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

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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

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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 $\alpha4\beta1$ (VLA-4) integrin. The $\alpha4$ 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, $\alpha4$ integrins $\alpha4\beta1$ (Alon *et al.*, 1995b) and $\alpha4\beta7$ (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 $\alpha4\beta1$ include VCAM-1 present on activated endothelium (Elices *et al.*, 1990; 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 $\alpha4$ 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 $\alpha4$ integrins.

The $\alpha4$ subunit contains three putative EF-hand-like sites, but no I-domain (Takada *et al.*, 1989), and the $\beta1$ 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 $\alpha4$ 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 $\alpha4$ integrins (Jakubowski *et al.*, 1995; Yauch *et al.*, 1997), we show here that mutations of $\alpha4$ 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-$\alpha4$, B-5G10 (Hemler *et al.*, 1987), A4-PUJ1 (Pujades *et al.*, 1996); anti-$\beta1$, 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.*, 1978) 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 C κ fusion protein (VCAM-1-k), and a rat monoclonal antibody to mouse C κ 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 C κ segment. Recombinant soluble VCAM-1 (rsVCAM-1) and a bivalent human VCAM-1 fusion protein $(VCAM\text{-}1)_2$-Ig were prepared as described elsewhere (Lobb *et al.*, 1991; Jakubowski *et al.*, 1995). Conjugation of $(VCAM\text{-}1)_2$-Ig with alkaline phosphatase (AP) was performed using conventional methods, and the resulting reagent $(VCAM\text{-}1)_2$-Ig-AP) was greater than 95% crosslinked and fully functional (Lobb *et al.*, 1995).

Site-directed mutagenesis was utilized to produce $\alpha4$ cDNA containing alterations within three putative divalent cation-binding domains as described previously (Masumoto and Hemler, 1993). Transfected K562 cells were enriched for $\alpha4\beta1$-positive cells by immunomagnetic bead selection (Dynal Co., New York, NY) using the anti-$\alpha4$ 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 $MnCl_2$ for 30 min at 4°C. Unbound VCAM-1-k was removed by two washes (also in the presence of 1 mM $MnCl_2$), 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-150 superfine column was utilized to assure that it was nonaggregated.

CS1 Peptide Cell-binding Assays

Aliquots of 1.5×10^5 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 $MnCl_2$, and 0.02% sodium azide. Then, to detect a CS1 ligand-induced conformational change in the $\beta1$ subunit (Pujades *et al.*, 1996), cells were incubated for 30 min at 4°C with mAb 9EG7 ($\sim$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

machine (Becton Dickinson, Oxnard, CA). For total β1 determinations, rat anti-human β1 mAb 13 was utilized.

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

Aliquots of 1.5×10^5 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 $\alpha4\beta1$ (Jakubowski *et al.*, 1995). Briefly, both monovalent and bivalent binding may occur yielding $\alpha4\beta1$/VCAM-Ig and $(\alpha4\beta1)_2$/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 $\alpha4\beta1$/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 $\alpha4\beta1$/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.

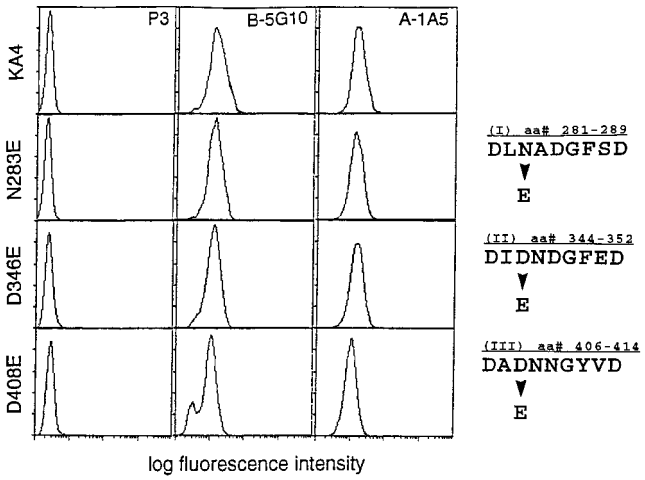

Figure 1. Flow cytometric analysis of $\alpha4$ and $\beta1$ present in K562 cells. Transfected K562 cells were stained using the control antibody P3, anti-$\alpha4$ MAb B-5G10, and anti-$\beta1$ MAb A-1A5, and cell surface levels were determined by flow cytometry. The sequences of the $\alpha4$ EF hand-like domains containing each mutation are indicated.

