Defining Extracellular Integrin α-Chain Sites That Affect Cell Adhesion and Adhesion Strengthening without Altering Soluble Ligand Binding

Cristina Pujades,*†‡ Ronen Alon,‡§ Robert L. Yauch,*‡ Akihide Masumoto,* Linda C. Burkly,¶ Chun Chen,∥ Timothy A. Springer,§ Roy R. Lobb,¶ and Martin E. Hemler*#

*Division of Tumor Virology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115; ‡Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel; §Center for Blood Research, Harvard Medical School, Boston, Massachusetts 02115; and ¶Biogen Inc., Cambridge, Massachusetts 02142

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It was previously shown that mutations of integrin α4 chain sites, within putative EF-hand-type divalent cation-binding domains, each caused a marked reduction in α4β1-dependent cell adhesion. Some reports have suggested that α-chain “EF-hand” sites may interact directly with ligands. However, we show here that mutations of three different α4 “EF-hand” sites each had no effect on binding of soluble monovalent or bivalent vascular cell adhesion molecule 1 whether measured indirectly or directly. Furthermore, these mutations had minimal effect on α4β1-dependent cell tethering to vascular cell adhesion molecule 1 under shear. However, EF-hand mutants did show severe impairments in cellular resistance to detachment under shear flow. Thus, mutation of integrin α4 “EF-hand-like” sites may impair 1) static cell adhesion and 2) adhesion strengthening under shear flow by a mechanism that does not involve alterations of initial ligand binding.

INTRODUCTION

The importance of cell adhesion mediated by members of the integrin family has been amply demonstrated in the context of development (Yang et al., 1993,1995; Fässler et al., 1995), platelet (Smyth et al., 1993) and leukocyte (Hemler, 1990; Springer, 1990) functions, tumor growth and metastasis (Brooks et al., 1994; Giancotti and Mainiero, 1994), and in many other areas of cell biology (Hynes, 1992). Subsequent to ligand binding and integrin clustering (Isenberg et al., 1987), there is a major reorganization of cytoskeletal proteins and associated signaling molecules (Hynes, 1992; Miyamoto et al., 1995). Thus, integrin-mediated cell adhesion can modulate vital cellular signaling pathways (Schlaepfer et al., 1994; Vuori and Ruoslahti, 1994), leading to regulation of gene expression, cell growth (Damsky and Werb, 1992; Juliano and Haskill, 1993), and programmed cell death (Meredith et al., 1993; Boudreau et al., 1995).

Specific integrin extracellular domains involved in ligand binding have been located (D’Souza et al., 1988; Smith and Cheresh, 1988; D’Souza et al., 1990; Smith and Cheresh, 1990; Diamond et al., 1993), mutated (Loftus et al., 1990; Takada et al., 1992; Michishita et al., 1993; Bajt et al., 1995), expressed as functional fusion proteins (Bergelson et al., 1994; Kern et al., 1994; Randi and Hogg, 1994; Zhou et al., 1994; Kamata and Takada, 1995), and crystallized (Lee et al., 1995). Within the integrin αIIb chain, putative “EF-hand”-type divalent cation-binding sites have been suggested to directly contact ligand (D’Souza et al., 1991; Gulino et al., 1992). However, a recently suggested β-propeller model
shows putative EF-hand-like sites on the face of the α-chain opposite to the proposed ligand-binding site (Springer, 1997).

To analyze ligand binding, we have chosen to study the α4β1 (VLA-4) integrin. The α4 integrins facilitate activation and recruitment of many leukocytes to sites of inflammation (Lobb and Hemler, 1994) and also play important roles in myogenesis (Rosen et al., 1992), melanoma metastasis (Qian et al., 1994), and hematopoiesis (Williams et al., 1991). In shear flow, α4 integrins α4β1 (Alon et al., 1995b) and α4β7 (Berlin et al., 1995) mediate initial adhesive interactions (tethering), followed by rolling adhesions of leukocytes on their respective ligands, vascular cell adhesion molecule 1 (VCAM-1) and MadCAM-1. Ligands for α4β1 include VCAM-1 present on activated endothelium (Elices et al., 1989; Rice et al., 1990; Schwartz et al., 1990) and the alternatively spliced CS1 region of fibronectin (Wayner et al., 1989; Guan and Hynes, 1990; Ferreira et al., 1990). Mouse embryos lacking α4 failed to undergo fusion of the allantois with the chorion during placentation and also failed to develop epicardium and coronary vessels (Yang et al., 1995), thus proving conclusively the in vivo relevance of α4 integrins.

