Role of ADMIDAS Cation-binding Site in Ligand Recognition by Integrin $\alpha_5\beta_1$*

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Integrin-ligand interactions are regulated in a complex manner by divalent cations, and multiple cation-binding sites are found in both $\alpha$ and $\beta$ integrin subunits. A key cation-binding site that lies in the $\beta$ subunit A-domain is known as the metal-ion dependent adhesion site (MIDAS). Recent x-ray crystal structures of integrin $\alpha_5\beta_1$ have identified a novel cation binding site in this domain, known as the ADMIDAS (adjacent to MIDAS). The role of this novel site in ligand recognition has yet to be elucidated. Using the interaction between the underlying hybrid domain, known as the ADMIDAS (adjacent to MIDAS).

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Integrins constitute a large family of $\alpha/\beta$ heterodimeric transmembrane receptors found in all metazoa (1). Cell-matrix and cell-cell interactions mediated by integrins are central to many fundamental biological processes such as embryonic morphogenesis, leukocyte trafficking, and platelet aggregation. Integrins can exist in either active (ligand competent) or inactive states is regulated intracellularly by the binding of cytoskeletal and signaling molecules (2, 3). Integrin-ligand interactions also require divalent cations and are regulated in a complex manner by changes in the concentrations of these ions. The effects of activation can be mimicked in vitro by cations such as Mn$^{2+}$ or Mg$^{2+}$, whereas Ca$^{2+}$ typically favors the inactive state. The different effects of these cations are related to their differential abilities to induce the integrin to undergo the shape changes involved in activation (4–6).

The molecular basis of integrin function has been greatly elucidated by x-ray crystal structures of the extracellular domains of $\alpha_5\beta_1$ in the unliganded and liganded states (7, 8). The overall structure of the heterodimer is that of a "head" on two "legs." The head region (where ligand binding takes place) comprises a seven-bladed $\beta$-propeller in the $\alpha$ subunit and a von Willebrand factor A-type domain in the $\beta$ subunit ($\betaA^1$; also referred to as "I-like domain"), an $\alpha$-$\beta$-fold, which is inserted by short N- and C-terminal linkers into a "hybrid" domain. The hybrid domain is a $\beta$-sandwich fold made up of the ~60 amino acid residues preceding and the ~90 residues following $\betaA$. Both tertiary and quaternary structural changes are observed upon the binding of a ligand mimetic peptide containing the RGD recognition sequence to the preformed integrin crystal (8), however, a pivotal conformational change appears to be an inwards movement of the $\alpha 1$ helix of $\betaA$. This shift of the $\alpha 1$ helix appears to be necessary for activation (6). We have also shown that $\alpha 1$ helix motion is linked to a movement in the $\alpha 7$ helix region and a swing of the hybrid domain away from the $\alpha$ subunit (9). These latter conformational changes were not observed in the liganded $\alpha_5\beta_1$ crystal structure (8), probably because of restraints imposed by lattice contacts (10, 11).

Six cation binding sites were found in the unliganded and eight in the liganded $\alpha_5\beta_1$ structures (7, 8). Four sites are present on the lower face of the $\alpha$ subunit $\beta$-propeller. Although originally thought to be EF-hand-like, these sites are now known to form $\beta$-hairpin loops (7, 12). All four hairpin loops are linked in a chain-like arrangement and the $\beta$-hairpin loop in blade 7 is probably important for stabilizing interactions with the underlying "thigh" domain. A fifth cation binding site is observed at the junction between the $\alpha$ subunit "thigh" and "calf" domains. Three sites are present on the top face of $\betaA$. The first, known as MIDAS (metal ion-dependent adhesion site), plays a central role in ligand recognition. One of the carboxylate oxygens of the aspartic acid side chain of RGD coordinates directly to a metal ion bound at the MIDAS, thus

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1 The abbreviations used are: $\betaA$, $\beta$ subunit von Willebrand factor A-domain; $\alphaA$, $\alpha$ subunit von Willebrand factor A-domain; MIDAS, metal-ion dependent adhesion site; ADMIDAS, adjacent to MIDAS; LIMBS, ligand-associated metal-binding site; mAb, monoclonal antibody; tro561-Fc, recombinant soluble integrin heterodimer containing C-terminal truncated $\alpha$, and $\beta$ subunits (residues 1–613 and $\beta$ residues 1–455) fused to the $\alpha$ region of human IgG1; $\alphaB$-Fc, recombinant soluble integrin heterodimer containing full-length extracellular domains of $\alpha$, and $\beta$ subunits (residues $\alpha$ residues 1–951 and $\beta$ residues 1–708); CHO, Chinese hamster ovary.

