Integrins \(\alpha_6\beta_1\) and \(\alpha_\beta_2\) are involved in localization of leukocytes at mucosal sites. Although both \(\alpha_6\beta_1\) and \(\alpha_\beta_2\) utilize the \(\beta_2\) chain, they have distinct binding specificities for E-cadherin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), respectively. We found that mutation of the metal ion-dependent adhesion site (MIDAS) in the \(\alpha_6\) A-domain (D190A) abolished E-cadherin binding, as did mutation F298A on the A-domain surface near the MIDAS cleft. A docking model of the A-domain with E-cadherin domain 1 indicates that coordination of the \(\alpha_6\) MIDAS metal ion by E-cadherin Glu\(^{31}\) and a novel projection of Phe\(^{298}\) into a hydrophobic pocket on E-cadherin provide the basis for the interaction. The location of the binding site on the \(\alpha_6\) A-domain resembles that on other integrins, but its structure appears distinctive and particularly adapted to recognize the tip of E-cadherin, a unique integrin ligand. Additionally, mutation of the \(\beta_2\) MIDAS motif (D140A) abolished \(\alpha_6\beta_1\) binding to E-cadherin and \(\alpha_\beta_2\)-mediated adhesion to MAdCAM-1, and \(\alpha_\beta_2\) chain mutations that abrogated binding of \(\alpha_\beta_2\)\(\beta_3\) to vascular cell adhesion molecule-1 and fibronectin similarly reduced \(\alpha_\beta_2\) interaction with MAdCAM-1. Thus, although specificity can be determined by the integrin \(\alpha\) or \(\beta\) chain, common structural features of both subunits are required for recognition of dissimilar ligands.

Integrins are heterodimeric glycoproteins consisting of non-covalently associated \(\alpha\) and \(\beta\) subunits that play diverse roles in cell-cell and cell-matrix interactions. Integrins of the \(\beta_1\), \(\beta_2\), and \(\beta_\text{sub}\) subfamilies are critical for leukocyte homing (1, 2). For example, binding of integrin \(\alpha\beta_7\) to mucosal addressin cell adhesion molecule-1 (MAdCAM-1)\(^1\) on intestinal endothelial cells is required for the homing of naive lymphocytes to Peyers patches (2). Integrins are also thought to play a role in micro-environmental homing or retention of lymphocytes at particular sites within a tissue (2). For instance, integrin \(\alpha_6\beta_1\) binds to epithelial cadherin (E-cadherin) and is involved in retention of lymphocytes within the epithelium (3–6).

Many integrin ligands are members of the immunoglobulin superfamily (such as VCAM-1 and MAdCAM-1) or possess domains that resemble the immunoglobulin fold (e.g. E-cadherin and the type III repeats of fibronectin). A unifying feature of these ligands is an integrin-binding surface containing an exposed acidic amino acid. For example, \(\alpha_6\beta_1\) and \(\alpha_\beta_2\) bind to VCAM-1 and MAdCAM-1, respectively, on the face of domain 1 formed by the C, F, and G \(\beta\)-strands, centered on an aspartate residue in the loop connecting the C and D strands (7–9).

Domain 1 of E-cadherin contains a glutamate residue at the tip of the BC-loop essential for \(\alpha_6\beta_1\) binding (10, 11), and the tenth type III repeat of fibronectin has an acidic residue within an RGD sequence on the FG-loop required for \(\alpha_6\beta_1\) binding (12). Other integrin ligands such as collagen I and ICsb that are not known to adopt immunoglobulin-like folds also possess vital acidic residues for \(\alpha_6\beta_1\) and MAdCAM\(_2\) binding, respectively (13, 14).

Approximately one-third of known \(\alpha\) chains (\(\alpha_1\), \(\alpha_2\), \(\alpha_6\), \(\alpha_7\), \(\alpha_{11}\), \(\alpha_{14}\), \(\alpha_{16}\), \(\alpha_M\), and \(\alpha_S\)) contain an inserted domain of \(\sim200\) amino acids related to the A-domains of von Willebrand factor. For those integrins tested, the A-domain is critically involved in ligand binding. For instance, isolated recombinant \(\alpha_2\), \(\alpha_{14}\), \(\alpha_M\), and \(\alpha_S\) A-domains share ligand binding properties with their parent integrins (15–18). The crystal structures of a number of integrin A-domains (\(\alpha_2\), \(\alpha_6\), \(\alpha_7\), \(\alpha_{11}\), \(\alpha_{14}\), \(\alpha_M\), and \(\alpha_S\)) reveal a common “Rossmann fold” in which a six-stranded parallel \(\beta\)-sheet is flanked by seven \(\alpha\)-helices (19–22). Five conserved amino acids (DXXXS, T, and D) come together with two or three water molecules at the top of the domain to chelate a metal ion in what has been termed the metal ion-dependent adhesion site (MIDAS). Mutation of these residues abolishes metal binding to the \(\alpha_M\) A-domain (23) and severely curtails or eliminates ligand binding activity for all integrins tested (23–28). In one crystal form of the \(\alpha_M\) A-domain, a glutamate residue from a neighboring A-domain penetrates the MIDAS cleft and provides a sixth coordination site for the metal ion (19, 29). This “open” conformation of the A-domain is proposed to reflect the active ligand-bound conformation. Because most integrin ligands possess a critical exposed acidic residue, bona fide ligand recognition may use a similar mechanism (19). Crystal structures in which no ligand mimetic is bound in the MIDAS cleft have a “closed” conformation that likely reflects the inactive form of the A-domain (29, 30). Comparison of the unbound \(\alpha_2\) A-domain with the recently crystallized complex of this A-domain bound to a collagenous triple peptide confirms the model (31). This complex and mutational analyses of the \(\alpha_2\), \(\alpha_{14}\), and

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‡ To whom correspondence should be addressed: Brigham and Women's Hospital, Smith Bldg., Rm. 538D, One Jimmy Fund Way, Boston, MA 02115. Tel.: 617-525-1105; Fax: 617-525-1010; E-mail: jhiggins@rics.bwh.harvard.edu. The atomic coordinates of the human E-cadherin domain 1 and \(\alpha_6\) A-domain models are available upon request.

