Specific Cysteines in β3 Are Involved in Disulfide Bond Exchange-dependent and -independent Activation of αIIbβ3*

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Disulfide bond exchange among cysteine residues in epidermal growth factor (EGF)-like domains of β3 was suggested to be involved in activation of αIIbβ3. To investigate the role of specific β3 cysteines in αIIbβ3 expression and activation, we expressed in baby hamster kidney cells normal αIIb with normal β3 or β3 with single or double cysteine substitutions of nine disulfide bonds in EGF-3, EGF-4, and β-tail domains and assessed αIIbβ3 surface expression and activation state by flow cytometry using P2 or PAC-1 antibodies, respectively. Most mutants displayed reduced surface expression of αIIbβ3. Disruptions of disulfide bonds in EGF-3 yielded constitutively active αIIbβ3, implying that these bonds stabilize the inactive αIIbβ3 conformer. Mutants of the Cys-567–Cys-581 bond in EGF-4 were inactive even after exposure to αIIbβ3-activating antibodies, indicating that this bond is necessary for activating αIIbβ3. Disrupting Cys-560–Cys-583 in the EGF-3/EGF-4 or Cys-608–Cys-655 in β-tail domain resulted in αIIbβ3 activation only when Cys-560 or Cys-655 of each pair was mutated but not when their partners (Cys-583, Cys-608) or both cysteines were mutated, suggesting that free sulfhydryls of Cys-583 and Cys-608 participate in αIIbβ3 activation by a disulfide bond exchange-dependent mechanism. The free sulfhydryl blocker dithiobisnitrobenzoic acid inhibited 70% of α-LIBS6 antibody-induced activation of wild-type αIIbβ3 and had a smaller effect on mutants, implicating disulfide bond exchange-dependent and -independent mechanisms in αIIbβ3 activation. These data suggest that different disulfide bonds in β3 EGF and β-tail domains play variable structural and regulatory roles in αIIbβ3.

Integrin αIIbβ3 mediates platelet aggregation by serving as a receptor for fibrinogen and von Willebrand factor. Like other integrins, the affinity of αIIbβ3 for its ligands is tightly regulated by cellular events (inside-out signaling). αIIbβ3 is inactive in resting platelets, but following activation by inside-out signals it undergoes conformational changes resulting in ligand binding to its large globular head, which further modifies the conformation leading to clustering of the αIIbβ3 receptors, tyrosine phosphorylation, and cytoskeleton rearrangement (1–6). Although the precise mechanism by which αIIbβ3 is activated is incompletely understood, recent crystallographic and electron microscopic data suggest that during activation αIIbβ3 changes from a low affinity bent conformation with a closed headpiece to a high affinity extended conformation with an open headpiece. Several intermediate affinity conformations have been proposed to be in equilibrium between these extreme conformational states (7–9). Physiologic agonist-induced inside-out signaling involves separation of the cytoplasmic tails of αIIb and β3 that results in rearrangement of the extracellular domain of the integrin, culminating in ligand binding (10, 11). Activation of αIIbβ3 can also be induced directly by antibodies to ligand-induced binding sites (LIBS)2 without inside-out signaling (12, 13).

Recent studies suggest that exofacial disulfide exchange is involved in the conformational changes that follow αIIbβ3 activation. Both αIIb and β3 subunits contain highly conserved cysteine residues that form disulfide bonds. β3 contains 56 cysteines, of which 31 are located in four epidermal growth factor (EGF)-like domains and 8 are located in the carboxyl-terminal β-tail domain (βTD) (7). Several naturally occurring cysteine substitutions in the EGF-like domains of β3 (C549R, C560F, C560R, and C598Y) that cause Glanzmann thrombasthenia, a severe bleeding disorder, as well as several artificial cysteine substitutions exhibit constitutively active αIIbβ3 (14–18). All cysteines in β3 had been assumed to be disulfide bonded (19), but a recent report suggested that some exist as free sulfhydryls exhibiting properties of a redox site directly involved in integrin activation (20). Moreover, the reducing agent dithiothreitol was shown to activate αIIbβ3 and cause slow progressive platelet aggregation by a mechanism involving both disulfide bond reduction and disulfide bond exchange (21). Two other reports by one of us showed that extracellular free thiols and enzymatically catalyzed disulfide bond exchanges are required for acquisition of a ligand binding conformation of αIIbβ3 (22, 23). In this context it is notable that αIIbβ3 harbors an endogenous thiol isomerase activity (24).

