Evidence That the Integrin β3 and β5 Subunits Contain a Metal Ion-dependent Adhesion Site-like Motif but Lack an I Domain*

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The amino-terminal domain of each integrin β subunit is hypothesized to contain an ion binding site that is key to cell adhesion. A new hypothesis regarding the structure of this site is suggested by the crystallization of the I domains of the integrin α5 and αM subunits (Lee, J.-O., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) Cell 80, 631–638; Qu, A., and Leahy, D. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10277–10281). In those proteins, an essential metal ion is bound by a metal ion-dependent adhesion site (MIDAS). The MIDAS is presented at the apex of a larger protein module called an I domain. The metal ligands in the MIDAS can be separated into three distinctly spaced clusters of oxygenated residues. These three coordination sites also appear to exist in the integrin β3 and β5 subunits. Here, we examined the putative metal binding site within β3 and β5 using site-directed mutagenesis and ligand binding studies. We also investigated the fold of the domain containing the putative metal binding site using the PHD structural algorithm. The results of the study point to the similarity between the integrin β subunits and the MIDAS motif at two of three key coordination points. Importantly though, the study failed to identify a residue in either β subunit that corresponds to the second metal coordination group in the MIDAS. Moreover, structural algorithms indicate that the fold of the β subunits is considerably different than the I domains. Thus, the integrin β subunits appear to present a MIDAS-like motif in the context of a protein module that is structurally distinct from known I domains.

Integrins are αβ heterodimers that mediate cell adhesion (1, 2). Integrins participate in development and tissue remodeling and are linked to several diseases. The integrins bind to many adhesive and extracellular matrix proteins. The focal points of this study are the αvβ3 and αvβ5 integrins, both of which recognize the Arg-Gly-Asp (RGD) tripeptide motif. The αvβ3 integrin binds to at least nine adhesive proteins and has two important biological functions. First, αvβ3 mediates the adhesion of osteoclasts to the bone surface (3), an event often considered to be the first step in bone resorption (4). Second, the αvβ3 integrin is expressed on the surface of angiogenic endothelial cells, where it is required for cell survival and further vessel development (5–7). It has been suggested that inhibitors of the αvβ3 integrin could be applied as antagonists of osteoporosis and tumor angiogenesis. The biological function of the αvβ5 integrin is less clear. This integrin can mediate cell adhesion to vitronectin. The αvβ5 integrin is also required for the internalization of adenovirus (8, 9), and it may be associated with angiogenesis (7).

All integrins require divalent cations to bind their ligands. An important clue to the structural basis for ion binding was revealed by the crystal structures of the I domains from the integrin α5 and αM subunits (10, 11). Each I domain spans approximately 200 residues and is homologous to an “inserted” domain in a number of other proteins including von Willebrand factor (12). In α5 and αM, the I domain is necessary and sufficient for ligand contact. These I domains contain a metal binding site called a MIDAS (metal ion-dependent adhesion site). This ion binding site consists of five liganding residues that can be separated into three groups. Each group of coordinating residues is located at separate positions within the primary amino acid sequence (10, 11). The first coordination group consist of the DXSXS sequence, where D is aspartate, X is any amino acid, and S is serine. The aspartate and both serines coordinate with metal ion. The second group, or coordination point, is a single threonine located 69 amino acids from the DXSXS. The third group is comprised of a single aspartate 102 residues from the DXSXS.

Interestingly, the DXSXS sequence is also present in the integrin β subunits (13), suggesting that they may also contain the MIDAS (10). If correct, this would provide a common structural basis for the regulation of all integrins by divalent metal ions. It would also imply that all integrins are regulated in a similar manner by metal ion. Despite this hypothesized similarity, integrins behave differently with respect to metal ions. For example, we recently demonstrated that the type of divalent ion present in the culture media regulates the way that the αvβ3 and αvβ5 integrins are organized on the cell surface (14). In fact, the same ion can direct the two integrins to completely different locations on the cell. This distinction suggests that the metal binding site within the β subunits is likely to have subtle but important structural differences that have an impact on receptor function.