\(^1\)The abbreviations used are: MAdCAM-1, mucosal addressin cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MIDAS, metal ion-dependent adhesion site; ICAM, intercellular cell adhesion molecule; mAb, monoclonal antibody; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; TBS, Tris-buffered saline; BSA, bovine serum albumin; MFI, mean fluorescence intensity; WT, wild-type.
αM integrins have revealed other residues on the upper surface of the A-domains surrounding the MIDAS cleft that contribute to the affinity and specificity of ligand binding (26, 29, 32–34).

This mechanism provides an elegant explanation of ligand binding by A-domain-containing integrins. However, most integrins do not possess an A-domain in the α chain, yet they are also dependent upon acidic residues in their ligands and have a similar requirement for divalent cations. A potential explanation is that the proposal that all integrin β chains have a region that adopts an A-domain-like fold (19, 35, 36). Indeed, all β chains possess a relatively conserved region of ~250 amino acids that contains a DXXSX sequence, which, together with conserved downstream oxygenated residues, could form a complete MIDAS site. In fact, mutation of such residues in β1, β2, β3, β5, and β6 integrins abolishes ligand binding activity (35, 37–45). However, it remains likely that non-A-domain-containing integrin α chains also contribute directly to ligand binding. The seven repeats present in the N-terminal region of all integrin α chains are proposed to fold into a “β-propeller” of seven four-stranded β-sheets (46), and mutations in the α chain on the upper surface of the predicted second, third, and fourth blades of the propeller, for example, diminish the binding of αβ2 to VCAM-1 (47–49). An alternative model proposes that this region of the α chain forms “EF-hand-type” domains, but also postulates a role in ligand binding (50).

Here, we analyze the role of α and β chains in the ligand binding activity of the β7 integrins αβ7β7 and αβ7β7. This is important for a number of reasons. First, the ligand for α7β7, E-cadherin, is a unique integrin counter-receptor. Although a critical glutamate residue (Glu176) required for integrin binding has been identified, its structural context is unlike that of other ligands of integrins with an A-domain (10, 11). Such integrins bind to counter-receptors that contain critical acidic residues presented on relatively flat surfaces (e.g. β2 integrin ligands ICAM-1 and -2) or on collagenous structures (9, 31). However, Glu176 in E-cadherin is on a highly exposed loop akin to similar loops containing critical acidic residues in VCAM-1, MadCAM-1, and fibronectin that bind to integrins lacking an α chain A-domain (11). Also, α7 has an X-domain of 55 amino acids immediately N-terminal to the A-domain that has 18 charged residues within a stretch of 21 amino acids and contains a post-translational cleavage site (see Fig. 1A) (51). For these reasons, there exists doubt as to the role of the A-domain in E-cadherin binding. Therefore, it is of great interest to determine if the α7 A-domain is involved and what role the X-domain plays. Second, the β7 chain must be important in defining binding specificity since αβ7 binds to MadCAM-1, whereas αβ7β7 does not (52–54); yet αβ7β7 and αβ7β7 utilize the β7 chain, but they have non-overlapping ligand binding specificities for E-cadherin and MadCAM-1, respectively (54). Also, in a number of animal models of colitis and graft-versus-host disease, the administration of antibodies to either αβ7 or α7β7 ameliorates the development of intestinal inflammation (55–58), and αβ7β7 potentially has a role in rejection of epithelial tissue within allografts (59). Therefore, there is considerable interest in the structural basis for ligand recognition by these integrins because of their important role in leukocyte trafficking and to aid the design of therapeutic small molecule inhibitors.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA-manipulating enzymes were from New England Biolabs Inc. (Beverly, MA); oligonucleotides were from Integrated DNA Technologies Inc. (Corvalis, IA); and other chemicals from Sigma. Purified human E-cadherin-Fc fusion protein was described previously (2).

**Generation of Transfected K562 and CHO Cell Lines**—K562 cells were maintained as described (11) and express no endogenous α7 or β7 chains detectable by flow cytometry or immunoprecipitation (data not shown). These cells were transfected by electroporation of plasmid DNA encoding of pAPRM8 or pAPRM8α7β7, 10 μg of pAPRM8α7β7, and 1 μg of pGKpuro vectors (73). After selection in 2.5 μg/ml puromycin for 2 weeks, single cells expressing αβ7β7 or αβ7β7 as determined by staining with anti-αβ7 or anti-α7 mAbs, respectively, were obtained using an Epics Elite ESP flow cytometer equipped with the Autoclone module (Coulter Corp., Miami, FL). For αβ7β7 transfecteds, mAb e7-1 was used, except for the mutants α7ΔGlu163, Glu166, and α7Glu166 (60) or anti-α7 mAb 7, which was used to confirm expression of αβ7β7.

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stain strongly with this mAb, for which BerACT-8 was used. For α5β1 transfectants, mAb B5E2 was used. K562 cells stably transfected with 1 μg of pGKpuro vector alone (K562 control cells) were used as controls. CHO cells were transfected with pBJ-1/α5 and pBJ-1/β1, selected, and maintained essentially as described previously (48, 49). Cell-surface Labeling and Immunoprecipitation—K562 or CHO transfectants were suspended at 106 cells/ml in phosphate-buffered saline with 0.2 mg/ml EZ-Link sulfo-L-cysteine labeling reagent (Pierce) and incubated for 30 min at 4 °C. Subsequently, aliquots were incubated with monoclonal antibodies for 1.5 h at 4 °C. Then, 10 μl of protein G-Sepharose resin was added, and the incubation at 4 °C was continued for a further 1.5 h. Immobilized complexes were washed six times with TBS and 0.1% Triton X-100, 1 mM MgCl2, and 1 mM CaCl2.

RESULTS

Model of the α5 Integrin A-Domain—To facilitate selection of residues for mutagenesis and to aid interpretation of the results, a three-dimensional model of the α5 chain A-domain was generated. A FASTA screen (75) of the Swiss Protein Database with the human α5 A-domain protein sequence revealed that it was most closely related to the A-domain of human αM (CD11b), with 38% identity over 192 amino acids. The alignment of the A5 α-domain sequence with that of αM revealed a single insertion of two amino acids in α5 compared with αM (Fig. 1A). Substitution of human α5 residues into the human αM crystal structure in the open conformation (1IDO (19)) produced a model of the α5 A-domain (Fig. 1B). No major changes in the backbone structure are necessary to accommodate the α5 protein sequence, and the conserved MIDAS residues Asp383, Ser192, Thr281, and Asp285 form a potential metal ion coordination site in a cleft at the top of the domain, as in known integrin A-domain structures. A notable difference from the α5 (and α1) structure in the model is the extended α1-β1 loop at the base of the A-domain due to the insertion of two amino acids mentioned above. The length of this loop is thus similar to that in the α1 and α2 A-domains, but is unique in that a disulfide bond that may rigidify the region is predicted to lie between the α1-β1 loop and the start of the β2-strand (Fig. 1, A and B).