Laminar Flow Assays

Polystyrene dishes were coated with rsVCAM-1 (in PBS containing 10 mM $NaHCO_3$, pH 8.5) and quenched with human serum albumin as described previously (Alon *et al.*, 1995b). Coated dishes were assembled as the lower wall in a parallel wall flow chamber and mounted on the stage of an inverted phase-contrast microscope. K562 cells (200–400,000 cells/ml) resuspended in Hanks' balanced salt solution containing 10 mM HEPES (pH 7.4), 1 mM $MgCl_2$, and 2 mM $CaCl_2$, were perfused through the flow chamber at room temperature at different flow rates to obtain the indicated specific shear stresses at the chamber wall. Tethering was determined by counting, from videotape images, cells in a given field of view (0.43 mm^2) during the first 15–60 s of continuous flow (Alon *et al.*, 1995b; Kassner *et al.*, 1995). Complete inhibition by anti-$\alpha4$ blocking antibodies and lack of any interaction with surfaces coated with human serum albumin was verified at all shear stresses tested in this study. For controlled force detachment assays, tethered cells allowed to accumulate at low shear flow (0.30–0.45 dynes/cm^2) were subjected to increasing shear flows in controlled increments [20–50% increases, each lasting 10 s generated by a programmable syringe pump (Harvard Apparatus, Natick, MA)]. The number of cells remaining bound after each 10-s interval of shear was determined and expressed as a percentage of the initially accumulated cells tethered to rsVCAM-1 at the lowest shear conditions.

RESULTS

Synthesis of Mutated $\alpha4$ Integrins

Conservative mutations within three different putative divalent cation-binding domains in the $\alpha4$ subunit were prepared and expressed as described previously (Masumoto and Hemler, 1993). Wild-type $\alpha4$ and mutant $\alpha4$ 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-5G10 and goat anti-mouse secondary antibody (Figure 1). Synthesis of $\alpha4$ (middle column) was nearly