To examine the hypothesized ion binding site within β3 and β5, the putative metal coordinating residues within each sub-
Evaluation of the Putative MIDAS in β3 and β5

EXPERIMENTAL PROCEDURES

Sequence Alignment and Structural Predictions—Amino acid sequences of β3 (residues 107–292) and β5 (residues 109–296) were each aligned with the I domain sequences of α5 (residues 125–310) and αM (residues 129–318) using the multiple sequence alignment program ClustalW (15). Without further manipulation, the putative metal ligands in both β3 and β5 subunits were aligned with the I domain sequences of the MIDAS motifs of α5 and αM.

The structures of β3, β5, and the I domains of α5 and αM were analyzed using the PHD algorithm (16, 17). The algorithm compares a predicted protein to the I domain sequences of α5, αM, and NMR structures and then assigns the probability that a residue is likely to be part of a metal ion binding site that controls ligand contact. The similarity in spacing and function of these aspartic acids within the MIDAS of β3 and β5 subunits indicates in bold lettering. All mutations were confirmed by dideoxy chain termination. The apparent affinity for cation was determined as the concentration of cation that supported the maximal ligand binding.

Ligand binding to cells expressing αvβ3 and αvβ5 and mutant forms of β3 was measured with 125I-vitronectin (Vn) using a tracer format (21). For that purpose, vitronectin was purified from bovine serum previously (22) and labeled to high specific activity (150,000 cpm/ng) with Na125I (Amersham Corp.). Cells expressing the mutants of β3 were harvested with EDTA/PBS and washed with Binding Buffer containing 0.5 mM MgCl2 and 0.02 mM CaCl2. Cells (10^7/ml) were incubated with 0.5 nM 125I-Vn and increasing concentrations of unlabeled Vn in cation-supplemented Binding Buffer at 14 °C for 70 min. Specific binding was determined by subtracting the EDTA-sensitive binding from total binding. The affinity of αvβ5 for vitronectin was derived using Scatchard analysis (20).

To measure the apparent affinity of integrin for Mg2+, Fab-9 binding was measured as a function of Mg2+ concentration. The apparent affinity for cation was taken as the concentration of cation that supported half-maximal ligand binding.

Cell Adhesion Assays—Cell adhesion assays were performed as described previously (23). Briefly, Fab-9 (50 nM) or vitronectin (6 nM) were coated on 96-well plates by an overnight incubation at 4 °C. The plates were then blocked with 1% bovine serum albumin. Cells (1 × 10^5 cells/well) were harvested with EDTA/PBS and resuspended in Binding Buffer containing the appropriate cations as described for soluble ligand binding. Cells were allowed to adhere at 37 °C for 45–60 min. Non-adherent cells were removed by gentle washing, and adherent cells were detected by a colorimetric assay for lysosomal acid phosphatase (24). Color absorbance was detected at 540 nm.

Measuring the Binding of Antibody AP5—The binding of mAb AP5 to 293 cells was measured as described previously (25). Briefly, cells were incubated with 50 μg/ml FITC-conjugated AP5 in the presence of varying concentrations of Ca2+ and analyzed by FACS. Mean fluorescence intensity was determined per 10,000 cells.

RESULTS

Sequence and Structural Comparison between the I Domain of αM and the Amino-terminal Domain of β3 and β5—As a first step toward characterizing the putative metal binding site in β3 and β5, their sequences were compared with the I domain of αM. The sequence of β3 (residues 107–292) was aligned with the I domain of αM using the multiple sequence alignment program ClustalW (15). The sequence of β5 was incorporated into this alignment using the extensive identity between β3 anti-mouse secondary antibody (Caltag). All cells used for binding studies exhibited stable integration of the β subunit cDNA into the genome.

