Ligand Binding to Integrin $\alpha_{IIb}\beta_3$ Is Dependent on a MIDAS-like Domain in the $\beta_3$ Subunit*

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Substitution of $\beta_3$ residue Asp$^{119}$, Ser$^{121}$, or Ser$^{123}$ results in a loss of the ligand binding function of integrin $\alpha_{IIb}\beta_3$. Homologous residues in other integrin $\beta$ subunits are similarly critical for ligand binding function. This DXSXS motif is also present in the I domain of certain integrin $\alpha$ subunits, where it constitutes a portion of the unique metal ion-dependent adhesion site (MIDAS). In this report, we have utilized the crystal structure of the recombinant $\alpha_\text{IIb}$ I domain to produce a three-dimensional model of the homologous region in the integrin $\beta_3$ subunit. We performed mutagenesis of candidate amino acid residues predicted from this model to be involved in cation coordination and ligand binding. We report the identification of Asp$^{217}$ and Glu$^{220}$ as residues essential for the ligand binding function of $\alpha_{IIb}\beta_3$. Alanine substitution of these residues did not affect receptor expression but abolished the binding of activation-dependent (PAC1) and -independent (OPG2) ligand mimetic antibodies. In our proposed model, $\beta_3$ Asp$^{217}$ is analogous to a metal-coordinating residue in the $\alpha_\text{IIb}$ MIDAS domain, while Glu$^{220}$ does not correspond to a functional MIDAS domain residue. Substitution of the highly conserved $\beta_3$ residue Thr$^{197}$ corresponding to a critical MIDAS metal-coordinating Thr residue did not affect ligand binding function, suggesting that this region of $\beta_3$ adopts a structure that is very similar to but not identical to that of the MIDAS domain. These data support a functional linkage between these two sequences and further define a common feature of ligand binding to integrins.

Platelet adherence to components of the subendothelial matrix, to other platelets, and to other cells plays a fundamental role in normal hemostasis. These platelet-adhesive interactions are mediated in large part through the major platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa). Elucidation of the mechanism of ligand recognition is central to the understanding of ligand interactions in general. Significant progress has been made in the identification of potential ligand binding sites in $\alpha_{IIb}\beta_3$. Previous studies indicate that the minimal ligand binding structures are located in the amino-terminal half of $\alpha_{IIb}\beta_3$ (1-6). The identification of more discrete regions within each of the subunits has benefited from the use of small ligand mimetic peptides as probes of ligand binding sites. Peptides derived from the carboxyl terminus of the fibrinogen $\gamma$ chain cross-link to a region of $\alpha_{IIb}$ defined by residues Ala$^{296}$-Met$^{314}$ (7). This location is particularly noteworthy, since it encompasses the second divergent cation-binding repeat of $\alpha_{IIb}$ (8). Peptides derived from this sequence inhibit ligand binding and directly bind fibrinogen in a cation-dependent manner (9), supporting a role in ligand binding.

Two distinct regions within $\beta_3$ have been implicated in ligand binding function of the receptor. Arg-Gly-Asp (RGD)-containing peptides cross-link to a discrete region of $\beta_3$ defined by residues Asp$^{109}$-Glu$^{171}$ (10). In addition, monoclonal antibodies (mAbs) specific for this region inhibit ligand binding and platelet aggregation (11, 12). Strong evidence for a direct interaction of this region in ligand binding comes from genetic analysis of a variant $\alpha_{IIb}\beta_3$ characterized by complete loss of ligand binding function (13). The molecular basis for this defect is substitution of Asp$^{119}$ → Tyr in mature $\beta_3$ (14). This Asp residue is absolutely conserved in all the integrin $\beta$ subunits, suggesting that this residue may play a role in ligand binding to all integrins. This hypothesis has been substantiated by the observation that substitution at residues homologous to $\beta_3$ Asp$^{119}$ in other integrin $\beta$ subunits abrogates ligand binding function (15-17). Moreover, substitution at this residue exerts dominant negative effects blocking the binding of RGD-dependent ligands as well as RGD-independent ligands, further suggesting that this residue may play a role in a common mechanism of ligand binding.

