Unique Disulfide Bonds in Epidermal Growth Factor (EGF) Domains of β3 Affect Structure and Function of αIIbβ3 and αvβ3 Integrins in Different Manner

Ronit Mor-Cohen‡1, Nurit Rosenberg‡, Yulia Einav‡, Ehud Zelzion‡, Meytal Landau‡, Wissam Mansour‡, Yulia Averbukh‡, and Uri Seligsohn

From the ‡1Amalia Biron Research Institute of Thrombosis and Hemostasis, Chaim Sheba Medical Center, Tel-Hashomer 52621 and ‡2Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel and ‡3Mathematical Biology Unit, Faculty of Sciences, Holon Institute of Technology, Holon 58102, Israel

Received for publication, October 6, 2011, and in revised form, February 1, 2012. Published, JBC Papers in Press, February 3, 2012, DOI 10.1074/jbc.M111.311043

The β3 subunit of αIIbβ3 and αvβ3 integrins contains four epidermal growth factor (EGF)-like domains. Each domain harbors four disulfide bonds of which one is unique for integrins. We previously discerned a regulatory role of the EGF-4 Cys-560–Cys-583 unique bond for αIIbβ3 activation. In this study we further investigated the role of all four integrin unique bonds in both αIIbβ3 and αvβ3. We created β3 mutants harboring serine substitutions of each or both cysteines that disrupt the four unique bonds (Cys-437–Cys-457 in EGF-1, Cys-473–Cys-503 in EGF-2, Cys-523–Cys-544 in EGF-3, and Cys-560–Cys-583 in EGF-4) and transfected them into baby hamster kidney cells together with normal αv or αIIb. Flow cytometry was used to measure surface expression of αIIbβ3 and αvβ3 and their activity state by soluble fibrinogen binding. Most cysteine substitutions caused similarly reduced surface expression of both receptors. Disrupting all four unique disulfide bonds by single cysteine substitutions resulted in variable constitutive activation of αIIbβ3 and αvβ3. In contrast, whereas double C437S/C457S and C473S/C503S mutations yielded constitutively active αIIbβ3 and αvβ3, the C560S/C583S mutation did not, and the C523S/C544S mutation only yielded constitutively active αIIbβ3. Activation of C523S/C544S αvβ3 mutant by activating antibody and dithiothreitol was also impaired. Molecular dynamics of C523S/C544S β3 in αIIbβ3 but not in αvβ3 displayed an altered stable conformation. Our findings indicate that unique disulfide bonds in β3 differrently affect the function of αIIbβ3 and αvβ3 and suggest a free sulphydryl-dependent regulatory role for Cys-560–Cys-583 in both αIIbβ3 and αvβ3 and for Cys-523–Cys-544 only in αvβ3.

Integrins are a family of non-covalently associated αβ heterodimeric receptors that mediate cell-matrix and cell-cell interactions. The β3 integrin subfamily includes αIIbβ3, the fibrinogen receptor, and αvβ3, the vitronectin receptor. Whereas αIIb binds exclusively to β3, αv can form complexes with β1, β3, β5, β6, and β8 (1).

αIIbβ3 is confined to the megakaryocyte/platelet lineage and mediates platelet aggregation through interactions with fibrinogen and von Willebrand factor (2). αvβ3 is expressed in various cell types such as endothelial cells, vascular smooth muscle cells, osteoclasts, platelets, and certain subpopulations of leukocytes and tumor cells (3, 4). The ligands for αvβ3 overlap those of αIIbβ3 but also include others such as osteopontin, plasmin, matrix metalloproteinase-2, and prothrombin (4–7). αvβ3 is implicated in various physiological and pathologic processes such as cell adhesion, migration, angiogenesis, wound healing (8, 9), bone resorption (10), arterial restenosis (11), and tumor metastasis (12).

The affinity of αIIbβ3 for its ligands is tightly regulated by cellular events and is inactive in resting platelets. After activation by inside-out signaling, αIIbβ3 undergoes conformational changes resulting in ligand binding to its large globular head that further modifies the conformation leading to clustering of the αIIbβ3 receptors, tyrosine phosphorylation, and cytoskeleton rearrangement (2, 13–15). Although less studied, affinity regulation of αvβ3 by intracellular signals was also reported (16). The physiologic significance of αvβ3 affinity modulation is not certain, but its activation increases adhesion and migration to ligands such as fibrinogen, vitronectin, osteopontin, and prothrombin (6, 17, 18). Moreover, activation of αvβ3 in certain cancer cells augments formation of metastases (12, 19). Both αIIbβ3 and αvβ3 can be activated directly by certain activating antibodies such as anti-ligand-induced binding site 6 (LIBS6)2 antibody without involving inside-out signaling (16, 20).

This article contains supplemental Figs. 1 and 2 and Table S1.*

1 To whom correspondence should be addressed: The Amalia Biron Research Institute of Thrombosis and Hemostasis, Chaim Sheba Medical Center, Tel-Hashomer 52621, Israel. Tel.: 972-3-5302105; Fax: 972-3-5351568; E-mail: ronit.cohen@sheba.health.gov.il.

2 The abbreviations used are: LIBS, ligand-induced binding site; BHK, baby hamster kidney; MD, molecular dynamics; r.m.s.d., root mean square deviation.
The nature of the conformational changes required for integrin activation is controversial. Several studies suggested that integrins are in a state of equilibrium between a low affinity bent conformation with a closed headpiece and a high affinity extended conformation with an open headpiece. Intermediate affinity conformations have also been suggested (21–23). Other studies proposed an alternative model in which activation involves release of a “deadbolt” created by an interface between the membrane proximal β-tail domain and the α7 helix of the ligand binding βA domain (24–26).