In the present study we analyzed the role of specific disulfide bonds in the exofacial domain in promoting αIIbβ3 activation.

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The abbreviations used are: LIBS, ligand-induced binding site; BHK, baby hamster kidney; DTNB, dithiobisnitrobenzoic acid; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; WT, wild type; βTD, β-tail domain.

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We created mutations in cysteine residues that disrupted nine disulfide bonds in the EGF and β-tail domains of β3 and expressed them in baby hamster kidney (BHK) cells. It will be shown that different cysteines play different roles in the activation of αIβ3 involving disulfide bond exchange-dependent or -independent mechanisms.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco modified Eagle’s medium, L-glutamine, and fetal calf serum were from Biological Industries (Beit-Haemek, Israel). Lipofectamine reagent and G418 were from Invitrogen. Hygromycin was from Roche Applied Science. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody P2 against αIβ3 complex was obtained from Immuno-tech (Marseille, France). FITC-conjugated fibrinogen mimetic murine monoclonal antibody PAC-1 was obtained from Dr. Mark Ginsberg (Dept. of Medicine, University of California, San Diego, La Jolla, CA). The activating monoclonal antibody PT25-2 was from Takara Bio Inc. (Shiga, Japan). The membrane impermeant-free sulfhydryl blocker dithiobisnitrobenzoic acid (DTNB) was from Sigma.

Construction of Expression Vectors for Mutant cDNAs—cDNAs of αIβ or β3 in pcDNA3 vector were gifts from Dr. Peter Newman from the Blood Center of Wisconsin, Milwaukee. cDNA of αIβ was subcloned to the PvuII site of pcP4E4 mammalian expression vector carrying the hygromycin resistance gene as a selection marker (Invitrogen) as previously described (25). Substitutions of selected cysteine residues by serine or other residues were created in the pcDNA3/β3 vector using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using two overlapping oligonucleotide primers containing single base pair substitutions (available upon request). Correct incorporation of the mutations into the pcDNA3/β3 vectors was verified by DNA sequencing. For creation of double mutants containing two cysteine substitutions together, we first introduced one mutation into normal pcDNA3/β3 vector and then used the mutant pcDNA3/β3 clone as a template for introducing the second mutation.

Co-transfection of αIβ and β3 cDNAs—BHK cells were grown in Dulbecco’s modified Eagle’s medium supplemented by 2 mg/ml L-glutamine and 5% fetal calf serum. The cells were co-transfected with 1 μg of normal or mutated forms of pcDNA3/β3 and 1 μg of normal pcP4E4/αIβ using Lipofectamine reagent. The transfected cells were grown in a selection medium containing 0.7 mg/ml G418 and 0.5 mg/ml hygromycin. Mock cells were transfected with both pcP4E4 and pcDNA3 and were selected in the same medium. At least two different transfections for each mutant or wild-type (WT) construct were performed and used for flow cytometry.

Flow Cytometry of Transfected BHK Cells—Transfected BHK cells were harvested with phosphate-buffered saline supplemented with 1 mM EDTA, pelleted, and incubated in Dulbeco’s modified Eagle’s medium for 30 min at room temperature. Cells were pelleted again, resuspended in phosphate-buffered

| TABLE 1 |
| --- |
| **Natural and artificial mutations created for disruption of nine disulfide bonds in β3** |
| **Domain** | **Disulfide bond** | **Mutations created** |
| EGF-3 | Cys-536–Cys-544<sup>a</sup> or Cys-523–Cys-544<sup>b</sup> | C536S, C544S, C523S, C544S/C536S |
| EGF-3/EGF-4 | Cys-560–Cys-583 | C560S, C560F, C560S/C560F |
| EGF-4 | Cys-567–Cys-581 | C567S, C581S, C575S/C581S |
| EGF-5 | Cys-575–Cys-586 | C575S, C586S, C575S/C586S |
| EGF-6 | Cys-588–Cys-598 | C588S, C598S, C588S/C598S |
| βTD | Cys-608–Cys-655 | C608S, C655S, C608S/C655S |
| Cys-614–Cys-635 | C614S, C615S |
| Cys-617–Cys-631 | C617S |

<sup>a</sup> Substitutions identified in patients with Glanzmann thrombasthenia are depicted in bold letters.

