

Amino Acid Residues in the PSI Domain and Cysteine-rich Repeats of the Integrin β_2 Subunit That Restrain Activation of the Integrin $\alpha_X\beta_2$ *

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Qun Zang and Timothy A. Springer‡

From the Center for Blood Research, Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

The leukocyte integrin $\alpha_X\beta_2$ (p150,95) recognizes the iC3b complement fragment and functions as the complement receptor type 4. $\alpha_X\beta_2$ is more resistant to activation than other β_2 integrins and is inactive in transfected cells. However, when human α_X is paired with chicken or mouse β_2 , $\alpha_X\beta_2$ is activated for binding to iC3b. Activating substitutions were mapped to individual residues or groups of residues in the N-terminal plexin/semaphorin/integrin (PSI) domain and C-terminal cysteine-rich repeats 2 and 3. These regions are linked by a long range disulfide bond. Substitutions in the PSI domain synergized with substitutions in the cysteine-rich repeats. Substitutions T4P, T22A, Q525S, and V526L gave full activation. Activation of binding to iC3b correlated with exposure of the CBR LFA-1/2 epitope in cysteine-rich repeat 3. The data suggest that the activating substitutions are present in an interface that restrains the human α_X /human β_2 integrin in the inactive state. The opening of this interface is linked to structural rearrangements in other domains that activate ligand binding.

The integrin $\alpha_X\beta_2$ (p150,95, CD11c/CD18) is one of four integrins that are restricted in expression to leukocytes and have different α subunits associated with a common integrin β_2 subunit (1, 2). $\alpha_X\beta_2$ is also known as the complement receptor type 4. Integrin $\alpha_X\beta_2$ is expressed on the surface of macrophages, monocytes, granulocytes, and certain activated and B lymphocyte subpopulations (3–6). Upon activation, $\alpha_X\beta_2$ binds to its ligands, complement component iC3b (7–9) and fibrinogen (5, 10), and mediates leukocyte adherence to endothelium and other cells, possibly by binding to intercellular adhesion molecule 1 (ICAM-1) (4, 11–13).

Comparisons among leukocyte integrins suggest that $\alpha_X\beta_2$ has the highest barrier to activation of ligand binding. On cells that coexpress the integrins $\alpha_X\beta_2$ and Mac-1 ($\alpha_M\beta_2$), stronger cellular activation is required to activate $\alpha_X\beta_2$ than $\alpha_M\beta_2$ for binding to the ligand iC3b (8). When transfected into COS or 293T cells, the integrins LFA-1 ($\alpha_L\beta_2$) and $\alpha_M\beta_2$ are active in binding ligands; however, $\alpha_X\beta_2$ is not (14). Construction of chimeric α_M and α_X α subunits showed that many reciprocal exchanges activated ligand binding, suggesting that structural

perturbations released restraints that otherwise held $\alpha_X\beta_2$ in an inactive state (14). Interestingly, although $\alpha_X\beta_2$ expressed on COS-7 cells could not bind to the ligand iC3b, an interspecies hybrid, $\alpha_X\beta_2$, comprised of chicken β_2 and human α_X subunits was constitutively active in binding iC3b (7). By contrast, human α_M /chicken β_2 and human α_M /human β_2 integrin heterodimers bound iC3b equally well. Studies with mAb¹ map ligand binding to the I domain of the $\alpha_X\beta_2$ α_X subunit (7). These findings suggest that an intersubunit restraint on α_X conformation is loosened with the chicken β_2 subunit so that α_X can more readily adopt the conformation that binds iC3b. It may be significant in light of this finding and the finding that $\alpha_X\beta_2$ is more difficult to activate than $\alpha_L\beta_2$ or $\alpha_M\beta_2$ that the association between α_X and β_2 is more difficult to disrupt with denaturing conditions than the association between α_L and β_2 or between α_M and β_2 (15).

A key question of current integrin research is the nature of the structural alterations in “inside-out signaling” that enables ligand binding by the extracellular domain in response to signals impinging on the cytoplasmic/transmembrane domains. Electron microscopy reveals an overall integrin structure of a globular head region connected to the cell membrane by two stalk regions (16). The head region binds ligand and contains domains from the N-terminal portions of both the α and β subunits. Seven 60-amino acid repeats in the N-terminal half of the α subunit have been predicted to fold into a β -propeller domain (17). A subset of integrin α subunits, including the α_X subunit, contains an I domain inserted between β -sheets 2 and 3 of the β -propeller domain. The I domain has a structure like small G proteins with a metal ion-dependent adhesion site at the top of the domain where ligand is bound (18, 19). A conformational change at the MIDAS that regulates ligand binding is linked structurally to a large movement of the C-terminal α -helix that connects the bottom of the I domain to the β -propeller domain (19–23). A domain in the β subunit has a predicted fold that is like the I domain and a MIDAS-like site (18, 24–26). This β subunit I-like domain associates with the side of the α subunit β -propeller domain at β -sheets 2 and 3 (27, 28) and is thus near to the α subunit I domain, which links to β -sheets 2 and 3 at the top of the β -propeller domain.