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explaining the absolute dependence of ligand binding on divalent cations (8). Occupancy of the MIDAS also induces conforma-
tional changes associated with activation of this domain. The second site lies adjacent to the MIDAS and is therefore termed ADMIDAS. The third is termed LIMBS (ligand-associated metal-binding site). The MIDAS and LIMBS sites were occupied only in the liganded structure (8). Residues involved in the cation coordination of the MIDAS and ADMIDAS sites are shown in Table I.

We have previously used the interaction between integrin αβ1 and fibronectin as a model system to identify and charac-
terize cation-binding sites that can support or modulate ligand recognition (13). Our results showed that several classes of cation-binding sites could be identified. Occupancy of the first class by Mg2+ or Mn2+ was required for ligand binding (ligand-
competent sites). The second class was implicated in the allo-
tropic inhibition of Mn2+-supported ligand binding by Ca2+ (inhibitory sites), whereas the third class was involved in the stimulation of Mg2+-supported ligand binding by Ca2+ (stimulatory sites). Recently we have shown that the ligand competent site for both Mg2+ and Mn2+ is the MIDAS of the β1 domain (4). The identity of the two classes of Ca2+-binding regulatory sites is currently unclear. In addition, how occupancy of these sites affects conformational movements has not yet been investigated.

Here we have examined the role of the ADMIDAS site in ligand binding by αβ1. Our findings provide evidence that the ADMIDAS is a member of the class of Ca2+-binding inhibitory sites and, while not essential for ligand binding, the ADMIDAS may have a role in stabilizing the active conformation through an effect on the α1 helix of βA. The ADMIDAS is also important for the transduction of cation-induced conformational changes from βA to the underlying hybrid domain.

**EXPERIMENTAL PROCEDURES**

**Monoclonal Antibodies and Proteins**—Rat mAbs 16 and 13 recognizing the human α- and β1 subunits, respectively, were gifts from Dr. K. Yamada (NIDCR, National Institutes of Health). Mouse anti-human α- and β1 mAbs 1D16 was a gift from Dr. E. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA). Mouse anti-human α- and mouse anti-human β1 mAbs 12G10 and 8E3 were purified as described (14, 4). A Fab fragment of 12G10 was prepared as previously described (9). Mouse anti-human β1 mAb 15/7 was a gift from P. Stephens, M. Robinson, and H. Kirby, Celltech Chiroscience, UK.

**Expression Vector Construction and Mutagenesis**—C-terminal truncated human α- and β1 constructs encoding α- and β1 residues 1–465 fused to the hinge regions and C12 and C13 domains of human IgG1 (a5(1–613)-Fc and β1(1–455)-Fc) were generated as previously described (16). C-terminal truncated constructs containing the full-length extracellular domains (a5(1–951)-Fc and β1(1–708) Fc) were produced as before (16). To aid the formation of heterodimers, the C13 domain of the α- construct contained a “knob” mutation, whereas the C13 domain of the β1 constructs carried a “knob” mutation as described (16, 17). The mutations in the β1 subunit were carried out using oligonucleotide-directed PCR mutagenesis, as described (9). Oli-
gonucleotides were synthesized by MWG Biotech (Southhampton, UK). The presence of the mutations (and the lack of any other changes to the wild-type sequence) was verified by DNA sequencing.

**Transfection**—Chinese hamster ovary cell L7611 variants (16) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% non-essential amino acids (growth medium). Cells were detached using 0.05% (w/v) trypsin, 0.02% (w/v) EDTA in phosphate-buffered saline, and plated overnight into 6-well culture plates (Costar). Approximately 1 μg of wild-type or mutant β1(1–455)-Fc with 1 μg of wild-type a5(1–613)-Fc DNA, or 1 μg of wild-type or mutant β1(1–708)-Fc with 1 μg of wild-type a5(1–951)-Fc DNA were used in transfection of the cells using LipofectAMINE PLUS reagent (Invitrogen, Paisley, Scotland) according to the manufacturer’s instructions. After 4 days, supernatants were harvested by centrifugation at 1000 × g for 5 min.