<sup>b</sup> Displayed in the αvβ3 crystal structure (7).

<sup>c</sup> Proposed by Springer and coworkers (30, 31).
saline supplemented with 1 mM MgCl₂ and 1 mM CaCl₂ (5 × 10⁵ cells/100 μl), and incubated for 30 min at room temperature with either 20 μl of FITC-conjugated P2 antibody or 20 μl of FITC-conjugated PAC-1 antibody. The cells were diluted to 5 × 10⁵ cells/ml and analyzed for surface fluorescence by flow cytometry (Coulter, EPICS, Luton, UK). To measure ligand binding after activation of αⅢbβ3 by anti-LIBS6 or PT25-2 antibodies, FITC-conjugated PAC-1 was added to cells suspended in phosphate-buffered saline supplemented with 0.25 mM MnCl₂ (5 × 10⁵ cells/100 μl) in the presence of 1 μl of anti-LIBS6 or 1 μg of PT25-2. These experiments were repeated in the presence of 2.5 mM DTNB, which inhibited free sulfhydryls. The base line for nontspecific binding of the antibodies was measured in mock cells. PAC-1 binding to αⅢbβ3 was expressed as percent of binding obtained with P2. The effects of anti-LIBS6 and DTNB were compared by two-tailed, paired t test analysis.

Alignment of EGF Domains and a Model of the EGF-3 Domain of αⅢb—
An initial alignment of 2125 EGF-like domains was taken from the Pfam data base (26) (accession number PF07974). Sequences derived from a whole genome shotgun were discarded, as were sequences of variant and mutant proteins. The remaining data set of 1462 sequences was used to generate a hidden Markov model (27) subsequently utilized to collect and align 1856 EGF domain sequences from the annotated SwissProt data base (28). From this alignment, a smaller multiple sequence alignment of representative sequences was obtained, including the EGF-3 and EGF-4 domains of human integrin αⅢb and additional human EGF domains for which the three-dimensional structure is available. A model of the EGF-3 domain of αⅢb was constructed using the program NEST with default parameters (29) based on the NMR structure of the EGF-3 domain in the α2 subunit (Protein Data Bank code 1L3Y) (30).

RESULTS
Effect of αⅢb Cysteine Substitutions on the Expression and Activation State of αⅢb—Fig. 1 displays the nine disulfide bonds in EGF-3, EGF-4, and αⅢb- tail domains that were disrupted. The pairing of cysteines was derived from the αⅢb crystal structure (7). Because Cys-544 was proposed to be bonded with Cys-523 rather than Cys-536 (30, 31), we also mutated the cysteine residues of the Cys-523–Cys-544 alternative pair (Fig. 1B). In most instances, we mutated each one of the cysteines of the pairs as well as both cysteines (Table 1). A total of 27 mutations were created, most with cysteine to serine substitutions; some were replicas of natural mutations detected in Glanzmann thrombasthenia patients (C549R, C560R, C560F) (14–16). All mutants were co-expressed with normal αⅢb in
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FIGURE 3. Activation of αIIbβ3 in WT and mutants of β3 by anti-LIBS6 antibody. PAC-1 binding to BHK cells expressing either WT αIIbβ3 or αIIbβ3 harboring mutations that disrupt the bonds in EGF-3 (A), the Cys-560–Cys-583 bond (B), the bonds in EGF-4 (C), and the Cys-608–Cys-655 bond in βTD (D). Samples were assessed by flow cytometry with or without anti-LIBS6 antibody and expressed as percent of αIIbβ3 expression measured with P2 antibody. Results are given as mean ± S.E. of at least three experiments.

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BHK cells and analyzed by flow cytometry. The extent of surface expression of all αIIbβ3 mutants was 17–100% of WT, except for C575S and C586S mutants, which expressed <10% of WT and hindered us from analyzing their activation state (Fig. 2A). In all but one pair (Cys-536/Cys-544), the double mutants were expressed better than the corresponding single mutants, suggesting that hydrogen bonds between the substituting serine residues stabilized the αIIbβ3 structure, compensating for the loss of disulfide bonds. The natural C549R mutation caused reduced surface expression of αIIbβ3 similarly to the corresponding C549S artificial mutation, suggesting that the disruption of the Cys-549–Cys-558 bond and not the bulky Arg residue present in the natural mutation caused the reduced expression. In contrast, the natural C560R and C560F mutations caused αIIbβ3 surface expression that was lower than the corresponding artificial C560S mutation, suggesting that the bulky substituting residues (Arg and Phe) interfered with the expression of αIIbβ3.