Many proteins contain cysteine residues that form disulfide bonds. Disulfide bonds are commonly considered to play a structural role by assisting protein folding and stabilizing the mature protein. However, for some disulfides, an alternative functional role as redox switches that change the function of their resident protein when they are oxidized or reduced has been established. The functional disulfides are less stable and often form in “forbidden” regions of primary or secondary structures in which they put strain on the protein structure (27, 28). There are two types of functional disulfides: catalytic and allosteric bonds (29). The catalytic bonds are located in the active site of enzymes that mediate thiol/disulfide exchanges (30), whereas the allosteric bonds regulate the function of the protein in which they reside by mediating a change when they are reduced (open) or oxidized (closed) (28, 29, 31). Disulfide bonds can have different configurations based on the angles between the six atoms comprising the bond; there is a typical configuration for each structural, catalytic, and allosteric bond (29, 32).

The β3 subunit contains a cysteine-rich domain comprising four epidermal growth factor (EGF)-like domains at the extracellular portion of the molecule. Each of these EGF-like domains contains eight cysteines that form four disulfide bonds in a 1–5, 2–4, 3–6, and 7–8 pattern except for EGF-1, which lacks the 2–4 disulfide bond (33, 34). The 2–4, 3–6, and 7–8 bonds are conserved in other EGF domains, whereas the 1–5 bond is unique for integrins (Fig. 1B) (35, 36). Cysteines in the β3 subunit were shown to be involved in the conformational changes that occur during activation of αIIbβ3, which requires extracellular free thiols and catalyzed disulfide bond exchanges (37–39). The reducing agent dithiothreitol (DTT) activates purified αIIbβ3 and causes slow progression of platelet aggre-
gation by a mechanism involving both disulfide bond reduction and disulfide bond exchange (40). αIIbβ3 and αvβ3 were also shown to have an endogenous thiol isomerase activity that could be attributed to CXXC sequences in β3 typical for oxidoreductases (41).

In a previous study we examined the role of specific cysteines in the β3 EGF-like domains in surface expression and activation of αIIbβ3. We identified cysteines that have a primary structural role and others that have a functional role. We also identified a free sulphydryl-dependent regulatory role for the integrin unique Cys-560–Cys-583 bond in EGF-4 (42). In this study we further investigated the role of integrin unique disulfide bonds in all four β3 EGF-like domains in αIIbβ3 and in αvβ3. We show that the unique bonds in β3 play different roles in regulating αIIbβ3 and αvβ3 function.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Dulbecco modified Eagle’s medium (DMEM), l-glutamine, and fetal calf serum were purchased from Biological Industries (Beit-Haemek, Israel). Lipofectamine reagent and G418 were from Invitrogen, hygromycin was from Roche Diagnostics, human fibrinogen and DTT were from Sigma, and FITC-conjugated monoclonal antibody P2 against αIIbβ3 was from Immunotech (Marseille, France). FITC-conjugated monoclonal antibody 23C6 against αvβ3 was obtained from ebioscience (San Diego, CA), and FITC-conju-gated rabbit anti-human fibrinogen antibody was from Dako (Glostrup, Denmark). The activating monoclonal antibody anti-LIBS6 was kindly provided by Dr. Mark Ginsberg (Department of Medicine, University of California, San Diego, La Jolla, CA).

**Construction of Expression Vectors for Wild Type (WT) and Mutant cDNAs**—cDNA of human αv in pcDNA1neo was kindly provided by Dr. David Cheresh from the Moores Cancer Center, University of California, San Diego, La Jolla, CA. It was subcloned to the BamHI and XbaI sites of pCDNA3 carrying the G418 resistance as a selection marker. cDNAs of human αIIb and β3 in pCDNA3 vector were kindly provided by Dr. Peter Newman from the Blood Center of Wisconsin, Milwaukee, WI. cDNA of β3 was subcloned into the PvuII site of pCEP4 mammalian expression vector carrying the hygromycin resistance gene as a selection marker (Invitrogen). Substitutions of selected cysteine residues by serine or other residues were created in the pCEP4/β3 vector using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) using two overlapping oligonucleotide primers containing single base pair substitution (available upon request). Correct incorporation of the mutations into the pCEP4/β3 vectors was verified by DNA sequencing. For creation of double mutants containing two cysteine substitutions together, we first introduced one mutation into normal pCEP4/β3 vector and then used the mutant pCEP4/β3 clone as a template for introducing the second mutation.

**Co-transfection of αv or αIIb cDNAs with β3 cDNAs**—Baby Hamster Kidney (BHK) cells were grown in DMEM supplemented with 1 mM MgCl2 and 1 mM CaCl2 (5 × 105 cells/100 μl), and incubated for 30 min at room temperature. Cells were then pelleted again, resuspended in PBS supplemented with 1 mM MgCl2 and 1 mM CaCl2 (5 × 105 cells/100 μl), and incubated for 30 min at room temperature with either 5 μl of FITC-conjugated 23C6 antibody or 20 μl of FITC-con-
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jugated P2 antibody. Cells were then diluted to $5 \times 10^5$ cells/600 µl and analyzed for surface fluorescence by flow cytometry (Coulter, EPICS, Luton, UK). For soluble fibrinogen binding, cells were suspended in PBS, 1 mM MgCl$_2$, 1 mM CaCl$_2$ (non-activating conditions) or PBS supplemented with 0.25 mM MnCl$_2$ and 1 µl of anti-LIBS6 antibody (activating conditions) and then incubated for 45 min at room temperature with 100 µg/ml human fibrinogen. Subsequently, cells were pelleted, washed once with PBS, resuspended in PBS, and incubated for another 20 min at room temperature with 10 µl of FITC-conjugated rabbit anti-human fibrinogen. Subsequently, cells were washed again with PBS, resuspended in 600 µl of PBS, and analyzed by flow cytometry. For measuring fibrinogen binding after DTT treatment, the cells were incubated with 10 mM DTT and analyzed by flow cytometry using FITC-conjugated P2 antibody. Cells were then diluted to 5 × 10$^5$ cells/ml of anti-LIBS6 antibody (activating conditions) and then incubated for 45 min at room temperature with 100 µg/ml human fibrinogen. Subsequently, cells were pelleted, washed once with PBS, resuspended in PBS, and incubated for another 20 min at room temperature with 10 µl of FITC-conjugated rabbit anti-human fibrinogen. Subsequently, cells were washed again with PBS, resuspended in 600 µl of PBS, and analyzed by flow cytometry. For measuring fibrinogen binding after DTT treatment, the cells were incubated with 10 mM DTT in PBS for 5 min at 37 °C and washed once with PBS before adding fibrinogen. Nonspecific binding of the antibodies was measured in mock cells. Fibrinogen binding to αβ3 and αIIbβ3 was expressed as percent binding obtained with 23C6 or P2 antibodies, respectively.