The α4 subunit contains three putative EF-hand-like sites, but no I-domain (Takada et al., 1989), and the β1 subunit may contain a single cation-binding “MIDAS” motif analogous to that seen in an I-domain (Lee et al., 1995). A prototype EF-hand motif contains 12 amino acids, with oxygen-containing residues at positions 1, 3, 5, 9, and 12 providing coordination sites for divalent cations (Strynadka and James, 1989). The EF-hand-like motifs found within all integrin α chains lack the position 12 coordination site, but nonetheless appear to bind divalent cations (D’Souza et al., 1991; Gulino et al., 1992). These sites have been difficult to study in the context of an intact integrin, because mutations within or nearby often cause loss of integrin expression (Masumoto and Hemler, 1993; Wilcox et al., 1995). However, conservative mutations could be made (at position 3) within each of the three α4 EF-hand-like divalent cation sites while still retaining expression (Masumoto and Hemler, 1993). These mutations had pronounced negative effects on cell adhesion that were assumed to result from diminished ligand binding (Masumoto and Hemler, 1993). Now that it has become feasible to analyze direct ligand binding to α4 integrins (Jakubowski et al., 1995; Yauch et al., 1997), we show here that mutations of α4 EF-hand-like sites each had no effect on soluble bivalent or monovalent ligand binding, and did not alter cell tethering to VCAM-1 under shear flow. Nonetheless, they greatly reduced adhesion strengthening under shear. These results suggest that extracellular integrin EF-hand sites regulate cell adhesion by a mechanism independent of ligand binding.

MATERIALS AND METHODS

Antibodies, Cells, and Integrin Ligand Proteins

Monoclonal antibodies utilized were anti-α4, B-5G10 (Hemler et al., 1987), A-PUJ1 (Pujades et al., 1996); anti-β1, A-1A5 (Hemler et al., 1983), TS2/16 (Hemler et al., 1984), monoclonal antibody (mAb) 13 (Akiyama et al., 1989), 9EG7 (Lenter et al., 1993), and the negative control antibodies P3 (Lemke et al., 1976) and J-2A2 (Hemler and Strominger, 1982). B-5G10 was directly conjugated to fluorescein isothiocyanate (FITC, Pierce, Rockford, IL) according to the manufacturer’s instructions. Antihuman IgG FITC-conjugated was purchased from Sigma (St. Louis, MO) and an antihuman Fc receptor (CD32) antibody was previously generated in our laboratory.

The CS-1 peptide (GPEILDVPST) derived from fibronectin was synthesized at the Dana-Farber Molecular Biology Core Facility. Purified VCAM-mouse Cκ fusion protein (VCAM-1-k), and a rat monoclonal antibody to mouse Cκ were obtained from Dr. Philip Lake (Sandoz Co., East Hanover, NJ). Briefly, VCAM-1-k was produced as a soluble protein from sf9 cells containing all seven human VCAM domains, except that the transmembrane and cytoplasmic portions of domain 7 have been replaced by a 100 aa mouse Cκ segment. Recombinant soluble VCAM-1 (rsVCAM-1) and a bivalent human VCAM-1 fusion protein (VCAM-1-lg) were prepared as described elsewhere (Lobb et al., 1991; Jakubowski et al., 1995). Conjugation of (VCAM-1-lg) with alkaline phosphatase (AP) was performed using conventional methods, and the resulting reagent (VCAM-1-lg-AP) was greater than 95% crosslinked and fully functional (Lobb et al., 1995).

Site-directed mutagenesis was utilized to produce α4 cDNA containing alterations within three putative divalent cation-binding domains as described previously (Masumoto and Hemler, 1993). Transfected K562 cells were enriched for α4-positive cells by immunomagnetic bead selection (Dynal Co., New York, NY) using the anti-α4 mAb B-5G10. All K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics, with 2.0 mg/ml G418 (Life Technologies, Gaithersburg, MD) also included for transfected cells.