Antibodies and Synthetic Peptides—The anti-αvβ3 mAb LM609 was purchased from Chemicon. Non-specific mouse IgG was obtained from Calbiochem. Monoclonal antibody P1F6 (anti-αvβ5) was purchased from BD Biosciences. Mammalian expression vector pcDNA3 (Invitrogen). Mutated constructs of human αM were expressed in 293 cells using the retroviral packaging system kindly provided by Dr. Jason H. Wasylyk (15). Without further manipulation, the putative metal ligands in both β3 and β5 subunits were aligned with the I domain sequences of the MIDAS motifs of α5 and αM.

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and β5. The DXXSXS motif and an aspartic acid residue representing the third coordination group in the MIDAS align well in all three proteins. The β subunits diverge from the MIDAS motif at the middle metal coordinating position (Thr-209 in αM). The β subunits contain a small disulfide-bonded loop in this region, a structure absent in the I domains. Within this disulfide-bonded loop, β5 contains two threonine residues (Thr-182 and Thr-183) that could be homologues of the coordinating motif at the middle metal coordinating position (Thr-209 in αM and 107–295) were aligned using the program ClustalW (15). The positioning of the MIDAS residues in αM and α5 are identical, so only αM is shown. The amino acid residues that coordinate metal ion in αM and the predicted coordinating residues in β3 and β5 are boxed. These same regions were subjected to structural prediction using the PHD algorithm (16, 17). The predicted structures were compared with the actual structure of αM as determined by crystallographic data (10). β strands are represented as hatched rectangles; α helices are shown as shaded rectangles, and turns/random coils are left open. Secondary structural elements according to the αM crystal structure are labeled as β sheet strands A–F and α helices I–7 (10). Crystal structure is abbreviated as X and predicted structure as P. The position and length of each element in the figure is shown to scale.

**Fig. 1. Sequence alignment and structural prediction of the αM I domain with β3 and β5.** The I domains of αM, α5, and β3, and the corresponding regions in β3 (residues 107–292) and in β5 residues (109–295) were aligned using the program ClustalW (15). The positioning of the MIDAS residues in αM and α5 are identical, so only αM is shown. The amino acid residues that coordinate metal ion in αM and the predicted coordinating residues in β3 and β5 are boxed. These same regions were subjected to structural prediction using the PHD algorithm (16, 17). The predicted structures were compared with the actual structure of αM as determined by crystallographic data (10). β strands are represented as hatched rectangles; α helices are shown as shaded rectangles, and turns/random coils are left open. Secondary structural elements according to the αM crystal structure are labeled as β sheet strands A–F and α helices I–7 (10). Crystal structure is abbreviated as X and predicted structure as P. The position and length of each element in the figure is shown to scale.

### Expression of the Mutant Forms of β3 and β5 in 293 Cells

To probe the structure of the ion binding site within each β subunit, we mutated putative metal liganding residues to alanine. In β3 these are Asp-121, Asn-186, and Ser-190, and in β5 the putative coordinating residues are Asp-217, Asn-265, and Ser-266. Because of their proximity to the last putative coordination residue, we also mutated Glu-220 and Glu-223 within β5 and Glu-223 within β5. Each cDNA construct was transfected into 293 cells. Following antibiotic selection and FACs sorting, transfected cells were found to express nearly equivalent levels of each of the mutated integrins on the cell surface (Fig. 3). One mutant, β3 E220A, was not expressed on the cell surface, even though repeated attempts were made to transfect this mutant. To confirm proper heterodimer forma-
tion, the mutant integrins were immunoprecipitated from lysates of 125I-labeled cells using antibodies against αvβ3 (LM609) and αvβ5 (P1F6). Each antibody immunoprecipitated αv and the relevant β subunit in an approximate 1:1 stoichiometry, confirming that the mutated subunits complex with αv (data not shown).

