Mutation of Putative Divalent Cation Sites in the α^4 Subunit of the Integrin VLA-4: Distinct Effects on Adhesion to CS1/Fibronectin, VCAM-1, and Invasin

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Abstract. To investigate the functional significance of putative integrin divalent cation binding sites, several mutated α^4 subunit cDNAs were constructed. Mutants contained the conservative substitution of Glu for Asp or Asn at the third position in each of three putative divalent cation sites. Transfection of wild-type or mutated α^4 into K562 cells yielded comparable expression levels and immunoprecipitation profiles. However, for all three α^4 mutants, adhesion to CS1/fibronectin was greatly diminished in either the presence or absence of the stimulatory anti-β_1 mAb TS2/16. Constitutive adhesion to vascular cell adhesion molecule (VCAM) 1 was also diminished but, unlike CS1 adhesion, was restored upon TS2/16 stimulation. In contrast, adhesion to the bacterial protein invasin was minimally affected by any of the three mutations. For each of the mutants, the order of preference for divalent cations was unchanged compared to wild-type α^4, on CS1/fibronectin ($Mn^{2+} > Mg^{2+} > Ca^{2+}$), on VCAM-1 ($Mn^{2+} > Mg^{2+} = Ca^{2+}$) and on invasin ($Mg^{2+} = Ca^{2+}$). However for the three mutants, the efficiency of divalent cation utilization was decreased. On VCAM-1, 68–108 μM Mn^{2+} was required to support half-maximal adhesion for the mutants compared with 14–18 μM for wild-type α^4. These results indicate (*a*) that three different ligands for VLA-4 show widely differing sensitivities to mutations within putative divalent cation sites, and (*b*) each of the three putative divalent cation sites in α^4 have comparable functional importance with respect to both divalent cation usage and cell adhesion.

NEARLY all integrin ligand binding activities are completely inhibited by EDTA, but can be restored by the addition of divalent cations. Each integrin α subunit has three or four putative divalent cation sites (24, 36, 74) that partly resemble the EF-hand consensus structure seen in other divalent cation binding proteins such as calmodulin, troponin c, and paravalbumin (68). From the studies of calmodulin and troponin C, an EF-hand is a loop structure in which acidic amino acids (Asp or Glu), together with other oxygen containing amino acids (at positions 1, 3, 5, 7, 9, 12), provide 6 or 7 coordination sites for a divalent cation (68). Conservative mutations, or alanine substitutions, at positions 1, 3, and 12 within troponin C EF-hands emphasize that those residues are critical for both function and cation binding (5, 53). In another study, conservative mutations at position 9 of the EF-hand of the *Escherichia Coli* D-glucose and D-galactose receptor caused a change in both cation selectivity and affinity (16).

Each of the three putative divalent cation sites of the integrin α^4 subunit has the amino acid sequence Asp-X-Asp(or Asn)-X-Asp(or Asn)-Gly-X-X-Asp (Table I), which partly resembles the EF-hand motif (25, 69, 70). These sites are likely to be important for integrin function because (*a*) ligand binding has been mapped to that region (9, 63), (*b*) ~3–4 cations were found to bind to a single integrin (64), (*c*) a synthetic peptide derived from a putative cation binding site inhibited ligand binding activity in a calcium-dependent manner (10), (*d*) divalent cations directly bound to a recombinant fragment of α^{IIb} containing putative cation sites (23), and (*e*) a mutation (Asp to Glu) at EF-hand position 5 abolished the fibrinogen binding activity of an 11-amino acid peptide spanning divalent cation site II of integrin α^{IIb} (10). Furthermore there are several examples of divalent cations having an impact on activation-specific integrin epitopes (11, 20, 77), and also different divalent cations (eg., Ca^{2+}, Mg^{2+}, Mn^{2+}) can dramatically alter the specificity of ligand binding (14, 21, 36). The putative divalent cation sites within integrins lack an acidic residue at EF-hand position 12, that is critical for cation coordination in other molecules (68, 74). Thus it has been hypothesized that the missing coordination residue might be supplied by integrin ligands (7, 74), which often contain aspartic acid at a critical position (3, 38, 39, 52, 66, 78). However, despite these results and speculations, direct evidence has not yet been provided for a functional role of putative α subunit divalent cation binding sites in the context of an intact integrin heterodimer.

The integrin VLA-4 is expressed on circulating blood cells (e.g., lymphocytes, monocytes, and eosinophils), thymic

Table I. Putative Divalent Cation Binding Sites of α^4

Domain	Residue number	Amino acid sequence
		1 2 3 4 5 6 7 8 9
I	281–289	D L N A D G F S D
II	344–352	D I D N D G F E D
III	406–414	D A D N N G Y V D

and bone marrow progenitors (75), fetal myoblasts (57), some melanoma cells (54), and several other cell types. Ligands for VLA-4 include vascular cell adhesion molecule (VCAM)[1]-1, a molecule expressed on activated endothelium (2, 13, 31, 34, 51, 55, 61), and the alternatively spliced cell attachment domain, CS1, within the Hep II region of fibronectin (19, 22, 79). The VLA-4/VCAM-1 interaction is important for hematopoiesis (47, 59, 72), fetal myogenesis (54) and also may play a critical role in the migration of mononuclear leukocytes to inflammatory sites (34) and in the pathogenesis of diseases such as arthritis (40, 48, 76), atherosclerosis (8), and autoimmune encephalomyelitis (81). The VLA-4/CS1 interaction may be important during embryogenesis (12), bone marrow and thymic progenitor cell maturation (56, 72, 75, 80), and localization of sensitized T lymphocytes to antigenic sites (17). Like VLA-4 (an $\alpha^4\beta_1$ heterodimer), the $\alpha^4\beta_7$ heterodimer has also been found to mediate adhesion to VCAM-1 and to fibronectin (6, 58).

Several integrins, including VLA-4, interact with invasin, an outer membrane protein from *Yersinia pseudotuberculosis* that mediates bacterial penetration into mammalian cells (33). Surprisingly, VLA-4 on lymphocytes had a high constitutive activity for adhesion to invasin (15), whereas adhesion to VCAM-1 and CS1/fibronectin is markedly more dependent on functional activation of the integrin (4, 45, 62, 78).

As with other integrins, VLA-4–mediated adhesive functions require divalent cations. The function of VLA-4 is moderately supported by Mg^{2+}, enhanced in the presence of Mn^{2+}, and sometimes partially suppressed in the presence of Ca^{2+} (45). Notably, the presence of Ca^{2+} suppressed the function of partly active but not fully active VLA-4, and suppressed adhesion to CS1/fibronectin but not VCAM-1 (45). These results point out not only a critical mechanistic difference between VCAM-1 and CS1 as VLA-4 ligands, but also suggest that variations within divalent cation sites have a pivotal role in the manifestation of different states of constitutive activity.

A homology modeling study has demonstrated that a putative divalent cation site from the integrin α^4 subunit might use positions 1, 3, 5, 7, and 9 to form a structure similar to conventional EF-hands (74). To directly explore the functional importance of putative divalent cation sites for VLA-4 in particular, and for integrins in general, we have mutated key residues within three different sites in the α^4 subunit and stably expressed these constructs in K562 cells. These transfectants were then used to carry out a detailed analysis of VLA-4 adhesion to CS1/fibronectin, VCAM-1, and invasin. Remarkably, we found that divalent cation site mutagenesis had a different influence on cell adhesion to each ligand. Also we found that each divalent cation site appeared to contribute comparably towards integrin function.

1. *Abbreviations used in this paper*: HBSA, heat-denatured BSA; MBP, maltose binding protein; VCAM, vascular cell adhesion molecule.

Materials and Methods

Antibodies and Other Proteins and Reagents

Monoclonal antibodies used were as follows: anti-VLA-1, TS2/7 (28); anti-VLA-2, 5E8 (83); anti-VLA-3, J143 (18); anti-VLA-4, B-5G10 (30), HP1/7 (60), HP1/2 (60), L25 (46); anti-VLA-5, mAb 16 (1); anti-VLA-6, BQ16 (a gift from Dr. M. Liebert, University of Michigan, Ann Arbor, MI); anti-VLA-β_1, A-1A5 (27), TS2/16 (28); and the negative control antibodies P3 (35) and J-2A2 (26).