A second potential ligand-interactive site in $\beta_3$ is defined by residues Ser$^{211}$-Gly$^{222}$. Peptides corresponding to this sequence bound fibrinogen and blocked its binding to $\alpha_{IIb}\beta_3$ (18). Antibodies directed against this peptide also blocked the binding of adhesive proteins to purified receptor (18). In addition, two natural receptor variants characterized by loss of ligand binding function contain substitutions at Arg$^{214}$ (19, 20), further implicating this region in receptor function. Despite the identification of these putative ligand contact points, the relationship, if any, between these discrete sites and the precise molecular mechanism by which these sites mediate recognition may provide additional insights into integrin-ligand interactions in general.

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ligand binding remains to be determined. A paradigm of ligand binding to all integrins is an absolute dependence upon divalent cations. Thus it is significant that the Asp\(^{119}\) to Tyr mutation in \(\beta_3\) also alters the conformation of \(\alpha_1 \beta_3\) in a manner consistent with loss of bound divalent cation (13). This observation led to the hypothesis that this region may play a divalent cation binding site (14). Indeed, a synthetic \(\beta_3\) peptide corresponding to \(\beta_3\) residues Met\(^{118}\)–Ile\(^{131}\) directly binds the luminescent calcium analog terbium (21). Substitution of Asp\(^{119}\) by alanine in this peptide substantially reduces the terbium/peptide interaction, providing support for a role of this region in both ligand recognition and cation binding. 

Mutational analysis of residues proximal to Asp\(^{119}\) assigned critical functional roles to Ser\(^{121}\) and Ser\(^{123}\) in the ligand binding function of \(\alpha_1 \beta_3\) (22). Similarly, a role in ligand binding function has been reported for a corresponding Ser residue in \(\beta_3\) (17). Together Asp\(^{119}\), Ser\(^{121}\), and Ser\(^{123}\) of \(\beta_3\) compose a DXSX\(^{s-3}\) sequence that is absolutely conserved in all the integrin \(\beta\) subunits. The divalent cation dependence of integrin function together with the high degree of conservation of these functionally significant residues forms the basis of the hypothesis that ligands interact with divalent cations bound to this highly conserved site in the \(\beta\) subunits (14). The presence of critical oxygenated residues in integrin ligands (14, 23–27) and the displacement of divalent cations following ligand binding (21, 28) support this view.

The essential DXSX\(^{s-3}\) motif in the integrin \(\beta\) subunits is also highly conserved in the inserted (I) domain present in six of the integrin \(\alpha\) subunits. These 200 amino acid residues in domains homologous to the A domain of von Willebrand factor are critical for both cation and ligand binding to I domain-containing integrins (29). High resolution crystal structures of isolated recombinant \(\alpha_m\) (30) and \(\alpha_l\) (31) I domains clearly establish that this DXSX\(^{s-3}\) sequence constitutes a portion of a unique metal coordination site designated the metal ion-dependent adhesion site (MIDAS) (30). The conservation of the metal-coordinating consensus sequence DXSX\(^{s-3}\) and the similarity of hydropathy plots between this region of \(\beta_3\) and the \(\alpha_m\) I domain suggested that this conserved region of the integrin \(\beta\) subunits may also adopt an I domain fold (30, 32). To test this hypothesis, we have utilized the crystal structure of the recombinant \(\alpha_m\) I domain to model the homologous region in the integrin \(\beta_3\) subunit and performed mutagenesis of candidate amino acid residues predicted to be involved in cation coordination and ligand binding. We report the identification of Asp\(^{217}\) and Glu\(^{220}\) as residues essential for ligand binding function of \(\alpha_m \beta_3\). Asp\(^{217}\) is analogous to a metal-coordinating residue in the I domain MIDAS motif. However, Glu\(^{220}\) does not correspond to a functional \(\alpha_m\) MIDAS domain residue, indicating that the two regions may adopt a similar but not identical structure. These results establish a functional linkage between these two sequences and further define a novel cation-binding motif essential for integrin receptor function.

