and β\textsubscript{3}G708L mutants is due to increased affinity rather than valency. As a further control, the α\textsubscript{IIb}G975L and β\textsubscript{3}G708N 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, α\textsubscript{IIb}G972L, α\textsubscript{IIb}G976L, and β\textsubscript{3}G708L, as well as the GAAKR mutant, but not the nonactivating α\textsubscript{IIb}G975L mutant, bound LIBS6 mAb to essentially maximal levels, i.e., comparably to binding in the presence of Mn\textsuperscript{2+} and RGD (Fig. 5A). The same activating Leu mutants and the GAAKR mutant, but not α\textsubscript{IIb}G975L, 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 Mn\textsuperscript{2+} and RGD (Fig. 5B). The same results were obtained in EDTA as in Ca\textsuperscript{2+}, 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 β\textsubscript{3}N305T bound D3 and other anti-α\textsubscript{IIb}β\textsubscript{3} LIBS antibodies at maximal levels in Ca\textsuperscript{2+}, i.e., comparably to binding in the presence of Mn\textsuperscript{2+} and RGD (data not shown) (33). Similarly, the β\textsubscript{3} wedge mutant bound anti-β\textsubscript{3} 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, β\textsubscript{3}G708N Exhibits Increased Ligand Binding Activity as a Consequence of Increased Affinity Rather Than Increased Valency.** Above, we showed that the β\textsubscript{3}G708N mutation has no detectable effect on ligand binding by α\textsubscript{IIb}β\textsubscript{3} in 293T cell transfectants. Li et al. (11) have shown that β\textsubscript{3}G708N mutation in CHO transfectants leads to increased ligand binding compared to wild type. We confirmed that in CHO α\textsubscript{IIb}β\textsubscript{3} transfectants, β\textsubscript{3}G708N increased binding to the multivalent ligand-mimetic PAC-1 IgM, although to a level <20% of that of the wedge mutant β\textsubscript{3}N305T (Fig. 6A). The β\textsubscript{3}G708N mutant also showed increased binding to PAC-1 Fab (Fig. 6B). Binding to multimeric PAC-1 IgM and monomeric PAC-1 Fab by the β\textsubscript{3}G708N mutant was comparably increased (Fig. 6B). In contrast, the tetrameric α\textsubscript{IIb}W967C 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 β\textsubscript{3}G708N in CHO cells is due to an effect on affinity rather than valency.

To determine whether the β\textsubscript{3}G708N mutation induced macromolecular clustering, 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 α\textsubscript{IIb}β\textsubscript{3} transfectants. Under basal conditions in Ca\textsuperscript{2+}, the vast majority of the cells expressing wild-type and β\textsubscript{3}G708N α\textsubscript{IIb}β\textsubscript{3} demonstrated relatively evenly distributed cell-surface integrin (Fig. 6C). Only 4% and 5% of cells, respectively, showed any substantial macromclustering. We also ranked the cells by expression of α\textsubscript{IIb}β\textsubscript{3} and quantitated clustering in the 10% highest expressing cells in each group. These cells, which expressed about three to five more α\textsubscript{IIb}β\textsubscript{3} than the mean, also showed comparable macromclustering for wild type (9%) and β\textsubscript{3}G708N (12%). The presence of similar but small subpopulations of cells with macromclustering illustrates the importance of population analysis in addition to showing micrographs of individual cells. After treatment with cytochalasin-D to decrease integrin cell-surface diffusivity (37), integrins remained unclu-
Wild-type microscopy studies of integrin clustering on the cell surface. Cells expressing (between the N-terminal regions of the integrin suggests that activation is a consequence of disruption of the with activation-restraining disulfides. This finding strongly mutations map to the same interface as previously defined mutagenesis to perturb TM domain association. The activating state (8). Here, we use the converse approach of Leu scanning into this interface maintained the integrin in the low-affinity unit TM domains and showed that disulfide bonds introduced teine scanning mutagenesis to identify the This investigation has yielded insights into the role that TM Discussion

A better, demonstrating valency rather than affinity regulation. The integrin dimer is the only example in which we have been able to capture homo-

Fig. 6. Ligand binding and clustering of selected αbbβ3 mutations stably expressed in CHO cells. (A and B) Ligand binding. PAC-1 IgM (A) and PAC-1 Fab (B) binding of CHO cell transfectants. Binding was determined as the MFI of PAC-1 staining as a percentage of MFI of staining with AP3 mAb. (C) Confocal microscopy studies of integrin clustering on the cell surface. Cells expressing wild-type αbbβ3 or mutant αbbβ3G708N were incubated with Cy3-AP3 mAb in the absence (control) or presence of cytochalasin-D (Cyto D), anti-mouse IgG (Abs), or anti-mouse IgG/cytochalasin-D (Cyto D + Abs) at 37°C for 30 min, followed by fixation, and subjected to confocal microscopy.

tered on the vast majority of cells (Fig. 6C). Only a slight increase in the number of cells with macroclustering was seen, and the percentages were similar for wild type (13%) and β3G708N (16%). On the other hand, crosslinking cell-surface αbbβ3 with primary and secondary antibodies promoted significant αbbβ3 macroclustering (76% for wild type, and 73% for β3G708N) in a manner that was further enhanced by concomitant cytochalasin-D treatment (96% macroclustering for wild type, and 94% for β3G708N) (Fig. 6C). Importantly, under all conditions, no substantial difference was observed in the propensity of wild-type and β3G708N αbbβ3 to form cell-surface macroclusters.

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 with activation-restraining 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 of 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, αbbβ3W967C, 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, αbbβ3W967C 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 (αbbβ3GAAKR), and introduction of a glycan wedge into the I-like hybrid domain interface (β3N305T) increase monomeric affinity for ligand. Thus, the αbbβ3W967C mutation increases valency, whereas all other activating mutations in the TM domains characterized here, including αbbβ3G708N, 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 αbbβ3 under a variety of conditions.

The two most activating mutations we found in the αbbβ3 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 αbbβ3 is in the interface with β3 in the membrane (8). Homomeric association of truncated αbbβ3 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 αβ3, GXXXG motif being involved in heterodimerization with β3 and homomerization with αβ3. Furthermore, the findings that (i) G972L and G976L mutations are activating (here) and (ii) decrease αβ3 homomerization (25) are compatible with activation by separation of αβ3 and β3 TM domains but are incompatible with activation by αβ3 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 homomeric 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 macro-clustering 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.

We thank Drs. M. H. Ginsberg, S. Shattil, M. Handa, and L. K. Jennings for generously providing antibodies; J. Bennett for providing CHO cell lines; Aideen Mulligan for laboratory management assistance; Jessica Martin for secretarial assistance; and Drs. M. H. Ginsberg and M. L. Dustin for reviewing the manuscript. This work was supported by National Institutes of Health Grant HL48675 (to T.A.S. and J.T.).