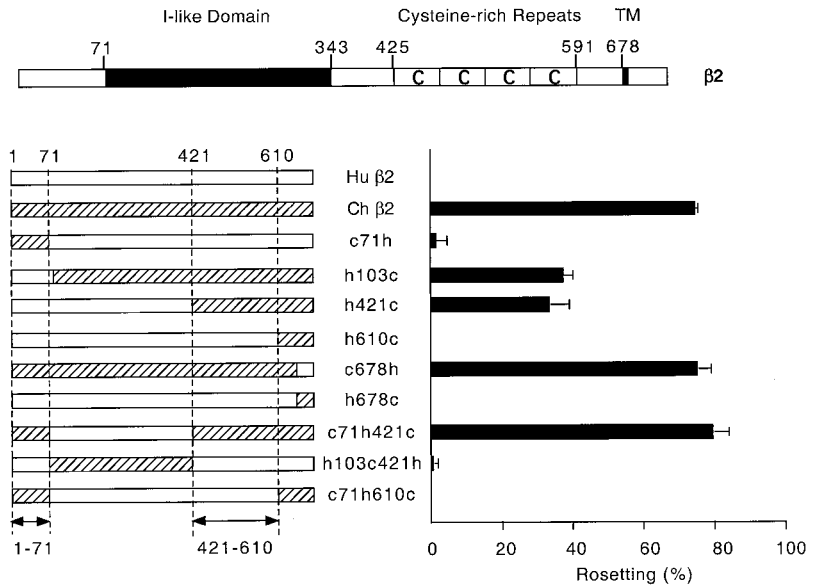
The stalk regions provide the crucial link between the signals impinging on the α and β subunit transmembrane and cytoplasmic domains and the conformational changes that occur in the ligand-binding head region. In the α subunit, the stalk region appears to consist of the region C-terminal to the predicted β -propeller domain. The stalk region is predicted to consist of domains with a two-layer β -sandwich structure (29).

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‡ To whom correspondence should be addressed: Center for Blood Research, Dept. of Pathology, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Tel.: 617-278-3200; Fax: 617-278-3030; E-mail: springer@sprgsi.med.harvard.edu.

¹ The abbreviations used are: mAb, monoclonal antibody; MIDAS, metal ion-dependent adhesion site; FBS, fetal bovine serum; c, chicken; h, human; m, mouse.

FIG. 1. Regions 1–71 and 421–610 of the chicken β_2 subunit activate $\alpha_x\beta_2$ binding to iC3b. The indicated human/chicken β_2 chimeras were cotransfected with the human α_x subunit into 293T cells. The percentage of transfected cells rosetting with >10 iC3b-sensitized erythrocytes was determined with microscopy. Rosetting was calculated as the percentage of rosetting cells divided by the percentage of 293T cells expressing $\alpha_x\beta_2$ as determined by immunofluorescence flow cytometry. Results are averages of three experiments. Bars indicate standard deviation. *Hu*, human; *Ch*, chicken



Four subregions of the α_M stalk have been defined with mAb epitopes, three of which react with mAbs whether or not the β subunit is coexpressed. In the β subunit, the stalk region appears to consist of the cysteine-rich regions that precede and follow the I-like domain, *i.e.* residues 1–103 and 342–678 in β_2 . These cysteine-rich regions are linked by a long range disulfide bond defined in β_3 that is predicted to link Cys-3 and Cys-425 in β_2 (30). The N-terminal cysteine-rich region of residues ~1–50 shares sequence homology with membrane proteins including plexins, semaphorins, and the *c-met* receptor (31). This region has two predicted α -helices and has been termed the “PSI domain” for plexins, semaphorins, and integrins. The segment from residues 425 to 590 has a cysteine content of 20% and is composed of four cysteine-rich repeats. The first repeat is less similar to the others and at its N-terminal end contains the cysteine that disulfide bonds to the PSI domain. Several monoclonal antibodies that activate integrins or report conformational changes have been mapped to the C-terminal region of the β subunit that includes the cysteine-rich repeats (28, 32–37) and to the N-terminal cysteine-rich region (33). Many of these mAbs recognize epitopes that become exposed after integrin activation. One of these, mAb KIM127 to the β_2 subunit, is not dependent on association with the α subunit for reactivity and indeed reacts better with the free β_2 subunit than with the integrin $\alpha\beta$ heterodimer (38). Thus, structural changes in the stalk region that include exposing antibody epitopes on the integrin β subunit are associated with integrin activation.

Here, we have defined regions of the integrin β_2 subunit involved in regulating ligand binding by $\alpha_x\beta_2$. Ligand binding is activated when the human α_x subunit is complexed with the chicken β_2 subunit (7). We hypothesized that this reflects a release of structural contacts between the human α_x and human β_2 subunits that normally restrain $\alpha_x\beta_2$ in a nonligand binding conformation. To map these contacts within the β_2 subunit, we have utilized chicken/human β_2 chimeras. We map the key differences and provide direct evidence that residues that restrain ligand binding by β_2 are present in both the N-terminal cysteine-rich PSI domain and the C-terminal cysteine-rich repeats.

MATERIALS AND METHODS

Cell Lines and Monoclonal Antibodies—293T cells (human renal epithelial transformed cells) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (Life Technologies, Inc.), 2 mM glutamine, and 50 μ g/ml gentamicin. The mouse anti-human α_x mAb

CBRp150/2E1 (7) and the anti-human β_2 mAbs KIM185 (39) and CBR LFA-1/2 (40) have been previously described.