Monovalent VCAM-1-k Cell-binding Assays

Cells were washed with 5 mM EDTA in Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl) and then incubated with increasing amounts of VCAM-1-k in the presence of 1 mM MnCl2 for 30 min at 4°C. Unbound VCAM-1-k was removed by two washes (also in the presence of 1 mM MnCl2), and then cells were incubated with FITC-conjugated goat anti-mouse κ affinity-purified antibody (Caltag Co., San Francisco, CA) for 30 min at 4°C, washed, and fixed with 2% paraformaldehyde. Binding of VCAM-1-k (Pujades et al., 1996) was quantitated using a FACScan machine (Becton Dickinson Co., Mountainview, CA), and at least 3000–5000 cells were analyzed for each mean fluorescence intensity (MFI) determination. Background binding, obtained by incubating cells with VCAM-1-k in the presence of 5 mM EDTA, was subtracted and was typically not more than 5–10% of the maximum total MFI units obtained. VCAM-1-k isolated from a Sephadex G-50 superfine column was utilized to assure that it was nonaggregated.

CS1 Peptide Cell-binding Assays

Aliquots of 1.5 × 105 cells were washed in TBS and then incubated with CS1 peptide (0–2 mM) for 15 min at 37°C in TBS containing 5% bovine serum albumin (BSA). 0.1 mM MnCl2, and 0.02% sodium azide. Then, to detect a CS1 ligand-induced conformational change inM Tris-HCl (pH 7.4), cells were incubated for 30 min at 4°C with mAb 9EG7 (~2 μg/ml), washed twice with TBS containing 2% BSA and 0.02% sodium azide, and then incubated for 30 min at 4°C with FITC-conjugated goat anti-rat IgG (Sigma). Finally, cells were washed twice and analyzed using a FACScan.
Distinct Integrin Cation and Ligand Sites

Indirect Bivalent (VCAM-1)$_2$-Ig Cell-binding Assays

Aliquots of 1.5 x 10$^5$ cells were preincubated for 15 min at 4°C in TBS containing 5% BSA, 2% human serum, and 5 μg/ml of mouse anti-human Fc receptor (CD32) mAb to block nonspecific antibody binding. Cells were then incubated with (VCAM-1)$_2$-Ig for 30 min at 4°C in TBS containing 2 mM MnCl$_2$, washed twice by suspension in TBS/MnCl$_2$, and finally incubated with FITC-anti-human IgG (Sigma) for 30 min at 4°C (in TBS/MnCl$_2$) before analysis of at least 3000–5000 cells using a FACScan machine (Becton Dickinson). Sodium azide (at 0.02%) was included throughout to prevent ligand and integrin internalization.

Theoretical analysis of the interaction of a bivalent ligand with a monovalent receptor has been described elsewhere (Perelson and DeLisi, 1980) and adapted recently to the binding of (VCAM-1)$_2$-Ig to α4β1 (Jakubowski et al., 1995). Briefly, both monovalent and bivalent binding may occur yielding α4β1/VCAM-Ig and (α4β1)$_2$/VCAM-Ig complexes, respectively, with K$_1$ defining the monovalent binding constant, and K$_2$ defining the conversion of monovalent to bivalent binding. With increasing ligand concentrations, a bell-shaped binding curve can be obtained, provided that suitably stringent washing conditions select only for bivalently bound (VCAM-1)$_2$-Ig. The (VCAM-1)$_2$-Ig concentration at which the peak MFI value is achieved for a given bell curve defines the dissociation constant for monovalent α4β1/VCAM-Ig complexes, K$_1$. The washing procedure described above was shown previously to yield bell-shaped curves, consistent with predominantly bivalent binding of (VCAM-1)$_2$-Ig (Jakubowski et al., 1995). Furthermore, this method was used successfully to estimate the affinity of α4β1/ligand interactions, as modulated by Mn$^{2+}$ and mAb TS2/16 (Jakubowski et al., 1995).

Direct Bivalent (VCAM-1)$_2$-Ig Cell-binding Assays

A high sensitivity direct cell-binding assay using AP-coupled VCAM-1-Ig has been described (Lobb et al., 1995). Briefly, 96-well filtration plates (Millipore, Bedford, MA) were preincubated for 1 h at 25°C with phosphate-buffered saline containing 1% BSA and 0.1% Tween 20, drained, using a vacuum manifold, and then washed twice with assay buffer (TBS containing 0.1% BSA, 2 mM glucose, and 10 mM HEPES). Then after 10$^5$ cells were incubated for 1 h at 4°C with increasing amounts of (VCAM-1)$_2$-Ig-AP in the presence of 2 mM MnCl$_2$, the plate was drained and washed twice rapidly with assay buffer containing 2 mM MnCl$_2$. Then alkaline phosphatase substrate (10 mg/ml 4-nitrophenyl phosphate in 0.1 M glycine, 1 mM ZnCl$_2$, and 1 mM MgCl$_2$ at pH 10.5) was added for 25 min at 25°C. After the reaction was stopped with 3 M NaOH, the OD at 405 nm was determined. Background values obtained in the absence of (VCAM-1)$_2$-Ig-AP (typically = 0.5 OD) were subtracted, and the mean of triplicate determinations (± SD) is presented.