Recombinant soluble VCAM-1 (rsVCAM-1) (42), and CS1 peptide conjugated with BSA (CS1-BSA) were gifts from Dr. R. Lobb (Biogen Co., Cambridge, MA) and Dr. T. Shimo-Oka (Iwaki Glass Co., Tokyo, Japan), respectively. Coupling was carried out using the sulfo-MBS reagent (37), acting on a cysteine added to the amino-terminal end of the CS1 peptide. An invasin–MBP (maltose binding protein) fusion protein containing the carboxy-terminal 479 amino acids of invasin (41) was obtained from Dr. R. R. Isberg (Tufts University, Boston, MA). Human fibronectin was obtained from Telios Pharmaceuticals (La Jolla, CA).

Site-directed Mutagenesis and Transfections

Full-length α^4 cDNA in Bluescript (70) was subjected to site-directed mutagenesis using the Muta-Gene phagemid in vitro mutagenesis kit (Bio-Rad, Richmond, CA), as recommended. Oligonucleotides representing bases 976–1002 (for Asn^{283} to Gln or Glu mutation), 986–1003 (for Asp^{285} to Glu mutation), 1170–1187 (for Asp^{346} to Glu mutation), and 1355–1371 (for Asp^{408} to Glu mutation) were used as primers. Base substitutions at sites of mutation were confirmed by DNA sequencing (82). Mutated α^4 inserts were then excised and ligated into the pFNeo expression vector, as previously described (13). K562 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, L-glutamine and antibiotics (complete media). 7.5×10^6 K562 cells were electroporated with 25 μg of the different pFNeo-α^4 constructs, and 48 h after transfection, cells were placed in complete media containing 1.5 mg/ml of geneticin (G-418 sulfate; GIBCO BRL, Gaithersburg, MD). Geneticin-resistant cells were analyzed by flow cytometry for VLA-4 surface expression using a FACScan machine, as previously described (13), and enriched by selection with immunomagnetic beads when necessary.

The control cell line, 4RL, is derived from K562 cells transfected with α^4 in which the 80/70 cleavage site was abolished due to mutation of Arg^{558} to Leu (73).

Immunoprecipitation

Cells were surface labeled with ^{125}I using lactoperoxidase and lysed in the presence of 1.0% Triton X-100. Immunoprecipitation and SDS-PAGE analyses (on 7% polyacrylamide gels) were carried out as previously described (29).

Cell Adhesion Assays

Cell attachment to ligand proteins was carried out in the presence or absence of stimulatory or blocking antibodies as previously described (6, 45). Briefly, cells were labeled by incubation with the fluorescent dye BCECF (Molecular Probes, Inc., Eugene, OR) and then 5×10^4 cells were added to 96-well microtiter plates (Flow Labs, Inc., McLean, VA) that had been coated with protein ligands and blocked with 0.1% heat-denatured BSA (HBSA). After 20–25 min of incubation at 37°C, unbound cells were removed by washing. Then, cells remaining attached to the plate were analyzed using a Fluorescence Concentration Analyzer machine (Millipore Corp., Bedford, MA). After subtraction of background cell binding, values for cells bound/mm^2 were calculated. Background cell binding (typically <2%) was assessed using BSA-coated wells in the experiments for CS1 or for VCAM-1, and using MBP-coated wells in the experiments for invasin-MBP fusion protein. RPMI media (containing 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+}) was used as an assay buffer unless otherwise indicated. Results are reported as the mean with the standard deviation of triplicate determinations. As previously shown (45) adhesion of α^4-transfected cells to CS1-BSA or to the FN33/66 fragments of fibronectin was nearly identical in terms of cation dependence and maximal adhesion supported. Thus, CS1-BSA was used for most experiments due to its greater availability.

The mAbs TS2/16, HP1/2, and mAb 16 were added at a final concentration of 3.0, 1.0, and 1.0 μg/ml, respectively. To analyze the effects of divalent cations, cells were washed with 1 mM EDTA in PBS after labeling with BCECF, then washed once with TBS, and resuspended in TBS containing

Table II. Site-directed Mutagenesis in Putative Divalent Cation Sites of α^4

Mutants				Results of transfection	
Domain	Position	Residue number	Mutation	Expression	Stability
I	3	283	N → Q	–	
I	3	283	N → E	++	*stable (N283E)*
I	5	285	D → E	±	unstable
II	3	346	D → E	++	*stable (D346E)*
III	3	408	D → E	++	*stable (D408E)*

0.1% HBSA and 2 mM glucose. $CaCl_2$, $MgCl_2$, or $MnCl_2$ were then added to the plated cells in each well, and TBS was used instead of RPMI in all subsequent procedures.

Results

Construction and Expression of Mutated α^4

Five different conservative mutations in the putative divalent cation sites of the α^4 subunit were designed to minimize disruption of the association of $\alpha\beta$ subunits (Table II). Although transfection into K562 cells was done at least 4 or 5 times for each mutant, one of the mutations (N283Q) was not expressed at all, and another (D285E) was expressed at levels too low and too unstable for functional analysis. In contrast, substitution of Glu for Asp or Asn at the third position of each divalent cation site yielded mutant α^4 for which stable transfectants could successfully be established. These transfectants, mutated in cation sites I, II, and III were designated N283E, D346E, and D408E, respectively, and were expressed at levels comparable to wild-type α^4 in K562 cells (called KA4), as detected by immunofluorescence staining using VLA-4–specific mAb B-5G10 (Fig. 1, panels *KA4*, *N283E*, *D346E*, and *D408E*). In comparison, the mock-transfected cell (KpF) expressed barely a trace level of α^4 (Fig. 1, panel *KpF*). Additional staining comparing three different VLA-4–specific mAbs (HP1/7, HP1/2, L25) that define distinct epitopes of α^4, yielded identical profiles for each transfectant (data not shown). Also, no marked alterations in the expression of endogenous VLA-5 were observed among the five transfectants used in this study, and no expression of VLA-1, -2, -3, or -6 was observed (data not shown).

Analysis of VLA-4 expression by immunoprecipitation using an anti-α^4 mAb yielded nearly identical patterns of α^4 and β_1 proteins for each transfectant (Fig. 2, lanes *b–e*). In addition anti-β_1 mAB immunoprecipitated the β_1 subunit in association with both transfected α^4 and the endogenously expressed α^5 from KA4 cells (Fig. 2, lane *a*) and from each mutant transfectant (not shown).

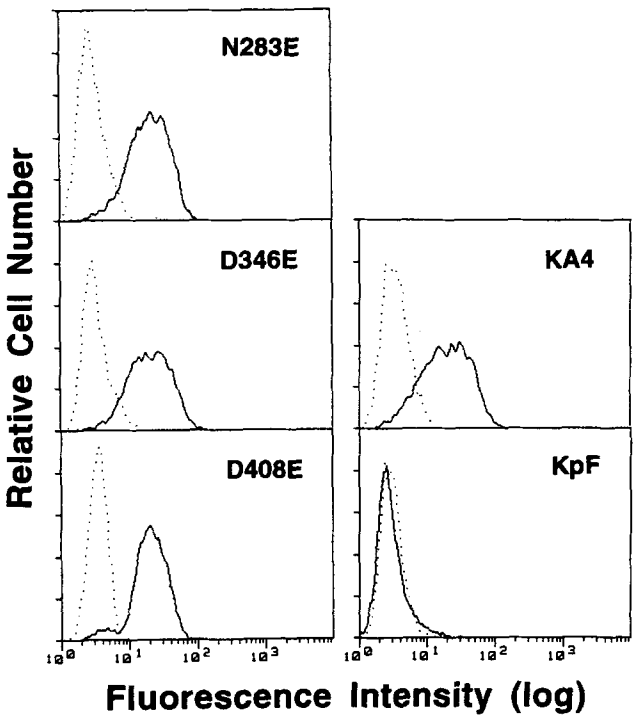

Figure 1. Cell surface expression of VLA-4 heterodimers on transfected K562 cells. Transfected K562 cells stained with control mAb J-2A2 (*dotted line*) or anti-VLA-4 mAb B-5G10 (*solid line*) were measured by flow cytometry.