Human/Chicken or Human/Mouse Chimeric β_2 Constructs—Human or chicken β_2 cDNA were inserted in vector Ap^rM8 (41). Chimeras and substitution mutants were generated by polymerase chain reaction overlap extension (42). Briefly, 5’ and 3’ end primers were designed to include unique restriction sites. Mutations were introduced by a pair of inner complementary primers. After a second round of polymerase chain reaction, the products were digested and ligated with the corresponding predigested plasmids. All constructs were verified by DNA sequencing.

Transfection—Plasmids for transfection were purified by QIAprep Spin Kit or Maxi Kit (Qiagen, Chatsworth, CA). 293T cells were transiently transfected with human α_x and wild-type or mutant β_2 constructs using calcium phosphate (43, 44). Medium was changed after 7–11 h. Cells were harvested for analysis 48 h after transfection.

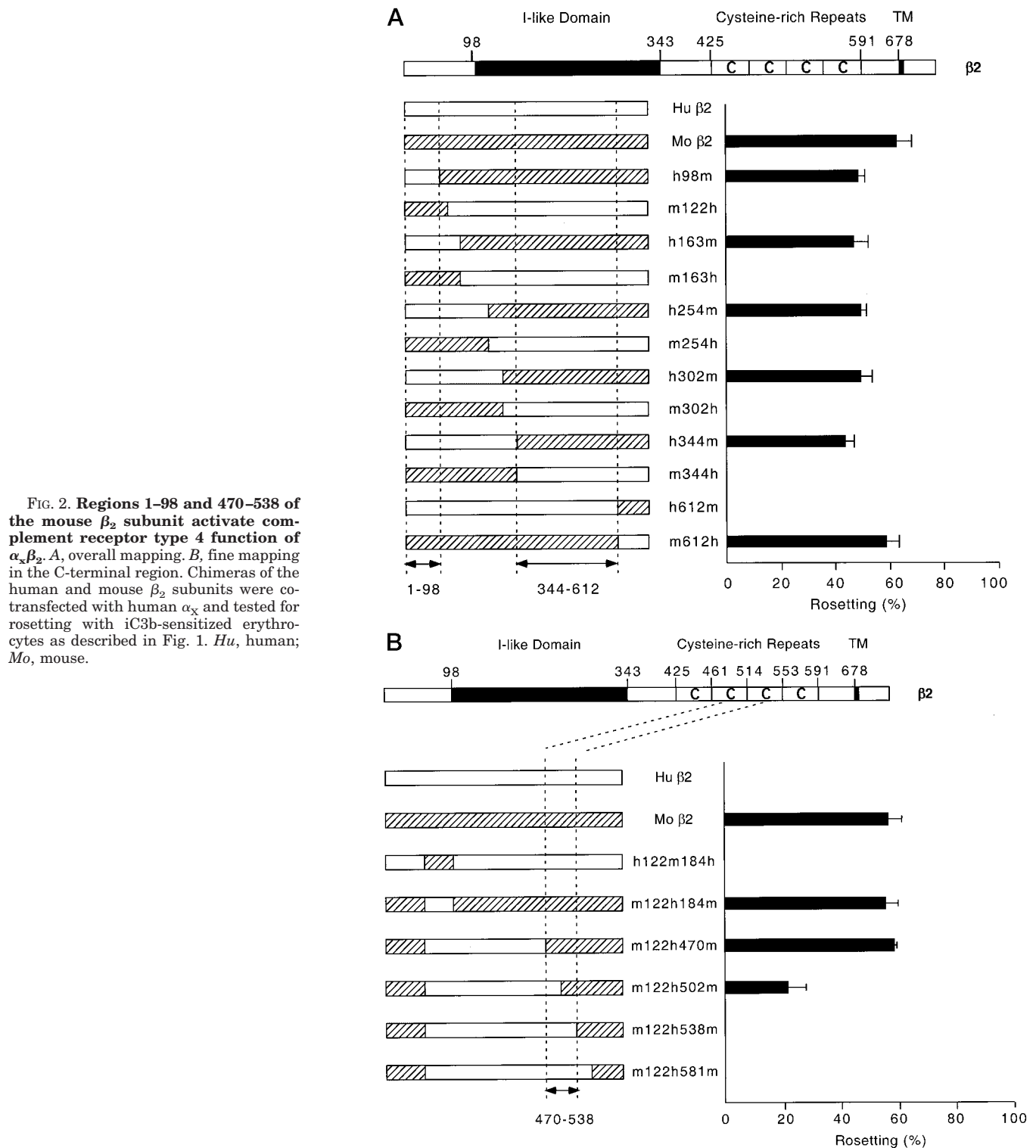
Flow Cytometry—Cells were washed twice with L15 medium supplemented with 2.5% fetal bovine serum (L15/FBS). Cells (10^6) were incubated with 50 μ l of primary antibody (20 μ g/ml purified mAb, or 1:100 dilution of ascites) on ice for 30 min. Cells were then washed three times with L15/FBS, followed by incubation with 50 μ l of a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Zymed Laboratories Inc., San Francisco, CA) for 30 min on ice. After washing three times with L15/FBS, cells were resuspended in 200 μ l of cold phosphate-buffered saline and analyzed on a FACScan (Becton Dickinson, San Jose, CA). Antigen expression is presented as mean fluorescence intensity of cells.

iC3b-coated Erythrocyte Binding Assay—As described previously (7, 14), sheep erythrocytes (Colorado Serum Co., Denver, CO) were washed, resuspended to 6×10^8 cells/10 ml in buffer 1 (Hanks’ balanced salt solution, 15 mM HEPES, pH 7.3, and 1 mM $MgCl_2$), and sensitized with 80 μ l of IgM anti-Forssman mAb M1/87 culture supernatant for 1 h at room temperature (E-IgM). The cells were then washed and resuspended in 1.8 ml of buffer 2 (Hanks’ balanced salt solution, 15 mM HEPES, pH 7.3, 1 mM $MgCl_2$, and 1 mM $CaCl_2$), supplemented with 200 μ l of C5-deficient human serum (Sigma). After incubation at 37 $^\circ$ C for 1 h, the resulting E-IgM-iC3b were washed twice and resuspended in 6 ml of buffer 2.