Assessing the Ligand Binding Function of Mutated Forms of β3—The ligand binding function of each mutated form of αvβ3 was measured using the model ligand Fab-9 which has been characterized previously (18, 19, 27). This ligand was chosen because the binding affinity of soluble vitronectin for αvβ3 on 293 cells was too low to yield reproducible binding data. In these binding studies, the metal concentration was set to an optimal level. Wild-type αvβ3 bound to 125I-Fab-9 with an affinity of 9 ± 3 nM (n = 10). Within the detectable range of binding, the mutation of β3 residues D119A and D217A abolished binding of soluble Fab-9 to the cell surface (Fig. 4). Surprisingly, cells expressing the T182A and T183A mutations bound to 125I-Fab-9 with an affinity identical to that of wild-type αvβ3. To determine whether the mutations T182A and T183A had a more subtle effect on cation-dependent ligand binding, we measured their apparent affinities for Mg2+. The apparent affinities of T182A or T183A for Mg2+, as reported by Fab-9 binding, were identical to wild-type αvβ3 (Table 1). Thus, unlike the corresponding threonines within αv and αM, neither of the candidate threonines within β3 appear to be crucial metal ligands.

Cell adhesion is a multimeric interaction between clustered integrins and a non-diffusible matrix. Therefore, it can often proceed even when the affinity between integrin and ligand is very low. Consequently, we measured the effect of mutations within the β3 subunits on cell adhesion to immobilized Fab-9. Surprisingly, cells expressing β3 mutants at Asp-119 and Asp-217 adhered to Fab-9 (Fig. 5), even though they failed to bind soluble ligand. The mutated forms of β3 supported adhesion to a level that usually reached approximately 40% that of wild-type β3. More importantly, both mutations also exhibited a shift in the apparent affinity for metal ion. This was measured by determining the level of metal ions that supported half-maximal adhesion. The study was done with Mn2+ because it has the highest affinity for the integrin. The D119A mutation exhibited an apparent affinity for ion that was approximately 6–10-fold lower than that of wild-type β3. The mutation at Asp-217 was even more deleterious, exhibiting an apparent affinity for metal that was 15–20-fold lower than wild-type β3. These are the first data to demonstrate that mutations at putative metal-coordinating residues within an integrin β subunit shift the ion response curve. This can be interpreted to indicate that Asp-119 and Asp-217 contribute to metal binding affinity.

Assessing the Ligand Binding Function of Mutant Forms of the β5 Subunit—The binding of vitronectin to wild-type αvβ5 on 293 cells was initially characterized in conditions containing 500 μM Mg2+ and 20 μM Ca2+. Under these cation concentrations, the binding of vitronectin was specific and saturable with a Kd of 9 nM. Vitronectin binding to cells expressing wild-type αvβ5 could be completely inhibited with function-blocking mAb P1F6 or GRGDSP peptide (data not shown). Each mutant of αvβ5 was evaluated for its ability to bind soluble vitronectin. The binding of vitronectin was assayed as a function of the concentrations of Mg2+ or Mn2+ (Fig. 6). The titration of Mn2+ was carried out to only 5 mM because artifactual binding of vitronectin was detected above this concentration. In this experiment, the data are expressed as a percentage of maximal binding to wild-type αvβ5 which was always measured in parallel. Cells expressing alanine mutations at Asp-121 and Asp-220 failed to bind to soluble Vn in either Mg2+ or Mn2+. In the radioligand binding assay that was employed, we were only able to detect vitronectin binding to integrin when the Kd was below 500 nM. Since wild-type αvβ5 has a Kd of 9 nM for soluble
identical results were obtained. Ligand binding to cells expressing mutant affinities (we were unable to identify a residue in and Ser-190 to alanine had no effect on ligand binding. Thus, determined as the concentration of Mg$^{2+}$ was used as a competitor. Each point is the average of triplicate data points. Scatchard plots (bound (B) versus bound/free (B/F)) and calculated affinities ($K_d$) are shown in the insets. The correlation coefficients of each Scatchard plot are as follows: wild-type (WT), $r^2 = 0.97$; T182A, $r^2 = 0.92$; and T183A, $r^2 = 0.85$. No specific binding was detected for D119A and D217A. Each figure is representative of at least two experiments in which identical results were obtained. Ligand binding to cells expressing mutant αβ3 was always done in parallel with cells expressing WT αβ3.