Molecular Dynamics Simulation—The atomic coordinates of the EGF-1 to β-tail domain (E2B) fragment comprising residues 435–690 of the β3 subunit were obtained from the Protein Data Bank, PDB ID 3IJE, for αβ3 integrin (33) and PDB ID 3FCS for αIIbβ3 integrin (34). The addition of the six missing residues (477–482) to the 3FCS protein structure and incorporation of the C523S/C544S double mutation for both proteins were performed using the Swiss-PDB Viewer program (43). WT and mutant molecular dynamics (MD) simulations for each protein were carried out using GROMACS 4.0.7 package of programs (44) with 53a6 force field (45).

The proteins were immersed in triclinic boxes filled with SPC (46) water molecules that extended to at least 12 Å between the molecule and the edge of the box. Na$^+$ and Cl$^-$ ions were added to neutralize the system at a physiological salt concentration of 100 mM. The system, protein, water, and ions were energy-minimized using the steepest descent algorithm with a force tolerance of 1000 KJ mol$^{-1}$ nm$^{-2}$. A 40-ps simulation with position restraints was conducted at 300 K to “soak” the water molecules into the protein. The system was further simulated for a 1-ns unrestrained equilibration simulation. Starting with the final structure of the preliminary simulation, the MD simulation was set up. Position restraint was applied to the first and last Ca atoms of the E2B fragment to prevent it from adopting conformations that are not plausible with its orientation within the whole αIIbβ3 or αβ3 integrin. The MD simulations were run under NPT conditions using Berendsen’s coupling algorithm for keeping the temperature and pressure constant ($p = 1$ bar; $\tau_p = 0.5$ ps; $T = 300$ K; $\tau_T = 0.1$ ps) (47). During the MD runs, the LINCS algorithm (48) was used to constrain the lengths of all bonds; the water molecules were restrained using the SETTLE algorithm. A 12 Å cutoff was used for the van der Waals interactions. The long-range electrostatic interactions were treated by the particle mesh Ewald method (49). The coordinates were saved every 1 ps. The MD simulation time-step was 2 fs. Total simulation time for each protein was 30 ns.

Trajectory Analysis—Trajectories obtained from various simulations were analyzed using GROMACS package of programs, including root mean square deviation (r.m.s.d.) calculations that analyze the extent to which the simulated structure differs from the original one and cluster analysis, which was performed with an r.m.s.d. cut-off value of 0.4 nm (50).

For the calculation of the domain contribution to the total r.m.s.d. of the proteins, the individual r.m.s.d. for each domain was normalized to the value of the whole protein mass. Visual analysis of the trajectories was performed using Visual Molecular Dynamics program (50).

Analysis of Unique Disulfide Bonds Configuration—The configurations of the four unique disulfide bonds, Cys-437–Cys-457, Cys-473–Cys-503, Cys-523–Cys-544, and Cys-560–Cys-583, taken from the αIIbβ3 crystal structure (PDB 3FCS) and the αβ3 crystal structure (PDB 3IJE) were analyzed by the Disulfide Bond Analysis software available at the Adult Cancer Program Lowy Cancer Research Centre, The University of New South Wales, Sydney, Australia (32). In the 3IJE structure of αβ3, the sulfur atoms of cysteines 473 and 503 are not covalently connected, probably because of incorrect assignment during structure determination (51). We, therefore, used the original structure-factor file from the PDB 3IJE to re-refine cysteines 473 and 503. In this new refined structure, the sulfur atoms are covalently connected, being 2.1 Å apart, whereas in the original refinement, they are 3.3 Å apart. We then used the new coordinates to predict the type of disulfide bond.

RESULTS

Surface Expression of αβ3 and αIIbβ3—To determine the importance of the four β3 unique disulfide bonds for the structure and function of αIIbβ3 and αβ3, we disrupted the Cys-437–Cys-457, Cys-473–Cys-503, Cys-523–Cys-544, and Cys-560–Cys-583 disulfide bonds shown in Fig. 1A by replacing Cys by Ser residues. The putative bonds link the non-conserved integrin unique cysteines 1 and 5 in each EGF domain as shown in Fig. 1B. The cysteine pairs forming the disulfide bonds were derived from the αIIbβ3 crystal structure, PDB 3FCS (34) and the αβ3 crystal structure, PDB 3IJE (33). We mutated the eight cysteines of the four pairs as well as both cysteines in each pair. We also mutated Cys-567 and Cys-575 to Ser to analyze the disruption of the 2–4 and 3–6 conserved disulfide bonds in EGF-4 (Fig. 1B). Altogether we created 14 different β3 mutants as shown in Supplemental Table 1.