Generation of K562 Cells Expressing Mutant α5β1 Integrins—To localize sites on the human α5β1 integrin that are involved in binding to E-cadherin, we generated a series of site-directed mutants of both α (Fig. 1A) and β (Fig. 1B) chains. Mutations were made in the DNAX MIDAS motifs of both α5 (D190A) and β1 (D140A) chains and in a number of residues predicted by the model to be solvent-exposed at the top of the α5 A-domain surrounding the MIDAS site (G193A, D199A, and E202A). A final mutant lacking the entire charged B loop at the base of the A-domain due to the insertion of two amino acids mentioned above. The length of this loop is thus similar to that in the α1 and α2 A-domains, but is unique in that a disulfide bond that may rigidify the region is predicted to lie between the α1-β1 loop and the start of the β2-strand (Fig. 1, A and B).

Binding data for variations in integrin expression level between clones, binding was calculated relative to that of an α5β1 reference mAb (Fib504) according to the following equation: % wild-type binding = 100 × (a – b(1 – d)(e – f))/g(h – b), where a = MFI of test reagent on test K562 clone, b = MFI of isotype-matched negative control on test K562 clone, c = MFI of Fib504 on test cell, d = MFI of negative control mAb Y13.238 on test cell, e = MFI of test reagent on reference K562-WT transfectant cell, f = MFI of isotype-matched negative control on K562-WT cell, g = MFI of Fib504 on K562-WT transfectant cell, and h = MFI of negative control mAb Y13.238 on K562-WT transfectant cell.
The role of α and β chains in ligand binding by β7 integrins

The structure of human E-cadherin (ECAD) is schematically depicted in Figure 1A. The X-domain of ECAD is shown in blue, the A-domain in green, and the Y-domain in orange. The post-translational cleavage site E is indicated with a triangle. The triangle indicates the ΔGlu176 deletion; and the ΔGlu163–Glu166 deletion is italicized. The δ-strands in the A-domain crystal structure (19) are indicated, and the MIDAS resides are underlined. Residues in boldface are those mutated in this study; the triangle indicates the ΔGlu176 deletion; and the ΔGlu163–Glu166 deletion is italicized. Mutations that influence binding of E-cadherin are labeled with asterisks. The post-translational cleavage site of αE is indicated with a vertical arrow, and a predicted disulfide bond between Cys225 and Cys228 is marked. B, ribbon diagram of the αE A-domain model based on the crystal structure of the αE A-domain in a heptamer conformation (19) as described under “Results.”

Mapping of anti-αEβ7 monoclonal antibody epitopes is shown in Figure 1B. The first line contains αE chain to associate with β7 chain as determined by densitometry was within 2-fold of that determined for wild-type αEβ7 in all cases, with the exception of αE(Glu230→Asp) and αE(Asp42→Glu) residues. The location of αE helices and β-strands in the αE A-domain crystal structure (19) are indicated, and the MIDAS residues are underlined. Residues in boldface are those mutated in this study; the triangle indicates the ΔGlu176 deletion; and the ΔGlu163–Glu166 deletion is italicized. Mutations that influence binding of E-cadherin are labeled with asterisks. The post-translational cleavage site of αE is indicated with a vertical arrow, and a predicted disulfide bond between Cys225 and Cys228 is marked. B, ribbon diagram of the αE A-domain model based on the crystal structure of the αE A-domain in a heptamer conformation (19) as described under “Results.”

Anti-β7 mAb Fib504 (Fig. 2A). The ratio of co-immunoprecipitated αE chain to β7 chain as determined by densitometry was within 2-fold of that determined for wild-type αEβ7 in all cases, with the exception of αE(R159S/R160S)β7, confirming that these mutations do not have a major impact on heterodimer formation. The mutation R159S/R160S appeared to reduce the ability of the αE chain to associate with β7 chain, as expected, resulting from loss of the charged region (data not shown). However, this mutant, which displayed evidence of proteolytic degradation, was expressed at a low level, and less αE chain was precipitated with an anti-β7 chain mAb than for the other mutants. Another mutant with a deletion of the entire X-domain was not expressed at the cell surface and therefore was excluded from the analysis. This suggests that large deletions within the X-domain have some impact on the ability of the αE chain to associate with β7 and to be transported to the cell surface.

Mapping of anti-αEβ7 monoclonal antibody epitopes. To probe the overall conformation of the mutant αEβ7 integrins on the cell surface and to map antibody-binding epitopes, the reactivity of each mutant with a panel of anti-αEβ7 mAbs was determined by flow cytometric analysis and compared with that of anti-β7 mAb Fib504 as a reference (see “Experimental Procedures”). mAbs Fib21, Fib30, and Fib504, which recognize three distinct epitopes on the β7 chain (65), all bound equally well to all the mutant αEβ7 integrins (Fig. 3A). Thus, none of the mutations, including that of the β7 MIDAS motif (D140A), had any detectable influence on the conformation of the β7 chain. This analysis also supported the use of Fib504 as a reference mAb for subsequent analysis of anti-αE mAbs. All the mutant αEβ7 integrins were recognized as well as the wild type by multiple mAbs that recognize different epitopes of αEβ7, and that do not recognize denatured protein in immunoblotting (Refs. 63 and 71 and this study), suggesting that the mutant αEβ7 integrins maintain their basic structure. Indeed, no significant difference was seen in the recognition by mAbs LF61 and ABB1.3 of any of the mutants (Fig. 3B).