Cell Adhesion

Cell adhesion was performed as described previously (Masumoto and Hemler, 1993; Yauch et al., 1997). Briefly, BCECF-AM (Molecular Probes, Eugene, OR) labeled cells were added to 96-well plates previously coated overnight with rsVCAM-1 and blocked with 0.1% heat-denatured BSA for 45 min at 37°C. Plates were centrifuged at 500 rpm for 2 min and analyzed in a Cytofluor 2300 measurement system (Millipore Corp.) to determine total cellular fluorescence. Plates were incubated for an additional 15 min at 37°C, washed three to four times with adhesion media, and fluorescence was reanalyzed to determine the fraction of cellular fluorescence remaining. Background binding to heat-denatured BSA alone was typically <5% and was subtracted from experimental values. Data are expressed as the mean of triplicate determinations ± SD.

RESULTS

Synthesis of Mutated α4 Integrins

Conservative mutations within three different putative divalent cation-binding domains in the α4 subunit were prepared and expressed as described previously (Masumoto and Hemler, 1993). Wild-type α4 and mutant α4 proteins (designated N283E, D346E, and D408E) were present at the surface of K562 cells at comparable levels as detected by flow cytometry using mAb B-SG10 and goat anti-mouse secondary antibody (Figure 1). Synthesis of α4 (middle column) was nearly...
equivalent to expression of \( \alpha_4 \) (right column), indicating that \( \alpha_4 \beta_1 \) (VLA-4) is the major \( \beta_1 \) integrin synthesized on these cells. Additional staining with directly conjugated FITC-B5G10 mAb confirmed that wild-type \( \alpha_4 \) and the N283E, D346E, and D408E mutants were synthesized at comparable levels. Mock-transfected and untransfected K562 cells showed no detectable \( \alpha_4 \) synthesis. The N283E, D346E, and D408E mutations caused minimal disruption of the overall \( \alpha_4 \beta_1 \) structure. As previously shown (Masumoto and Hemler, 1993), these mutant \( \alpha_4 \) proteins retained identical levels of three \( \alpha_4 \) epitopes (called A, B, and C) that were mapped to \( \alpha_4 \) regions flanking the N283E, D346E, and D408E mutation sites (Kamata et al., 1995; Schiffer et al., 1995).

**Binding of Soluble Ligands to K562-\( \alpha_4 \) Transfectants**

Here, we first utilized a monovalent soluble VCAM-1 fusion protein (VCAM-1-k) to generate comparable binding curves for transfectants synthesizing wild-type \( \alpha_4 \) and the N283E, D346E, and D408E mutants (Figure 2A). This binding was dependent on \( \text{Mn}^{2+} \), and no binding was observed in the presence of \( \text{Ca}^{2+} \) or \( \text{Mg}^{2+} \), in agreement with previous soluble VCAM-1 binding results (Jakubowski et al., 1995; Lobb et al., 1995). Also, VCAM-1-k did not bind to nontransfected K562 cells, confirming specificity for \( \alpha_4 \beta_1 \). Although we refer to soluble VCAM-1-k as “monovalent,” it potentially contains two binding sites for \( \alpha_4 \beta_1 \) (VCAM domains 1 and 4; Osborn et al., 1992; Vonderheide and Springer, 1992). However, for the assay time (30 min) and temperature (4°C) utilized, domain 4 is not expected to make much of a contribution (Needham et al., 1994).