All β3 mutants were expressed in BHK cells alone, giving rise to a chimeric complex with the endogenous hamster αv or with normal human αv and αIIb. The surface expression was analyzed by flow cytometry using αIIbβ3- or αβ3-specific antibodies. Because the 23C6 antibody against αβ3 recognizes the chimeric as well as the human αβ3 (data not shown), we substracted the expression obtained for BHK cells only transfected with human β3 from the expression obtained by BHK cells transfected with both human β3 and αv. The effect of cysteine substitutions on surface expression of human αIIbβ3 versus αβ3 is shown in Fig. 2. Disruption of the integrin unique disulfide bonds by most mutants resulted in moderate reductions of 3

L. Levin, E. Zelzion, Y. Tsfadia, E. Nachliel, M. Gutman, and Y. Einav, manuscript in preparation.
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Most cysteine substitutions had similar effects on the expression of αIIbβ3 and αvβ3. A two-way analysis of variance test that compared the expression of all αIIbβ3 mutants with all αvβ3 mutants yielded no statistically significant different between the integrins (p = 0.23). However, there were some mutations that displayed a different effect on the two receptors; disrupting the Cys-473–Cys-503 bond in EGF-2 resulted in a significantly reduced surface expression of αvβ3 relative to αIIbβ3 (p < 0.05), whereas disruption of the Cys-437–Cys-457 bond only by the C457S mutation resulted in a significantly reduced expression of αIIbβ3 compared with αvβ3 (p < 0.01).

Effect of β3 Cysteine Substitutions on Activation State of αvβ3 and αIIbβ3—The activation state of mutated αvβ3 and αIIbβ3 receptors was determined by binding of soluble fibrinogen to BHK cells under non-activating conditions (Fig. 3). Excluded were the conserved C567S and C575S mutants because their expression was too low to enable reliable fibrinogen binding measurements (Fig. 2).

Cells harboring the unique disulfide bond disruptions in EGF-1 and EGF-2 exhibited a profound increase in fibrinogen binding to both αvβ3 and αIIbβ3 mutant receptors compared with the WT receptors (p < 0.0001) (Fig. 3) implying that these mutations give rise to constitutively active receptors. However, the extent of activation varied between αvβ3 and αIIbβ3; disrupting the Cys-437–Cys-457 bond in EGF-1 by any of the three mutations resulted in a greater increase in fibrinogen binding to the αIIbβ3 mutants when compared with the αvβ3 mutants (C437S and C457S, p < 0.01; C437S/C457S, p < 0.05). Disrupting the Cys-473–Cys-503 bond in EGF-2 also resulted in a profound increase in fibrinogen binding to both αIIbβ3 and αvβ3, respectively (Fig. 2).

FIGURE 1. Disulfide bonds in the EGF domains of the β3 subunit. A, shown is a ribbon representation of EGF-1 (white), EGF-2 (black), EGF-3 (light gray), and EGF-4 (gray) domains of β3 (PDB code 3UJE). The four cysteine pairs that were disrupted in the four EGF domains are represented by spheres. B, shown is sequence alignment of the four EGF domains of β3 based on the crystal structure of αvβ3 (33). Depicted are the three typical disulfide bonds formed between the conserved C2, C3, C6, and C7-C8 cysteines (white boxes) and the disulfide bond formed between the unique integrin C1-C5 cysteines (gray boxes). EGF-1 lacks C2 and C4 and, thus, lacks the C2-C4 bond. The EGF domain boundaries are assigned by their numbering in the SwissProt data base. The cysteines that were mutated in this study are shown in parentheses.

FIGURE 2. Surface expression of normal or mutant αIIbβ3 and αvβ3 integrins. Surface expression of αIIbβ3 or αvβ3 in BHK cells expressing normal or mutated β3 together with normal αIIb or αv were measured by flow cytometry using FITC-conjugated P2 antibody against αIIbβ3 or FITC-conjugated 23C6 antibody against αvβ3. The disulfide bonds that were disrupted by the mutations are depicted above the bars. The EGF domains that contain the disulfide bonds are shown below the bars. Error bars represent the mean ± S.E. of at least three experiments. Statistically significant differences were calculated by a two-tailed, unpaired t test. Asterisks denote a p value of <0.05.
Overall, in contrast to the similar effect of the mutations on surface expression of αIIbβ3 and αvβ3, the effect of the mutations on the activation state of αIIbβ3 and αvβ3 differed significantly by two-way analysis of variance test \((p < 0.0001)\).
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A

![Graph A showing Fibrinogen binding compared to Fibrinogen + LIBS6 for different conditions.

B

![Graph B showing Fibrinogen binding compared to Fibrinogen + LIBS6 for different conditions.

C

![Graph C showing fold increase in fibrinogen binding under activating conditions.

R² = 0.9258

Fibrinogen binding in non-activating conditions, % of receptor expression
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The C523S/C544S αvβ3 mutant that was not active under non-activating conditions was the only exception. It was only partially activated by anti-LIBS6 and Mn2⁺, yielding less than 50% fibrinogen binding compared with WT αvβ3 (p < 0.001) (Fig. 4B). Exclusion of the C523S/C544S αvβ3 mutant from the regression analysis depicted in Fig. 4C improved the fit of the inverse correlation (R² = 0.9604).

To examine whether the C523S/C544S αvβ3 mutant could be further activated, we amplified the activation conditions by adding 10 mM DTT together with anti-LIBS6 and Mn2⁺ (Fig. 5). DTT indeed increased significantly by 1.75-fold fibrinogen binding to C523S/C544S αvβ3 compared with WT αvβ3 (p < 0.05). However, the binding of fibrinogen was still only 56% of that of the binding observed in WT αvβ3. This difference was of borderline statistical significance (p = 0.059). These data indicated that even in the presence of three different activators, the C523S/C544S αvβ3 mutant could not be activated to the same extent as WT αvβ3.