The mutagenesis of αE mapped five of the eight anti-αEβ7 mAbs tested to the αE A-domain (Fig. 3B). mAb αE7-1 bound similarly to all the A-domain mutants except one, αE(G230A/
V231A)β₇, to which its binding was reduced by >80%. Thus, the epitope of this mAb is highly likely to encompass Gly²²⁰ and/or Val³³¹. Similarly, mAb αE7-2 binding was abolished only to αEβ₇(E325A)β₇; mAb αE7-3 binding was abolished only to αEβ₇(F298A)β₇; and mAb HML-1 and 2G5 binding was reduced by ~80 and 60%, respectively, only to αEβ₇(G193A)β₇. Gly¹⁹³, Gly²²⁰, Val³³¹, Phe³³⁶, and Glu³³⁹ form a cluster of exposed residues surrounding the MIDAS-chelated metal ion at the top of the A-domain model (see Fig. 8A). Since αE7-1, αE7-2, αE7-3, HML-1, and 2G5 all block αEβ₇-mediated adhesion to E-cadherin, whereas LF61 and ABB1.3 do not (3, 5, 63), these results provide a preliminary mapping of the E-cadherin-binding site on αEβ₇ to the top of the A-domain surrounding the MIDAS site.

Interestingly, removal of the cleavage site within the unique αE X-domain (αEβ₇R159S/R160S)β₇) reduced the binding of mAb BerACT-8 by 70%, whereas all the other mAbs tested bound normally (Fig. 3B). Immunoprecipitation of αEβ₇ R159S/R160S)β₇ with BerACT-8 suggests that the residual binding of this mAb is due to recognition of the small proportion of αEβ₇(F298A)β₇ with a cleaved αE chain (data not shown). These findings suggest that the loss of BerACT-8 binding to the R159S/R160S mutant is not directly due to the loss of the two arginine residues, but rather that BerACT-8 recognizes an epitope that is fully exposed only when the X-domain is cleaved. However, the precise location of the BerACT-8 epitope was not determined in this study.

**Mutation of the MIDAS Motif of the α₇ or β₇ Chain Abolishes Adhesion to E-cadherin**—The functional activity of the αEβ₇ mutants was tested by measuring the adhesion of the K562 transfectants to E-cadherin-Fc fusion protein immobilized on microtiter plate wells as described previously (5, 11). Thirty to forty percent of K562-αEβ₇ WT cells adhered to E-cadherin-Fc at saturating concentrations, whereas K562 control cells did not adhere (data not shown). Strikingly, mutation of the DXSXS sequence to AXXSXS within the MIDAS motif of either the αE or β₇ (D190A) or β₇ (D140A) chain completely abolished detectable adhesion of transfected K562 cells to E-cadherin-Fc-coated wells (Fig. 4A). Thus, the coordination of metal ions by the MIDAS residues of the αE A-domain and the proposed A-like domain of β₇ are likely to be critical for αEβ₇-mediated adhesion to E-cadherin.

**Phe³³⁶ at the Top of the α₇ A-Domain Is Critical for Adhesion to E-cadherin**—K562 cells expressing α₇β₇-containing mutations G193A, D199A, R202A/D205A, G230A/V231A, D240A, P311H/E345A/T346A, E325A, and Y354W in the α₇ A-domain adhered to E-cadherin-Fc similarly to K562-αEβ₇ WT transfectants (Fig. 4A). Assays conducted at lower coating concentrations of E-cadherin-Fc or without manganese also did not reveal any differences from the wild type (data not shown). In contrast, K562-αEβ₇(F298A)β₇ cells did not adhere to E-cadherin-Fc at any concentration tested (p < 0.01 versus the wild type by Dunnett’s multiple comparison test). Phe³³⁶ is predicted to be prominently exposed at the top of the α₇ A-domain close to the MIDAS metal ion coordination site and thus is likely to be involved directly in binding to E-cadherin. The lack of structural data for the potential A-like domain of integrin β chains precluded a reliable similar analysis of the β₇ chain.

Neither removal of the cleavage site from the X-domain of the αE chain (R159S/R160S) nor deletion of a single residue within the charged region (ΔGlu³³⁹) altered adhesion to E-cadherin-Fc (Fig. 4A). The results suggest that the fine structure of the charged region of the X-domain is not crucial for binding to E-cadherin and that post-translational cleavage in this region is not a prerequisite for adhesion to E-cadherin. Furthermore, an antiserum to the charged region of the X-domain that recognizes αEβ₇ on the cell surface by flow cytometry does not inhibit the adhesion of K562-αEβ₇ WT transfectants to E-cadherin-Fc (data not shown), indicating that this region does not participate directly in the binding of αEβ₇ to E-cadherin.

**Direct Binding of Soluble E-cadherin-Fc to K562-αEβ₇ Transfectants Confirms and Extends Definition of the E-cadherin-binding Site**—Adhesion assays are not simply a measure of direct receptor to ligand binding because stable adherence of cells to immobilized ligands may require post-receptor events including modulation of the cytoskeleton. It is possible that mutation of αEβ₇ might affect signaling through the integrin and such downstream events rather than alter the direct binding to E-cadherin. To rule out this possibility and to provide a quantitative assay for direct binding of αEβ₇ to E-cadherin, we made use of the ability of soluble E-cadherin-Fc to bind directly to αEβ₇ (5). The binding of biotinylated E-cadherin-Fc to the surface of K562-αEβ₇ transfectants could be detected by flow
cytometry using a neutravidin-conjugated Alexa 488 secondary reagent (Fig. 4B). No such binding was seen to K562 control cells. Saturation of binding was achieved between 50 and 100 μg/ml E-cadherin-Fc (data not shown). The binding of 100 μg/ml biotinylated E-cadherin-Fc to K562 cells transfected with each mutant form of αEβ7 was assessed. In each case, the background binding of biotinylated human IgG1 was subtracted, and the results are expressed as the percentage of the binding seen to wild-type αEβ7 after correcting for the level of surface expression determined using anti-β7 chain mAb Fib504.

The results (Fig. 4C) confirm those obtained in adhesion assays. Mutation of the MIDAS motif of either the αE or β7 chain completely abrogated the ability of E-cadherin-Fc to bind αEβ7 (p < 0.01 versus the wild type by Dunnett’s multiple comparison test). Also, the critical importance of Phe298 for binding to E-cadherin was reinforced. No direct binding of E-cadherin-Fc to αEβ7 could be detected if this amino acid was mutated to alanine (p < 0.01). In addition, the direct binding assay highlighted the previously undetected role of Glu325 in binding to E-cadherin. Mutation of this amino acid to alanine unexpectedly increased the binding of E-cadherin-Fc to αEβ7 2-fold over the wild type (p < 0.01). This residue is exposed on the surface of the αE A-domain near the MIDAS site, and it forms a crucial part of the epitope of the adhesion-blocking mAb αE7-2 (Fig. 3B). As in the adhesion assays, mutation of other residues in the A-domain, mutation of the X-domain cleavage site (R159S/R160S), and removal of a single amino acid within the charged region (ΔGlu176) did not alter E-cadherin-Fc binding.