For comparison of purified wild-type heterodimers with het-
erodimers containing the ADMIDAS or LIMBS mutations in β1, 75-cm2 flasks of subconfluent CHOL7611 cells were transfected with 5 μg of wild-type or mutant β1 and hybrid proteins, and 5 μg of wild-type α- construct as described above. After 4 days, culture supernatants were harvested by centrifugation at 1000 × g for 5 min. Wild-type or mutant heterodimers were purified using Protein A-Sepharose essentially as described before (16). Concentration measurements of wild-type or mutant heterodimers were performed using a purified αβ- Fc standard of known concentra-
tion (a gift from P. Stephens, M. Robinson, and H. Kirby, Celltech Chiroscience, UK).

**Effect of ADMIDAS Mutations on 3Fn6–10 Binding**—Solid phase ligand binding assays using eitherFc captured or directly coated inte-
grin were performed essentially as previously described (6, 9, 13, 16). In these assays 3Fn6–10 coupled to sulfo-NHS LC biotin was used at 0.1 μg/ml, unless stated otherwise. Measurements obtained were the mean ± S.D. of four replicate wells.

**Surface Plasmon Resonance**—Experiments were performed using the BIAcore 3000 (Biacore AB). Running buffer was 150 mM NaCl, 25 mM Tris-Cl, 1 mM MnCl2, pH 7.4. 3Fn6–10 coupled to biotin maleimide (Sigma) was bound to the surface of a streptavidin-coated chip (Biacore AB). Dilutions of wild-type or mutant β1-Fc or αβ-Fc with the D137A or D138A mutations in the running buffer were injected at 10 μl/min 25 °C, into flow cells containing approximately 300 response units of 3Fn6–10 fragment. The same buffer containing 5 mM EDTA in place of MnCl2 was used to regenerate the surface after each injection. No binding was observed when samples were injected in the presence of EDTA. All measurements were baseline-corrected by subtracting the sensorgram obtained with that from a control flow cell coated with streptavidin alone. Kinetic parameters were determined by fitting the data to a 1:1 Langmuir binding model using BIAevaluation software version 3.1.

**Effect of Divalent Cations on 15/7 Binding**—96-well plates (Costar ½-area EIA/RIA, Corning Science Products, High Wycombe, UK) were coated with goat anti-human γ1 Fc (Jackson Immunochemicals, Strat-
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![Image](71x278 to 292x737)

**FIG. 1.** Effect of β1 subunit D137A and D138A ADMIDAS mutations on binding of the 3Fn6–10 fibronectin fragment to αβ1-Fc. A, Fc-captured integrin; B, directly adsorbed integrin. Binding of 3Fn6–10 was measured in 1 mM Mn²⁺ in the absence of mAbs (open bars) or presence of the activating mAbs TS2/16 (cross-hatched bars) or 12G10 (diagonally hatched bars). The mutation E140A was used as a control. The non-function perturbing mAb K20 had no effect on 3Fn6–10 binding (data not shown).

![Image](326x526 to 553x737)

**FIG. 2.** Effect of ADMIDAS mutations on binding of 3Fn6–10 fibronectin fragment to αβ1-Fc. Binding of 3Fn6–10 to wild-type integrin or D137A or D138A mutants was measured in the absence of mAbs (open bars) or presence of the activating mAbs TS2/16 (cross-hatched bars) or 12G10 (diagonally hatched bars). The non-function perturbing mAb K20 had no effect on 3Fn6–10 binding (data not shown). Assay was performed using Fc-captured integrin.

for 30 min at room temperature (50 μl/well). Wells were then washed four times with buffer A, and color was developed using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (50 μl/well). Absorption at 405 nm was measured using a plate reader (Dynex Technologies). Background binding to mAbs to wells incubated with supernatant from mock-transfected cells was subtracted from all measurements. Measurements obtained were the mean ± S.D. of four replicate wells.