To assay the binding of $\alpha_x\beta_2$ to iC3b, 293T cells transfected with recombinant $\alpha_x\beta_2$ were plated on 12-well polylysine-coated plates for at least 4 h prior to the experiment. After washing with buffer 2, the cells were incubated together with 200 μ l of E-IgM-iC3b for 30 min at 37 $^\circ$ C. Unbound erythrocytes were removed by washing three times, and rosettes (>10 erythrocytes/293T cell, >100 cells examined) were scored with microscopy.

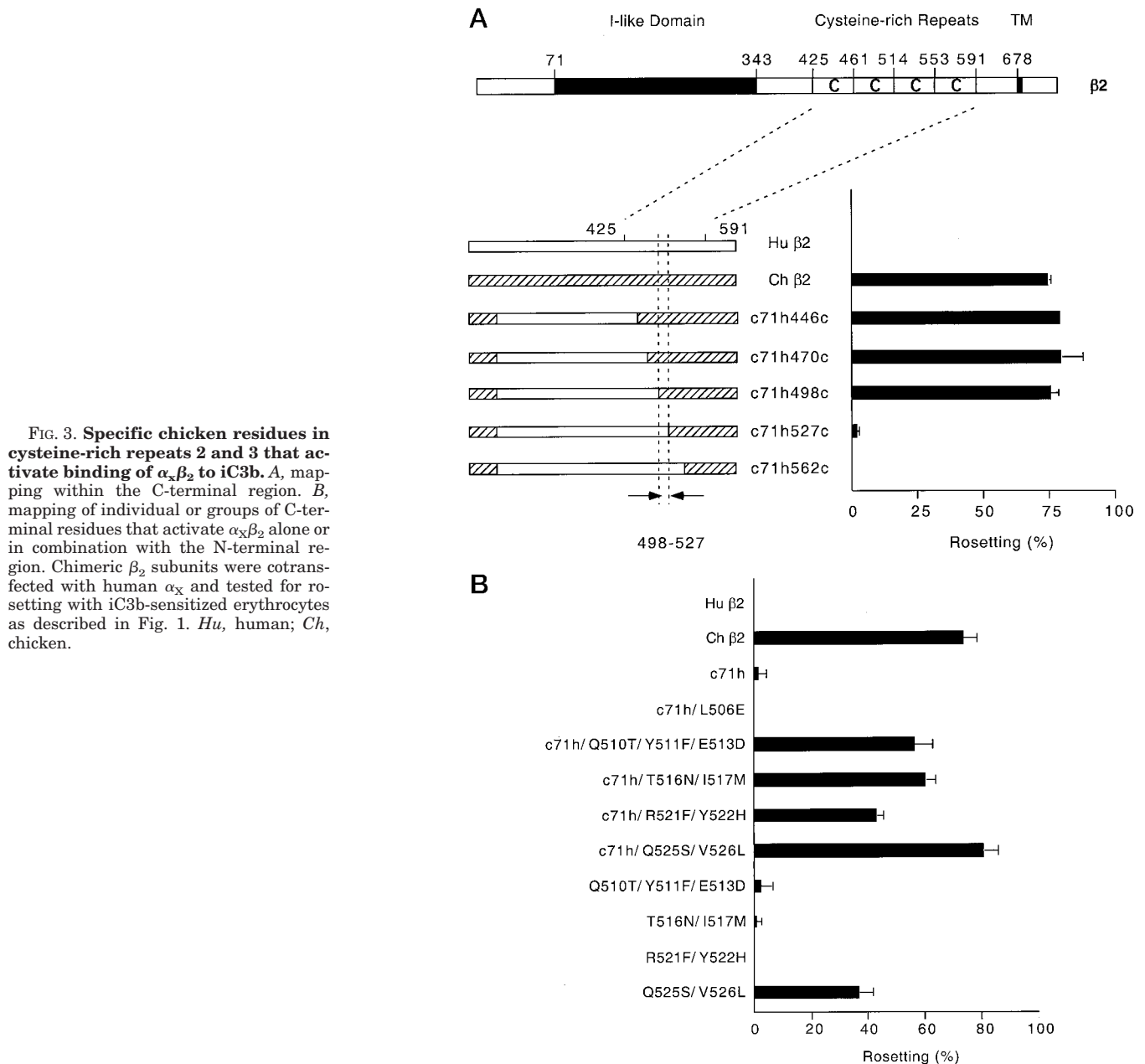
RESULTS

Cysteine-rich Regions of the β_2 Subunit Regulate Integrin $\alpha_x\beta_2$ Binding to iC3b—To locate regions in the integrin β_2 subunit that restrain activation of $\alpha_x\beta_2$, interspecies human/chicken chimeric β_2 subunits were made. Chimeras were