The CS1 peptide, in the presence of 0.1 mM \( \text{Mn}^{2+} \), gives a dose-dependent induction of the mAb 9EG7 epitope on the integrin \( \beta_1 \) subunit (Pujades et al., 1996). By this detection method, wild-type \( \alpha_4 \) (in KA4 cells), and all three \( \alpha_4 \) mutants, bound comparable levels of CS1 peptide titrated from 0 to 2 mM (Figure 2B). This binding was \( \alpha_4 \beta_1 \) dependent, since CS1 did not induce the 9EG7 epitope on untransfected K562 cells. Also, CS1 peptide induction of the 9EG7 epitope was blocked by the anti-\( \alpha_4 \) blocking mAb A4-PUJ1 (our unpublished observations). In experiments reported elsewhere, a pair of \( \alpha_4 \) cysteine mutants showed diminished binding of both VCAM-1-k and CS1 peptide (Pujades et al., 1996), confirming that the methods utilized here are indeed capable of detecting alterations in ligand binding.

**Binding of Bivalent (VCAM-1)\( _2 \)-Ig to K562 Transfectants**

To assess further the effects of \( \alpha_4 \) mutations on ligand binding, a bivalent (VCAM-1)\( _2 \)-Ig chimeric protein was utilized, which contains the first two domains of human VCAM-1 fused to part of the human IgG1 heavy chain (Jakubowski et al., 1995). As described in MATERIALS AND METHODS, titration of cells with (VCAM-1)\( _2 \)-Ig, accompanied by sufficient washing (to remove the monovalent component), should yield a

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**Figure 2.** Indirect analysis of soluble monovalent ligand binding. Cells were tested for binding by VCAM-1-k (A) or CS1 peptide (B). For VCAM-1-k binding, cells were incubated for 30 min at 4°C, with increasing amounts of VCAM-1-k, in the presence of 1 mM MnCl\( _2 \), and then stained with FITC-antimouse \( \kappa \) chain antibody and analyzed by flow cytometry. For each experiment, VCAM-k binding in the presence of 5 mM EDTA was used to obtain the background signal (typically <5–10% relative to the maximal signal), which was then subtracted. For this experiment, fluorescence intensity (MFI units) due to VCAM-1-k binding were corrected by a factor of 1.3 for the D346E and D408E mutants, because the surface level of wild-type \( \alpha_4 \) was 1.3-fold greater than that of the mutants. The level of CS1 peptide binding was determined indirectly by measuring induction of the 9EG7 epitope, as described in MATERIALS AND METHODS. The 9EG7 epitope expression results are presented as a fraction of the total \( \beta_1 \) subunit (measured using mAb 13).
bell-shaped curve (representing bivalent binding) in which the peak of the bell curve corresponds to the monovalent dissociation constant ($K$). Indeed, titration of our K562 transfectants did yield bell-shaped curves, with apices in the vicinity of 100 nM in all cases (Figure 3). Notably, determination of $K$ is independent of differences in peak height that result from up to twofold variation in $a$ levels. Also, the binding was completely $a$ dependent, since $(VCAM-1)_2$ bound negligibly to nontransfected K562 cells (our unpublished observations).

In Figure 3, $(VCAM-1)_2$ binding was measured indirectly using a FITC-conjugated antihuman Ig reagent. In another experiment (Figure 4), binding was assessed directly by utilizing an alkaline phosphatase-coupled $(VCAM-1)_2$-AP reagent and a filter aspiration method for rapid, efficient removal of unbound ligand (Lobb et al., 1995). Again, three different K562 transfectants yielded similar bell-shaped curves; this time each with apices of $\sim$10 nM. In a control experiment, only a low level of background binding was seen for untransfected K562 cells (typically <0.1 OD unit). Together these results suggest that the apparent dissociation constant for monovalently bound $(VCAM-1)_2$-Ig in a 1:1 complex with a single receptor molecule is essentially identical for the wild-type and mutant $a$ levels were not more than twofold.

In a previous study, titration of Mn$^{2+}$ revealed pronounced deficiencies in adhesion to VCAM-1 mediated by the N283E, D346E, and D408E mutants (Matsumoto and Hemler, 1993). Here, ligand binding was measured over a wide range of Mn$^{2+}$ concentrations, with soluble $(VCAM-1)_2$-AP held constant at the suboptimal dose of 4 nM. As shown in two separate experiments (Figure 5A and 5B), there were no consistent differences between $(VCAM-1)_2$-AP binding to wild-type $a$ and mutants D346E or D408E. When Mn$^{2+}$ was held constant at 0.1 mM, binding of $(VCAM-1)_2$-Ig-AP (incubated at 4 nM) was again not diminished for any of the mutants compared with wild-type $a$ (Figure 5D). Binding to mutant D346E was a little elevated in Figure 5D, but this appears to represent experimental variation rather than a conclusive result. Whereas Mn$^{2+}$ supported ligand binding to wild-type and mutant $a$ to a similar extent (Figure 5, A, B, and D), a static cell adhesion assay revealed a pronounced difference in Mn$^{2+}$ effects (Figure 5C). To obtain comparable levels of cell adhesion, approximately 10-fold more Mn$^{2+}$ was required for D408E as compared with wild-type $a$. This cell adhesion result obtained using D408E (Figure 5C) essentially confirms
the greater requirement for Mn\(^{2+}\) that was seen previously for cell adhesion by all three cation site mutants (Masumoto and Hemler, 1993).