**MATERIALS AND METHODS**

**Antibodies and Reagents—**The IgM, murine mAb PAC1 binds specifically to activated \(\alpha_m \beta_3\) (33) and was provided by Dr. Sanford Shattil (The Scripps Research Institute, La Jolla, CA). The \(\alpha_m \beta_3\) complex-specific mAb OPG2 (34) was provided by Dr. Thomas Kunicki (The Scripps Research Institute, La Jolla, CA). The \(\alpha_m \beta_3\)-specific mAb D57 and the activating anti-\(\beta_3\) mAb anti-LIBS2 have been previously described (35, 36). mAbs D57 and OPG2 were biotinylated using biotin-N-hydroxysuccinimide (Pierce) according to the manufacturer’s directions. Anti-D57 and D57 fluorophore were 1:1 fluorescent protein ratio of 2.6 to fluorescence isothiocyanate-carbodiimide (Calbiochem). Fluorescein isothiocyanate-conjugated goat anti-mouse IgM was obtained from Tago (Burlingame, CA). The peptidomimetic Ro 43-5054 (37) was generously provided by Beat Steiner (Hoffman-LaRoche, Basel, Switzerland). Oligonucleotides were synthesized on a model 391 DNA synthesizer (Applied Biosystems Inc., Foster City, CA).

**Mutagenesis—**Expression constructs encoding wild type \(\alpha_m \beta_3\), CD20, and wild type \(\beta_3\), CD3a, have been previously described (36). A 2.6-kilobase pair fragment of \(\beta_3\) encoding the I domain and a portion of the 3′ untranslated sequence was removed from CD3a by digestion with HindIII and DraI and ligated into the expression vector pcDNA3 (Invitrogen, La Jolla, CA) that had been digested with XhoI, blunt-ended with T4 polymerase, and subsequently digested with HindIII. The resulting construct was designated pc3A. Site-directed mutagenesis of selected \(\beta_3\) residues was performed by splice overlap extension as described (38). Polymerase chain reaction-generated fragments containing mutations were digested with KpnI and BstXI, and the resulting 448-base pair fragment was gel-purified and ligated to KpnI/BstXI-digested pc3A. All ligated polymerase chain reaction fragments were sequenced in their entirety to verify the introduction of the mutation and the absence of any other substitutions. DNA constructs were then purified on Qiagen columns (Chatsworth, CA) prior to transfection.

**Cell Culture and Transfection—**Chinese hamster ovary cells obtained from the American Type Culture Collection (Rockville, MD) were maintained in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) containing 10% fetal bovine serum, 1% nonessential amino acids, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were transiently transfected using Lipofectamine (Life Technologies Inc.) as described previously (39). Functional analysis of cells was routinely performed 48 h after transfection.

**Flow Cytometry—**Surface expression of transfected integrins was analyzed by flow cytometry as described (40). PAC1 binding was analyzed by two-color flow cytometry as described in detail (39). PAC1 binding was analyzed only on the subset of cells positive for \(\alpha_m \beta_3\) expression that were gated utilizing biotinylated D57. The binding of OPG2 was performed essentially as described for PAC1 binding, except biotinylated OPG2 was used and the activating mAb anti-LIBS6 was omitted. OPG2 binding was analyzed on the subset of cells positive for \(\alpha_m \beta_3\) expression that were gated utilizing fluorescein isothiocyanate-conjugated D57. Analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA).