named according to the species origin of their segments. For example, h103c indicates that residues 1 to 103 are human and residues 104 to the C-terminal end are chicken. Each construct was cotransfected with the human α_x subunit into 293T cells. Proper expression was confirmed by immunostaining with antibody CBRp150/2E1 to the α_x subunit. All human/chicken β_2 chimeras studied here were expressed as well as human β_2 in $\alpha_x\beta_2$ complexes. The percentage of 293T transfectants expressing $\alpha_x\beta_2$ ranged from 68 to 85% for chimeras and wild type in all experiments described below. Transfectants were assayed

for activation of ligand binding by rosetting with erythrocytes sensitized with iC3b (E-IgM-iC3b). The percentage of rosetting cells was normalized to the percentage of $\alpha_x\beta_2^+$ cells for each construct. Transfectants expressing hybrid $\alpha_x\beta_2$ (human α_x /chicken β_2) but not transfectants expressing human $\alpha_x\beta_2$ formed rosettes with E-IgM-iC3b, confirming previous observations with COS-7 cell transfectants (7). The chimeras mapped activation of ligand binding by chicken β_2 to two regions, residues 1–71 and residues 421–610 (Fig. 1). The importance of residues 421–610 was shown by activation of iC3b



rosetting by chimeras h103c and h421c but not by chimeras h610c, h103c421h, and c71h610c. Residues 1–71 were not sufficient by themselves to activate iC3b binding as shown with chimera c71h; however, they augmented rosetting when present in combination with residues 421–610 (Fig. 1). Thus, with residues 421–610 of chicken origin in chimeras h103c and h421c, about 40% of transfectants rosetted with E-IgM-iC3b. With both residues 1–71 and 421–610 of chicken origin in chimeras c678h and c71h421c, about 80% of transfectants rosetted. This was the same level as with the wild-type chicken β_2 subunit. Thus, activation was a synergistic effect of the N- and C-terminal cysteine-rich regions of the chicken β_2 subunit.

Activation of $\alpha_x\beta_2$ by Regions in the Mouse β_2 Subunit—We found that $\alpha_x\beta_2$ heterodimers containing human α_x and mouse β_2 subunits were activated for binding to iC3b almost as well as those containing human α_x and chicken β_2 subunits (Fig. 2A). Human/mouse β_2 chimeras showed that the region containing residues 344–612 was activating (Fig. 2A). The h98m chimera was less activating than mouse β_2 , suggesting that the N-terminal cysteine-rich region contributed to activation. Fur-

thermore, the m122h, m163h, m254h, m302h, and m344h chimeras showed that the N-terminal cysteine-rich region was not sufficient for activation, similar to the results with the chicken β_2 subunit.

The activating region in the C-terminal cysteine-rich region in the mouse β_2 subunit was defined with a further series of chimeras (Fig. 2B). These narrowed activation by the C-terminal cysteine-rich region to residues 470–538 since chimera m122h470m was activating, whereas m122h538m was not (Fig. 2B). Furthermore, residues in two different segments, 470–502 and 502–538, were activating because chimera m122h502m was partially activating, whereas m122h470m was fully activating, and m122h538m was not activating.

Chicken Residues in Cysteine-rich Repeats 2 and 3 Activate $\alpha_x\beta_2$ —Mapping of the C-terminal cysteine-rich repeat region of chicken β_2 was refined with five further chicken/human chimeric β_2 constructs. Each construct contained N-terminal residues 1–71 and various lengths of the C-terminal cysteine-rich repeats from chicken β_2 (Fig. 3A). Rosetting of chimeras c71h446c, c71h470c, and c71h498c with E-IgM-iC3b was sim-