\(\alpha 4\beta 1\)-Dependent Tethering and Adhesion Strengthening on VCAM-1

To determine whether the N283E, D346E, and D408E \(\alpha 4\) mutations can affect \(\alpha 4\beta 1\)-dependent cell tethering under flow conditions, the various K562 transfecteds were perfused into a flow chamber containing rsVCAM-1 immobilized at different densities, and tethering was monitored. Wild-type \(\alpha 4\) and the \(\alpha 4\) mutants showed comparable tethering efficiencies (in 1 mM MgCl\(_2\), 2 mM CaCl\(_2\)) regardless of whether rsVCAM-1 was present at intermediate (Figure 6A) or high (Figure 6, B and C) density. When these same experiments were carried out in the presence of 0.1 mM Mn\(^{2+}\) and low-density rsVCAM-1, again there were no consistent differences in tethering (Figure 6D).

Although the initial cell attachment to rsVCAM-1 was not affected by the N283E, D346E, and D408E \(\alpha 4\) mutations, subsequent adhesion strengthening was markedly altered. Transfected K562 cells were allowed to interact (tether) to rsVCAM-1 in low shear flow (i.e., 0.30–0.45 dynes/cm\(^2\)). Then, after an average of ~50 tethered cells had accumulated during a 15- to 30-s interval, the shear stress was incrementally elevated, and adherent cells were tested for ability to resist increasing detaching shear forces. Compared with the N283E, D346E, or D408E transfecteds, the KA4 cells showed much higher resistance to detaching shear forces (Figure 7A–C). Remarkably, on high-density rsVCAM-1 (Figure 7B), when 80% of initially tethered mutant cells had detached from the substrate (at 2

![Figure 5. Mn\(^{2+}\) effects on binding of (VCAM-1)_2-Ig-AP. Cells were incubated with 4 nM (VCAM-1)_2-Ig-AP, and \(\alpha 4\)-specific binding was determined as described in the legend to Figure 4. (A and B) Wild-type \(\alpha 4\) was synthesized at comparable levels to D408E (A) and D346E (B), and in two separate experiments binding was determined over a range of MnCl\(_2\) concentrations. (C) Cell adhesion to VCAM-1, coated at 2 \(\mu\)g/ml, was measured for KA4 and D408E cells at various MnCl\(_2\) concentrations. (D) Binding of (VCAM-1)_2-Ig-AP was carried out in 0.1 mM MnCl\(_2\). Results were normalized relative to wild-type \(\alpha 4\) binding (given an arbitrary value of 1.0). Each point represents the mean of two to three experiments. Typical OD\(_{405}\) values for specific (VCAM-1)_2-Ig-AP binding ranged from 0.5 to 1.0. Also, data in D were adjusted for differences in \(\alpha 4\) surface levels, which varied from each other by not more than 1.5-fold.](link)
blood T cells in the presence of Mn$^{2+}$ (30 nM) for VCAM-1 binding to peripheral levels (100 nM, 10 nM) are both within range of a published value. Notably, our two estimated VCAM-1-binding constants ($100 \text{nM}, 10 \text{nM}$) are both within range of a published value ($30 \text{nM}$) for VCAM-1 binding to peripheral blood T cells in the presence of Mn$^{2+}$ (Jakubowski et al., 1995).