Adhesion of K562 Transfectants to CS1 and to VCAM-1

Neither KA4 cells nor any of the mutants adhered to CS1 in the absence of stimulation by the anti-β_1 antibody TS2/16 (Fig. 3). In the presence of TS2/16, KA4 cells showed moderate adhesion, whereas N283E cells and D346E cells adhered minimally to CS1, and adhesion by D408E cells was at an intermediate level. A somewhat different result was observed for adhesion to VCAM-1. KA4 cells readily adhered to VCAM-1, and this adhesion was amplified only 1.3–1.8 fold by TS2/16 (Fig. 4). In contrast, N283E cells and D346E cells (and to a lesser extent D408E cells) showed much less adhesion to VCAM-1 in the absence of stimulation, but TS2/16 stimulation brought them up to a comparable level of adhesion as seen for KA4 cells. Adhesion of all of these cells to CS1 or to VCAM-1 was entirely mediated by VLA-4 because the mock-transfected (KpF) cells did not adhere to either CS1 or VCAM-1, regardless of TS2/16 stimulation.

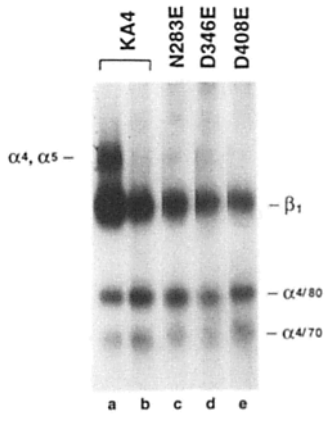

Figure 2. Immunoprecipitation of VLA-4 from transfected K562 cells. Equivalent amounts of KA4, N283E, D346E, and D408E cells were radiolabeled with ^{125}I, extracted with Triton X-100, and then immunoprecipitated using either the anti-β_1 mAb A-1A5 (lane *a*), or the anti-α^4 mAb B-5G10 (lanes *b–e*). The negative control antibody P3 yielded blank lanes from each cell extract (not shown).

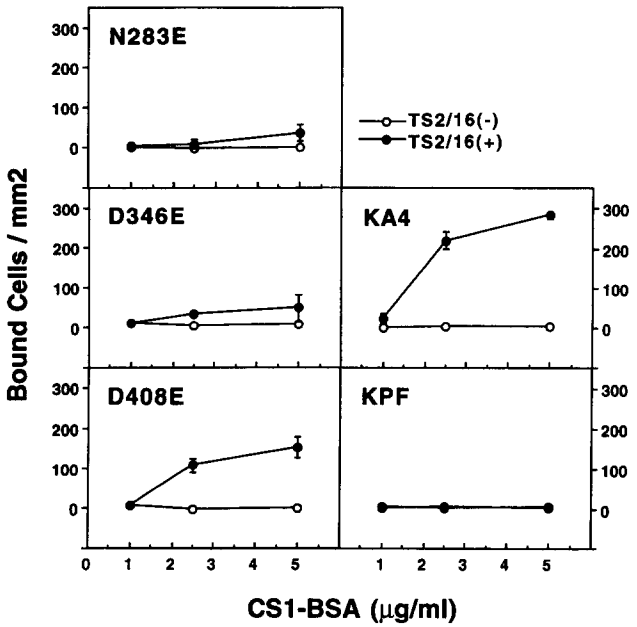

Figure 3. Adhesion of transfected K562 cells to CS1 peptide. Cells (KpF, KA4, N283E, D346E, and D408E) were tested for adhesion to microtiter plates coated with various amounts of CS1-BSA in the presence or absence of TS2/16 as described in Materials and Methods.

Comparison of VLA-4-mediated Adhesion of K562 Transfectants to CS1, VCAM-1, and Invasin

For invasin adhesion experiments, plates were coated with low levels of invasin-MBP fusion protein to avoid VLA-4-independent background adhesion, and also cells were preincubated with anti-α^5 mAb 16 to block the slight contribution of VLA-5 to adhesion. Using these experimental conditions, cell adhesion was almost completely inhibited by the anti-α^4 mAb HP1/2 (Fig. 5 *C*) thus confirming that VLA-4 plays a major role. In the absence of TS2/16 stimulation, adhesion of each mutant transfectant to invasin was only partially diminished, down to a level that was 62–71% relative to that seen with KA4 cells. With TS2/16 stimulation, adhesion by the mutants was 90–98% relative to KA4 cells.

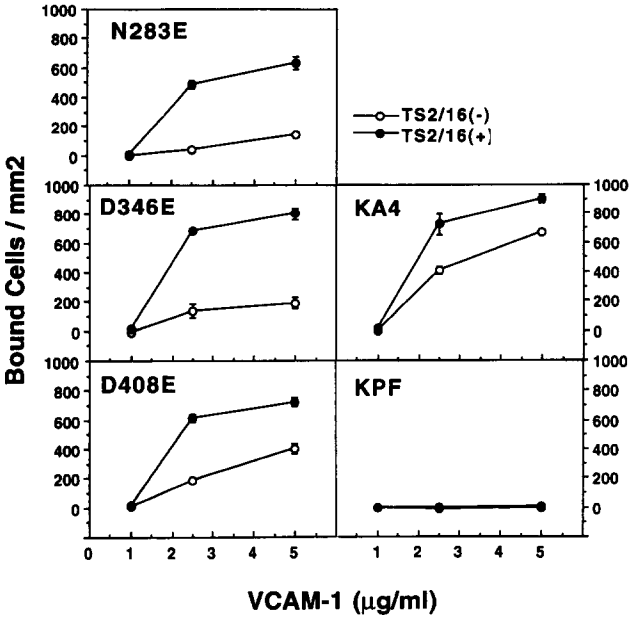

Figure 4. Adhesion of transfected K562 cells to VCAM-1. Cells (KpF, KA4, N283E, D346E, and D408E) were tested for adhesion to microtiter plates coated with various amounts of rsVCAM-1 in the presence or absence of TS2/16 as described in Materials and Methods.

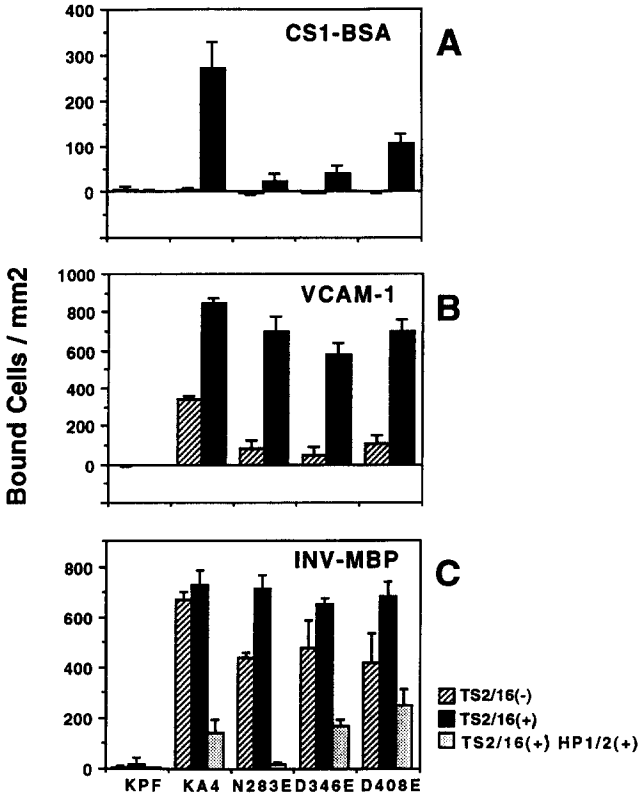

Figure 5. Comparison of VLA-4-mediated adhesion of transfected K562 cells to its three ligands. Cells (KpF, KA4, N283E, D346E, and D408E) were tested for adhesion to microtiter plates coated with 2.5 μg/ml of CS1-BSA, 2.5 μg/ml of rsVCAM-1, or 0.4 μg/ml of invasin-MBP fusion protein (*INV-MBP*) in the presence or absence of TS2/16 as described in Materials and Methods. HP1/2 was added where indicated, and cells were preincubated with mAb 16 for invasin experiments.