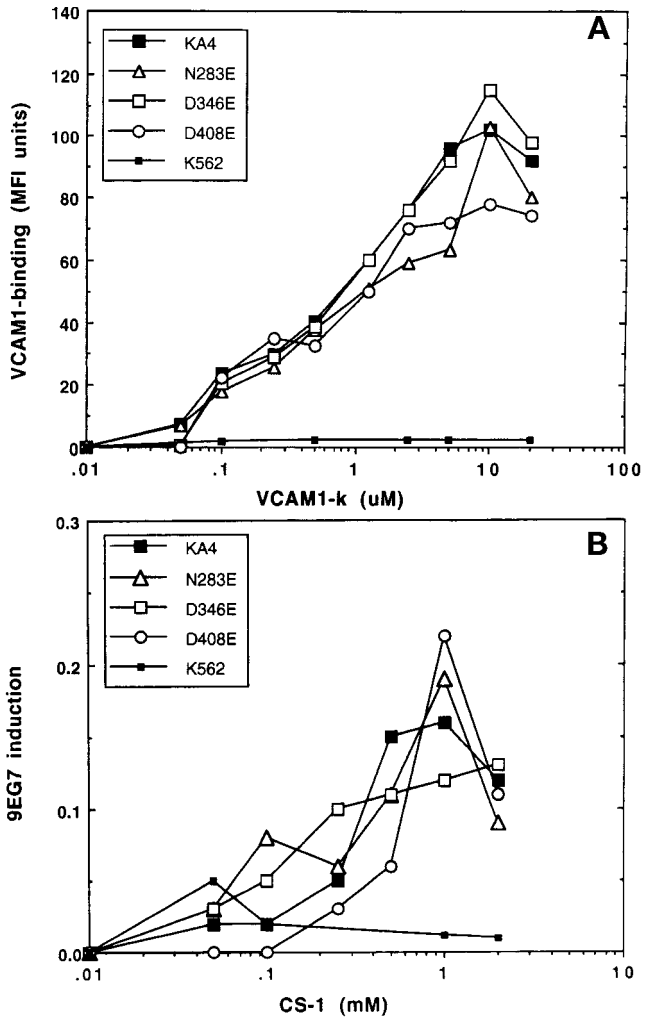

Figure 2. Indirect analysis of soluble monovalent ligand binding. Cells were tested for binding by VCAM-1-k (A) or CS1 peptide (B). For VCAM-1-k binding, cells were incubated for 30 min at 4°C, with increasing amounts of VCAM-1-k, in the presence of 1 mM $MnCl_2$, and then stained with FITC-antimouse κ 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 α4 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 β1 subunit (measured using mAb 13).

equivalent to expression of β1 (right column), indicating that α4β1 (VLA-4) is the major β1 integrin synthesized on these cells. Additional staining with directly conjugated FITC-B5G10 mAb confirmed that wild-type α4 and the N283E, D346E, and D408E mutants were synthesized at comparable levels. Mock-transfected and untransfected K562 cells showed no detectable α4 synthesis. The N283E, D346E, and D408E mutations caused minimal disruption of the overall α4β1 structure. As previously shown (Masumoto and Hemler, 1993), these mutant α4 proteins retained identical levels of three α4 epitopes (called A, B, and C) that were mapped to α4 regions flanking the N283E, D346E, and D408E mutation sites (Kamata *et al.*, 1995; Schiffer *et al.*, 1995).

Binding of Soluble Ligands to K562-α4 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 α4 and the N283E, D346E, and D408E mutants (Figure 2A). This binding was dependent on Mn^{2+}, and no binding was observed in the presence of Ca^{2+} or 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 α4β1. Although we refer to soluble VCAM-1-k as "monovalent," it potentially contains two binding sites for α4β1 (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 Mn^{2+}, gives a dose-dependent induction of the mAb 9EG7 epitope on the integrin β1 subunit (Pujades *et al.*, 1996). By this detection method, wild-type α4 (in KA4 cells), and all three α4 mutants, bound comparable levels of CS1 peptide titrated from 0 to 2 mM (Figure 2B). This binding was α4β1 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-α4 blocking mAb A4-PUJ1 (our unpublished observations). In experiments reported elsewhere, a pair of α4 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 $(VCAM\text{-}1)_2$-Ig to K562 Transfectants

To assess further the effects of α4 mutations on ligand binding, a bivalent $(VCAM\text{-}1)_2$-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 $(VCAM\text{-}1)_2$-Ig, accompanied by sufficient washing (to remove the monovalent component), should yield a