**Comparison of Epitope Expression by Wild-type and Mutant Heterodimers**—Plates were coated with anti-human Fc and blocked as described above. The blocking solution was removed and cell culture supernatants were added (25 μl/well) for 1–2 h. All supernatants were assayed in triplicate, and supernatant from mock-transfected cells was used as a negative control. The plate was washed 3 times with buffer A with 1 mM NaN₃ (buffer B) (200 μl/well), and anti-i5 or anti-iβ1 mAbs (5 μg/ml) were added (50 μl/well). The plate was incubated for 2 h and then washed 3 times in buffer B. Peroxidase-conjugated anti-rat or anti-mouse secondary antibodies (1:1000 dilution in buffer B; Jackson Immunochemicals) were added (50 μl/well) for 30 min, the plate was washed four times in buffer B, and color was developed as described above. All steps were performed at room temperature. Results shown are representative of three separate experiments.

In each assay involving a comparison between different heterodimers the binding of mAb 8E3 (5 μg/ml) was used to normalize for any differences between the amounts of the different heterodimers bound to the wells. For example, normalized absorbance of wells coated with mock supernatant, 

\[ \text{Absorbance of wells coated with wild-type integrin} \]

was calculated as:

\[ \frac{\text{Absorbance of wells coated with wild-type integrin} - \text{Absorbance of wells coated with wild-type integrin}}{\text{Absorbance of wells coated with wild-type integrin}} \]

**RESULTS**

**ADIMIDAS Mutants Have Low Constitutive Activity and Show Reduced Expression of Activation Epitopes**—The ADMIDAS site contains residues Ser¹⁴, Asp¹³, and Ala¹⁴¹ (or Asp²⁰⁵) (see Table 1). The side chains of both Asp¹³ and Asp²⁰⁵ contribute two carboxylic oxygens to cation coordination, hence mutation of either residue would be expected to
CHO L761h cells were transfected with α5(1-613)-Fc and wild-type or mutant β1(1-455)-Fc. Cell culture supernatants were analyzed for reactivity with anti-α5- and anti-β1-mAbs (used at 5 μg/ml) by sandwich enzyme linked immunosorbent assay. The assay was performed in the presence of 1 mM Mn²⁺. The E140A mutant was used as a control. Results shown are from one experiment, representative of at least three separate experiments.

### Table II

| mAb | Epitope | β1 Mutant |
|-----|---------|-----------|
|     |         | D137A     | D138A     | E140A     |
| α5 subunit | β-Propeller |           |           |           |
| PID6 | W3       | ++        | +++       | +++       |
| 16   | W2/W3    | ++        | +++       | +++       |
| SNACA52 | W2      | ++        | +++       | +++       |

| β1 subunit | A domain |       |       |
| 1            | 207-218  | +   | +     |
| TS2/16       | 207-218  | +++ | +++   |
| 4B4          | 207-218  | +++ | +++   |
| P4C10        | 207-218  | +++ | +++   |
| 12G10        | 207-218, 154/155 | ++⁺ | +    |

| Hybrid domain | 82-87 |       |       |
| JB1A         | 370/371/417 | +   | +     |
| 15/7         | 370/371/417 | +⁻ | +⁻    |
| HUTS-4       |         |      | +     |

| PSI domain   | 14-55  |       |       |
|             |       | +++   | +++   |

* +++, reactivity 80-100% of wild-type integrin.
* +, reactivity 50-80% of wild-type integrin.
* +, reactivity 20-50% of wild-type integrin.
* +/−, reactivity <20% of wild-type integrin.

The other ADMIDAS residues contribute only a backbone carbonyl (except in the case of Asp157, which also participates in cation coordination at the MIDAS site). Hence to selectively test the role of the ADMIDAS site we made mutants D137A and D138A. For these studies we employed a recently described system for expression of recombinant soluble α5β1 (16). We mainly used a truncated version of α5β1, α5(1–613) β1(1–455), fused to the Fc region of human IgG1 (hereafter referred to as tro5β1-Fc). This heterodimer contains the α subunit β-propeller and thigh domain with the β subunit A, PSI, and hybrid domains (7), and retains the ligand-binding properties of the full-length receptor (16). The advantages of this system have been described previously (6, 9).

The ligand binding activity of the wild-type or mutant integrins was tested after either capture of the integrin onto a 96-well plate coated with anti-human Fc polyclonal antibody, or by directly coating the purified integrin onto the plate (16). Wild-type tro5β1-Fc had low activity when Fc captured but had high activity after direct adsorption (Fig. 1, A and B); the activating mAb 12G10 (15) restored the activity of Fc-captured integrin but did not further increase the activity of directly coated receptor. In contrast, both ADMIDAS mutants had very low activity after either Fc capture or direct adsorption. 12G10 only partially rescued the activity of either Fc-captured or directly adsorbed D137A and D138A mutants (in the case of directly adsorbed integrin to ~15 and ~70% of wild-type levels, respectively).