**TABLE I**
The apparent affinities of β3 mutants T182A and T183A for magnesium (Mg$^{2+}$)

| Cell line | Apparent affinity for Mg$^{2+}$ | n |
|-----------|---------------------------------|---|
| WT        | 0.5                             | 4 |
| T182A     | 0.5, 0.7                        | 2 |
| T183A     | 0.5, 0.7                        | 2 |

vitronecin, we conclude that mutations at Asp-121 and Asp-220 cause at least a 55-fold reduction in the affinity of the integrin for vitronectin. These data are consistent with the role of each aspartate in metal coordination and with nearly identical data obtained for β3 (see above). The mutation of Asn-186 and Ser-190 to alanine had no effect on ligand binding. Thus, we were unable to identify a residue in β5 that corresponds to the metal coordinating threonine (Group 2) in αi and α3. It is also interesting to note that the mutation of Glu-223 to alanine eliminated the ability of αβ5 to bind soluble vitronectin. Although this residue is not homologous to any of the metal ligands in the MIDAS, our data indicate that it has a role in ligand binding function. It may, in fact, substitute for the missing second metal ligand (see “Discussion”).

The ability of each mutant form of αβ5 to mediate adhesion to immobilized vitronectin was also measured (Table II). The substitution of alanine at Asp-121 and Glu-223 of β5 resulted in complete abrogation of cell adhesion, whereas alanine substitutions at β5 residues Asn-186 and Ser-190 had no effect on the ability of the cells to adhere to vitronectin. In contrast, β5 containing D220A mediated cell adhesion, although the absolute level of adhesion at saturation was lower than wild-type αβ5. The apparent affinity of this mutant form of αβ5 for metal ion was 5–50-fold lower than that exhibited by wild-type αβ5 (45–62 versus 1–10 μM). This observation is consistent with a role for Asp-220 in coordinating metal ion and is also consistent with the fact that the homologous residue in β3 (Asp-217) contributes to metal binding affinity.

**Mutations at Asp-119 and Asp-217 of β3 Fail to Disrupt the Function of the Inhibitory Ca$^{2+}$-binding Site**—Integrins contain two classes of ion binding sites, one that promotes ligand binding, called a Ligand Competent site, and another that inhibits ligand binding, called an Inhibitory site (27–30). The monoclonal antibody AP5 binds to the amino-terminal domain of the β3 subunit and reports the occupation of the Inhibitory Ca$^{2+}$-binding site (25). As an extension of the present study, we measured the effect of each point mutation within β3 on the sensitivity of the binding of the AP5 antibody to Ca$^{2+}$. As shown in Fig. 7, the binding of AP5 to wild-type αβ3 and to both β3 D119A and β3 D217A was blocked by Ca$^{2+}$. Thus, Asp-119 and Asp-217 are not part of the Inhibitory ion binding site.

**DISCUSSION**
The primary objectives of this study were to examine the possibility that the amino-terminal portion of the integrin β
subunit contains a MIDAS-like metal binding site and to assess whether this motif in the integrin β subunit is positioned at the apex of an I domain structure. The simplest step in this analysis involved a comparison of the two structures. The I domains and the amino-terminal portion of the integrin β subunits have similar hydrophathy profiles (10) and also exhibit some sequence homology, particularly at residues known to ligand with metal. Both observations suggest the potential for a common fold. Here, we examined this possibility in more detail using the PHD algorithm, which generates a predicted structure based on the propensity of individual residues within a given local environment to exist in a helix, a sheet, or a disordered loop. Importantly, the algorithm relies on known crystal and NMR structures to predict tertiary structure from the primary sequence. It is reported to have a success rate of approximately 70% (17). The PHD algorithm correctly predicted 10 of 13 structural elements within the I domains of αv and αm, attesting to its ability to identify the major elements within an I domain. In contrast, the algorithm predicted that only 2–3 of the 13 I domain elements are present in the corresponding positions of β3 and β5. Although the β subunits appear to have some sequence similarity with the I domains, an in-depth analysis using a sophisticated algorithm suggests that the three-dimensional structure of the integrin β subunits is likely to be significantly different from that of the I domains. Based on this analysis it does not appear that the integrin β subunits contain an I domain-like region. This does not exclude the possibility that a metal-binding MIDAS motif could be presented in the context of a different backbone structure.