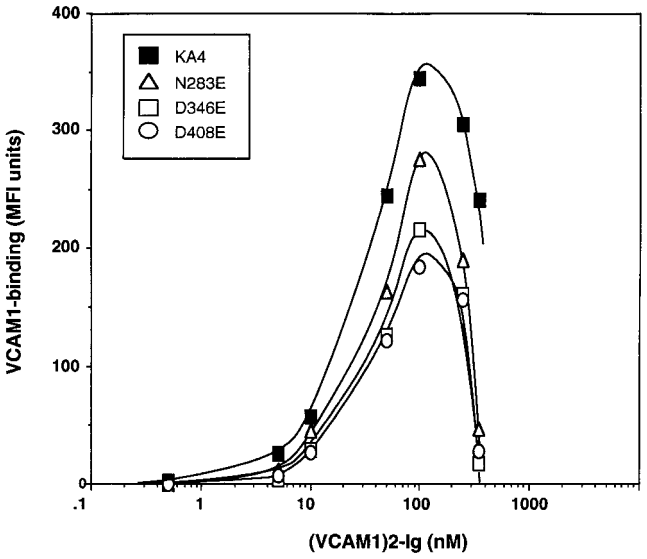

Figure 3. Indirect bivalent $(VCAM\text{-}1)_2$-Ig binding as a function of ligand concentration. Cells were incubated with increasing amounts of $(VCAM\text{-}1)_2$-Ig in the presence of 2mM $MnCl_2$. After washing, the cells were incubated with FITC-antihuman IgG and analyzed using a FACscan. Prior to incubation with the ligand, nonspecific binding was blocked with anti-Fc receptor antibodies and human serum. All experiments were done at 4°C and with 0.02% sodium azide to minimize receptor and/or ligand internalization. Background binding, determined in the presence of 5 mM EDTA, was subtracted (and was typically < 10 MFI units). At the time of this experiment, differences among wild-type and mutant $\alpha 4$ levels were not more than twofold.

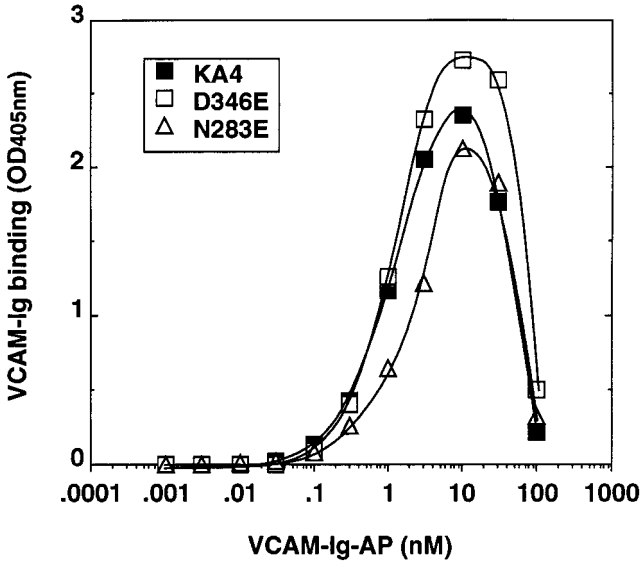

Figure 4. Direct bivalent binding of $(VCAM\text{-}1)_2$-Ig-AP to $\alpha 4\beta 1$. Cells were incubated with increasing amounts of $(VCAM\text{-}1)_2$-Ig-AP in the presence of 2mM $MnCl_2$. After washing, cells were incubated with AP substrate and color development was determined at OD 405 nm. Background binding to mock-transfected K562 cells (typically <0.1 OD units) was subtracted. At the time of this experiment, differences among cell surface levels of $\alpha 4$ wild-type, D346E, and N283E were not more than twofold.

bell-shaped curve (representing bivalent binding) in which the peak of the bell curve corresponds to the monovalent dissociation constant (K_{-1}). 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 $K_{-1} = \sim 100$ nM is independent of differences in peak height that result from up to twofold variation in $\alpha 4$ levels. Also, the binding was completely $\alpha 4\beta 1$ dependent, since $(VCAM\text{-}1)_2$-Ig bound negligibly to nontransfected K562 cells (our unpublished observations).

In Figure 3, $(VCAM\text{-}1)_2$-Ig 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 [$(VCAM\text{-}1)_2$-Ig-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 ~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 $(VCAM\text{-}1)_2$-Ig in a 1:1 complex with a single receptor molecule is essentially identical for the wild-type and mutant forms of $\alpha 4\beta 1$. Thus, these mutations do not affect the apparent intrinsic affinity of individual VCAM-1 domains for $\alpha 4\beta 1$.

In a previous study, titration of Mn^{2+} revealed pronounced deficiencies in adhesion to VCAM-1 mediated by the N283E, D346E, and D408E mutants (Masumoto and Hemler, 1993). Here, ligand binding was measured over a wide range of Mn^{2+} concentrations, with soluble $(VCAM\text{-}1)_2$-Ig-AP held constant at the suboptimal dose of 4 nM. As shown in two separate experiments (Figure 5,A and 5B), there were no consistent differences between $(VCAM\text{-}1)_2$-Ig-AP binding to wild-type $\alpha 4$ and mutants D346E or D408E. When Mn^{2+} was held constant at 0.1 mM, binding of $(VCAM\text{-}1)_2$-Ig-AP (incubated at 4 nM) was again not diminished for any of the mutants compared with wild-type $\alpha 4$ (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 $\alpha 4$ 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 $\alpha 4$. This cell adhesion result obtained using D408E (Figure 5C) essentially confirms