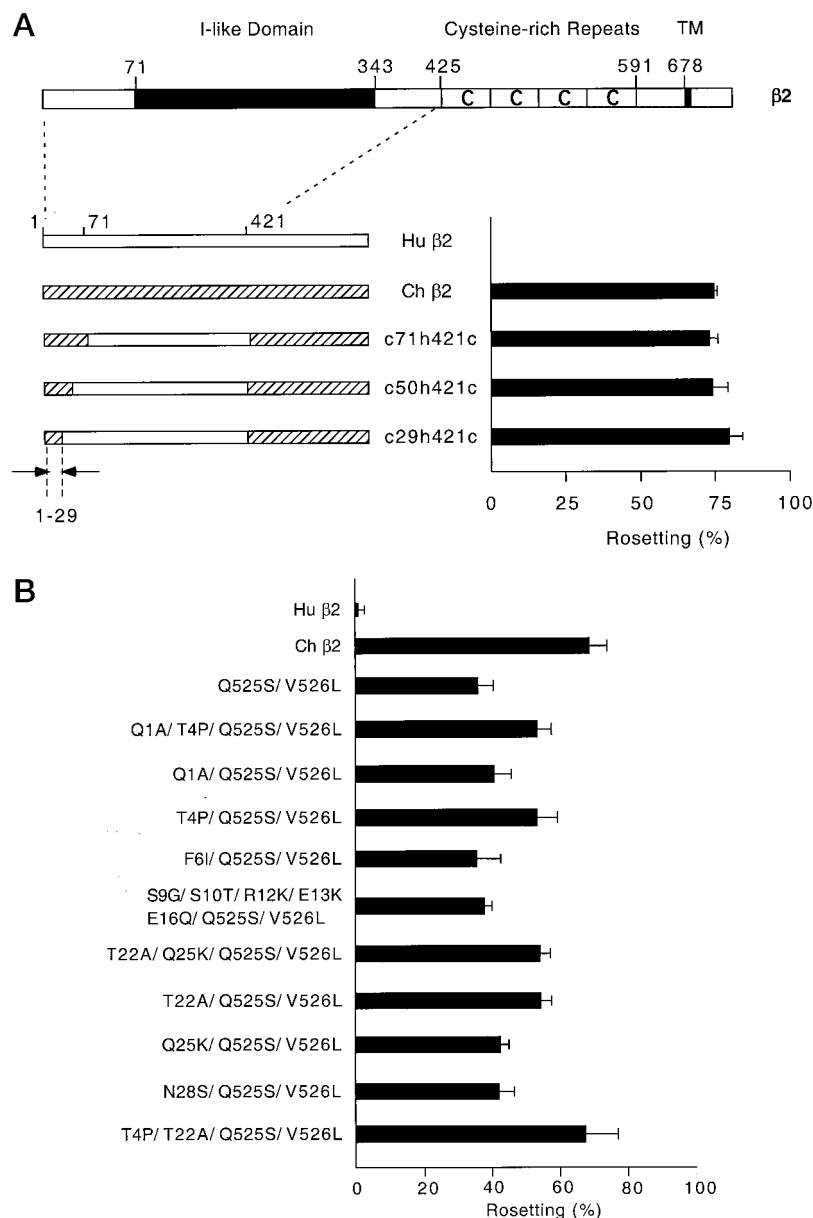


FIG. 4. Chicken residues 4 and 22 of the β_2 subunit activate iC3b binding in synergy with residues 525 and 526. *A*, fine mapping of the N-terminal region in synergy with the C-terminal region. *B*, mapping of individual N-terminal residues in synergy with residues 525 and 526. Details are as described in Fig. 1. *Hu*, human; *Ch*, chicken.

ilar to that of wild-type chicken β_2 . However, chimeras c71h527c and c71h562c did not bind to iC3b. Therefore, residues within region 498–527 can activate binding to the ligand iC3b.

Within the activating region defined in chicken β_2 of 498–527, 11 residues differ between human and chicken. Groups of one to three chicken amino acid residues in this region were introduced into the human β_2 subunit and their effect on binding to iC3b was examined (Fig. 3*B*). In combination with chicken residues 1–71, four groups of amino acid substitutions were activating: Q510T/Y511F/E513D in repeat 2 and T516N/I517M, R521F/Y522H, and Q525S/V526L in repeat 3. Chimera c71h/Q525S/V526L was as active as chicken β_2 . The four activating groups of residues were also tested in the absence of any other chicken residues. In this situation, only the mutation Q525S/V526L was activating, and its activity was reduced compared with c71h/Q525S/V526L (Fig. 3*B*).

Residues 4 and 22 in the N-terminal Cysteine-rich Region of Chicken β_2 Activate $\alpha_x\beta_2$ in Synergy with the C-terminal Cysteine-rich Region—Mapping of the N-terminal cysteine-rich region was refined with three chicken/human chimeras that included different portions of the N-terminal region in

combination with the synergistic C-terminal segment (Fig. 4*A*). All three β_2 chimeras, c71h421c, c50h421c, and c29h421c, activated binding to E-IgM-iC3b to the same extent as chicken β_2 . Thus, residues within the first 29 amino acids of the β_2 subunit are sufficient to synergistically activate $\alpha_x\beta_2$.

In region 1–29 of the β_2 subunit, 11 residues differ between the human and chicken. Groups of these residues were substituted with chicken sequence in combination with the mutation Q525S/V526L in the C-terminal cysteine-rich region in each construct (Fig. 4*B*). Most of the mutants rosetted E-IgM-iC3b no better than the parent Q525S/V526L mutant. However, mutants Q1A/T4P/Q525S/V526L and T4P/Q525S/V526L but not Q1A/Q525S/V526L were more active than Q525S/V526L, implicating the substitution T4P in activation. Similarly, mutants T22A/Q25K/Q525S/V526L and T22A/Q525S/V526L but not Q25K/Q525S/V526L were more active than Q525S/V526L, implicating T22A. Moreover, the combination of mutations T4P and T22A was even more active, and the mutant T4P/T22A/Q525S/V526L was as active as chicken β_2 (Fig. 4*B*). Therefore, four chicken residues, two each in the N-terminal and C-terminal cysteine-rich regions of β_2 , are sufficient to maximally activate iC3b rosetting by $\alpha_x\beta_2$.

Activating Mutations Expose the CBR LFA-1/2 Epitope in the C-terminal Cysteine-rich Region of β_2 —Several mAbs that activate β_2 integrins map to the C-terminal cysteine-rich region of the β_2 subunit (28, 37). The mouse/human substitutions recognized by these mAbs map very near to the substitutions Q525S/V526L that activate $\alpha_X\beta_2$. Specifically, mAb KIM185 recognizes residues 581–604 and mAb CBR LFA-1/2 recognizes residues 534 and 536.² Recognition by mAb CBR LFA-1/2 correlates with the activation status of β_2 integrins; $\alpha_1\beta_2$ and $\alpha_M\beta_2$, which are active in 293T cell transfectants, are recognized well by CBR LFA-1/2, whereas $\alpha_X\beta_2$, which is inactive in 293T cells, is recognized poorly² (Fig. 5). We examined the effect of activating mutations on expression of CBR LFA-1/2 and KIM185 epitopes (Fig. 5). The KIM185 epitope was expressed equally well by wild-type and mutant $\alpha_X\beta_2$. By contrast, activating mutations induced exposure of the CBR LFA-1/2 epitope (Fig. 5). The Q525S/V526L mutation partially exposed the CBR LFA-1/2 epitope, whereas the T4P/T22A/Q525S/V526L mutation maximally exposed the epitope, *i.e.* to the same level as seen with KIM185 mAb. Exposure of the CBR