**Molecular Modeling—**A set of models for the potential MIDAS domain of \(\beta_3\) were produced using homology modeling based on the suggested topological similarity between the integrin \(\alpha_m\) I domain and the integrin \(\beta\) subunits (30). Secondary structure prediction was performed on the amino acid sequence of \(\beta_3\), Tyr\(^{110}\)–Leu\(^{294}\) using the program PHD (41). The sequence alignment used for homology modeling was derived in two stages. First, the amino acid sequences of those proteins with similar sequence to the \(\alpha_m\) I domain found using BLAST (42) (26 in total) were multiply aligned against each other using Clustal W (43). Similarly, proteins with sequence similar to \(\beta_3\) (30 in total) were multiply aligned against each other. Second, these two sequence alignments were aligned against each other as two “profiles” within Clustal W. Thus, the integrity of each of the two original alignments was maintained in combining them to obtain the final alignment. This alignment was then manually adjusted so that 1) \(\alpha_m\) Thr\(^{299}\), which has a hydroxyl group coordinating with Mg\(^{2+}\), corresponded with \(\beta_3\) Thr\(^{297}\) that is completely conserved among the integrin \(\beta\) subunits; 2) \(\alpha_m\) Asp\(^{262}\), which coordinates the Mg\(^{2+}\) ion via a water molecule was aligned with the totally conserved \(\beta_3\) Asp\(^{217}\); 3) wherever possible, no insertion or deletion occurred within the crystallographically determined secondary structural elements of \(\alpha_m\), and 4) there was reasonable agreement between the predicted secondary structure of \(\beta_3\) and the determined structure of \(\alpha_m\).

**Homology modeling was performed using the program MODELLER (44). Two disulfide bonds are predicted in \(\beta_3\), Tyr\(^{115}\)–Leu\(^{294}\) (45). These two bonds were restrained to be present in the model and are indicated in Fig. 1. A set of 10 models was generated. Discrepancies between the sequence alignment and the resulting models were identified by consistent bond length and bond angle violations in the corresponding regions of the models, and the sequence alignment was adjusted to remove them. The alignment was modified in an iterative manner until no consistent bond length and bond angle violations were observed across the set of 10 models. Further adjustment of the alignment was performed to minimize the number of buried charge residues not involved in salt bridges. The final alignment is shown in Fig. 1.

**RESULTS**

**The Conserved Region of \(\beta_3\) Adopts an I Domain-like Fold—**Ligand binding to all integrins is dependent upon divalent cations. Substitution of Asp\(^{119}\) in \(\beta_3\) abolishes ligand binding
A MIDAS-like Motif in β3 Is Essential for Ligand Binding

Fig. 1. Sequence alignment used for modeling. Amino acid sequences are shown for both αm (CD11b) and β3 (CD61). The residues known to coordinate metal in the CD11b I domain and the corresponding β3 residues are shown as white characters on a black background. CD11b Sec str x-ray, the crystallographically determined secondary structure of CD11b (30). CD61 Sec str model, the secondary structural elements of CD61 as determined by PROCHECK (67). CD61 Sec str PHD, the secondary structural elements predicted for CD61 on the basis of its amino acid sequence by the program PHD (41). The names of the secondary structural elements and the positions of the disulfide bonds are shown by Sec str/disulfides.

function of β3 integrin and alters a divergent cation conformation of α1β3β3 (14). The similarity of hydropathy plots between this region of β3 and the region of the I domain containing the MIDAS motif suggested that this region of β3 may adopt a similar fold. To test the prediction that this region has a structure functionally equivalent to a MIDAS domain, we built a three-dimensional model of the region of β3 defined by Tyr110-Leu294. The proposed β3 structure has secondary structural elements very similar to those in the I domain with a central β-sheet composed of five parallel β strands and one antiparallel β strand surrounded by seven α-helices (Fig. 2). The proposed model brings two pairs of cysteine residues into close proximity, where they can form the predicted disulfide bonds (45). In general, the model places hydrophilic residues on the outside and hydrophobic residues on the inside of the structure. One exception is in the βD strand, immediately preceding Asp217. Here two arginine residues, Arg214 and Arg216 (that are not conserved among the integrin β subunits) appear to be buried, although the model provides salt bridge partners (Glu220 and Asp242, respectively) to neutralize them. The buried position of Arg214 might help explain the sensitivity of this site to substitutions (19, 20). The cluster of residues Asp193, Ser212, and Ser213 that have been previously implicated in ligand binding are located at the top of the structure in a loop between βA and α1 and form part of the potential MIDAS-like motif (Fig. 2).