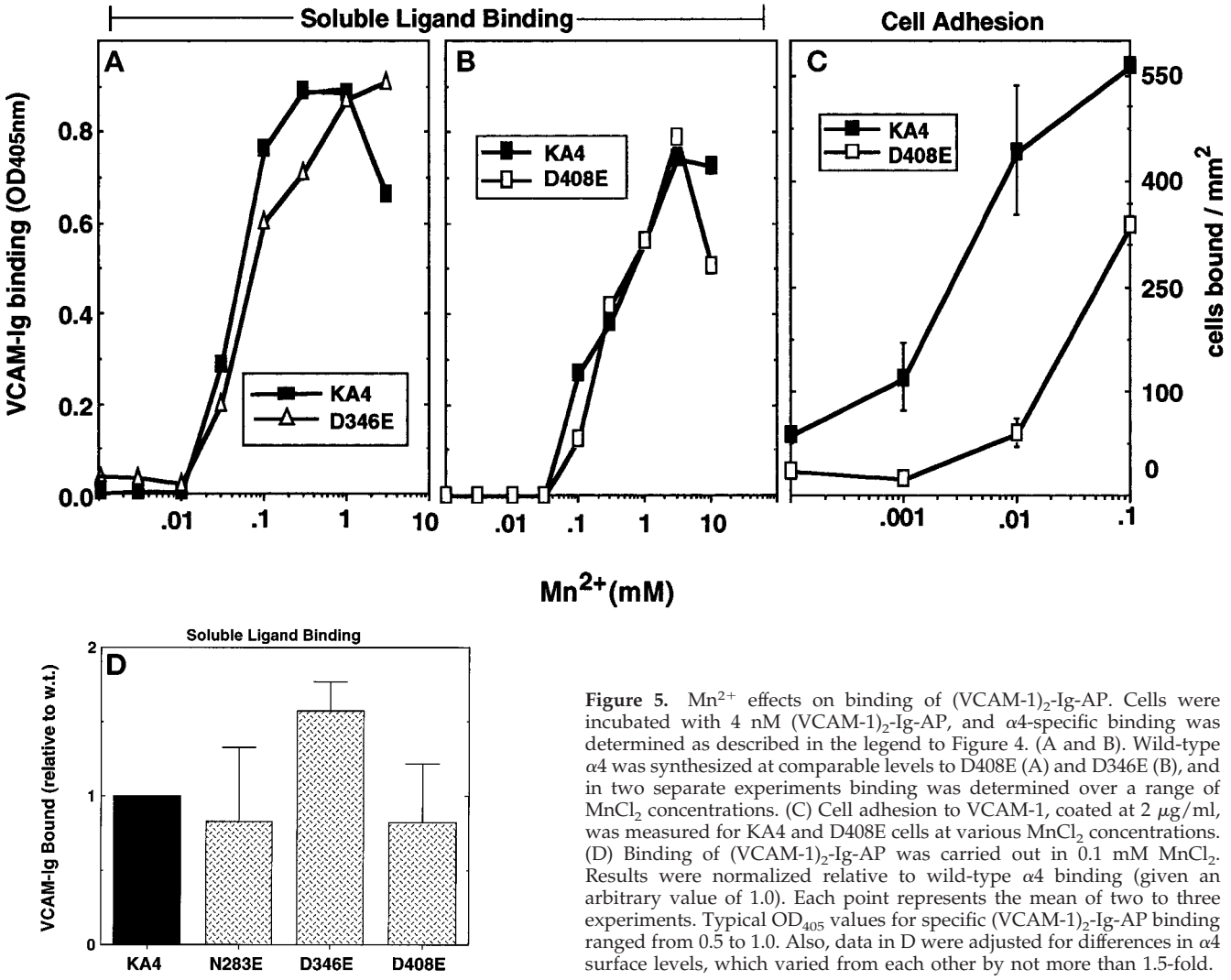

Figure 5. Mn^{2+} effects on binding of $(VCAM\text{-}1)_2$-Ig-AP. Cells were incubated with 4 nM $(VCAM\text{-}1)_2$-Ig-AP, and $\alpha 4$-specific binding was determined as described in the legend to Figure 4. (A and B). Wild-type $\alpha 4$ was synthesized at comparable levels to D408E (A) and D346E (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 μg/ml, was measured for KA4 and D408E cells at various $MnCl_2$ concentrations. (D) Binding of $(VCAM\text{-}1)_2$-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 $(VCAM\text{-}1)_2$-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.

the greater requirement for Mn^{2+} that was seen previously for cell adhesion by all three cation site mutants (Masumoto and Hemler, 1993).

α4β1-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 transfectants 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 transfectants, 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

dynes/cm^2), more than 90% of KA4 cells remained adherent. At intermediate rsVCAM-1 density, KA4 cells were less resistant to detaching forces, but were still substantially more resistant to detachment than the mutant transfectants (Figure 7A). At low-density rsVCAM-1, in the presence of 0.1 Mn^{2+} (instead of 1 mM Mg^{2+}, 1 mM