LFA-1/2 epitope correlated with activation of binding to iC3b (Fig. 5). Therefore, the mutations cause structural rearrangements in the stalk region that lead to exposure of the CBR LFA-1/2 epitope and are linked to activation of ligand binding. Furthermore, binding of the CBR LFA-1/2 and KIM185 mAbs demonstrates that the mutations do not disrupt the structure of the cysteine-rich repeats.

DISCUSSION

Among the β_2 integrins, $\alpha_X\beta_2$ is the most resistant to activation and to dissociation of its α and β subunits. Here, we have identified specific amino acid residues that restrain $\alpha_X\beta_2$ in a conformation in which it does not bind its ligand, iC3b. We extended previous observations with the chicken β_2 subunit (7) by showing that pairing of human α_X with β_2 from another species, the mouse, also activates binding to iC3b. Interspecies β_2 subunit chimeras associated with human α_X subunits demonstrated that the C-terminal cysteine-rich repeats from mouse or chicken were sufficient for partial activation and that the N-terminal cysteine-rich PSI domain was insufficient for activation but synergized with the C-terminal cysteine-rich repeats.

Activating substitutions in the N-terminal region were localized within the PSI domain (Fig. 6). Human/chicken substitutions T4P and T22A each synergized with the C-terminal region and, when present together, gave augmented synergy. PSI domains in integrins contain six cysteines that form intradomain disulfide bonds and one cysteine that forms a long range interdomain disulfide (30, 31). Each of the activating substitutions neighbors a cysteine residue (Fig. 6). The substitution T4P neighbors Cys-3, which forms the long range disulfide bond to Cys-425, which is at the beginning of the C-terminal cysteine-rich repeats (Fig. 6). Thus, the two regions in which activating substitutions are found, the PSI domain and cysteine-rich repeats, are linked by a disulfide bond and must be neighboring domains in the three-dimensional structure of the integrin β subunit.

Activating substitutions within the C-terminal region localized to cysteine-rich repeats 2 and 3 (Fig. 6). One segment containing activating mouse substitutions localized wholly within repeat 2, whereas another included portions of repeats 2 and 3 (Fig. 6). Fine mapping of three groups of chicken substitutions that activated $\alpha_X\beta_2$ in synergy with chicken residues in the PSI domain showed that one group mapped to repeat 2 and two groups mapped to repeat 3 (Fig. 6). One pair of substitutions that was sufficient for activation of $\alpha_X\beta_2$, Q525S/V526L, mapped to repeat 3. We cannot exclude the presence of activat-

² C. Lu, M. Ferzly, J. Takagi, and T. A. Springer, manuscript in preparation.

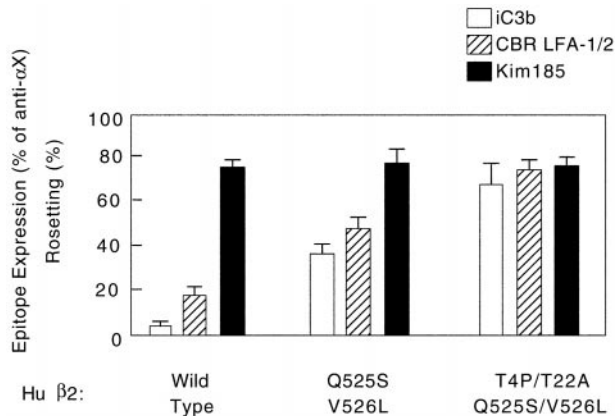


FIG. 5. Activation of iC3b binding by individual amino acid substitutions in the PSI domain and cysteine-rich repeat 3 correlates with exposure of the CBR LFA-1/2 epitope. Mutant or wild-type β_2 subunits were cotransfected with human α_X into 293T cells. The transfectants were immunostained with mAbs KIM185 or CBR LFA-1/2 followed by immunofluorescence flow cytometry. Epitope expression is normalized to the percentage of cells binding mAb CBR p150/2E1 to the α_X subunit. Binding to iC3b was quantified as described in Fig. 1. *Hu*, human.

FIG. 6. Sequence differences of the human β_2 PSI domain and cysteine-rich repeats with the mouse and chicken and localization of activating mutations. Only residues that differ in the mouse or chicken sequences are shown. *Underlined residues*: tested, no effect. *Black inverse residues*: activating. *Gray inverse residues*: activating in synergy with other residues. Underlining or inverse font is extended to include the group of residues that was tested. The long range disulfide bond between Cys-3 and Cys-425 is shown. *Dots* above the sequence mark residue positions that are multiples of ten. *hu*, human; *mo*, mouse; *ch*, chicken.