The D137A and D138A mutations were also made in a construct containing the full-length extracellular domains of α5 and β1 (hereafter referred to as α5β1-Fc). The mutations had little or no effect on the expression of α and β subunit epitopes, apart from low expression of the 15/7 activation epitope in the D138A mutant (data not shown). After Fc capture, the wild-type α5β1-Fc had constitutively high ligand binding activity but the mutants displayed little or no activity (Fig. 2). Activating mAbs TS2/16 and 12G10 had little effect on the ligand binding activity of the wild-type receptor but partially (D137A) or completely (D138A) restored the activity of the ADMIDAS mutants (Fig. 2).

These results show that under conditions where the wild-type receptor is fully active (either constitutively or after mAb-or coating-induced activation), the ADMIDAS mutants have low activity. Because activity can be rescued (at least in part) by activating mAbs, the ADMIDAS mutants are not defective in ligand binding but instead the mutations appear to inhibit ligand binding by inactivating the receptor (i.e. shifting the equilibrium toward the inactive state). Consistent with this interpretation, the D137A and D138A tro5β1-Fc mutants showed decreased expression of activation epitopes, such as that recognized by mAb 12G10 (Table II). Interestingly, 154/155 and Asp157 and Asp158 lie at the top of the α helix of βA and we have previously shown that a movement of this helix is important for activation (6). The 12G10 epitope lies partly in the α helix and changes in expression of this epitope parallel to a shift in α (6). The D137A and D138A tro5β1-Fc mutants also showed a dramatic reduction in the binding of the 15/7 and HUTS-4 mAbs. The low expression of 12G10, 15/7, and HUTS-4 activation epitopes appears to correlate with the low ligand binding activity of the tro5β1-Fc D137A and D138A mutants. (This correlation was only approximate, however, because the D138A mutant had lower expression of these epitopes but higher ligand binding activity than the D137A mutant.)

The ADMIDAS Mutations Increase the Dissociation Rate of Fibronectin from α5β1—To gain further insight into why the ADMIDAS mutants were defective in ligand binding we used surface plasmon resonance to examine the kinetics of the interaction of 3Fn6–10 with wild-type and mutant α5β1-Fc (Fig. 3, A–C). The data showed that the ADMIDAS mutants had similar association rate constants but much faster dissociation rate constants than the wild-type receptor (Table III). These results show that whereas ligand binds to the ADMIDAS mutants at a similar rate to the wild-type integrin, the complex rapidly dissociates after binding takes place. (These rapid off-rates may preclude detection of ligand binding to the mutants...
in solid phase assays, where there is a considerable time delay between removing unbound ligand and detecting bound ligand.) Hence, these findings support the suggestion that the ADMIDAS site is important for the stabilization of the active (ligand-competent) state of the integrin. Furthermore, the rapid dissociation rate observed for the D138A mutant was decreased approximately 10-fold in the presence of a Fab fragment of 12G10 (Fig. 3D), demonstrating that the effect of the mutation could be overcome by stabilizing the active conformation.

ADMIDAS Mutants Have a Similar Divalent Cation Dependence of Ligand Binding to Wild-type Integrin—Fibronectin binding to \( \alpha_\beta_1 \) is supported by Mn\(^{2+} \) and Mg\(^{2+} \) but only very weakly by Ca\(^{2+} \) (13). We compared the effect of these ions on binding of the 3Fn6–10 fragment to wild-type \( \alpha_\beta_1 \)-Fc and the D138A mutant in solid phase assays. In these, and all subsequent ligand-binding assays shown, we used Fc-captured integrin activated by 12G10, to measure ligand binding under similar conditions for wild-type and mutant receptors. The results (Fig. 4, A and B) showed that ligand binding to the D138A mutant was modulated by divalent cations in a very similar manner to the wild-type integrin. The only significant difference between the mutant and wild type was that Mg\(^{2+} \) ions showed a reduced ability to support ligand binding to the mutant relative to Mn\(^{2+} \). No ligand binding was observed in the absence of cations. Results obtained for the D137A mutant were analogous to those for D138A (data not shown).