In comparison, there was no adhesion to CS1 without TS2/16 stimulation, and adhesion of N283E, D346E, and D408E cells to CS1 was still greatly diminished (8, 14, 39% relative to KA4 cells, respectively) when stimulated by TS2/16 (Fig. 5 *A*). Adhesion of N283E, D346E, and D408E cells to VCAM-1 was diminished (26, 15, and 34%, respectively, relative to KA4 cells) without TS2/16 stimulation, but upon TS2/16 stimulation was restored to 83, 68, and 83%, respectively, relative to KA4 cells (Fig. 5 *B*).

In conclusion, as the result of mutations in the putative divalent cation sites of α^4, VLA-4-mediated adhesion to CS1 was greatly diminished regardless of stimulation, adhesion to VCAM-1 was diminished but restored upon VLA-4 stimulation, and adhesion to invasin was minimally affected.

Effects of Divalent Cations on Adhesion

To obtain optimal conditions for analysis of the effects of

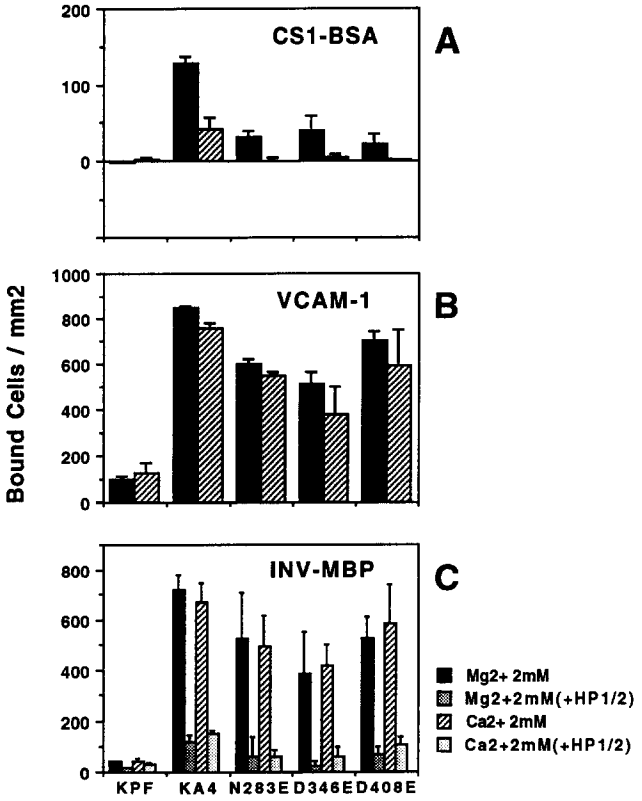

Figure 6. Effects of Mg^{2+} and Ca^{2+} on adhesion of transfected K562 cells to its three ligands. Cells (KpF, KA4, N283E, D346E, and D408E) were incubated with TS2/16, and tested for adhesion to microtiter plates coated with 2.5 μg/ml of CS1-BSA, 2.5 μg/ml of rsVCAM-1, or 0.4 μg/ml of invasin-MBP fusion protein (*INV-MBP*) in the presence of 2.0 mM Mg^{2+} or 2.0 mM Ca^{2+} as described in Materials and Methods. Cells were preincubated with mAb 16 for invasin experiments, and mAb HP1/2 was added where indicated.

divalent cations on adhesion, TS2/16 was included in all assays (Figs. 6 and 7). In the wild-type α^4 transfectant (KA4 cells), Ca^{2+} was much less effective than Mg^{2+} in supporting adhesion to CS1, and in the mutants, Ca^{2+} was totally ineffective (Fig. 6 *A*). In contrast, Ca^{2+} was as effective as Mg^{2+} in supporting adhesion to either VCAM-1 or to invasin (Fig. 6, *B* and *C*). Notably, these results obtained using saturating levels of Mg^{2+} or Ca^{2+} (2 mM) are comparable to the results obtained in Figs. 3–5 using RPMI media (Mg^{2+} and Ca^{2+} each at 0.5 mM).

Although adhesion in the presence of 1 mM Mn^{2+} was somewhat increased (Fig. 7 *C*) relative to adhesion in the presence of 2 mM Mg^{2+} (Fig. 7 *A*), the mutant transfectants were still markedly deficient in their ability to adhere to CS1. When adhesion to fibronectin was analyzed, the mutants were similarly found to be deficient both in the presence of 2 mM Mg^{2+} (Fig. 7 *B*) and in the presence of 1 mM Mn^{2+} (Fig. 7 *D*). For these experiments, cells were preincubated with the anti-α^5 mAb 16 to block VLA-5–mediated adhesion, and thus we were able to obtain cell adhesion that was almost completely inhibited by anti-α^4 mAb HP1/2 (Fig. 7, *B* and *D*), indicating that the observed adhesion was largely VLA-4 mediated.

When adhesion of VCAM-1 was analyzed over a wide range of Mn^{2+} concentrations (Fig. 8), the titration profiles for K562 cells containing N283E, D346E, and D408E mutations were similar, each showing half-maximal adhesion (ED50 values) at 55–85 μM Mn^{2+}. In contrast, K562 cells containing wild-type α^4 (KA4) had an ED50 value of approximately 10 μM, and K562 cells (4RL) containing α^4 with an unrelated mutation (abolishing the 80/70 cleavage site) (73) had a similar ED50 values (~12 μM). From four different experiments such as shown in Fig. 8, average ED50 values were obtained (Table III) showing that the efficiency of Mn^{2+} utilization was altered 4–7-fold by mutations N283E, D346E, and D408E.

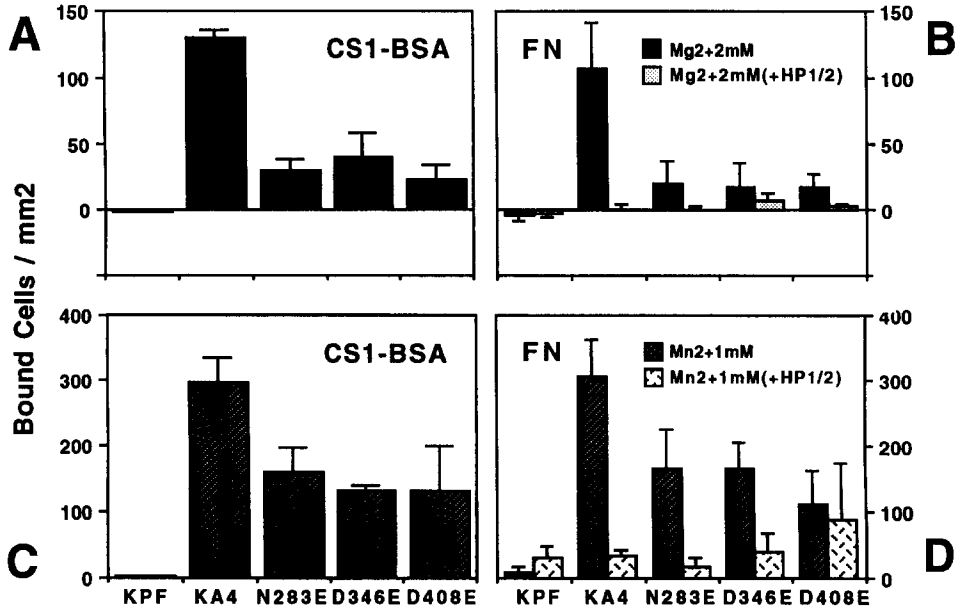

Figure 7. Comparison between adhesion to CS1 and to fibronectin. Cells (KpF, KA4, N283E, D346E, and D408E) were incubated with TS2/16, and tested for adhesion to microtiter plates coated with 2.5 μg/ml of CS1-BSA or 1.0 μg/ml of fibronectin in the presence of 2.0 mM Mg^{2+} or 1.0 mM Mn_6^+ as described in Materials and Methods. The dose of fibronectin used was near saturating with respect to integrin-dependent adhesion. At higher doses (not shown), non-integrin dependent adhesion was observed. Cells were preincubated with mAb 16 for fibronectin experiments, and HP1/2 was added where indicated.