**Molecular Dynamics Analysis**—To test whether the profound difference between the effects of the C523S/C544S mutation on αIIbβ3 and αvβ3 stemmed from altered effects of the αIIb and αv on the β3 structure, we performed MD analysis of the mutated β3 E2B fragments comprising the four EGF domains and the β3 tail domain derived from the αIIbβ3 and the αvβ3 crystal structures. We traced the modulation of the proteins with time by examining the backbone r.m.s.d. relative to the initial structure (Fig. 6A). The simulated E2B fragment of the C523S/C544S αIIbβ3 mutant underwent major structural modulation until 10 ns and stabilizes around an average value of 1.2 nm up to the end of the simulation. The same E2B fragment, taken from the C523S/C544S αvβ3 mutant, showed an unstable mode of conformational change; the r.m.s.d. value rose rapidly to above 1 nm within 5 ns, it stayed high up to 10 ns, and then declined and fluctuated around 0.7 nm up to the end of the simulation. The resulting αIIbβ3 E2B structure was substantially different from the original one, whereas the final αvβ3 E2B structure resembled the starting conformation (Fig. 6A and supplemental Fig. 1).

Cluster analysis showed that the mutant αIIbβ3 E2B fragment demonstrated few conformations during the simulation and seemed to be locked in a stable conformation from a 10-ns time frame (Fig. 6B). In contrast, the mutant αvβ3 E2B fragment showed six populated clusters with no significant preference by one of them. The most abundant cluster was found within a 17–26-ns interval.

Fig. 6C presents the extent by which each domain contributed to the total protein r.m.s.d. There was a striking difference between αIIbβ3 and αvβ3; whereas in αvβ3 the effect of all domains was similar, in αIIbβ3 there was variable contribution of the domains. In the E2B fragment of αIIbβ3, EGF-2 accounted for most of the structural changes, whereas EGF-4 contributed the less. The other three domains had an intermediate and equal contribution to the protein conformation dynamics. Consistent with the r.m.s.d. of the whole protein (Fig. 6A) and the stable abundant cluster (Fig. 6B), the r.m.s.d. values of all domains barely fluctuated from 10 ns until the end of the simulation. In the E2B fragment of αvβ3, all five domains exhibit identical r.m.s.d. patterns throughout the simulation, once again showing no significant conformational changes from the original structure.

To further understand the structural differences between the mutated β3 E2B fragments of αIIbβ3 and αvβ3, we superimposed the most abundant conformation of each mutant fragment and the relevant simulated WT fragments (Fig. 6D). In αIIbβ3, the location of the 544 residue in the mutant fragment resembled that of its original location in the WT fragment, whereas the mutated 523 residue moved further apart from its original WT position and from its 544 partner, causing a change in the orientation of the beginning of the EGF-3 domain and especially of the EGF-2 domain. Thus, whereas in WT αIIbβ3 there is an angle of ~90° between EGF-2 and EGF-3, in the mutant these two domains are positioned almost on the same line. In the αvβ3 mutant the shifting of the 523 residue from its original position and from its 544 partner is less pronounced. Thus, the angle between EGF-2 and EGF-3 and the resulting orientation of the EGF-2 domain is just slightly different from the WT protein.

Collectively, the MD simulations of the isolated E2B fragment of β3 showed that the C523S/C544S double mutation affected the β3 subunit differently depending on its α subunit partner. The αIIbβ3 mutant gained a new highly stable structure, whereas the αvβ3 mutant was unstable and fluctuated mostly around its starting conformation.

**Classification of Unique Disulfide Bonds**—Table 1 summarizes the results obtained by the Disulfide Bond Analysis software. The EGF-1 Cys-437–Cys-457 and the EGF-2 Cys-473–Cys-503 disulfide bonds in both αIIbβ3 and αvβ3 structures are...
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A

C523S/C544S αvβ3

B

C523S/C544S αvβ3

C523S/C544S αIIbβ3

C

C523S/C544S αvβ3

D

WT and mutant αvβ3

C523S/C544S αIIbβ3

WT and mutant αIIbβ3
TABLE 1  
Classification of the four unique disulfide bonds by their configuration

| EGF domain | Disulfide bond | Pairing | Configuration | Classification |
|------------|----------------|---------|---------------|----------------|
| EGF-1      | 437–457        | 1–5     | ~/~ RHHook    | Catalytic      |
| EGF-2      | 473–503        | 1–5     | ~/~ RHHook    | Catalytic      |
| EGF-3      | 523–544        | 1–5     | LHHook (a/bβ3 )| Unclassified  |
| EGF-4      | 560–583        | 1–5     | LHHook        | Unclassified   |

*According to a/bβ3 crystal structure, PDB 3FCS (34).
*According to a/bβ3 crystal structure, PDB 3FE (33).

in a ~/- RHHook configuration that is typical for a catalytic disulfide bond. The EGF-3 Cys-523–Cys-544 bond in the a/bβ3 structure is ~ RHStaple, which is a typical allosteric configuration, but in the a/bβ3 structure it is ~ LHHook, which was not classified to any type of disulfide bond. The EGF-4 Cys-560–Cys-583 disulfide bond is in ~ LHHook configuration in both a/bβ3 and a/bβ3 structures.

DISCUSSION

The aim of this study was to evaluate the importance of the unique disulfide bonds in the four EGF-like domains of the a/b subunit for the structure and function of a/bβ3 and a/bβ3. For this purpose we created mutants in which each of the four unique disulfide bonds in the four EGF-like domains was disrupted by cysteine to serine substitutions. We determined the surface expression and activation state of the mutants by using specific antibodies for each integrin or fibrinogen, respectively. Because free sulfhydryls were shown to play a role in a/bβ3 activation by disulfide bond rearrangement (37–40, 42), we compared the effect of substituting both cysteines to the effect of substituting only one cysteine yielding free sulfhydryls in their cysteine partners.