Asp217 and Glu220 Are Required for Ligand Binding Function—Metal coordination in the αm I domain is provided in part by the DXSXS sequence and two noncontiguous amino acid residues (Thr209 and Asp242) located carboxyl-terminal to the DXSXS motif (30). It is likely that oxygenated residues critical for metal coordination would be conserved in all the integrin β subunits. Alignment of the corresponding regions of the known integrin β subunits served to identify a number of conserved candidate residues (Fig. 3). To identify residues essential for ligand binding, we performed alanine mutagenesis and examined the effect of these substitutions on ligand binding function following transient co-transfection of Chinese hamster ovary cells with a mutant β subunit and wild type α1β3.

To determine the effect of these substitutions on the ligand binding function of the expressed mutant receptors, we examined the capacity of the transfectants to bind the mAb PAC1 by flow cytometry. PAC1 is a murine IgM that binds specifically to the activated conformation of α1β3β3, and its binding is blocked by macromolecular ligands as well as ligand mimetic peptides (33, 46, 47). PAC1 also fails to bind to ligand binding-defective mutants (22, 48). The binding of PAC1 to transfected cells was assayed by flow cytometry in the absence or presence of mAb anti-LIBS6 that acts directly upon α1β3β3, provoking high affinity ligand binding function (49). Cells expressing wild type α1β3β3 bound PAC1 in the presence of the activating mAb anti-LIBS6 (Fig. 4). The binding of mAb PAC1 was specific, since it was completely blocked by the α1β3β3-specific peptide mimetic Ro 43–5054. Alanine substitution of a number of highly conserved oxygenated residues in β3 did not affect heterodimer formation or receptor processing, since all mutants were expressed on the cell surface as assayed with the anti-α1β3β3-specific mAb D57 (Table 1). However, alanine substitution of Asp217 or Glu220 resulted in a loss of PAC1 binding (Fig. 4). These two mutants readily bound anti-LIBS6 as assayed by flow cytometry (data not shown). Therefore, the lack of PAC1 binding was not due to failure of anti-LIBS6 to bind to these mutants. Furthermore, the loss of ligand binding to these two mutants was not a generalized effect, since alanine substitution of a number of other highly conserved residues with oxygenated side chains had no effect on ligand binding function (Table 1).

To substantiate the results obtained with mAb PAC1, the effect of these substitutions on the binding of another ligand mimetic mAb, OPG2 was also examined by flow cytometry. OPG2 is a murine IgG1k that blocks the binding of adhesive protein ligands to α1β3β3, and its binding is blocked by RGD peptides (34). However, in contrast to PAC1, the binding of mAb OPG2 is activation-independent. Consistent with the results obtained with PAC1, OPG2 failed to bind to cells expressing the β3 mutants Asp217 → Ala or Glu220 → Ala (Fig. 5). Alanine substitution of other conserved residues had no effect on the binding of mAb OPG2 (Table 1).

Asp217 in β3 is found at the end of βD strand in the proposed model and is predicted to be the functional equivalent of Asp217 in the αm I domain (30). In contrast, β3 Glu220 does not appear to have a functional equivalent in the I domain MIDAS motif. The loss of ligand binding function of α1β3β3 after substitution of β3 Glu220 suggested that Glu220 might be involved in metal coordination.
investigate this hypothesis, two additional sets of models were generated. In the first set, the OE1 atom of Glu\textsuperscript{220} was taken to form a hydrogen bond with a coordinating water molecule; a distance restraint of 2.5–3.3 Å was therefore imposed between these two atoms throughout generation of this model. In the second set, the OE1 atom of Glu\textsuperscript{220} was taken to coordinate directly with the metal ion; a distance restraint of 1.8–2.2 Å was therefore imposed between these two atoms throughout generation of this model. Good quality models could be obtained with Glu\textsuperscript{220} interacting with the metal ion either directly or indirectly via a water molecule. This interaction appears to be facilitated by Pro\textsuperscript{219} becoming cis (it is trans in the model depicted in Fig. 2 generated without Glu\textsuperscript{220} being restrained) and is aided by the conformational freedom of Gly\textsuperscript{221} and Gly\textsuperscript{222}. These results support a hypothesis that Glu\textsuperscript{220} could participate in metal coordination.