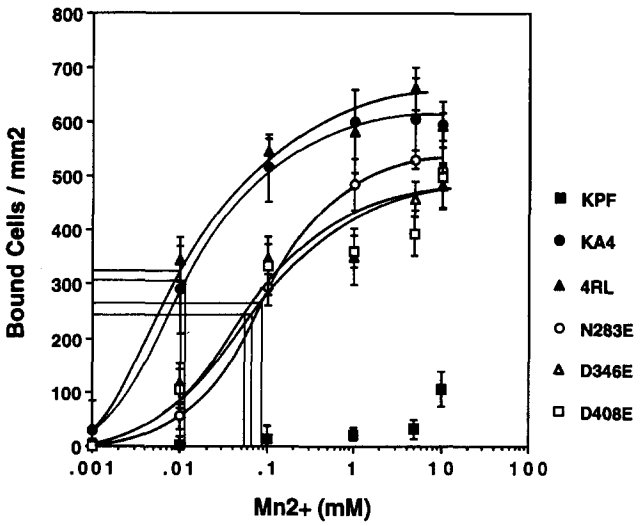

Figure 8. Effect of Mn^{2+} on cell adhesion to VCAM-1. Cells (KpF, KA4, 4RL, N283E, D346E, and D408E) were tested for adhesion to microtiter plates coated with 2.5 μg/ml rsVCAM-1 in the presence of the indicated concentrations of Mn^{2+}, and in the absence of the antibody TS2/16, as described in Materials and Methods.

In a control experiment, ED50 values were determined for K562 cells expressing different levels of wild-type α^4, varying over a 10-fold range. Although maximal adhesion varied with expression, ED50 values were comparable to those seen in Table III (not shown). Thus, the ED50 values appear to be independent of the overall adhesive state of the cell. When adhesion assays were carried out using invasin instead of VCAM-1, or Mg^{2+} instead of Mn^{2+}, ED50 values were elevated by several fold for mutations N283E, D346E, and D408E (not shown), consistent with results in Table III.

Discussion

Contrasting Effects of Mutations on Adhesion to Different VLA-4 Ligands

In this study, we have established that each of the three putative divalent cation binding sites in the α^4 subunit play critical roles in determining VLA-4–mediated adhesive activity to its ligands. Conservative mutation of these sites did not disturb heterodimer assembly with endogenous β_1, but did clearly affect ligand binding activity. Depending on which VLA-4 ligand was used in cell adhesion assays, these three mutations resulted in (*a*) nearly complete loss of function, (*b*) partial loss of function, or (*c*) minimal effect on function. The nearly complete loss of VLA-4–mediated adhesion to CS1/fibronectin could not be overcome by the addition of function-stimulating agents. Adhesion to VCAM-1 was also substantially diminished in all three mutants, but this effect could be overcome by the addition of the stimulatory anti-β_1 antibody TS2/16. In contrast to the other ligands, the bacterial protein invasin supported a high level of VLA-4–mediated adhesion that was not dependent on stimulatory agents, and was only minimally influenced by position 3 mutations. Together these results emphasize the versatility of a single integrin, VLA-4, able to interact in a distinct manner with three different ligands.

Table III. ED50/Mn^{2+} for Each Transfectant in Adhesion to VCAM-1

Cell	ED50/Mn^{2+}
	μM
KA4	18 ± 6
4RL	14 ± 6
N283E	108 ± 21‡
D346E	90 ± 17‡
D408E	68 ± 14*

Values of ED50/Mn^{2+} (50% maximal effective dose of Mn^{2+}) in adhesion to VCAM-1 were determined as shown in Fig. 8. The results are the mean ± SE of four independent experiments done in triplicate. Statistical differences from control (KA4 cell) were determined by *t* test.
* $p < 0.05$.
‡ $p < 0.01$.

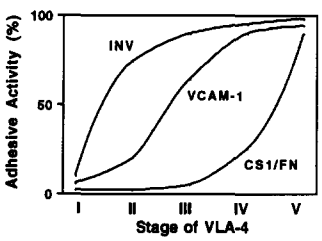

Figure 9. Schematic diagram depicting multiple activation states of VLA-4.

Perhaps the simplest explanation for these results is that weaker interactions are more susceptible to partial disabling of the ligand binding site thus causing them to drop below the threshold of the adhesion assay. Shown schematically in Fig. 9 are five arbitrary stages of VLA-4 activity. Wild-type VLA-4 in transfected K562 cells is at stage III, and can be pushed up to stage IV or V upon the addition of TS2/16 or Mn^{2+}. In contrast, mutated VLA-4 has been shifted to stage II, where interaction with invasin is retained, VCAM-1 is diminished, and CS1/FN is absent. Stimulation of the mutated VLA-4 can restore stage III or IV activity, as adhesion to invasin is still maintained at a high level, VCAM-1 is somewhat restored, and CS1/FN is still largely absent. Even in the presence of both Mn^{2+} and TS2/16, the mutated VLA-4 could not reach stage V. These results confirm and extend previous results (45) showing that putative divalent cation sites are likely to play a critical role in determining the activation state of VLA-4.

To develop this model further, direct ligand binding affinity measurements would be helpful. However, we have been unable to obtain such measurements using fibronectin (or FN 40 fragment), CS1 peptide, VCAM-1, or invasin because the interactions of the former with VLA-4 are too weak (especially in the mutants), because there is a large integrin-independent binding component for CS1 and invasin, and because sufficient VCAM-1 material is not available. At present we predict that invasin would have the highest affinity, followed by VCAM-1, and then CS1/fibronectin. Consistent with this, the affinity of invasin for VLA-5 has been found to be two orders of magnitude higher than the affinity determined for VLA-5/fibronectin (50).

Regardless of the precise affinity constants, it is clear that changing Asn or Asp to Glu in position 3 of each of the putative divalent cation sites had a dramatic effect on adhesion, especially for weaker ligands such as CS1 or fibronectin, or VCAM-1 in the absence of integrin stimulation. We predict

that VLA-4 interactions with ligands weaker than CS1, such as the Leu-Asp-Val tripeptide (78) would have an even higher threshold for activation, leading to a greater requirement for stimulation, and an even greater sensitivity to mutagenesis such as performed here.

The effects of our mutations on VLA-4–dependent adhesion to intact fibronectin were very similar to the effects on adhesion to CS1 peptide. Thus, other regions within fibronectin reported to be involved in VLA-4–mediated adhesion (32, 44, 49) either are functionally indistinguishable from the CS1 region, or are not functioning in our assays.

The constitutively high adhesion-supporting properties of invasin are in striking contrast to the other VLA-4 ligands, and consistent with previous suggestions that adhesion to invasin does not require cellular activation (15). This aspect of the invasin/VLA-4 interaction places invasin-bearing bacteria outside of the normal activation dependent constraints on other VLA-4 ligands, and thus may favor the spread of the bacteria. We have also found that, although VLA-4 and VLA-5 are present in nearly equal amounts on α^4-transfected K562 cells, the major contribution towards adhesion to invasin is provided by VLA-4. It remains to be determined why VLA-5 would be a very high affinity receptor for invasin in some studies (50), but relatively ineffective in ours and other (15) studies. One possibility is that fusion proteins containing different portions of invasin (41) could have altered integrin specificities.