Effects of Disulfide Bond Disruptions on a/bβ3 and a/bβ3 Expression—Our results summarized in Table 2 show that disruptions of the unique bonds in the EGF domains of a/b resulted in mild reductions of surface expression of both a/bβ3 and a/bβ3, implying that these bonds do not play a primary structural role in the EGF folding. This is consistent with them being non-conserved bonds that do not exist in EGF domains of other proteins (36). In contrast, disruptions of the two disulfide bonds that are conserved in all EGF domains, i.e. Cys-567–Cys-581 (C2-C4 of EGF-4) and Cys575–Cys586 (C3-C6 of EGF-4), by creating C567S and C575S, respectively, resulted in a profound reduction of a/bβ3 and a/bβ3 surface expression compared with the WT receptors. This indicates that these conserved bonds are important for the EGF-4 folding and their disruption, especially of the Cys-575–Cys-586 bond, exerts a deleterious effect on the EGF-4 structure. These results are consistent with our previous study in which we demonstrated a smaller effect on a/bβ3 expression by disruption of non-conserved disulfide bonds when compared with disruption of conserved disulfide bonds (42). Our present findings indicate that this phenomenon also exists in a/bβ3 integrin.

In our study most mutations had a similar effect on surface expression of a/bβ3 and a/bβ3. These results are of particular interest as several mutations in a/b were previously shown to exert a more deleterious effect on a/bβ3 expression than on a/bβ3 expression, i.e. H280P, S162L, R216G (52), L196P (53), and R216A (54), suggesting that the expression of a/bβ3 is more strictly regulated than a/bβ3. Notably, none of these reported mutations disrupted disulfide bonds in the EGF domains. Thus, our results suggest that the different a subunits of each receptor have no substantial effect on the structural outcome of disulfide bond disruptions in EGF domains of the common b subunit.

Few mutations did exert a different effect on a/bβ3 and a/bβ3 expression (Table 2). The C457S mutation, which disrupts the EGF-1 Cys-437–Cys-457 disulfide bond, yielded significantly reduced expression of a/bβ3 compared with a/bβ3 (Fig. 2). Additionally, all three mutations disrupting this bond resulted in only 40–50% of WT a/bβ3 surface expression, contrasting with 65–85% of WT a/bβ3 surface expression. Thus, although this bond is not conserved, it seems important for the EGF-1 folding, and its disruption impairs the formation of a/bβ3 more than a/bβ3.

Surprisingly, all three mutations disrupting the unique EGF-2 Cys-473–Cys-503 disulfide bond displayed the opposite effect, resulting in less expression of a/bβ3 compared with a/bβ3, suggesting that this bond is more important for the formation of the a/bβ3 complex than for the a/bβ3 complex.

Effects of Disulfide Bond Disruptions on a/bβ3 and a/bβ3 Function—Several studies showed that disruptions of disulfide bonds in the EGF domains of a/b destabilize the inactive conformer of a/bβ3 and give rise to constitutively active receptor (42, 55, 56). We also identified a regulatory role for the unique Cys-560–Cys-583 disulfide bond in EGF-4 (42). In this study we addressed the question of whether other unique disulfide bonds in a/b have a regulatory role in a/bβ3 activation and whether they have a similar role in a/bβ3 activation. Our data demonstrate a diverse effect of disulfide bond disruptions on a/bβ3 and a/bβ3 activation (Table 2). Disruptions of the unique EGF-1 Cys-437–Cys-457 and EGF-2 Cys-473–Cys-503 bonds by all single mutants and double mutants resulted in constitutively active a/bβ3 and a/bβ3 (Fig. 3), suggesting that these bonds are important for stabilization of the inactive conformers of both receptors and that the presence of free sulfhydryls is not necessary for the activation. The more profound

FIGURE 6. Molecular dynamics simulations of a/bβ3 and a/bβ3 protein fragments. A, shown is r.m.s.d. values (nm) of the backbone atoms from their initial coordinates as a function of simulation time. The r.m.s.d. of the mutated b3 fragments of a/bβ3 (lower panel) and a/bβ3 (upper panel) are shown. Notable was a significant conformational change in the mutated b3 fragment of a/bβ3, whereas the mutated b3 fragment of a/bβ3 returned to its original conformation. B, cluster distribution throughout the simulation was calculated with a r.m.s.d. cut-off value of 0.4 nm. It shows a stable dominant cluster in the a/bβ3 mutated fragment (lower panel) and several clusters with none being dominant in the a/bβ3 mutated fragment (upper panel). C, shown is the relative contribution of the different domains to the total protein r.m.s.d. The domains are color-coded. In a/bβ3 (lower panel), the domains contribute differently to the conformational change, whereas in a/bβ3 (upper panel) all domains changed to the same extent. D, superposition of mutant and WT most abundant conformations of b3 fragments of a/bβ3 (lower panel) and a/bβ3 (upper panel). The mutant fragments are shown in red, and the WT fragments are shown in blue. The focus is on the EGF-3 domain, which is presented in solid colors. The end of EGF-2 (left) and the beginning of EGF-4 (right) are presented in transparent colors. The WT Cys-523 and Cys-544 residues and the mutated Ser-523 and Ser-544 residues are indicated.
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**Table 2**