**Fig. 3.** Epitope mapping of anti-αEβ7 monoclonal antibodies. The binding of anti-β7 (A) or anti-αEβ7 (B) mAbs to K562 cells expressing mutant forms of αEβ7 was determined by flow cytometry. Binding is expressed as the percentage of binding obtained with anti-β7 mAb Fib504 and is corrected for variations in the expression level on different clones as described under “Experimental Procedures.” None of the antibodies bound to K562 control cells. Each bar represents the mean ± S.D. determined from two to four different K562 clones analyzed in two to five separate experiments. DE176, ΔGlu176. *, significant difference versus αEβ7 WT by Dunnett’s multiple comparison test (p < 0.01).
The Role of α and β Chains in Ligand Binding by β7 Integrins

Mutation of the MIDAS Motif of the β7 Chain Abolishes αβ7-mediated Adhesion to MadCAM-1—A role for the MIDAS motif within the potential A-like domain of the β7 chain has been proposed for αβ7-mediated adhesion to MadCAM-1 based on β7/β7 chimera analysis and mapping of the adhesion-blocking antibody ACT-1 epitope to this region of the β7 chain (60). Transfection of both human α4 and β7 chains into K562 cells conferred the ability to adhere to MadCAM-1-Ig fusion protein, but not to human IgG1, as described by others (60). Adhesion to MadCAM-1-Ig could be blocked by anti-human α4β7 antibody ACT-1, and K562 control cells were unable to adhere to MadCAM-1-Ig (data not shown). After transfection of K562 cells with α4 and the β7 chain containing the D140A mutation within the MIDAS motif, we obtained clones that expressed the mutant α4β7(D140A) as assessed by flow cytometry. A comparison of the staining obtained with mAbs to at least two different epitopes on the α4 chain (B5G10, B5E2, and HP1/2) (76), three different epitopes on the β7 chain (Fib21, Fib30, and Fib504) (65), and an antibody that recognizes cell-surface β7 chain only in the context of α4β7 (ACT-1) (60, 77) showed that the binding of these mAbs was unaffected and confirmed that the D140A mutation did not grossly affect the structure of α4β7 (Fig. 5A). In addition, immunoprecipitation of wild-type α4β7 and α4β7(D140A) with either mAb ACT-1 or Fib504 demonstrated that heterodimerization was not affected (Fig. 5B) (data not shown). Despite this, K562-α4β7(D140A) cells were unable to adhere to MadCAM-1-Ig, even when the mutant integrin was expressed at higher levels than wild-type α4β7 (Fig. 5C). Thus, an intact MIDAS motif within the A-like domain of the β7 chain is required for the adhesion of α4β7 to MadCAM-1 as well as for the adhesion of α4β7 to E-cadherin.

Mutations of the α4 Chain That Reduce α4β7-mediated Adhesion Also Reduce αβ7-mediated Adhesion to MadCAM-1-Ig—A number of residues in the second, third, and fourth repeats of the α4 chain have been implicated in the binding of α4β7 to VCAM-1 and fibronectin (47–49). To determine if the same residues are involved in the binding of α4β7 to MadCAM-1, we transfected CHO cells with previously described mutant α4 chains together with the wild-type β7 chain. CHO cells were used in this case because K562 cells were found to express endogenous human α4 chain at significant levels upon transfection with β7 (data not shown). We generated CHO cells expressing α4β7 with single amino acid substitutions in the α4 chain (Y187A, G190A, and Y202A) (48) or in which predicted residues 112–131; and R3a, residues 151–164) (49). All the mutants were expressed at levels greater than that of wild-type E-cadherin-Fc directly binds to K562-α4β7 WT, WT, but not to K562 control cells. The binding of 50 μg/ml biotinylated human E-cadherin-Fc or human IgG1 to transfected K562 cells was determined by flow cytometry in the presence of 1 mM each MnCl2, MgCl2, and CaCl2, using a neutravidin-conjugated Alexa 488 secondary reagent. C, the direct binding of 100 μg/ml E-cadherin-Fc to K562-α4β7 mutants was analyzed as described for B. Binding is expressed as the percentage of binding obtained with anti-β7 mAb Fib504 as described under “Experimental Procedures.” Each bar represents the mean ± S.D. determined from two to four different K562 clones analyzed in two to three separate experiments. Asterisks indicate that no binding above that to IgG1 could be detected (p < 0.01 versus the wild type by Dunnett’s multiple comparison test); double asterisks indicate a significant increase in binding over the wild type (p < 0.01). E176, ΔGlu176.

**Fig. 4. Analysis of the E-cadherin binding capacity of mutant α4β7 integrins.** A, adhesion of K562-α4β7 mutants to E-cadherin-Fc. In all cases, flow cytometry to determine integrin expression level was conducted within 30 h of the assay; E-cadherin-Fc was coated at saturating concentrations between 20 and 100 ng/ml; and the background adhesion to the same concentration of human IgG1 was subtracted. Each bar represents the mean ± S.E. pooled from four to nine determinations in triplicate from two to four independent experiments involving two to four different K562 clones. The results were combined as described under “Experimental Procedures.” The lack of a bar indicates that no adhesion above that to IgG1 could be detected. *, p < 0.01 versus the wild type by Dunnett’s multiple comparison test. B, E-cadherin-Fc directly binds to K562-α4β7 WT, WT, but not to K562 control cells. The binding of 50 μg/ml biotinylated human E-cadherin-Fc or human IgG1 to transfected K562 cells was determined by flow cytometry in the presence of 1 mM each MnCl2, MgCl2, and CaCl2, using a neutravidin-conjugated Alexa 488 secondary reagent. C, the direct binding of 100 μg/ml E-cadherin-Fc to K562-α4β7 mutants was analyzed as described for B. Binding is expressed as the percentage of binding obtained with anti-β7 mAb Fib504 as described under “Experimental Procedures.” Each bar represents the mean ± S.D. determined from two to four different K562 clones analyzed in two to three separate experiments. Asterisks indicate that no binding above that to IgG1 could be detected (p < 0.01 versus the wild type by Dunnett’s multiple comparison test); double asterisks indicate a significant increase in binding over the wild type (p < 0.01). ΔE176, ΔGlu176.