| Receptor              | \( k_{on} \) \( 10^6 \text{ M}^{-1} \text{s}^{-1} \) | \( k_{off} \) \( 10^{-1} \text{ s}^{-1} \) | \( K_D \) nM |
|-----------------------|-----------------------------|-----------------------------|---------|
| Wild-type             | 2.39 ± 0.101                | 0.31 ± 0.065                | 1.3     |
| D137A mutant          | 2.41 ± 0.69                | 2.12 ± 0.40                | 8.8     |
| D138A mutant          | 3.38 ± 1.34                | 1.12 ± 0.14                | 3.3     |

The ADMIDAS lies very close to the MIDAS and therefore mutations in the ADMIDAS could influence cation binding to the MIDAS. The affinity of the MIDAS for a particular divalent cation is reflected by the concentration of the ion that supports half-maximal ligand binding (18) (provided that the ligand concentration is low, Ref. 19). This assumption is valid because the MIDAS cation is essential for ligand binding and therefore ligand binding is directly proportional to the fraction of integrin occupied by cation. We therefore tested whether the D138A mutation affected the concentrations of Mn\(^{2+} \) or Mg\(^{2+} \) required for half-maximal ligand binding. The EC\(_{50} \) values for

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**Fig. 3.** Effect of ADMIDAS mutations on the kinetics of \( \alpha_\beta_1 \)-Fc binding to the 3Fn6–10 fibronectin fragment. Biotinylated 3Fn6–10 (~300 response units) was bound to a streptavidin-coated chip and wild-type \( \alpha_\beta_1 \)-Fc (A), D137A mutant (B), or D138A mutant (C) in a running buffer containing 1 mM Mn\(^{2+} \) was injected over the surface for 500 s and the dissociation phase was followed for 600 s. Traces show increasing concentrations (in nanomolar) of \( \alpha_\beta_1 \)-Fc analyte. D, D138A mutant (17 nm) was injected over the 3Fn6–10-coated chip after preincubation with the Fab fragment of mAb 12G10 (100 nM, thick trace) or with running buffer alone (thin trace). The dissociation rate constant \( (k_{off}) \) in the presence of 12G10 is \( \sim 1 \times 10^{-4} \text{ s}^{-1} \) compared with \( \sim 1 \times 10^{-2} \text{ s}^{-1} \) in the absence of 12G10. No integrin binding was observed in the presence of 3 mM EDTA (+EDTA).
Mn$^{2+}$ were 29 ± 4 and 25 ± 2 μM for the wild-type receptor and D138A mutant, respectively. The EC$_{50}$ values for Mg$^{2+}$ were 2.1 ± 0.4 and 2.3 ± 0.3 mM, respectively. Hence, these results showed that the D138A mutation had no significant effect on the apparent affinity of Mn$^{2+}$ or Mg$^{2+}$ binding to the MIDAS. Furthermore, the functional consequences of the ADMIDAS mutations appear not to be because of any affect on the MIDAS.

**ADMIDAS Mutants Show Reduced Allosteric Inhibition of Mn$^{2+}$-supported Ligand Binding by Ca$^{2+}$**—The crystal structure of α6β2 in the unliganded state showed that the ADMIDAS site could be occupied by Ca$^{2+}$. We have previously shown that one of the effects of Ca$^{2+}$ ions on α6β2-fibronectin interactions is an allosteric inhibition of ligand binding supported by Mn$^{2+}$ (13). We therefore tested whether the D138A mutation affected the ability of Ca$^{2+}$ ions to inhibit binding of the 3Fn6–10 fragment in the presence of a constant concentration of Mn$^{2+}$ (100 μM). The results (Fig. 5) showed that the D138A mutation reduced the ability of Ca$^{2+}$ to inhibit ligand binding. Similar results were obtained with the D137A mutant, whereas the E140A mutation had no effect (data not shown). The inhibition of Mn$^{2+}$-supported ligand binding by Ca$^{2+}$ had a multiphasic pattern suggesting that this inhibition was mediated by Ca$^{2+}$ binding to at least three separate sites of differing affinities (high, medium, and low). Comparison of the pattern of inhibition for the D138A mutant with that of the wild-type receptor showed that the effect of the medium affinity site was lost. Making the assumption that the concentration of Ca$^{2+}$ ions on the affinity site was lost. Mathematical modeling of the inhibition (not shown) indicated that this site has an apparent K$_D$ of 160 ± 30 μM (n = 4). Taken together, these findings show that the ADMIDAS is a selective, medium affinity Ca$^{2+}$-binding site that contributes to the allosteric inhibition of Mn$^{2+}$-supported ligand binding.