| Disrupted disulfide bond | Domain | Induced activation by anti-LIBS6 and Mn2⁺ | Proposed role in activators | Surface expression | Activation state | Proposed role in holotoxin activation | Proposed role in stabilizing inactive conformer |
|--------------------------|--------|------------------------------------------|-----------------------------|-------------------|----------------|--------------------------------------|---------------------------------------------|
| EGF-1                    |        | Highly active                            |                             | 65–80% of WT in all mutants | Stabilization of the inactive conformer | Stabilization of the incompact folded form | Stabilization of the inactive conformer |
| EGF-2                    |        | Highly active                            |                             | 65–80% of WT in all mutants | Stabilization of the inactive conformer | Stabilization of the inactive conformation | Stabilization of the inactive conformer |
| EGF-3                    |        | Highly active                            |                             | 65–80% of WT in all mutants | Stabilization of the inactive conformer | Stabilization of the inactive conformation | Stabilization of the inactive conformer |
| EGF-4                    |        | Highly active                            |                             | 65–80% of WT in all mutants | Stabilization of the inactive conformer | Stabilization of the inactive conformation | Stabilization of the inactive conformer |
| EGF-4                    |        | Highly active                            |                             | 65–80% of WT in all mutants | Stabilization of the inactive conformer | Stabilization of the inactive conformation | Stabilization of the inactive conformer |
| EGF-4                    |        | Highly active                            |                             | 65–80% of WT in all mutants | Stabilization of the inactive conformer | Stabilization of the inactive conformation | Stabilization of the inactive conformer |

**Activation of αllβ3 mutants**

Disruption of the Cys-560–Cys-583 bond in EGF-4 by mutating Cys-560 resulted in constitutive activation of αllβ3, but mutating its partner Cys-583 or both cysteines resulted in almost no activation of the receptor, suggesting that αllβ3 activation is dependent on generation of free sulfhydryls, probably in Cys-583. Disruption of this bond also had a variable effect on ανβ3 activation, giving rise to a very limited activation by mutating each Cys-560 or Cys-583 and no activation by mutating both cysteines. These results imply that ανβ3 activation is dependent on generation of free sulfhydryls in either Cys-560 or Cys-583. A similar differential activity between cysteines of disulfide pairs was shown for genotoxin in which mutating of Cys-96 or Cys-149 but not their respective cysteine partners abolished holotoxin activity, strongly suggesting that these disulfide bonds participate in sulfhydryl-dependent redox reactions driven by Cys-96 and Cys-149 (57). Taken together, our results support and extend our previous study (42) by implying a sulfhydryl-dependent regulatory role for the Cys-560–Cys-583 bond in the activation of both αllβ3 and ανβ3.

A remarkable difference between the receptors was demonstrated when the Cys-523–Cys-544 bond was disrupted. All single or double mutations gave rise to constitutively active αllβ3, whereas only the single mutations but not the double mutation gave rise to constitutively active ανβ3. These results suggest that activation of ανβ3 but not αllβ3 is dependent on the presence of free sulfhydryls in either Cys-523 or Cys-544. Moreover, although all other αllβ3 and ανβ3 mutant receptors could be further activated by anti-LIBS6 and Mn2⁺ to the same extent or even higher than WT receptors (Fig. 4), the C523S/C544S ανβ3 mutant was activated only to less than 50% that of WT ανβ3, supporting the notion that ανβ3 activation is dependent on free sulfhydryls in either Cys-523 or Cys-544. Interestingly, the addition of the reducing agent DTT further increased the activation of the C523S/C544S ανβ3 mutant but was still far below the extent of WT ανβ3 activation under the same conditions (Fig. 5). DTT was shown to activate integrins by a mechanism involving both reduction of disulfide bonds and induction of disulfide bond rearrangement (40). It is reasonable to assume that reduction of the disulfide bonds by DTT did occur in the C523S/C544S ανβ3 mutant, resulting in further activation of the receptor, but because free sulfhydryls in both Cys-523 and Cys-544 were absent, disulfide bond rearrangement could not occur, thereby preventing full activation of the receptor.

Anti-LIBS6 and Mn2⁺ are commonly used as activators of αllβ3 and have been also used for activation of ανβ3 (16, 20). The fact that all mutants could be further activated by these activators, even those that were highly activated under nonactivating conditions, indicates that these constitutively active receptors did not display their fully active conformer. These
results are consistent with studies showing that αIIbβ3 displays multiple conformational states (21, 22) and suggest that this is also the case in αvβ3.

**Different Effect of αIIb and αv Subunits on Structure of β3 Subunit—**Several mutations that do not disrupt disulfide bonds were shown to induce formation of constitutively active αIIbβ3 and αvβ3 including I719A, E749A, and D723R in the β3 tail (58, 59), N305T in the βI domain (60), and T562N in EGF-4 (61). In our study we compared for the first time the effect of disrupting the four unique disulfide bonds in the EGF domains of β3 in αIIbβ3 and αvβ3 and showed that indeed, most mutations yielded constitutively active αIIbβ3 and αvβ3. Nevertheless, some mutations displayed a different effect on the activation state of the two integrins. Because the most substantial difference between αIIbβ3 and αvβ3 was shown for the C523S/C544S double mutation, we sought a possible structural mechanism that agrees with this difference. The crystal structures of αIIbβ3 (34) and αvβ3 (33) display a similar structure of the common β3 subunit (supplemental Fig. 2). We, therefore, used the MD analysis to track changes in the structure of the mutated C523S/C544S β3, taken from each of the αIIbβ3 and αvβ3 crystal structures over time (Fig. 6). The MD analysis revealed a substantial difference between the structural changes of the mutated β3 from each receptor, which agrees with the functional differences shown by flow cytometry. The β3 subunit from αIIbβ3 changed considerably from the original structure and remained stable in its new conformation probably giving rise to αIIbβ3 activation (supplemental Fig. 1A). The most abundant structure of the mutated β3 subunit of αIIbβ3 displayed a substantial distance between the 523 and the 544 residues. Thus, the orientation of the EGF-2 domain relative to the EGF-3 domain changed in the mutant subunit and displayed an expanded angle relative to the WT subunit (Fig. 6D). The EGF-2/EGF-1 interface comprises the β-knee of the β3 subunit, and opening of this knee by rearrangements in the long loop connecting C1 to C2 in EGF-2 was shown to induce integrin activation (51). We propose that changing the orientation of EGF-2 by the C523S/C544S mutant has an effect on the EGF-2/EGF-1 interface, resulting in opening of the knee and activation of αIIbβ3. In contrast to αIIbβ3, the β3 subunit from αvβ3 exhibited an initial change but then returned to its original structure, probably yielding a non-active conformation (supplemental Fig. 1B). Compared with αIIbβ3, the change in the orientation of the EGF-2 domain in the most abundant structure of the mutated β3 subunit of αvβ3 was less pronounced, and the EGF-2/EGF-3 interface resembled the WT protein, consistent with its non-activating conformation (Fig. 6D). Collectively, these results strongly suggest that the αv or αIIb subunits differentially affect the structure of the same β3 subunit, resulting in a fundamental difference in the ability of the αIIbβ3 or αvβ3 integrins to be activated by the C523S/C544S mutation.