**DISCUSSION**

**Ligand-binding Similarities**

In previous studies (Masumoto and Hemler, 1993), the N283E, D346E, and D408E mutations caused a substantial decrease in cell adhesion to VCAM-1 and an even greater decline in adhesion to immobilized CS1 peptide. At that time it was assumed that diminished adhesion was due to decreased ligand binding. Indeed, results elsewhere suggest that EF-hand sites within the integrin $\alpha$IIb chain may be directly involved in fibrinogen binding (DSouza et al., 1991; Gulino et al., 1992). With the current results we now demonstrate that the binding of soluble ligands is not altered upon mutation of putative EF-hand sites in $\alpha 4\beta 1$. Wild-type $\alpha 4$ and the N283E, D346E, and D408E mutants each showed similar binding to monovalent and bivalent VCAM-1, in both indirect and direct binding assays, and also showed similar binding to CS1 peptide. Our indirect binding assay using bivalent VCAM-1 yielded a monovalent VCAM-1 binding constant of $\sim 100 \text{nM}$ for both wild-type and mutant $\alpha 4\beta 1$. In comparison, our direct bivalent VCAM-1-binding assay yielded a monovalent-binding constant of $\sim 10 \text{nM}$ for wild-type and mutant $\alpha 4\beta 1$. To explain this discrepancy, we surmise that the more prolonged washing steps in the indirect assay may have eluted some bound ligand, especially at lower doses, and thus shifted the ligand dose curve to the right. Notably, our two estimated VCAM-1-binding constants ($100 \text{nM}, 10 \text{nM}$) are both within range of a published value ($30 \text{nM}$) for VCAM-1 binding to peripheral blood T cells in the presence of Mn$^{2+}$ (Jakubowski et al., 1995).

Integrins each may have four to six putative divalent cation-binding sites, with ligands binding in the vicinity of these sites on both the $\alpha$ and $\beta$ chains (Loftus et al., 1994). Based on the crystal structure of an integrin $\alpha$-chain I-domain (Lee et al., 1995) and sequence comparisons with other proteins (Tuckwell et al., 1992), it seems that integrin divalent cation domains are all missing at least one coordination site, which could be provided by ligand. However, it has been difficult to visualize how a single ligand could provide missing coordination residues for four to six divalent cations, and it also seems unlikely that a single integrin would bind four to six ligands. Notably, the recently proposed $\beta$-propeller model places putative EF-hand sites on the opposite face of the molecule, away from ligand contact sites (Springer, 1997). The current results now reinforce the idea that $\alpha$-chain EF-hand-like sites may in fact not directly bind ligand.

The N283E, D346E, and D408E mutations also had minimal impact on K562 cell tethering to immobilized VCAM-1 under shear. Lack of an effect on tethering is consistent with no effect on ligand binding, because tethering, like ligand binding, primarily involves univalent interactions. In this regard, lipid bilayers containing P-selectin at a density below that required to support rolling yielded transient neutrophil tethers.
that dissociated with first-order kinetics, suggestive of univalent bonds (Alon et al., 1995a).

In earlier experiments, the N283E, D346E, and D408E mutants showed deficiencies in static cell adhesion to CS1 peptide or fibronectin, regardless of whether Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$ was present (Masumoto and Hemler, 1993). Also, deficiencies in adhesion to VCAM-1 were observed in the presence of Ca$^{2+}$ plus Mg$^{2+}$, or Mn$^{2+}$, and over a range of Mn$^{2+}$ concentrations. In contrast, the present experiments show no difference in tethering to VCAM-1 in the presence of Ca$^{2+}$ plus Mg$^{2+}$, or Mn$^{2+}$, and no consistent difference in ligand binding to CS1 peptide (in 0.1 mM Mn$^{2+}$) or to VCAM-1 (in 2 mM Mn$^{2+}$, 0.1 mM Mn$^{2+}$, or over a range of Mn$^{2+}$ levels). Previously it was shown that 6- to 10-fold more Mn$^{2+}$ was required to achieve half-maximal cell adhesion by the N283E, D346E, and D408E mutants compared with wild-type α4 (Masumoto and Hemler, 1993). That result has been confirmed here using the D408E mutant (Figure 5C). In sharp contrast, there was essentially no difference in the levels of Mn$^{2+}$ required to support half maximal binding of soluble VCAM-1 to mutant and wild-type α4 (Figure 5, A and B). Because there were essentially no consistent ligand binding or tethering differences between mutant and wild-type α4, under any conditions tested, we conclude that differences in static cell adhesion must involve something other than altered ligand binding.