**ADMIDAS Mutants Show No Change in the Ability of Ca$^{2+}$ to Modulate Mg$^{2+}$-supported Ligand Binding**—A second effect of Ca$^{2+}$ ions on α6β2-fibronectin interactions is modulation of Mg$^{2+}$-supported ligand binding. Ca$^{2+}$ ions can either stimulate or inhibit Mg$^{2+}$-supported ligand binding, dependent upon the Ca$^{2+}$ concentration (13). Low Ca$^{2+}$ concentrations markedly increase the affinity of Mg$^{2+}$ for the MIDAS site (and thereby stimulate ligand binding at low Mg$^{2+}$ concentrations) but at high Ca$^{2+}$ concentrations Ca$^{2+}$ ions compete directly with Mg$^{2+}$ for binding to the MIDAS and thereby inhibit ligand binding. The D138A mutation had little or no effect on the modulation of Mg$^{2+}$-supported ligand binding by Ca$^{2+}$ (Fig. 6). Similar results were obtained for the D137A mutant (not shown). These findings suggest that the ADMIDAS site is not a stimulatory Ca$^{2+}$-binding site.

**ADMIDAS Mutations Block Transduction of a Conformational Change to the Hybrid Domain—Asp137 and Asp138 lie in the α1 helix. We have previously shown that α1 helix movement is linked to a movement in the α7 helix region, and thereby to an outward swing of the hybrid domain, which exposes the 15/7 and HTUS-4 epitopes (9). Mn$^{2+}$ or Mg$^{2+}$ binding to the MIDAS is able to promote this conformational change (9). We therefore tested whether the ADMIDAS mutations affected induction of the 15/7 epitope by these cations (Fig. 7). The results showed that the D137A and D138A mutants had lower levels of 15/7 binding compared with the wild-type tr

![Image](92x325 to 272x737)

**FIG. 4.** Effect of divalent cations on the binding of 3Fn6–10 fibronectin fragment to wild-type trα5β1-Fc (A) and trα5β1-Fc with the D138A ADMIDAS mutation (B). Binding of 3Fn6–10 was measured in the presence of varying concentrations of Mn$^{2+}$ (●), Mg$^{2+}$ (■), or Ca$^{2+}$ (▲). Assay was performed using Fc-captured integrin in the presence of mAb 12G10.

**FIG. 5.** Effect of D138A ADMIDAS mutation on the inhibition of Mn$^{2+}$-supported ligand binding by Ca$^{2+}$—Binding of 3Fn6–10 to wild-type trα5β1-Fc (●) or trα5β1-Fc with the D138A mutation (■) was measured in the presence of a constant concentration of Mn$^{2+}$ (100 μM) and varying concentrations of Ca$^{2+}$. For ease of comparison, data for the D138A mutant were normalized to the same level of ligand binding as the wild-type integrin in the absence of Ca$^{2+}$. The level of ligand binding supported by 4 mM Ca$^{2+}$ alone is shown by the open symbols (○), wild type; (□) D138A mutant. Assay was performed using Fc-captured integrin in the presence of mAb 12G10.
type integrin in the absence of divalent ions and Mn\(^{2+}\) or Mg\(^{2+}\) failed to increase 15/7 binding. The control mutation E140A had no effect on the induction of 15/7 binding by these ions (data not shown). Similar results were obtained with the HUTS-4 mAb (data not shown). Hence these findings suggest that the ADMIDAS mutations block the pathway of conformational change from the MIDAS to the hybrid domain.