Notably, using an alanine scanning method, Donald et al. (62) identified a D552A mutation in the EGF-3 domain of β3 that constitutively activates αvβ3 but not αIIbβ3. This might be another example of a different effect of the αv or αIIb subunits on the β3 structure leading to a functional difference between the receptors. It is interesting that both the D552A and the C523S/C544S mutations are located in the same EGF-3 domain. Further investigation is needed to find out whether there are more structural differences between the β3 subunit of the two integrins that differently affect their function and whether these differences cluster in the EGF-3 domain.

**Possible Regulatory Role for Unique Disulfide Bonds in β3 EGF Domains as Redox Switches—**Disulfide bonds that form in primary and secondary regions that put strain on the protein structure are often involved in redox regulation (27, 28). However, the crystal structures of both αIIbβ3 and αvβ3 revealed that none of the four unique disulfide bonds analyzed in this study was located in such regions. Nevertheless, our results indicate that there is a free sulphydryl-dependent regulatory role for the EGF-3 Cys-523–Cys-544 in αvβ3 activation but not in αIIbβ3 activation. Interestingly, the configuration analysis (Table 1) supports our findings showing that this bond is −LHHook in αIIbβ3, which is unclassified, whereas in αvβ3 it is −RHStaple, classified as an allosteric bond that was shown to have a regulatory role in the function of several proteins. The EGF-4 Cys-560–Cys-583 disulfide bond is in the unclassified −LHHook configuration in both structures. This configuration displays a very short distance between the α-carbons similar to −RHStaple and thus might also have a sulfhydryl-dependent regulatory role as was indicated in our results.

The integrin unique disulfide bonds in EGF-1 (Cys-437–Cys-457) and EGF-2 (Cys-473–Cys-503) appear to be important for stabilizing the inactive αIIbβ3 and αvβ3 conformers. Interestingly, these bonds are classified as functional-catalytic (Table 1). Indeed, our results pointed to a functional role for these bonds, but there was no indication for a catalytic function. Notably, Cys-457 and Cys-473 are the first cysteines in the CXXC motif that were proposed to play a role in the endogenous thiol isomerase activity of β3 integrins (41). Further investigation is needed to find out whether these bonds are involved in catalysis of disulfide bond exchanges. Overall, we demonstrate a good correlation between the configurations of the studied disulfide bonds and their functional roles, showing that configuration analysis can be an important tool for predicting the role of disulfide bonds.

**Affinity Modulation of αvβ3 and Its Implications—**Numerous studies were carried out to discern the mechanisms of αIIbβ3 affinity modulation, whereas αvβ3 was less studied. αvβ3 can adhere to various ligands without prior activation, but its activation either by inside-out signaling or by activating antibodies promotes its adhesive and migratory properties and adenovirus-mediated gene transfer (16, 18, 63). Moreover, activation of αvβ3 by activating antibodies or by the activating C723R mutation induced angiogenesis and tumor metastasis (12, 19, 64), suggesting that affinity modulation of αvβ3 has a physiological and pathological importance. Our study suggests that like αIIbβ3, αvβ3 activation can be regulated by a disulfide bond exchange-dependent mechanism. This mechanism of affinity modulation might represent a rapid pathway of αvβ3 activation that can influence various processes involving αvβ3 such as cell migration, angiogenesis, adenovirus transfer, and tumor metastasis. Exploring the specific disulfide bonds involved in this pathway and identifying those that differently regulate αvβ3 and αIIbβ3 function can be of particular impor-
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tance for the development of specific drugs that target one receptor and not the other.

In conclusion, the results presented in this study and previous studies suggest that the unique disulfide bonds in the β3 subunit play a primary functional role in αIIbβ3 and αβ3 activation. We also suggest a free sulfhydryl-dependent regulatory role for the EGF-4 Cys-560–Cys-583 bond in both receptors and for the EGF-3 Cys-523–Cys-544 bond only in αβ3. The integrin-specific disulfide bonds in EGF-1 (Cys-437–Cys-457) and EGF-2 (Cys-473–Cys-503) play a role in stabilizing the inactive conformation of both receptors.

Acknowledgments—We thank Dr. David Cheresh from the Moores Cancer Center, University of California, San Diego, La Jolla, CA for providing anti-LIBS6 antibody, Dr. David Cheresh from the Moores Cancer Center, University of California, San Diego, La Jolla, CA for providing αv cDNA, and Dr. Peter Newman from the Blood Center of Wisconsin, Milwaukee, WI for providing αIIb and β3 cDNAs.

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