\( \alpha_7\beta_4 \) as determined by flow cytometry with anti-\( \alpha_7\beta_4 \) mAb ACT-1; anti-\( \beta_7 \) mAbs Fib21, Fib30, and Fib504; and anti-\( \alpha_4 \) mAbs B5G10 and B5E2 (data not shown). Immunoprecipitation with anti-\( \beta_7 \) mAb Fib504 from surface-biotinylated CHO transfectants also confirmed the presence of \( \alpha_7\beta_4 \) heterodimers for all the mutants (Fig. 6A). As previously found for \( \alpha_7\beta_4 \) (48, 49), none of the mutations altered the binding of mAb B5G10 to \( \alpha_7\beta_4 \). The binding of anti-\( \alpha_7\beta_4 \) mAb HP1/2 to the R2 mutant was reduced by \( \sim 85\% \), consistent with the similarly reduced binding of mAb HP2/1 (which recognizes the same epitope (76)) to \( \alpha_7\beta_4 \) (49).

Transfection with both wild-type \( \alpha_4 \) and \( \beta_7 \) chains conferred upon CHO cells the ability to adhere to MadCAM-1-Ig fusion protein and to MadCAM-1-transfected L1-2 cells, but not to human IgG1 or to untransfected L1-2 cells (Fig. 6, B and C) (data not shown). Approximately 40\% of CHO-\( \alpha_7\beta_4 \) cells adhered to MadCAM-1-Ig, and this could be blocked by anti-\( \alpha_7\beta_4 \) mAb ACT-1. Untransfected CHO cells were unable to adhere to MadCAM-1-Ig (Fig. 6B). A 2-fold greater percentage of CHO-\( \alpha_7\beta_4 \) (Y202A)\( \beta_7 \) cells adhered to MadCAM-1-Ig compared with CHO-\( \alpha_7\beta_4 \) WT cells, presumably due to the higher level of \( \alpha_7\beta_4 \) (Y202A)\( \beta_7 \) expression (Fig. 6B). However, CHO cells expressing \( \alpha_7\beta_4 \) (G190A)\( \beta_7 \) did not adhere to MadCAM-1-Ig even though they also had higher surface expression than wild-type \( \alpha_7\beta_4 \) transfectants. In addition, CHO-\( \alpha_7\beta_4 \) (Y187A)\( \beta_7 \) cells adhered less well than CHO-\( \alpha_7\beta_4 \) WT cells to MadCAM-1-Ig despite the higher cell surface expression of the mutant integrin. CHO cells expressing \( \alpha_7\beta_4 \) (R2)\( \beta_7 \) were unable to adhere to MadCAM-1-Ig, but the \( \alpha_7\beta_4 \) (R3a)\( \beta_7 \)-expressing cells adhered well. Similar results were obtained when the adhesion of MadCAM-1-expressing L1-2 cells to the CHO transfectants was assessed (Fig. 6C), although in this system, the defect in CHO-\( \alpha_7\beta_4 \) (Y187A)\( \beta_7 \) cell adhesion was more pronounced. In summary, the G190A and R2 mutations abolished \( \alpha_7\beta_4 \)-mediated adhesion to MadCAM-1, whereas the Y202A and R3a mutations did not substantially reduce adhesion, and Y187A had an intermediate effect.

**DISCUSSION**

Despite the distinctive nature of E-cadherin as the only integrin ligand known that is a cadherin and the presence of unique structural features within the \( \alpha_7 \) chain that could contribute to ligand binding, the E-cadherin-binding site includes the A-domain of the \( \alpha_7 \) chain, and its location appears similar to that defined for other integrin-ligand interactions. Mutation of the conserved MIDAS motif within the \( \alpha_7 \) A-domain (D190A) abolishes E-cadherin binding. This is consistent with the requirement for magnesium or manganese ions (5) and an expressed acidic residue within E-cadherin for \( \alpha_7\beta_4 \) binding (10, 11) and strongly suggests a direct role for a metal ion coordinated by the MIDAS motif in the interaction. This result also shows not alter the binding of anti-\( \alpha_7\beta_4 \) (ACT-1), anti-\( \alpha_4 \) (B5G10, B5E2, and HPI2), and anti-\( \beta_7 \) (Fib21, Fib30, and Fib504) mAbs to K562-\( \alpha_7\beta_4 \) transfectants. Antibody binding was determined as described under “Experimental Procedures.” None of the antibodies bound to K562 control cells. Each bar represents the mean ± S.D. determined from four different K562 clones analyzed in three separate experiments. B, the \( \beta_7 \) mutation D140A does not alter \( \alpha_7\beta_4 \) heterodimer formation. K562 cells transfected with wild-type \( \alpha_7\beta_4 \) or \( \alpha_7\beta_4 \) (D140A) or K562 control cells were surface-labeled with biotin. Proteins immunoprecipitated with anti-\( \alpha_7\beta_4 \) mAb ACT-1 were detected as described in the legend to Fig. 2. C, the mutation D140A in the \( \beta_7 \) chain MIDAS motif of \( \alpha_7\beta_4 \) abolishes detectable adhesion to MadCAM-1-Ig. The adhesion of K562 transfectants to human MadCAM-1-Ig or IgG1 was determined as described under “Experimental Procedures.” The expression level of \( \alpha_7\beta_4 \) (D140A) on the cell surface by flow cytometry was higher than that of the wild type. The data are the mean ± S.D. (\( n = 3 \)) and are representative of three separate experiments with two different clones.
that, contrary to previous concepts (9), A-domain-containing integrins do not require the critical acidic residue of the ligand to be on a flat surface since Glu31 of E-cadherin is on the highly exposed BC-loop at the top of domain 1 in a context most similar to that of the RGD sequence in fibronectin, rather than on a fairly level surface at the end of the C strand as in ICAM-1 and -2.

In contrast, the mutation R159S/R160S within the extra X-domain of the αE chain prevents the normal post-translational cleavage of the αE chain, but does not alter the binding of E-cadherin to αEβ7. Deletion of a single amino acid within the charged region of the X-domain also has no detectable effect on E-cadherin binding. Since an anti-X-domain antiserum also fails to influence adhesion to E-cadherin, we speculate that the αE X-domain does not contribute directly to the E-cadherin-binding interface. The X-domain may be important in optimizing the orientation of the A-domain for ligand binding since it is located at the junction between the second blade of the predicted β-propeller and the A-domain of αE. Such a role would be consistent with the presence of an X-domain in murine and rat αE chains, but its low sequence homology and poor length conservation compared with human αE (78, 79). Alternatively, the X-domain might be involved in interactions with yet unidentified αEβ7 ligands.