The activation state of mutant αIIbβ3 complexes was determined by binding of the fibrinogen mimetic antibody PAC-1. Cells harboring disulfide bond disruptions in EGF-3 displayed a pronounced increase in PAC-1 binding. Compared with WT cells, there was a 25–33-fold increase for Cys-536–Cys-544 or Cys-523–Cys-544 bonds and a 17–24-fold increase for the Cys-549–Cys-558 bond (Fig. 2B) (p < 0.01). The effect of disruptions of the Cys-560–Cys-583 bond located at the interface between EGF-3 and EGF-4 depended on the cysteine that was mutated (Fig. 2B). Although all substitutions of Cys-560 gave rise to a 22–24-fold increase in PAC-1 binding compared with WT cells (p < 0.01), a mutation in the partner cysteine C583S did not exhibit a significant increase in binding, and a double mutant (C560S/C583S) only yielded an 8-fold increased binding (p < 0.05).

Disruption of two disulfide bonds in EGF-4 domain also yielded variable results (Fig. 2B). Cells with the Cys-567–Cys-581 bond disruptions caused by mutations of Cys-567, Cys-581, or both displayed no significant increase in binding of PAC-1. In contrast, cells with Cys-588–Cys-598 bond disruptions displayed increased PAC-1 binding, a 10-fold increase for C588S (p < 0.05), a 23-fold increase for C598S (p < 0.001), and a 34-fold increase for C588S/C598S (p < 0.001). Disruption of the Cys-608–Cys-655 bond in the βTD by a C655S mutation displayed a 14-fold increase in binding PAC-1 (p < 0.001), whereas a mutation in its counterpart, C608S, or the double mutation C608S/C655S did not exhibit a significant increase in PAC-1 binding (Fig. 2B). Mutations of cysteines of two other disulfide bonds within βTD, C614S or C617S, yielded no significant increase in PAC-1 binding, which agrees with a previous report (32).

Activation of αIIbβ3 Mutants by Activating Antibodies—To examine whether the constitutively active mutants reached their maximum activation state, we used two activating antibodies, anti-LIBS6 and PT25-2. As expected, anti-LIBS6 antibody caused a 50-fold increase in binding of PAC-1 to WT αIIbβ3 (Fig. 3A). The effect of anti-LIBS6 antibody on the αIIbβ3 mutants varied considerably; all mutants in EGF-3 exhibited no significant increase in PAC-1 binding (Fig. 3A), whereas mutants of the Cys-560–Cys-583 bond located at the interface between EGF-3 and EGF-4 displayed increased PAC-1 binding (Fig. 3B). The increase was inversely related to the extent of constitutive activation. In the Cys-560 mutants that were strongly activated, anti-LIBS6 antibody only exerted a 1.3–1.8-fold increase in PAC-1 binding; in the counterpart
C583S mutant that was not constitutively active, PAC-1 binding increased by nearly 30-fold; and in the double C560S/C583S mutant, which was weakly constitutively active, anti-LIBS6 antibody caused a 5-fold increase in PAC-1 binding (p < 0.05). The Cys-567–Cys-581 bond mutants in EGF-4 that were constitutively inactive did not exhibit an increased PAC-1 binding induced by anti-LIBS6 antibody, whereas the constitutively active Cys-588–Cys-598 bond mutants displayed a 1.2–3-fold increase in PAC-1 binding (p < 0.05) (Fig. 3C). Anti-LIBS6 antibody also induced a further increase in PAC-1 binding to the Cys-608–Cys-655 mutants that was related to their constitutive activation state, a 2.6-fold increase for C655S, a 12-fold increase for C608S, and a 6.2-fold increase for C608S/C655S (p < 0.05) (Fig. 3D). All the above experiments were reproduced with PT25-2-activating antibody (data not shown). Because the epitope for anti-LIBS6 antibody is within residues 602–690 of β3 (13) and the epitope of PT25-2 is at the lower surface of the β-propeller of αIIbβ3 (33, 34), it appears that their effect on the mutants was not epitope-specific. Taken together, our data show that whereas mutants involving Cys-560–Cys-583, Cys-588–Cys-598, or Cys-608–Cys-655 bonds could be further activated by activating antibodies, mutants with Cys-536/Cys-523–Cys-544 or Cys-549–Cys-558 bond disruptions could not be further activated, and mutants involving Cys-567–Cys-581 bond disruption remained non-active.