**DISCUSSION**

Three main classes of cation-binding sites that affect integrin function have been deduced from studies on \(\alpha_5\beta_1\), \(\alpha_1\beta_2\), and \(\alpha_6\beta_4\) (13, 18, 20, 21). These are ligand-competent, stimulatory (or effector), and inhibitory sites. The major ligand-competent site has recently been shown to be the MIDAS of \(\beta A\) (6, 8). Using the prototypical interaction between \(\alpha_5\beta_1\) and fibrinectin, we now report (a) that the ADMIDAS site does not contribute directly to ligand binding but is important for stabilizing the active conformation of the integrin, (b) that the ADMIDAS is a Ca\(^{2+}\)-binding site involved in the allosteric inhibition of Mn\(^{2+}\)-supported ligand binding, and (c) the ADMIDAS is involved in transduction of a conformational change from the MIDAS through the C-terminal helix region of the \(\beta A\) domain to the underlying hybrid domain, implying an important role in receptor signaling.

In allocating functions to the various cation-binding sites in integrins, a central issue is which sites need to be occupied for ligand binding to occur; i.e. the identity of the ligand-competent sites. Both the MIDAS and LIMBS appear to fall into this category (8). The ADMIDAS, however, belongs to the category of Ca\(^{2+}\)-binding inhibitory sites. A surprising feature of the inhibition of ligand binding to \(\alpha_5\beta_1\) by Ca\(^{2+}\) is that Ca\(^{2+}\) acts as a competitive inhibitor of ligand binding supported by Mg\(^{2+}\) but not of ligand binding supported by Mn\(^{2+}\). This previously led us to suggest that there are separate ligand-competent sites for Mg\(^{2+}\) and Mn\(^{2+}\) (13). However, it is now clear that the major ligand-competent site for both Mg\(^{2+}\) and Mn\(^{2+}\) is the MIDAS (6). Why Ca\(^{2+}\) is able to displace Mg\(^{2+}\) but not Mn\(^{2+}\) from the MIDAS will be the subject of future investigations.

Whereas the ligand-competent sites typically bind Mn\(^{2+}\) or Mg\(^{2+}\), both the stimulatory and inhibitory sites appear to bind Ca\(^{2+}\) selectively (13, 20, 21). The \(\alpha_5\beta_4\) crystal structures showed that the ADMIDAS can be occupied by either Ca\(^{2+}\) or Mn\(^{2+}\). However, no insight into the specificity of this site should be inferred from these studies because the crystals were prepared in the presence of a high concentration of a single cation (5 mM Ca\(^{2+}\) or Mn\(^{2+}\)). The studies presented here show that the ADMIDAS preferentially binds Ca\(^{2+}\); this feature may be because of its high content of coordinating groups from negatively charged residues (22). In addition to the ADMIDAS, other sites appear to contribute to the inhibition of Mn\(^{2+}\)-supported ligand binding by Ca\(^{2+}\); these could include sites on \(\alpha\) subunit \(\beta\)-propeller.\(^2\) The LIMBS would appear to be a good candidate for a stimulatory Ca\(^{2+}\)-binding site because of its close proximity to the MIDAS. However, it is difficult to test this suggestion experimentally because LIMBS mutations block ligand binding to \(\alpha_5\beta_1\), and therefore it is not possible to check if the synergistic effects of low concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) are abrogated by LIMBS mutations.\(^3\)

A second major issue concerns the contribution of cation-
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The cation-binding sites regulate ligand binding mainly through effects on conformation of the head region. Hence, for example, the activating property of Mn$^{2+}$ on integrin function appears to be primarily through its effect on the βA domain rather than on straightening. This activating effect of Mn$^{2+}$, and of mAbs such as TS2/16, appears to be due mainly to a decrease in the dissociation rate (28–30).

In summary, we have established previously unidentified roles for the ADMIDAS site in regulation of ligand binding to αβ2. These findings also support our recently proposed model of activation of the head region involving movements of the α1 and α7 helices of βA (9). Control of this shape-shifting pathway may explain, in part, why the ADMIDAS site is absolutely conserved throughout all integrin β subunits sequenced to date (31, 32).

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The adaptation mechanism of integrins has recently been proposed to involve a switchblade-like straightening from a highly bent to an extended form (26, 27). Mn$^{2+}$ but not Ca$^{2+}$ is able to mediate this conformational rearrangement (27). However, here we have recapitulated the effects of divergent cations on the native receptor (with full-length extracellular domains) (13) using the truncated integrin (which lacks most of the ligand regions). Therefore, although cation occupancy and receptor bending/straightening may be linked, the effects of the different cations on activation and ligand binding are not dependent on unbending of the receptor. Instead, these data suggest that