Therefore, a series of biochemical studies were performed to further assess metal and ligand binding to the αvβ3 and αvβ5 integrins. As a first step, the ion specificity of the ligand binding event was tested. Indeed, both integrins have an ion preference that is remarkably similar to that reported for the I domain of αM (26). Although some minor differences exist between αvβ3 and αvβ5, transition state metal ions like Ce3+ and Mn2+, as well as the cation Mg2+, support ligand binding. Divalent ions like Ca2+ and Ba2+ were far less effective. Although we know the regulation of ligand binding to αv-integrins to be complex and that it can involve regulation by two classes of ion binding sites (27, 28, 30, 32), this simple test shows that the ligand binding event for αv-integrins has an ion specificity that is more similar to that of an I domain (26) than to an EF-hand (33).

A more detailed analysis of metal binding involved the mutation of the putative metal coordinating residues within β3 and β5. This approach identified two distantly spaced aspartic acid residues that greatly influence receptor function. These are Asp-119 and Asp-217 in αvβ3 and Asp-121 and Asp-220 in αvβ5. By sequence alignment, each aspartate appears to be a homologue of metal ligands in the MIDAS motifs of αi and αm. Substitution of any of these aspartates with alanine reduces the affinity of the αv-integrins for soluble ligands by at least 50-fold. Interestingly, mutation of Asp-119 and Asp-217 within β3 and Asp-220 in β5 did not completely abrogate receptor function because integrins with these mutations could still mediate cell adhesion. Despite the inability of each mutated integrin to bind soluble ligand, the ability of mutants at β3 residues Asp-119 and Asp-217 and β5 Asp-220 to mediate cell adhesion proves that these aspartates are not absolutely essential for ligand contact. It is important to emphasize that cell adhesion to an immobilized substratum is the summation of multivalent receptor–ligand contacts brought about by integrin clustering. In addition the ligand is immobilized and cannot freely diffuse; therefore, cell adhesion can often be observed even when the affinity between ligand and integrin is too low to measure in soluble ligand binding assays. Thus, another interpretation of this result is that each aspartate contributes to ligand binding affinity. We believe this to be a reasonable inference especially since FACS analysis indicates that each mutant is expressed on the cell surface at a level equivalent to
the wild-type integrin. However, because the mutant integrins fail to interact with soluble ligand, we are unable to provide a quantitative measure of the difference in ligand binding affinity.

It is also key to assess whether the mutations at putative metal coordination sites alter the affinity of the integrin for metal ion. Unfortunately, the inability to generate milligrams of recombinant integrin, and the relatively low affinity of the integrin for ion, makes a direct measure of this parameter nearly impossible. We were, however, able to assess metal binding affinity indirectly by measuring the apparent affinity of the integrin for metal ion as reported by ligand binding. This was accomplished by measuring cell adhesion across a range of metal ion. From this analysis it is evident that the mutation of Asp-119 and Asp-217 in β3, and Asp-220 in β5, reduces the apparent affinity of each integrin for metal ion. Mutation of each aspartic acid lowered the apparent affinity for either Mn$^{2+}$ or Mg$^{2+}$ by 10–20-fold. This is the first evidence we are aware of in which an aspartic acid within an integrin β subunit has been shown to influence metal ion affinity. The simplest interpretation of this finding is that each of these aspartic acid residues is part of a metal ion binding site. Without a direct measure of metal binding affinity to each mutant, and without a three-dimensional structure, these aspartates cannot be unequivocally assigned as metal ligands. Yet, because each of the apparent affinities that are listed were compiled from at least three separate experiments. Adhesion of cells containing the β5 mutations was always compared with the adhesion of 293 cells expressing wild-type αvβ5.