Effect of the Free Sulfhydryl Blocker DTNB on the αIIbβ3 Activation State—To study the role of free sulfhydryls on the activation state of the β3 mutants, we selected disulfide bonds from each one of the four regions, EGF-3, EGF-3/EGF-4 boundary, EGF-4, and βTD and examined the effect of adding 2.5 mM DTNB on PAC-1 binding to αIIbβ3. Fig. 4 shows that DTNB caused a consistent, but not statistically significant, decrease in PAC-1 binding in all mutants examined except for C560S/C583S and C588S/C598S mutants, which exhibited a slight but statistically significant decrease (Fig. 4, B and C). Notably, the constitutively active mutants remained considerably active after DTNB exposure. Thus, blocking free sulfhydryls with DTNB in the selected αIIbβ3 mutants had no or small effect on their constitutively activation state.

Inhibition of Anti-LIBS6 Antibody-induced Activation by DTNB—Because DTNB had only a slight effect on the activation state of the mutants examined, we also analyzed the effect of DTNB on activation induced by anti-LIBS6 antibody. As expected, anti-LIBS6 antibody-induced activation of WT αIIbβ3 measured by PAC-1 binding declined by 70% in the presence of 2.5 mM DTNB (Fig. 5) (p < 0.001). A less pronounced but statistically significant decline by 32, 32, and 35% was observed for C583S (Fig. 5A), C588S (Fig. 5B), and C608S (Fig. 5C) mutants, respectively (p < 0.005). In contrast, DTNB had a non-statistically significant inhibitory effect on anti-LIBS6 antibody-induced increase in PAC-1 binding to all other mutants examined. The EGF-3 and Cys-567–Cys-581 mutants that were not activated by anti-LIBS6 antibody were not tested with DTNB.

Alignment of EGF-like Domains—Our data show that substitutions of different cysteines in the EGF and β-tail domains elicit diverse effects on the activation state of αIIbβ3. Because EGF-like domains are conserved and abundant in proteins (35), we performed a sequence alignment analysis to seek a potential pattern in this diversity. Ten conserved EGF-like domains are presented in Fig. 6. All EGF domains share a distinctive motif of 6 conserved cysteines (C1–C6) that typically form three internal disulfide bonds in a 1–3, 2–4, and 5–6 pattern, although C1, C2, C3, and C4 display in some instances alternative patterns of disulfide bridges that have the potential of rearranging disulfide bonds (36, 37). In β integrins, 2 additional cysteines were iden-
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In this study, we evaluated the role of a series of disulfide bonds localized in the β3 EGF-3, EGF-4, and β-tail cysteine-rich domains in the expression and activation of αIIbβ3. For this purpose we created mutants in which one or both cysteines of the disulfide bonds were substituted and determined their effect on αIIbβ3 surface expression and activation state using antibodies specific for the αIIbβ3 complex (P2) or for its active conformer (PAC-1), respectively. Maximum activation of αIIbβ3 was achieved by using the LIBS-activating antibodies, i.e. anti-LIBS6 or PT25-2. Because free sulfhydryls were suggested to play a role in αIIbβ3 activation by disulfide bond rearrangements (20–24), we compared the effect of substituting both cysteines to the effect of substituting only one cysteine predictably yielding free sulfhydryls in their cysteine partners. We also examined the effect of DTNB, a sulfhydryl blocker, on the activation state of αIIbβ3 and on activation of αIIbβ3 by the activating antibodies.