**DISCUSSION**

We report that alanine mutagenesis of selected amino acids in β\textsubscript{3} that are highly conserved among the integrin β subunits has identified Asp\textsuperscript{217} and Glu\textsuperscript{220} as residues essential for the ligand binding function of α\textsubscript{IIb}β\textsubscript{3}. A model of this region of β\textsubscript{3} based on the crystal structure of the recombinant α\textsubscript{M}I domain predicts that this region may share many of the structural

**FIG. 2.** Ribbon diagram of lowest energy model of β\textsubscript{3} Tyr\textsuperscript{110}–Leu\textsuperscript{294}. β strand and α-helix assignments are as designated in Fig. 1. Predicted disulfide bonds (45) are shown in yellow. Enlargement shows proposed MIDAS-like motif. Oxygen atoms are shown as a blue ball (M). Generated using MOLSCRIPT and RASTER3D (68, 69).
elements of the I domain. This proposed model is consistent with the mutagenesis data and suggests that Asp217 and Glu220 are located in the D-a5 loop, where they can participate in the coordination of a metal ion together with the previously identified Asp119, Ser121, and Ser123. Asp217 but not Glu220 is analogous to a metal-coordinating residue in the MIDAS domain, indicating that region adopts a similar but not identical fold to that of the MIDAS motif.

Homology modeling suggests that β3 Asp217 is the functional equivalent of αM Asp242. In the Mg²⁺ form of the αM I domain, the carboxylate group of Asp242 contributes to metal coordination via a hydrogen bond to a coordinating water molecule and through a second hydrogen bond to the hydroxyl side chain of the directly coordinating residue Ser144. In the Mn²⁺ form of the αM I domain, the metal is coordinated slightly differently, since Asp242 directly coordinates the bound metal (32). Similarly, the Asp242 homologue in the β3 Mn²⁺ I domain structure, Asp239, directly coordinates the bound metal (31). Substitution of αM Asp242 in the isolated I domain results in loss of both cation binding and ligand binding (29). Furthermore, substitution of Asp242 in αM and substitutions of the equivalent residues in the I domains of α1 (Asp253), α2 (Asp253), and α5 (Asp254) all exert dominant negative effects, since each substitution results in the loss of binding of structurally distinct ligands to the intact receptor. Together these results indicate that the participation of this Asp residue in metal coordination is critical for ligand binding. The observed loss of function of α11β3 following substitution of β3 Asp217 is consistent with this residue being the functional equivalent of these I domain Asp

![Image](59x288 to 311x736)

**FIG. 4.** Substitution of β3 amino acid residue Asp217 or Glu220 results in loss of PAC1 binding. The binding of the α11β3 complex-specific mAb D57 or the ligand mimetic mAb PAC1 to Chinese hamster ovary cells transiently transfected with α11α5 and the indicated β3 subunit was examined by flow cytometry. Results are depicted as histograms with the log of the fluorescence intensity on the abscissa and the cell number on the ordinate. To determine PAC1 binding, transfected cells were incubated (activated) with 4 μM anti-LIBS 6 mAb followed by the addition of mAb PAC1 (IgM). Cells were washed, stained with fluorescein-conjugated goat anti-mouse IgM for 30 min, and analyzed.