Implications Regarding Divalent Cation Sites

Conservative changes of Asp or Asn to Glu at position 3 within sites I, II, and III were sufficient to allow VLA-4 expression, but alter adhesive function. These conservative mutations did not appear to cause gross changes in the overall integrin structure because (*a*) association between α^4 and β_1 was retained, (*b*) three distinct α^4 epitopes were unaltered, and (*c*) VLA-4–dependent adhesion to at least one ligand, invasin, was minimally affected. The observed functional alterations appeared to be specific for these mutations. For example, mutation of the α^4 80/70 cleavage site (73) had no effect on cell adhesion or cation utilization. Also, mutations of Cys^{278} (three residues away from site I), Cys^{717}, Cys^{767}, and Cys^{828} had no effect on either cell adhesion or divalent cation utilization (Pujades, C., J. Teixidó, and M. E. Hemler, unpublished observations).

The most likely interpretation of the functional effects of the N283E, D346E, and D408E mutations is that the presence of a larger amino acid (Glu) may have disrupted the tertiary structure at the cation binding site, and perhaps even prevented cation binding. In this regard, substitution of Glu for Asp in position 3 of a divalent cation site in troponin C yielded functionally inactive protein with concomitantly diminished cation binding capacity (5, 53).

Thus far, because we have elected to study putative divalent cation sites in the context of the intact VLA-4 heterodimer, it has been impractical to directly determine the stoichiometry of cation binding to the mutant integrins due to the very large amount of protein required, and the instability of the purified VLA-4 heterodimer (31).

Also, we have not yet been able to successfully express α^4 in which more radical mutations have been carried out (e.g., Asp to Ala at positions 1, 3, 5, or 9), and even some conservative mutations have yielded unstable integrins. For example, we mutated position 5 (Asp to Glu) within α^4 site I, but the resulting integrin was not sufficiently stable to allow functional studies (Table II). Likewise, we were unable to express mutated α^4 containing a conservative Asn to Gln mutation at position 3 within site I. These results emphasize that EF-hand residues not only may be important for integrin function, but also may play a role in heterodimer assembly and/or maturation. Thus, it appears that mutants which are expressed and also show functional alterations represent a narrow window between no expression and no functional effects.

Mutations within Sites I, II, and III Have Comparable Effects

Regardless of whether cation binding is eliminated from site I, II, or III, or merely altered, it is clear that the efficiency of divalent cation utilization has been similarly decreased for each position 3 mutant, and at the same time adhesive functions have been similarly influenced. In some instances the effects of the mutation in site II were slightly more obvious, and the effects of the site III mutation were a little less obvious, but overall, the effects were comparable.

Whereas all integrin α chains have 3–4 putative divalent cation sites, the extent to which each one contributes has not been clear. The noncompetitive inhibition of Mg^{2+} binding to VLA-2 by Ca^{2+} has suggested distinct sites for different cations (65). Other studies have found that α^{IIb} cation binding sites may be heterogeneous with respect to calcium binding affinity (23, 67). Our results argue against radical heterogeneity among the putative divalent cation sites. The divalent cation usage pattern was identical for each mutant and the relative order of effectiveness ($Mn^{2+} > Mg^{2+} > Ca^{2+}$, on CS1/fibronectin; $Mn^{2+} > Mg^{2+} = Ca^{2+}$, on VCAM-1) resembled that seen with wild-type α^4.

Many integrin ligands contain an essential aspartic acid residue, and these have been hypothesized to directly coordinate with integrin divalent cations (7, 74). However, it is difficult to reconcile this model with our results showing that all three putative cation sites may be similar in terms of divalent cation usage and contribution to VLA-4 function, as it appears unlikely that three ligands would bind at once to the same integrin. Instead, the "missing" cation coordination in integrin EF-hand sites might be provided by water molecules, and/or by having a single amino acid supply two coordination sites, as is found for other EF-hand structures (68). Because there is only a single putative EF-hand–type site within integrin β subunits (43), that is perhaps a better candidate for direct ligand–cation contact. Consistent with this idea, an Asp to Tyr mutation at position 1 of the putative β_3 EF-hand caused complete loss of ligand binding function (43), and as Asp to Ala mutation at position 1 of the β_1 EF-hand site caused complete loss of VLA-5 binding either to fibronectin or to invasin (71).

In conclusion, our results indicate that the three putative divalent cation sites within the VLA-4 α chain contribute similarly to the function of the integrin, and support the concept that a single integrin can adhere to multiple ligands, each regulated in a distinct manner. In future studies involving transgenic animals, these mutants will be useful for evaluating the in vivo functional consequences of expressing VLA-4 in which adhesion to CS1/fibronectin is diminished relative to adhesion to VCAM-1.