Residues predicted from our αE A-domain model to reside in loops close to the MIDAS cleft also were implicated in E-cadherin binding and suggest a wider interaction surface that is involved in ligand recognition at the top of the A-domain. The binding of five blocking monoclonal antibodies to αEβ7 mapped to a cluster of residues (Gly193, Glu207, Val211, Phe298, and Glu325) encircling the MIDAS on the surface of the A-domain (Fig. 7A). The mutation F298A abolished detectable binding of E-cadherin to αEβ7 and is located in the β7–αE loop. This hydrophobic residue is predicted to be prominently exposed on the top surface of the A-domain (Fig. 7B). The mutation E325A, in the αE–αE loop also at the top of the A-domain (Fig. 7B),
increased the direct binding of E-cadherin to αβ, 2-fold and thus also may be close to the cadherin-binding surface.

Using this information, we docked a model of human E-cadherin (11) onto the αE A-domain. Importantly, we utilized a model of the αK A-domain based on the open form of the αM A-domain, as this is likely to be the active conformation (31). The critical Glu^{31} residue in the BC-loop of E-cadherin domain 1 was maintained at a distance suitable for interaction with the magnesium ion chelated by the A-domain MIDAS motif. The E-cadherin molecule was then rotated relative to the A-domain structure to optimize the interaction and to minimize steric interference. Strikingly, the best orientation of the two structures places Phe^{298} of the αK A-domain into a hydrophobic pocket formed between the BC- and FG-loops of E-cadherin domain 1 (Fig. 8A). This interaction and the coordination of the MIDAS metal ion by Glu^{31} in E-cadherin provide the most important contributions to the contact between the two molecules. This is consistent with the finding that the mutation E31A in E-cadherin and the F298A and MIDAS mutation D190A in αβ all abolish detectable binding between the two proteins. Our previous study identified further residues in E-cadherin that contribute to αβ binding (11). Interestingly, mutation of Asn^{27}, Lys^{30}, and Glu^{89} in the BC- and FG-loops of E-cadherin reduces adhesion of K562-αβ, cells to E-cadherin-Fc. These amino acids are all found at the proposed interface between E-cadherin and the A-domain (Fig. 8B). Asn^{27} and Glu^{89} could potentially form a hydrogen bond and a salt bridge, respectively, with Arg^{331} in the A-domain, and Lys^{30} in E-cadherin could form another salt bridge with Asp^{196} in the A-domain. Arg^{331} was not mutated in this study, but the model predicts that it may be an important component of the E-cadherin-binding site. This proposal is consistent with the marked reduction (>90%) that mutation of Glu^{89} causes in K562-αβ adhesion to E-cadherin (11). We also examined the interaction of E-cadherin with an αβ A-domain model in the closed conformation that is likely to represent the inactive form (30, 31). E-cadherin residues Asn^{27}, Lys^{30}, and Glu^{89} all interact with a region of the A-domain that undergoes a marked conformational change upon transition between the two forms (19, 29–31) (Fig. 8B). Indeed, in the active form of the A-domain, Asp^{196} and Arg^{331} both move into more favorable positions for interaction with E-cadherin. Glu^{325} is also in this region of the A-domain, but is not predicted to make direct contacts with E-cadherin. This raises the possibility that the E325A mutation may increase binding to E-cadherin because it favors adoption of the active conformation.

The potential projection of Phe^{298} into a distinct pocket in E-cadherin domain 1 suggests a novel mode of integrin-ligand interaction that contributes to the specificity of αβ for E-cadherin. The equivalent of Phe^{298} in αA, Thr^{243}, is involved in binding to ICAM-1 and -2, but forms part of a relatively flat binding surface (20, 32, 33), and His^{258} of αB is involved in collagen I binding, but forms a hydrogen bond with the main chain of collagen (31, 34). Also, the αL and α2 integrin A-domains contain an extra four-amino acid α-C helix in the βL-αL loop that markedly alters the topology of the A-domain surface and regulates access to the collagen-binding site (21, 22, 31, 34, 80). Although hydrophobic residues are involved in all integrin A-domain interactions examined, the penetration of a prominent aromatic residue into a hydrophobic pocket of the ligand is not predicted for αL binding to ICAM-1 or -2 (20, 33, 81) and is not involved in α2 A-domain binding to collagen (31). Interestingly, the amino acids implicated in binding of the αM A-domain to E-cadherin are similar to residues on the closely related A-domain of αM that are involved in binding iC3b (see Fig. 1A). The residue equivalent to Phe^{298} in the αM A-domain is also phenylalanine, and the mutation F246K abolishes αM binding to iC3b (29), whereas the F246A mutation reduces binding by 50% (26). Strikingly, the mutation D273K, the residue in the αM A-domain corresponding to Glu^{325}, also leads to a doubling of ligand binding activity, in this case for iC3b (29). The results...
suggest that the $\alpha_E$ and $\alpha_M$ A-domains share common features for recognition, despite the dissimilarity of their ligands.

We found residues that could be mutated in the $\alpha_E$ A-domain with no detectable influence on E-cadherin binding, but that are important for ligand binding by $\alpha_M$ and $\alpha_L$ A-domains. The mutations G193A, D199A, and G230A/V231A in $\alpha_E$ did not cause a significant change in E-cadherin binding, whereas the mutations G143M, D149K, and E178A/E179A in $\alpha_M$ all reduced iC3b binding by 90% (29), and M140Q, E146A, and T175A in $\alpha_L$ all reduced ICAM-1 or -2 binding (32, 33). It is possible that in some instances, the more dramatic amino acid substitutions made in the studies of $\alpha_M$ are responsible for the detection of more important residues. For example, the M140Q mutation reduced ICAM-1 binding by 65% (32), but M140A had no effect on ICAM-1 or -2 binding in another study (33). Alternatively, it may be that E-cadherin has a smaller footprint on the $\alpha_E$ A-domain than iC3b on the $\alpha_M$ A-domain or ICAM on the $\alpha_L$ A-domain. Indeed, the docking model of E-cadherin with the $\alpha_E$ A-domain does suggest limited contact between the molecules. This is due in part to the position of the critical Glu$^{31}$ residue of E-cadherin at the tip of the first domain, in contrast to the flat surfaces of ICAM-1 and -2 that bind the $\alpha_L$ A-domain. It is also possible that other residues on the surface of the $\alpha_E$ A-domain that we did not mutate in this study contribute to E-cadherin binding. However, these results implicate common ligand-binding loops on the surface of all A-domains that surround the MIDAS cleft, but highlight integrin-specific differences that are critical to defining ligand binding specificity.