**FIG. 5.** Loss of mAb OPG2 binding following substitution of β3 Asp217 or Glu220. The binding of the activation-independent ligand mimetic mAb OPG2 to Chinese hamster ovary cells transiently transfected with wild type α11α5 and the indicated β3 mutant was examined by flow cytometry. In each panel, the binding of OPG2 to the indicated mutant (open histogram) is superimposed on the binding of OPG2 to cells transfected with wild type α11β3. Results are expressed as histograms of cell number versus log fluorescence intensity.

| Amino acid | Anti α11β3 | PAC1 | OPG2 |
|------------|------------|------|------|
| Ser108     | +          | +    | +    |
| Asp179     | +          | +    | +    |
| Thr182     | +          | +    | +    |
| Thr183     | +          | +    | +    |
| Thr195     | +          | +    | +    |
| Thr197     | +          | +    | +    |
| Asp208     | +          | +    | +    |
| Thr201     | +          | +    | +    |
| Glu205     | +          | +    | +    |
| Asp217     | +          | +    | +    |
| Glu220     | –          | –    | –    |
| Asp224     | +          | +    | +    |
| Glu233     | +          | +    | +    |
| Asp251     | +          | +    | +    |
| Asp259     | +          | +    | +    |
| Asp270     | +          | +    | +    |

**TABLE I** Summary of monoclonal antibody reactivity with mutant integrins

Chinese hamster ovary cells transiently transfected with wild type α11β3 and the indicated β3 mutant were analyzed for mAb reactivity by flow cytometry as described under "Materials and Methods." +, positive staining; –, staining not significantly different from negative control (mock-transfected cells).
residues and strongly suggests that its role in the ligand binding function of α1β3 is due to its participation in cation coordination.

The basis for the requirement of β3 Glu220 in the ligand binding function of α1β3 is less obvious. A Glu residue is absolutely conserved at this position in all the integrin β subunits. A Glu residue, separated from the coordinating αM Asp242 residue by a Gly, is conserved in αL (Glu44) but is less well conserved among other proteins in the A/Δ domain superfamily (30). The effect of substitution of this Glu residue in the I domain on intact αMβ2 receptor function has not been examined directly by mutagenesis. In the Mg2⁺ form of the αM I domain, Glu244 is hydrogen-bonded through its main chain carbonyl to a coordinating water molecule (30). However, one would expect the potential for this indirect main chain interaction with a coordinating water molecule to be the same for Ala as it is for Glu if the structure of these two domains is similar. In this regard, we note that the register at the top of βD cannot be assigned with certainty, since β3 (as well as other integrin β subunits) does not contain the Gly residue that is conserved in each of the integrin I domains. An alternative register makes this loop between βD and α5 more similar to that in the von Willebrand factor A3 domain2 that lacks this conserved Gly. This would have the effect of placing Asp217 one residue further up the loop, and Glu220 would then be placed in a position where it too could participate in metal coordination either directly or indirectly via a water molecule. Given the inherent limitations of the proposed model, it is also possible that Glu220 may participate directly in ligand binding in a manner distinct from a potential role in cation coordination. Precise functional assignment of this residue will ultimately require a high resolution crystal structure.

Ligand binding to α1β2, αMβ2, and αβ1β3 is critically dependent upon a conserved Thr residue within the I domain of the α subunits (50–53). In the crystal structure of the Mg2⁺ form of αM, Thr209 makes a strong bond to the metal via its side chain hydroxyl oxygen (30). In the Mn2⁺ form of the αM I domain, Thr209 does not coordinate the metal directly but rather hydrogen-bonds to a coordinating water molecule (32). Similarly, in the Mn2⁺ form of the αL I domain, the corresponding residue in αL, Thr206, is also hydrogen-bonded to a coordinating water molecule (31). In both of these I domains, this critical Thr residue is present on an extended loop between α3 and α4. Inspection of the corresponding loop region in the β3 structural model identified two potential residues, Thr195 and Thr197, to be spatially adjacent to the DXSXS sequence. While Thr195 is not well conserved, Thr197 is absolutely conserved among all the integrin β subunits. In contrast to the critical role that the conserved Thr residue located at this position plays in ligand binding to I domain-containing integrins, alanine substitution of β3 Thr195 or Thr197 had no effect on ligand binding, since both mutants readily bound the ligand mimetic mAb PAC1, suggesting that metal coordination may be different in these two structures.