TABLE I
C-terminal cysteine-rich repeats: variation
between species and activation

Repeat	Length	Human-chicken differences	Human-mouse differences	Activating substitutions
	<i>residues</i>	<i>%</i>	<i>%</i>	
1	36	47	22	ND ^a
2	53	23	17	+
3	39	38	26	+
4	39	46	36	-

^a ND, not determined.

ing substitutions in repeat 1 because all chimeras in which repeat 1 was mouse or chicken also contained repeats 2 and 3 from mouse or chicken, which were activating by themselves. However, the segments following the PSI domain and preceding repeat 1 were not activating. Furthermore, repeat 4 and more C-terminal segments were not activating. The species-specific differences in repeat 4 are greater than in repeats 2 and 3 (Table I); therefore, an insufficiency of species-specific differences cannot explain the lack of activation by repeat 4.

What is the mechanism of integrin activation by species-specific substitutions in the PSI domain and cysteine-rich repeats 2 and 3? Many other observations suggest that integrins are restrained in their resting state in a conformation that does not bind ligand and that a wide variety of perturbations can activate ligand binding. Our results suggest that the PSI domain and cysteine-rich repeats 2 and 3 have an important function in restraining integrins in their resting, inactive state. These restraints are overcome when the α and β subunits are from different species; therefore, it appears that there are direct interactions between these β subunit domains and the α subunit that constrain integrins in the inactive configuration. The substitutions are unlikely to disrupt the overall conformation of these domains because they are naturally occurring variations between species. Furthermore, we demonstrated that mAb CBR LFA-1/2, which binds to species-specific residues in repeat 3,² binds well when the activating mutations Q525S and V526L are present in repeat 3. Therefore, it appears that the activating mutations we have defined are within or near an interface between the β_2 and α_x subunits. The findings suggest that in resting integrins, there are contacts of the PSI domain and cysteine-rich repeats 2 and 3 with the α subunit and that these contacts help restrain ligand binding. It appears that certain species-specific substitutions disrupt this interaction and, thereby, lower the activation energy required for activation of ligand binding. Binding of iC3b by $\alpha_x\beta_2$ maps to the α_x I domain (7). Conformational shifts around the MIDAS in I domains regulate ligand binding and are linked to a large movement of the C-terminal α -helix of the I domain that connects to other integrin subunits (19–23). Therefore, it appears that an alteration in contacts in the stalk region between the α subunit and the PSI domain and the cysteine-rich repeats in the β subunit are linked to conformational rearrangements in the ligand-binding domains in the headpiece of integrins.

The loss of the restraints that keep $\alpha_x\beta_2$ in an inactive state appears to reflect an opening up of the $\alpha\beta$ interface in the stalk region based on exposure of the epitope for the mAb CBR LFA-1/2. This mAb can activate integrins $\alpha_L\beta_2$ and $\alpha_M\beta_2$ (40). It showed little reactivity with wild-type $\alpha_x\beta_2$; however, introduction of activating amino acid substitutions Q525S/V526L in cysteine-rich repeat 3 exposed the CBR LFA-1/2 epitope, and addition of the T4P/T22A substitutions fully exposed the epitope. Exposure correlated with iC3b binding. The CBR LFA-1/2 mAb maps to residues 534 and 536², and nearby residues 525 and 526, to which activating mutations map in repeat 3. It is unlikely that there is a significant conformational change in

this repeat because its structure is constrained by four disulfide bonds. Therefore, we envision a movement apart or change in orientation of the α and β subunits that exposes the CBR LFA-1/2 epitope in repeat 3.

Other studies also imply a structural restraint on integrin activation that is localized in the cysteine-rich regions of the β subunit. Activation of the integrin LFA-1 ($\alpha_L\beta_2$) expressed on COS cells was induced if the C-terminal cysteine-rich repeat region of the β_2 subunit was replaced by that of β_1 (45). A point mutation that introduces a N-glycosylation site into the beginning of cysteine-rich repeat 4 of the β_3 subunit activated integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ (46). Furthermore, disruption of the long range disulfide bond between the PSI domain and the cysteine-rich repeats resulted in increased ligand binding affinity of $\alpha_{IIb}\beta_3$ (47). Moreover, treatment with reducing agents, such as dithiothreitol, induced the active conformation of β_1 integrin (33) and increased platelet aggregation through the $\alpha_{IIb}\beta_3$ integrin (48). Recently, an anti- β_1 antibody with an activation-dependent epitope has been mapped to the N-terminal cysteine-rich region, suggesting a role of this region as a regulatory site for integrin activation (33). In addition, several monoclonal antibodies against the C-terminal cysteine-rich regions of β_1 (32, 34), β_2 (37), and β_3 (49) integrins have been described as activating mAbs with respect to their ability to promote ligand binding. A plausible explanation is that these mAbs selectively bind to the open conformation of the stalk region and thus stabilize integrins in this conformation and induce linked rearrangements in the ligand-binding domains. Indeed, activating mAbs to both the β_1 and β_2 cysteine-rich regions have been found to bind better to isolated β subunits than $\alpha\beta$ complexes, implying that they favor an open conformation (26, 37, 38, 50).

In contrast to domains in the globular headpiece of integrins, the stalk regions do not appear to directly bind ligand but instead appear to regulate ligand binding and to relay activation signals impinging on the cytoplasmic and transmembrane domains of the integrin α and β subunits. We have identified specific amino acid residues in the PSI domain and cysteine-rich repeats 2 and 3 of the β subunit that form part of the interface between the α and β subunits in the stalk region that restrains conformational movements in the ligand-binding headpiece. It would be very interesting to learn which regions of the α subunit participate in this interface and the molecular details of how structural alterations are communicated from one domain to another in integrins.

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