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References

1. Akiyama, S. K., S. S. Yamada, W.-T. Chen, and K. M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J. Cell Biol.* 109:863–875.
2. Allavena, P., C. Paganin, I. Martin-Padura, G. Peri, M. Gaboli, E. Dejana, P. C. Marchisio, and A. Mantovani. 1991. Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. *J. Exp. Med.* 173:439–448.
3. Altieri, D. C., J. Plescia, and E. F. Plow. 1993. The structural motif glycine 190-valine 202 of the fibrinogen τ chain interacts with CD11b/CD18 integrin ($\alpha M\beta 2$, Mac-1) and promotes leukocyte adhesion. *J. Biol. Chem.* 268:1847–1853.
4. Arroyo, A. G., P. Sánchez-Mateos, M. R. Campanero, I. Martín-Padura, E. Dejana, and F. Sánchez-Madrid. 1992. Regulation of the VLA integrin-ligand interactions through the $\beta 1$ subunit. *J. Cell Biol.* 117: 659–670.
5. Babu, A., H. Su, Y. Ryu, and J. Gulati. 1992. Determination of residue specificity in the EF-hand of troponin C for Ca^{2+} coordination, by genetic engineering. *J. Biol. Chem.* 267:15469–15474.
6. Chan, B. M. C., M. J. Elices, E. Murphy, and M. E. Hemler. 1992. Adhesion to VCAM-1 and fibronectin: comparison of $\alpha 4\beta 1$ (VLA-4) and $\alpha 4\beta 7$ on the human cell line JY. *J. Biol. Chem.* 267:8366–8370.
7. Corbi, A. L., L. J. Miller, K. O'Connor, R. S. Larson, and T. A. Springer. 1987. cDNA cloning and complete primary structure of the alpha subunit of a leukocyte adhesion glycoprotein, p150,95. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4023–4028.
8. Cybulsky, M. I., and M. A. Gimbrone. 1991. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science (Wash. DC).* 251:788–791.
9. D'Souza, S. E., M. H. Ginsberg, T. A. Burke, and E. F. Plow. 1990. The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its α subunit. *J. Biol. Chem.* 265:3440–3446.
10. D'Souza, S. E., M. H. Ginsberg, G. R. Matsueda, and E. F. Plow. 1991. A discrete sequence in a platelet integrin is involved in ligand recognition. *Nature (Lond.).* 350:66–68.
11. Dransfield, I., and N. Hogg. 1989. Regulated expression of Mg^{2+} binding epitope on leukocyte integrin α subunits. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3759–3765.
12. Dufour, S., J.-L. Duband, M. J. Humphries, M. Obara, K. M. Yamada, and J. P. Thiery. 1988. Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2661–2671.
13. Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M. E. Hemler, and R. R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell.* 60:577–584.
14. Elices, M. J., L. A. Urry, and M. E. Hemler. 1991. Receptor functions for the integrin VLA-3: fibronectin, collagen and laminin binding are differentially influenced by Arg-Gly-Asp peptide and by divalent cations. *J. Cell Biol.* 112:169–181.
15. Ennis, E., R. R. Isberg, and Y. Shimizu. 1993. Very late antigen 4-dependent adhesion and costimulation of resting human T cells by the bacterial $\beta 1$ integrin ligand invasin. *J. Exp. Med.* 177:207–212.
16. Falke, J. J., E. E. Snyder, K. C. Thatcher, and C. S. Voertlet. 1991. Quantitating and engineering the ion specificity of an EF-hand-like Ca^{2+} binding site. *Biochemistry.* 30:8690–8697.
17. Ferguson, T. A., H. Mizutani, and T. S. Kupper. 1991. The integrin-binding peptides GRGDSP and GPEILDVPST abrogate T cell mediated immune responses in vivo. *Proc. Natl. Acad. Sci. USA.* 88:8072–8076.
18. Fradet, Y., C. Cordon-Cardo, T. Thomson, M. E. Daly, W. F. Jr. Whitmore, K. O. Lloyd, M. R. Melamed, and L. J. Old. 1984. Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 81:224–228.
19. García-Pardo, A., E. A. Wayner, W. G. Carter, and O. C. Ferreira. 1990. Human B lymphocytes define an alternative mechanism of adhesion to fibronectin: the interaction of the $\alpha 4\beta 1$ integrin with the LHGPEILDV-PST sequence of the type III connecting segment is sufficient to promote cell attachment. *J. Immunol.* 144:3361–3366.
20. Ginsberg, M. H., A. Lightsey, T. J. Kunicki, A. Kaufmann, G. Marguerie, and E. F. Plow. 1986. Divalent cation regulation of the surface orientation of platelet membrane glycoprotein IIb. Correlation with fibrinogen binding function and definition of a novel variant of Glanzmann's thrombasthenia. *J. Clin. Invest.* 78:1103–1111.
21. Grzesiak, J. J., G. E. Davis, D. Kirchhofer, and M. D. Pierschbacher. 1992. Regulation of $\alpha_2\beta_1$-mediated fibroblast migration on type I collagen by shifts in the concentrations of extracellular Mg^{2+} and Ca^{2+}. *J. Cell Biol.* 117:1109–1117.
22. Guan, J.-L., and R. O. Hynes. 1990. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha 4\beta 1$. *Cell.* 60:53–61.
23. Gulino, D., C. Boudignon, L. Zhang, E. Concord, M. J. Rabiet, and G. Marguerie. 1992. Ca^{2+}-binding properties of the platelet glycoprotein IIb ligand-interacting domain. *J. Biol. Chem.* 267:1001–1007.
24. Hemler, M. E. 1990. VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu. Rev. Immunol.* 8:365–400.
25. Hemler, M. E. 1991. Structures and functions of VLA proteins and related integrins. *In* Receptors for Extracellular Matrix Proteins (A volume of Biology of Extracellular Matrix). R. P. Mecham and J. A. McDonald, editors. Academic Press, Inc., San Diego, CA. 255–299.
26. Hemler, M. E., and J. L. Strominger. 1982. Monoclonal antibodies reacting with immunogenic mycoplasma proteins present in human hematopoietic cell lines. *J. Immunol.* 129:2734–2738.
27. Hemler, M. E., C. F. Ware, and J. L. Strominger. 1983. Characterization of a novel differentiation antigen complex recognized by a monoclonal antibody (A-1A5): unique activation-specific molecular forms on stimulated T cells. *J. Immunol.* 131:334–340.
28. Hemler, M. E., F. Sánchez-Madrid, T. J. Flotte, A. M. Krensky, S. J. Burakoff, A. K. Bhan, T. A. Springer, and J. L. Strominger. 1984. Glycoproteins of 210,000 and 130,000 m.w. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. *J. Immunol.* 132:3011–3018.
29. Hemler, M. E., C. Huang, and L. Schwarz. 1987. The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight β subunit. *J. Biol. Chem.* 262:3300–3309.
30. Hemler, M. E., C. Huang, Y. Takada, L. Schwarz, J. L. Strominger, and M. L. Clabby. 1987. Characterization of the cell surface heterodimer VLA-4 and related peptides. *J. Biol. Chem.* 262:11478–11485.
31. Hemler, M. E., M. J. Elices, C. Parker, and Y. Takada. 1990. Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. *Immunol. Rev.* 114:45–65.
32. Iida, J., A. P. N. Skubitz, L. T. Furcht, E. A. Wayner, and J. B. McCarthy. 1992. Coordinate role for cell surface chondroitin sulfate proteoglycan and $\alpha 4\beta 1$ integrin in mediating melanoma cell adhesion to fibronectin. *J. Cell Biol.* 118:431–444.
33. Isberg, R. R., and J. M. Leong. 1990. Multiple $\beta 1$ chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. *Cell.* 60:861–871.
34. Issekutz, T. B., and A. Wyrkretowicz. 1991. Effect of a new monoclonal antibody, TA-2, that inhibits lymphocyte adherence to cytokine stimulated endothelium in the rat. *J. Immunol.* 147:109–116.
35. Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky. 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* 123:1548–1550.
36. Kirchhofer, D., J. J. Grzesiak, and M. D. Pierschbacher. 1991. Calcium as a potent physiological regulator of integrin-mediated cell adhesion. *J. Biol. Chem.* 266:4471–4477.
37. Kitagawa, T., and T. Aikawa. 1976. Enzyme coupled immunoassay of insulin using a novel coupling reagent. *J. Biochem.* 79:233–236.
38. Kloczewiak, M., S. Timmons, T. Lukas, and J. Hawiger. 1984. Platelet receptor recognition site on human fibrinogen: synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the τ chain. *Biochemistry.* 23:1767–1774.
39. Komoriya, A., L. J. Green, M. Mervic, S. S. Yamada, K. M. Yamada, and M. J. Humphries. 1991. The minimal essential sequence for a major cell type-specific adhesion site (CSI) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *J. Biol. Chem.* 266:15075–15079.
40. Laffón, A., R. García-Vincuña, A. Humbría, A. A. Postigo, A. L. Corbí, M. O. De Landázuri, and F. Sánchez-Madrid. 1991. Upregulated expression and function of VLA-4 fibronectin receptors on human activated T cells in rheumatoid arthritis. *J. Clin. Invest.* 88:546–552.
41. Leong, J. M., R. S. Fournier, and R. R. Isberg. 1990. Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1979–1989.
42. Lobb, R. R., G. Chi-Rosso, D. R. Leone, M. D. Rosa, B. M. Newman, S. Luhowskyj, L. Osborn, S. G. Schiffer, and C. D. Benjamin, I. G. Douglas, C. Hession, and E. P. Chow. 1991. Expression and functional characterization of a soluble form of vascular cell adhesion molecule 1 (VCAM1). *Biochem. Biophys. Res. Commun.* 178:1498–1504.