Effects of Disulfide Bond Disruptions on αIIbβ3 Structure and Function—Our results, summarized in Table 2, show that disruptions of different disulfide bonds in the β3 EGF-3, EGF-4, and β-tail domains had different effects on the structure and function of αIIbβ3. Disruption of most bonds in the β3 EGF-3 and EGF-4 domains by double or single mutants resulted in reduced surface expression of αIIbβ3 compared with WT αIIbβ3. However, the extent of the reduction was generally related to the extent of conservation of the disulfide bonds. Disruptions of disulfide bonds that are conserved in EGF domains (Fig. 6), e.g. Cys-549–Cys-558 (C5–C6 of EGF-3), Cys-567–Cys-581 (C1–C3 of EGF-4), and Cys-575–Cys-586 (C2–C4 of EGF-4), resulted in <50% surface expression of (8), or the Cys-608–Cys-655 bond (C) were activated with anti-LIBS6 antibody in the presence or absence of 2.5 mM DTNB. PAC-1 binding to the cells was assessed by flow cytometry and is presented as percent of αIIbβ3 expression measured by P2 antibody (mean ± S.E. of at least three experiments).
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Because the inactive state of αIβ3 depends on a specific conformation (7, 8), disruption of disulfide bonds in β3 is predicted to inflict structural changes that destabilize the inactive conformer and give rise to constitutively active αIβ3 (14, 18). This was indeed the case with disruptions of disulfide bonds in EGF-3: Cys-549–Cys-558, Cys-536–Cys-544, or Cys-523–Cys-544. All respective single or double mutants were presented at their fully active conformation because they could not be further activated by anti-LIBS6 or PT25-2-activating antibodies (Fig. 3A). Stabilization of an inactive conformer that prevents αIβ3 activation was observed in all mutants that disrupted the Cys-567–Cys-581 bond of the EGF-4 domain. All of these mutants were inactive and could not be activated by the activating antibodies, implying that the integrity of the Cys-567–Cys-581 bond is important for the activation of αIβ3.

So far, activation of αIβ3 by disulfide bond disruptions was mainly described for bonds in the EGF domains (14–18) but also in other regions such as the βTD (32). The βTD is unique for β integrins and contains four disulfide bonds, Cys-608–Cys-655, Cys-614–Cys-635, Cys-617–Cys-631, and Cys-663–Cys-687 (7). It was shown that deletions of the whole domain or parts of it as well as mutations disrupting the Cys-663–Cys-687 bond did not severely impair αIβ3 surface expression but resulted in a constitutively active integrin (32). These results suggest that this region is not essential for cell surface expression of αIβ3 but is important for maintaining the integrin in its resting conformation. Indeed, we showed that disruption of three βTD bonds, Cys-608–Cys-655, Cys-614–Cys-635, and Cys-617–Cys-631, did not severely impair αIβ3 expression. However, only disruption of the Cys-608–Cys-655 bond by the C655S mutant resulted in αIβ3 activation. Others have shown that disruptions of Cys-614–Cys-635 and Cys-617–Cys-631 by C635S and C631S mutations (32) did not result in αIβ3 activation. Our observation that C614S and C617S mutants were also inactive implies that disruption of the Cys-614–Cys-635 and Cys-617–Cys-631 bonds did not activate αIβ3. Taken together, expression of αIβ3 does not depend on the integrity of the βTD including its disulfide bonds. However, some disulfide bonds in this domain are involved in αIβ3 activation.

Effect of Free Sulfhydryls on the Activation of αIβ3—The role of free sulfhydryls and disulfide bond exchanges in αIβ3 activation was examined by analyzing the effects of double mutants versus substituting single cysteines, thereby allowing αIβ3 compared with WT, and in most individual mutants (C549S, C549R, C558S, C567S, C575S, and C586S) surface expression was <20% of WT (Fig. 2A). These results suggest that the conserved bonds are important for the EGF-3 or EGF-4 folding and that their disruption exerts a deleterious effect on the EGF structure. The Cys-588–Cys-598 bond (C5–C6 of EGF-3) was an exception; mutants that disrupted this bond displayed a relatively high expression of αIβ3, suggesting that although this bond is conserved, it does not play a primary structural role in EGF-4 folding.

Disruption of two non-conserved disulfide bonds that are unique for β integrins, i.e. Cys-560–Cys-583 bond and the Cys-523–Cys-544 bond described by Xiao et al. (9) (Fig. 6), yielded >50% surface expression of αIβ3 relative to WT, irrespective of the cysteine mutated. This finding suggests that disruption of non-conserved disulfide bonds has a smaller effect on the EGF structure than disruption of conserved disulfide bonds. It should be noted that the αvβ3 crystal structure implies a unique pairing of the non-conserved Cys-544 with the conserved Cys-536 (C2 of EGF-3) (7). However, our results show that expression of αIβ3 harboring the C536S mutant was extremely low compared with the partner C544S mutant (Fig. 2A). Such a difference between mutated cysteine pairs was not observed in any of the other pairs. Moreover, the αIβ3 harboring the C536S/C544S double mutant was expressed less than the C544S single mutant whereas in all other αIβ3 double mutants, expression was higher than the individual mutants. Thus, our results support the Cys-523–Cys-544 pairing proposed by Xiao et al. (9).