**TABLE II**

*The effect of point mutations in β3 on cell adhesion to vitronectin*

| Cell line | Mg$^{2+}$ | Mn$^{2+}$ |
|-----------|-----------|-----------|
| Wild-type | 80–180    | 1–10      |
| D121A     | No adhesion | No adhesion |
| N186A     | 125–350   | 2–3       |
| S190A     | 125–300   | 4–15      |
| D220A     | 1000–2500 | 45–62     |
| E223A     | No adhesion | No adhesion |

**FIG. 7.** Calcium inhibits the binding of AP5 to WT αvβ3 and to the D119A and D217A mutants. Human 293 cells expressing the noted integrin were incubated with 50 mg/ml FITC-labeled AP5. The concentrations of Ca$^{2+}$ were varied from 4 to 500 μM. The mean fluorescence intensity of 10,000 cells is presented for each point. Binding of AP5 to mutants T182A and T183A was also inhibited by Ca$^{2+}$ (not shown).

A final objective of the present study was to classify the putative ion binding site within the β subunits. There are two classes of metal ion binding sites on αvβ3 and on α5β1 (25, 27, 30). These two cation binding sites have opposing effects on ligand binding. One class of site(s), called Ligand Competent sites, must be occupied for ligand to bind (23, 27, 30). The second class of sites are called Inhibitory sites because, when occupied with Ca$^{2+}$, these sites interfere with ligand binding. The Inhibitory sites are allosteric to the ligand binding site and

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*This study also identifies an important distinction between the metal ligands in the MIDAS and in the β subunits. In the MIDAS motif, the second coordination group is a single threonine that coordinates with bound metal. Based on the alignment presented in Fig. 1, we hypothesized that the analogous threonine in β3 was at residue Thr-182 or Thr-183. Interestingly, the β5 subunit lacks this threonine, and we originally hypothesized that this difference in sequence at a metal ligand motif and require the association of both subunits for this function.

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*In conjunction with the present study, these data suggest a hypothesis regarding the RGD binding site. Collectively the two lines of data indicate that the DX5X5S motif form a metal binding site that is also part of the RGD binding cleft. In this respect, the integrin β subunits appear to contain a site that is similar to the MIDAS motif, where metal-coordinating residues are distinctly spaced in the primary sequence but come together in the tertiary structure of the protein to make contact with a metal ion. This similarity must be interpreted in the context of several key differences between the β subunits and the I domains. Structural algorithms indicate that the β subunits lack similarity to the I domains, so the β subunits are likely to contain a MIDAS within a different backbone. Within the putative metal binding domain, the β subunits contain two disulfide bonds, whereas the I domains do not. The two domains function differently as well. The αvβ3 and αvβ5 integrins bind the RGD motif and require the association of both subunits for this function.*
act by increasing the ligand dissociation rate (27). The data presented here provide the first mutational evidence that the Ligand Competent and Inhibitory sites are separate. The elimination of the Inhibitory cation binding site by mutation would be expected to decrease the ligand dissociation rate, thereby increasing overall ligand affinity. Inactivation of the Ligand Competent cation binding site would prevent ligand binding. Mutations at β3 Asp-119 and Asp-217 clearly belong to the class of sites called Ligand Competent sites. Importantly, the Inhibitory cation binding site appears to remain functional in both mutated integrins because the binding of the reporter antibody AP5 remains sensitive to Ca2+.

Addendum—While this paper was in review, reports on the same topic were published (Puzon-McLaughlin, W., and Takada, Y. (1996) J. Biol. Chem. 271, 20438–20443; Tozer, E. C., Liddleston, R. C., Sutcliffe, M. J., Smeeton, A. H., and Loftus, J. C. (1996) J. Biol. Chem. 271, 21978–21984; Goodman, T. G., and Bajt, M. L. (1996) J. Biol. Chem. 271, 23729–23736). In each report, similar mutations were made in other integrins, yielding similar data. It should be noted, however, that the interpretation of the data are somewhat different. Based on the data presented here, we are reluctant to classify the amino-terminal regions of β3 and β5 as "I domains." As discussed above, we believe there is sufficient reason to suspect that the β3 and β5 subunits bind to metal using a MIDAS-like motif but that the backbone of the domain containing the MIDAS is structurally distinct from the known conformation of the I domains.

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