We also found that mutation of residues in the $\alpha_4$ chain of
$\alpha_5 \beta_1$ can abrogate binding to MadCAM-1. The mutations G190A and Y187A substantially reduced adhesion of $\alpha_5 \beta_1$-expressing cells to MadCAM-1, whereas the mutation Y202A did not. In addition, the replacement of $\alpha_6$ residues 112–131 with the corresponding residues of $\alpha_5$ abolished MadCAM-1 recognition, whereas a similar substitution of residues 151–164 did not. The residues that influence adhesion lie on the upper surface of the second and third blades of the predicted $\beta$-propeller (46, 49), consistent with a role in ligand binding. Strikingly, this pattern of abrogation of $\alpha_L\beta_1$-mediated adhesion to MadCAM-1 is identical to that previously reported for $\alpha_L\beta_4$-mediated adhesion to VCAM-1 and the CS-1 fragment of fibronectin (48, 49). Although it remains possible that there are residues on $\alpha_6$ that interact with VCAM-1 and fibronectin but not with MadCAM-1 or vice versa, we clearly implicate a similar region of the $\alpha_4$ molecule in ligand binding for both $\alpha_5 \beta_1$ and $\alpha_6 \beta_7$.

Since $\alpha_6 \beta_4$ and $\alpha_7 \beta_4$ have distinct, non-overlapping ligand binding specificities for E-cadherin and MadCAM-1, respectively (54, 55) clearly the $\alpha_6$ chain must determine this difference in ligand binding specificity. Indeed, we have shown that mutations in both the $\alpha_5$ and $\alpha_6$ chains can abrogate the binding of these integrins to their ligands. Similarly, since $\alpha_5 \beta_1$ binds to MadCAM-1, whereas $\alpha_6 \beta_1$ binds to VCAM-1 but not MadCAM-1 (52–54), in this case, the $\beta_1$ chain must be important in defining specificity. As expected, mutation of the MIDAS motif of the $\beta_1$ chain (this study) or of the $\beta_1$ chain (47) abolishes ligand binding in both cases. Despite this, however, we have demonstrated that the $\beta_2$ chain MIDAS motif is critically involved in ligand binding for both $\alpha_5 \beta_1$ and $\alpha_6 \beta_1$ and that the $\alpha_6$ chain is critical in ligand binding for both $\alpha_5 \beta_1$ and $\alpha_6 \beta_1$. In each case, a similar binding site is implicated on the shared chain even when the integrin is engaged in interactions with different counter-receptors. These results indicate that changes in the combinations of $\alpha$ and $\beta$ chains that make up integrin heterodimers can yield different ligand binding specificities despite the utilization of similar binding surfaces on each chain.

Like the $\beta_1$ chain, $\beta_2$ can pair with $\alpha$ chains either containing or lacking a classical $A$-domain. It is known that the MIDAS motif of the $\beta_1$ chain is involved in ligand binding in the context of the $A$-domain-containing integrins $\alpha_5 \beta_1$ and $\alpha_6 \beta_1$ (43–45) and in ligand binding in the context of $\alpha$ chains that lack A-domains such as $\alpha_5 \beta_1$, $\alpha_6 \beta_1$, $\alpha_5 \beta_6$, $\alpha_6 \beta_6$, and $\alpha_5 \beta_7$ (35, 37–42). We have shown for the first time that the MIDAS motif of a single $\beta_1$ chain, $\beta_1^\prime$, is critical whether it is paired with an $A$-domain-containing ($\alpha_6$) or $A$-domain-lacking ($\alpha_5$) $\alpha$ chain. The results stress that in every case studied, the $\beta_1$ chain has a critical role to play in ligand binding, whatever the nature of the $\alpha$ chain. Because of this, it seems likely that the majority of integrin ligands will possess residues that are critical for interaction with $\alpha$ and $\beta$ chains. Such a mechanism has been proposed for the binding of the $\alpha_5 \beta_1$ integrin to fibronectin, where the RGD sequence in the tenth type III repeat together with a "synergy region" in the ninth type III repeat contribute to integrin binding (82). Similarly, along with the critical CD-loop in domain 1 of VCAM-1 and MadCAM-1, an "accessory site" in domain 2 has been implicated in the binding of the $\alpha_5 \beta_1$ and $\alpha_6 \beta_1$ integrins (9, 83–85). For these non-$A$-domain-containing integrins, it is interesting that although the primary binding site in the ligand includes a critical exposed acidic residue, the accessory site need not. Indeed, it has been suggested that the MIDAS site of the $\beta_1$ chain coordinates the acidic residue of the RGD sequence, whereas the synergy site interacts with the $\alpha_5$ chain (86). For $A$-domain-containing integrins containing MIDAS motifs in both the $\alpha$ and $\beta$ chains, it might be expected that two acidic residues within the ligand are vital for ligand binding. In general, however, only one such residue has been identified in these ligands. In some cases, it is possible that the necessary pairing of acidic residues might be achieved by dimerization of ligand. Indeed, both ICAM-1 and E-cadherin are thought to form dimers on the cell surface (87–89). It also remains possible that the $\beta_1$ chain A-like domain may regulate ligand binding by influencing the conformation of the $A$-domain in the $\alpha$ chain (30), but this is more difficult to reconcile with the similar role of the $\beta_1$ chain MIDAS in integrins with and without an $\alpha$ chain A-domain. Further structural data will be required to resolve!important reagents and for valuable discussions; Dr. Eugene Butcher for providing MadCAM-1-expressing L1-2 cells; and John Daley (Dana-Farber Cancer Institute) for help with autotolining.

Note Added in Proof—While this manuscript was under review, Ruiz-Velasco et al. also reported that mutation of $\alpha_4$ residue Tyr197 in $\alpha_5 \beta_1$ reduces MadCAM-1 binding (90).