FIGURE 6. Sequence alignment of 10 representative EGF domains of various human proteins. For proteins that comprise more than one EGF domain, the number of the domain is indicated. The 6 conserved cysteine residues (namely C1–C6) that characterize the EGF domain fold are highlighted by gray boxes, and the conserved C1–C3, C2–C4, and C5–C6 disulfide bonds are depicted. Additional cysteines that are unique for EGF-3 and EGF-4 domains of β integrins are shown in bold letters. The EGF domain boundaries are indicated by their numbering in the SwissProt database. The conventional residue numbers for Cys-536–Cys-544 and Cys-567–Cys-581 bonds are represented as proposed by Springer and coworkers (30, 31). The alternative disulfide bonds are depicted by dashed lines. According to this scheme, EGF-3 does not display a unique pattern of disulfide bonds and predicts Cys-523–Cys-544 and Cys-560–Cys-583 bonds in EGF-3 and EGF-4, respectively.
Table 2

| Domain       | Effect of DTNB on Anti-LIBS6 Activation | Effect of DTNB on Anti-LIBS6 Activation | Activation State | Expression Levels | Proposed Role in Structure and Function |
|--------------|----------------------------------------|----------------------------------------|------------------|-------------------|-----------------------------------------|
| EGF-3        | Stabilization of inactive conformer    | Stabilization of inactive conformer    | Further activated | >50%               |Constitutively active                        |
| EGF-4        | Stabilization of inactive conformer    | Stabilization of inactive conformer    | Further activated | >50%               |Constitutively active                        |
| βTD          | Stabilization of inactive conformer    | Stabilization of inactive conformer    | Further activated | >50%               |Constitutively active                        |

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formation of free sulfhydryls, and by use of DTNB. As discussed above, in the EGF-3 domain all single mutants and double mutants of each pair were constitutively active (Table 2), suggesting that all mutations caused destabilization of the inactive αllβ3 conformer and that it is not the formation of free sulfhydryls that gives rise to activation of αllβ3. In contrast, disruptions of the Cys-560–Cys-583 bond in the EGF-3/EGF-4 boundary and the Cys-608–Cys-655 bond in the βTD resulted in variable activation of αllβ3 depending on the mutated cysteine; mutating Cys-560 or Cys-655 resulted in αllβ3 activation but mutating their respective partners, Cys-583 or Cys-608, or both cysteines in each pair yielded weak or no αllβ3 activation. These results suggest that disruptions of Cys-560–Cys-583 or Cys-608–Cys-655 bonds were not sufficient to induce αllβ3 activation but rather that activation depends on generation of free sulfhydryls in Cys-583 and Cys-608, predictably augmenting disulfide bond rearrangements. Interestingly, all mutants of these bonds could be further activated by activating antibodies, implying that they were not presented at their fully active conformation. Disruption of the Cys-588–Cys-598 bond in EGF-4 displayed a unique feature; all mutants were constitutively active but the double C588S/C598S mutant was much more active than the individual C588S or C598S mutants (Fig. 2B), suggesting that the free sulfhydryls that were exposed in the single mutants interfered with full activation of αllβ3. Indeed, the single mutants could be further activated by activating antibodies (Fig. 3C), suggesting that they were not presented in their fully active conformer.

The free sulfhydryl blocker DTNB used at a concentration that was previously shown to completely block platelet aggregation (22) exerted only a minor effect on the activation state of WT and mutated αllβ3. Thus, the constitutively active αllβ3 mutants remained active in the presence of DTNB (Fig. 4). This finding suggests that maintenance of the already formed active αllβ3 conformer is minimally dependent on free sulfhydryls. This conclusion is supported by another study that showed that DTNB prevented agonist-induced platelet aggregation only when added before and not after the agonist (38).