Ca^{2+}), the KA4 cells were again more resistant to detachment compared with the mutant α4 transfectants (Figure 7C). If higher Mn^{2+} levels were utilized, differences in detachment resistance between mutant and wild-type cells became much less obvious. In another experiment (in 1 mM Mg^{2+}, 1 mM Ca^{2+}), transfected K562 cells were allowed 2 min of static contact with FN40 substrate, and then the shear stress was incrementally elevated. As indicated (Figure 7D), resistance to detaching shear forces was again markedly greater for KA4 cells compared with a representative mutant (N283E). These experiments (Figures 6 and 7) distinguish tethering and resistance to detachment (adhesion strengthening) as two distinct steps in α4β1-mediated adhesion, with only the latter affected by α-chain cation site mutations.

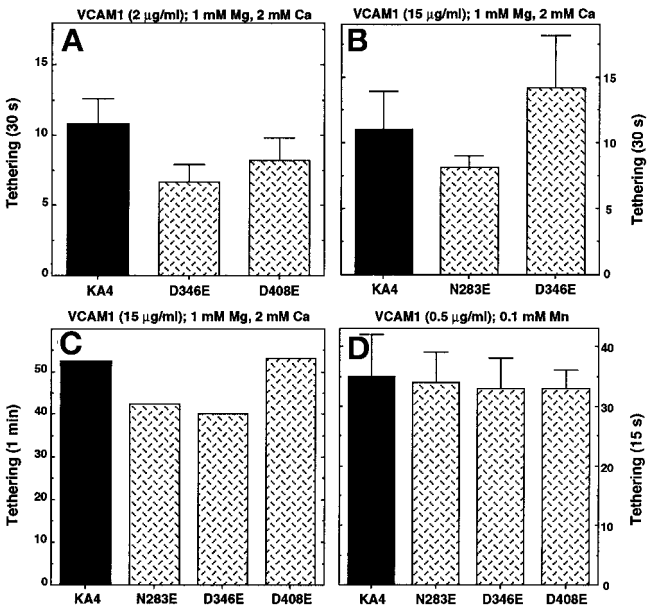

Figure 6. Tethering of K562 transfectants on rsVCAM-1. Into a parallel plate flow chamber, 2–5 $\times$ 10^5 cells were perfused at low wall shear stress (0.30–0.45 dyn/cm^2) and tethering to rsVCAM-1 was analyzed. The flow chamber was coated the indicated concentrations of rsVCAM. Experiments were carried out in 1 mM Mg^{2+}, 2 mM Ca^{2+} (A–C), or 0.1 mM Mn^{2+} (D). Tethering events were accumulated for 30 s (A and B), 1 min (C), or 15 s (D).