The fact that the critical I domain Thr residue coordinates the metal ion differently in the Mg2⁺ and Mn2⁺ forms suggests that these two forms may represent two different activation states of the receptor (31, 32). Indeed, Mn2⁺ can stimulate the binding of ligand to α1β3 and a number of other integrins in the absence of other agonists (54–59). In the present study, PAC1 binding to the expressed mutants was investigated in the presence of the activating mAb anti-LIBS6. Thus, one potential explanation for the observed lack of effect on PAC1 binding to the Thr197 → Ala mutant is that this residue may play an essential role in metal coordination in the unactivated conformation of the receptor but is not essential for coordination in the active conformation. However, substitution of Thr195 or Thr197 had no effect on the binding of OPG2, which does not require receptor activation. Thus it is unlikely that Thr197 participates in metal coordination in this structure. The present results cannot rule out that substitution of this Thr residue alone does not destabilize the coordination of bound cation sufficiently to inhibit ligand binding.

The identification of Asp217 and Glu220 as putative metal-coordinating residues provides an explanation for previous results implicating this region in ligand binding function (18) and the loss of ligand binding associated with mutations at Arg219 (19, 20). The current model places Arg214 in the middle of βD, which would bury that residue unless the carboxyl-terminal helix (α7) swings out from the position it occupies in the integrin I domain fold. An altered location of this helix is reasonable given its variable conformation in the integrin I domains. Therefore, while the proposed model does not support a direct role for this residue in metal coordination, the previously described Arg214 → Gln or Arg214 → Trp substitutions might both be predicted to produce alterations in the local structure, potentially destabilizing metal coordination mediated by Asp217. Such a structural alteration may also be the root cause of the impaired stability of the α11β3 heterodimer observed in these mutants (19, 20, 60).

The current results support a role for this region of β3 in ligand binding function as a consequence of its adopting a structure very similar to the MIDAS structure present in the I domains. In addition to the lack of a critical threonine residue for ligand binding located carboxyl-terminal to the DXSXS sequence in the β3 subunit, other significant differences exist in the ligand binding characteristics of I domain-containing integrins compared with α11β3. First, ligand binding to α11β3 is fully supported by Ca2⁺. In contrast, binding of ligands to recombinant I domains or intact I domain-containing integrins requires Mg2⁺ or Mn2⁺ and can be inhibited by Ca2⁺ (29, 55, 61, 62). The functional requirement for the negatively charged Glu220 but not for the uncharged Thr197 could explain why Ca2⁺, a larger ion with a greater tendency to form ionic bonds, can substitute for Mg2⁺ in the ligand binding function of α11β3 but not αMβ3. Second, the results presented here would indicate that I domain integrins potentially possess two MIDAS domains. The significance of two MIDAS domains and their interrelationship in ligand binding has not been addressed, yet mutations of critical residues in either the α or β subunit domains effectively abolish ligand binding to β2 integrins. Third, it is particularly noteworthy that isolated recombinant I domains can bind ligand directly (29, 51, 63–66). In contrast, high affinity ligand binding to α11β3 requires an intact heterodimer, since individual α11 or β3 subunits have not demonstrated ligand binding capacity.

In summary, we conclude that a highly conserved region in the integrin β subunits functions as a MIDAS-like domain and that β3 residues Asp217 and Glu220 play a role in ligand binding function via a likely contribution to a metal coordination sphere. In view of the ubiquitous divalent cation requirement for all integrin receptor function, it is likely that residues homologous to β3 Asp217 and Glu220 in other integrin β subunits will be assigned similar functional roles. The present results and the proposed model do not address the role of α11 in this structure; however, the cross-linking of ligand mimetic peptides to α11 substantiates the close proximity of regions of α11 to bound ligand. Accordingly, this presents the intriguing possibility that a residue located on α11 may supply a metal-coordinating ligand or interact directly with ligand. Such a

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2 R. Liddington, unpublished observations.
possibility might further explain the requirement of the α subunit for ligand binding and ligand recognition specificity in integrins that lack an I domain.

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