### Differences in Adhesion Strengthening

Although their ligand binding and tethering functions were not impaired, the α4β1 mutants showed a striking loss of resistance to detaching forces produced by high shear flow. In contrast, wild-type α4β1 resisted detachment, either from VCAM-1 (after cells had accumulated in low flow) or from FN40 (after a short period of static adhesion). Notably, mutant α4 integrins showed markedly deficient adhesion strengthening under the same cation conditions (1 mM Mg$^{2+}$, 2 mM Ca$^{2+}$) in which tethering was unaltered. Also in 0.1 mM Mn$^{2+}$, adhesion strengthening was greatly impaired for the mutant α4 integrins, whereas tethering and ligand binding were minimally altered.

Thus, it is now clear that adhesion strengthening, rather than ligand binding, is likely to be the critical parameter that causes diminished static cell adhesion...
as seen in the previous study (Masumoto and Hemler, 1993) and confirmed here. These results further emphasize that although cell adhesion and ligand binding are often regulated in parallel, the former is a multistep event subject to much more complex regulation. Furthermore, static adhesion may have an important adhesion strengthening component that can be regulated independent of ligand binding. The α4 EF-hand sites I, II, and III were all essential for adhesion strengthening, with no combination of only two sites being sufficient. Thus, these three α4 sites may act together as a functional unit. In this regard, a conservative mutation within one of the four EF-hand loops in troponin c caused 75% reduction in function (Babu et al., 1992).

In several respects, results obtained here for α4β1 EF-hand mutants parallel the results obtained previously for α4 cytoplasmic tail deletion and exchange mutants. If the α4 cytoplasmic domain was deleted or exchanged, adhesion strengthening was either reduced or increased, respectively, without altering tethering under shear (Alon et al., 1995b; Kassner et al., 1995) or ligand binding (Weitzman et al., 1997; Yauch et al., 1997). In the case of α4 tail deletion, a decrease in lateral diffusion may lead to diminished integrin assembly into clusters and thus diminished cell adhesion (Yauch et al., 1997). However, in the current studies neither constitutive α4β1 clustering nor ligand-induced clustering were obviously altered as determined by confocal microscopy (our unpublished observations). Thus, EF-hand-like sites may modulate adhesion strengthening by a mechanism somewhat different from integrin cytoplasmic domains. One possibility is that lateral interactions with other proteins may require α-chain EF-hand sites. In this regard, CD81 (a transmembrane-4 superfamily member), showed diminished associations with α4 D346E and D408E mutants, but retained association with α4 cytoplasmic tail mutants (Mannion et al., 1996). At present, it remains to be demonstrated whether altered associations with TM4SF proteins or any other transmembrane proteins may be responsible for reduced adhesion strengthening in the EF-hand mutants. Nonetheless, our results provide perhaps the first evidence that sites within integrin extracellular domains can regulate adhesion strengthening independent from ligand binding. Furthermore, these results, along with cytoplasmic tail results mentioned above, suggest that integrin extracellular and intracellular domains, in different ways, influence translation of ligand-binding events into subsequent adhesion strengthening events.

Integrin α-Chain Cation and Ligand-binding Sites

It was previously shown that a bacterial fusion protein containing 4 EF-hand-like divalent cation sites from the integrin α1β1 chain could bind to four molecules of calcium (Gulino et al., 1992). Elsewhere, position 3 within EF-hand loop II of troponin c was mutated from aspartate to glutamate, causing loss of both function and calcium binding (Babu et al., 1992). Thus, we assume that each of our EF-hand position 3 mutations (aspartate or asparagine to glutamate; N283E, D346E, and D408E) may also cause alterations in divalent cation binding. However, this point remains to be formally demonstrated and will require purification of α4β1 protein in large amounts sufficient to allow accurate assessment of a change in one cation site out of a likely total of four (one β and three α sites). At present it appears that cation binding to β1 was unaffected by our mutations, since there was no change in induction of the mAb 9EG7 epitope on the β1 subunit upon titration with Mn2+ (our unpublished results).

We have demonstrated here that integrin α-chain EF-hand-like sites can modulate cell adhesion and adhesion strengthening independent of ligand binding. Although our results suggest that EF-hand sites may not directly contact ligand, they nonetheless may be as important for integrin functions as direct ligand-binding sites. For example, it should be possible to design therapeutic agents distinct from standard ligand-binding antagonists that could inhibit integrin adhesion and signaling functions by acting at EF-hand sites. Finally, conclusions from this study should be applicable to the 15 other integrin α chains that each contain three to four similar EF-hand-like domains.

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REFERENCES


Distinct Integrin Cation and Ligand Sites