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 αIIb chain may be directly involved in fibrinogen binding (D'Souza *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 α4β1. Wild-type α4 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 ~100 nM for both wild-type and mutant α4β1. In comparison, our direct bivalent VCAM-1-binding assay yielded a monovalent-binding constant of ~10 nM for wild-type and mutant α4β1. 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 nM, 10 nM) are both within range of a published value (30 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 α and β chains (Loftus *et al.*, 1994). Based on the crystal structure of an integrin α-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 β-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 α-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

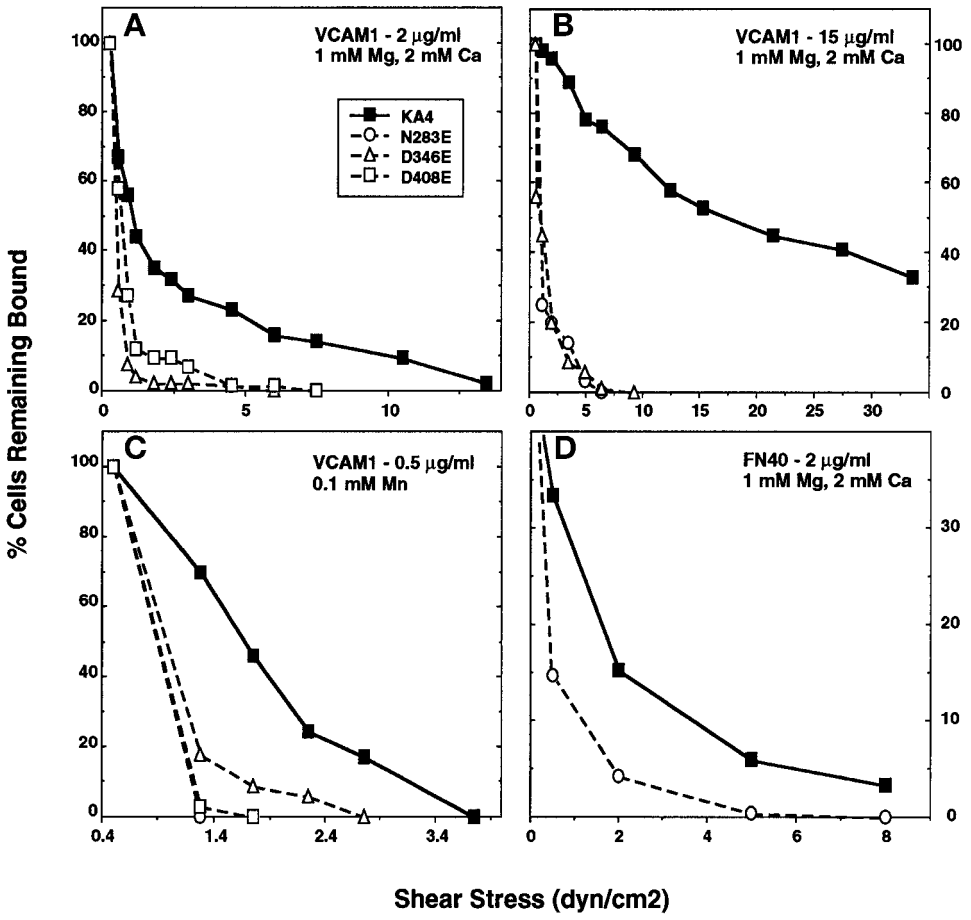

Figure 7. Shear resistance of K562 transfectants on rsVCAM-1 or FN40. Using initial conditions as in Figure 6, A, B, and D, an average of ~50 cells was allowed to accumulate in shear flow for 15 to 30 s on rsVCAM-1 coated at the indicated levels (A–C). Then cells were subjected to increasing shear stresses in small increments at 10-s intervals. After each 10-s interval, the percentage of cells remaining bound was determined at each shear. In D, cells were allowed to accumulate in static conditions for 2 min on FN40 (coated at 2 μg/ml). Then resistance to detachment was determined as for A–C. The values indicated in the detachment profiles (A, B, and D) are the mean of three to four experiments. Results in C are representative of four different experiments.

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+} 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 FN-40 (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 $\alpha4\beta1$ 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 $\alpha4\beta1$ 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 αIIb 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 $\alpha4\beta1$ 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 Mn^{2+} (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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