In contrast, DTNB inhibited 70% of PAC-1 binding to WT αllβ3 activated by anti-LIBS6 antibody (Fig. 5), suggesting that anti-LIBS6-induced activation of WT αllβ3 depends on the presence of free sulfhydryls and disulfide bond exchange. Conceivably, the residual 30% activity represented activation pathways that are independent of free sulfhydryls and disulfide bond exchange, consistent with previous reports that showed that the first stage of agonist-induced platelet aggregation was not inhibited by blockers of free sulfhydryls (22, 39). Interestingly, DTNB inhibited anti-LIBS6-induced activation of most mutants to a lesser extent than WT (Fig. 5). The most pronounced effect was shown for C583S, C588S, and the C608S mutants that were inhibited by ~30%. These data suggest that, unlike WT, the disulfide bond exchange-dependent component in the αllβ3 mutants was smaller than the disulfide bond exchange-independent component. It was previously shown that active αllβ3 contains two additional free sulfhydryls as compared with resting αllβ3 (21). It is possible that the disulfide bond exchange-independent step involved opening of existing disulfide bonds, a reaction that is not inhibited by
DTNB. Conceivably, these results suggest that activation of αIIbβ3 involves disulfide bond exchange-dependent as well as-independent mechanisms that are consistent with a model of multiple conformational states of αIIbβ3 (9).

Disulfide Bonds with a Possible Regulatory Effect—The results presented here do not support the suggestion of Kamata et al. (18) that all disulfide bonds in the EGF domains of β3 keep αIIbβ3 in an inactive state and that there is no unique cysteine that is critical for regulation of αIIbβ3 activation. Our results ascribe a role for the Cys-560–Cys-583 bond in regulating αIIbβ3 activation and suggest that free sulhydryl in reduced Cys-583 is involved in activating αIIbβ3 through disulfide bond rearrangements. The Cys-560–Cys-583 bond is unique to β integrins, and the crystal structure of αvβ3 suggests that it stabilizes a rigid interface between the EGF-3 and EGF-4 domains (7) (Fig. 6A). The NMR-based model proposed by Beglova et al. (30) and Takagi et al. (31) positioned the Cys-560–Cys-583 bond within the EGF-4 domain, predicting a shorter linker region between EGF-3 and EGF-4 that confers reduced flexibility between these domains. Thus, both the crystal structure and NMR studies indicate that the Cys-560–Cys-583 bond plays an important role in controlling the rigidity of the EGF-3/EGF-4 interface. It has been shown that the interconnections between EGF domains of integrins play a role in their activation and signal transmission (31, 40). The unique disulfide bonds linking EGF-3 to EGF-4 in β integrins, like Cys-560–Cys-583 in β3, were suggested to act similarly to calcium binding residues of EGF domains that operate in other proteins (40–42). Binding of calcium ions is assumed to regulate the function of these proteins by inducing local structural changes followed by reorganization of the EGF domains relative to each other as was shown for fibrillin-1 (41). It is possible that in β integrins, a similar regulation is induced by allosteric rearrangements of disulfide bonds that cause a change in the relative positions of tandem EGF domains, affording transmission of signal along the molecule as suggested by Chen and Hogg (37).

Another disulfide bond for which we provide evidence for a regulatory role is Cys-608–Cys-655 in the ΒTD. The ΒTD was suggested to be flexibly connected to EGF-4 by two weak hydrophobic bonds, and the interface between these domains was suggested to be involved in activation of integrins because it comprises the binding site for the activation-sensitive anti-β3 LIBS6 antibody and anti-β2 KIM185-activating antibody (40, 43). Conceivably, disruption of the Cys-608–Cys-655 bond located relatively close to the EGF-4/ΒTD interface (Fig. 1A) causes conformational changes involving disulfide bond rearrangements that lead to integrin activation. Together, our data suggest that interdomain contacts between EGF-3 and EGF-4 and between EGF-4 and ΒTD of β3 play a role in αIIbβ3 activation.

In conclusion, based on the results presented here and on other studies, we suggest that activation of αIIbβ3 is a dynamic multistep process involving disulfide bond exchange-dependent as well as-independent mechanisms. Some cysteines in β3 form disulfide bonds that have a fundamental structural role and their disruption can impair αIIbβ3 expression and/or activation manifested by constitutively active or non-active αIIbβ3. Other cysteine pairs have a regulatory role in αIIbβ3 activation, probably via their involvement in disulfide bond exchanges.

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