43. Loftus, J. C., T. E. O'Toole, E. F. Plow, A. Glass, A. L. Frelinger, and M. H. Ginsberg. 1990. A $\beta 3$ integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science (Wash. DC).* 249:915–918.
44. Massia, S. P., and J. A. Hubbell. 1992. Vascular endothelial cell adhesion and spreading promoted by the peptide REDV of the IIICS region of plasma fibronectin is mediated by the integrin $\alpha 4\beta 1$. *J. Biol. Chem.* 267:14019–14026.
45. Masumoto, A., and M. E. Hemler. 1992. Multiple activation states of VLA-4: mechanistic differences between adhesion to CS1/fibronectin and to VCAM-1. *J. Biol. Chem.* 268:228–234.
46. McIntyre, B. W., E. L. Evans, and J. L. Bednarczyk. 1989. Lymphocyte surface antigen L25 is a member of the integrin receptor superfamily. *J. Biol. Chem.* 264:13745–13750.
47. Miyake, K., I. L. Weissman, J. S. Greenberger, and P. W. Kincade. 1991. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J. Exp. Med.* 173:599–607.
48. Morales-Ducret, J., E. Wayner, M. J. Elices, J. M. Alvaro-Gracia, N. Zvaifler, and G. S. Firestein. 1992. $\alpha_4\beta_1$ integrin (VLA-4) ligands in arthritis: vascular cell adhesion molecule-1 expression in synovium and on fibroblast-like synoviocytes. *J. Immunol.* 149:1424–1431.
49. Mould, A. P., and M. J. Humphries. 1991. Identification of a novel recognition sequence for the integrin $\alpha 4\beta 1$ in the COOH-terminal heparin-binding domain of fibronectin. *EMBO (Eur. Med. Biol. Organ.) J.* 10:4089–4095.
50. Nhieu, G. V. T., and R. R. Isberg. 1991. The *Yersinia pseudotuberculosis* invasion protein and human fibronectin bind to mutually exclusive sites on the $\alpha 5\beta 1$ integrin receptor. *J. Biol. Chem.* 266:24367–24375.
51. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct cloning of vascular cell adhesion molecule 1 (VCAM1), a cytokine-induced endothelial protein that binds to lymphocytes. *Cell.* 59:1203–1211.
52. Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.
53. Putkey, J. A., H. L. Sweeney, and S. T. Campbell. 1989. Site-directed mutation of the trigger calcium-binding sites in cardiac troponin C. *J. Biol. Chem.* 264:12370–12378.
54. Rice, G. E., and M. P. Bevilacqua. 1989. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. *Science (Wash. DC).* 246:1303–1306.
55. Rice, G. E., J. M. Munro, and M. P. Bevilacqua. 1990. Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes: a CD11/CD18-independent adhesion mechanism. *J. Exp. Med.* 171:1369–1374.
56. Roldán, E., A. García-Pardo, and J. A. Brieva. 1992. VLA-4-fibronectin interaction is required for the terminal differentiation of human bone marrow cells capable of spontaneous and high rate immunoglobulin secretion. *J. Exp. Med.* 175:1739–1747.
57. Rosen, G. D., J. R. Sanes, R. LaChance, J. M. Cunningham, J. Roman, and D. C. Dean. 1992. Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. *Cell.* 69:1107–1119.
58. Rüegg, C., A. Postigo, E. E. Sikorski, E. C. Butcher, R. Pytela, and D. J. Erle. 1992. Role of integrin $\alpha 4\beta 7/\alpha 4\beta P$ in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J. Cell Biol.* 117:179–189.
59. Ryan, D. H., B. L. Nuccie, C. N. Abboud, and J. M. Winslow. 1991. Vascular cell adhesion molecule-1 and integrin VLA-4 mediate adhesion of human B cell precursors to cultured mouse marrow adherent cells. *J. Clin. Invest.* 88:995–1004.
60. Sánchez-Madrid, F., M. O. De Landazuri, G. Morago, M. Cebrian, A. Acevedo, and C. Bernabeu. 1986. VLA-3: a novel polypeptide association within the VLA molecular complex: cell distribution and biochemical characterization. *Eur. J. Immunol.* 16:1346–1349.
61. Schwartz, B. R., E. A. Wayner, T. M. Carlos, H. D. Ochs, and J. M. Harlan. 1990. Identification of surface proteins mediating adherence of CD11/CD18-deficient lymphoblastoid cells to cultured human endothelium. *J. Clin. Invest.* 85:2019–2022.
62. Shimizu, Y., G. A. Van Seventer, K. J. Horgan, and S. Shaw. 1990. Regulated expression and binding of three VLA ($\beta 1$) integrin receptors on T cells. *Nature (Lond.)* 345:250–253.
63. Smith, J. W., and D. A. Cheresh. 1990. Integrin ($\alpha V\beta 3$)-ligand interaction: identification of a heterodimeric RGD binding site on the vitronectin receptor. *J. Biol. Chem.* 265:2168–2172.
64. Smith, J. W., and D. A. Cheresh. 1991. Labeling of integrin $\alpha v\beta 3$ with $^{58}CO(III)$: evidence of metal ion coordination sphere involvement in ligand binding. *J. Biol. Chem.* 266:11429–11432.
65. Staatz, W., S. M. Rajpara, E. A. Wayner, W. G. Carter, and S. A. Santoro. 1989. The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg^{++}-dependent adhesion of platelets to collagen. *J. Cell Biol.* 108: 1917–1924.
66. Staatz, W. D., K. F. Fok, M. M. Zutter, S. P. Adams, B. A. Rodriquez, and S. A. Santoro. 1991. Identification of a tetrapeptide recognition sequence for the $\alpha_2\beta_1$ integrin in collagen. *J. Biol. Chem.* 266:7363–7367.
67. Steiner, B., D. Cousot, A. Trzeciak, D. Gillessen, and P. Hadvary. 1989. *J. Biol. Chem.* 264:13102–13108.
68. Strynadka, N. C., and M. N. G. James. 1989. Crystal structures of the helix-loop-helix calcium-binding proteins. *Annu. Rev. Biochem.* 58: 951–998.
69. Szebenyi, D. M. E., S. K. Obendorf, and K. Moffat. 1981. Structure of vitamin D-dependent calcium-binding protein from bovine intestine. *Nature (Lond.).* 294:327–332.
70. Takada, Y., M. J. Elices, C. Crouse, and M. E. Hemler. 1989. The primary structure of the $\alpha 4$ subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function: *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1361–1368.
71. Takada, Y., Y. Ylänne, D. Mandelman, W. Puzon, and M. H. Ginsberg. 1992. A point mutation of integrin β_1 subunit blocks binding of $\alpha_5\beta_1$ to fibronectin and invasin but not recruitment to adhesion plaques. *J. Cell Biol.* 119:913–921.
72. Teixidó, J., M. E. Hemler, J. S. Greenberger, and P. Anklesaria. 1992. Role of β_1 and β_2 integrins in the adhesion of human $CD34^{hi}$ stem cells to bone marrow stroma. *J. Clin. Invest.* 90:358–367.
73. Teixidó, J., C. M. Parker, P. D. Kassner, and M. E. Hemler. 1992. Functional and structural analysis of VLA-4 integrin α^4 subunit cleavage. *J. Biol. Chem.* 267:1786–1791.
74. Tuckwell, D. S., A. Brass, and M. J. Humphries. 1992. Homology modeling of integrin EF-hands: Evidence for widespread use of a conserved cation-binding site. *Biochem. J.* 285:325–331.
75. Utsumi, K., M. Sawada, S. Narumiya, J. Nagamine, T. Sakata, S. Iwagami, Y. Kita, H. Teraoka, H. Hirano, M. Ogata, T. Hamaoka, and H. Fujiwara. 1991. Adhesion of immature thymocytes to thymic stromal cells through fibronectin molecules and its significance for the induction of thymocyte differentiation. *Proc. Natl. Acad. Sci. USA.* 88:5685–5689.
76. van Dinther-Janssen, A. C. H. M., E. Horst, G. Koopman, W. Newmann, R. J. Scheper, C. J. L. M. Meijer, and S. T. Pals. 1991. The VLA-4/VCAM-1 Pathway is Involved in Lymphocytes Adhesion to Endothelium in Rheumatoid Synovium. *J. Immunol.* 147:4207–4210.
77. Van Kooyk, Y., P. Weber, F. Hogervorst, A. J. Verhoeven, G. A. Van Seventer, A. A. TeVelde, J. Borst, G. D. Keizer, and C. G. Figdor. 1991. Activation of LFA-1 through a Ca2+-dependent epitope stimulates lymphocyte adhesion. *J. Cell Biol.* 112:345–354.
78. Wayner, E. A., and N. L. Kovach. 1992. Activation-dependent Recognition by Hematopoietic Cells of the LDV Sequence in the V region of Fibronectin. *J. Cell Biol.* 116:489–497.
79. Wayner, E. A., A. García-Pardo, M. J. Humphries, J. A. McDonald, and W. G. Carter. 1989. Identification and characterization of the lymphocyte adhesion receptor for an alternative cell attachment domain in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.
80. Williams, D. A., M. Rios, C. Stephens, and V. P. Patel. 1991. Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. *Nature (Lond.).* 352:438–441.
81. Yednock, T. A., C. Cannon, L. C. Fritz, F. Sánchez-Madrid, L. Steinman, and N. Karin. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha 4\beta 1$ integrin. *Nature (Lond.).* 356:63–66.
82. Young, R. A., and R. W. Davis. 1983. Yeast RNA Polymerase II Genes: isolation with Antibody Probes. *Science (Wash. DC).* 222:778–782.
83. Zylstra, S., F. A. Chen, S. K. Ghosh, E. A. Repasky, U. Rao, H. Takita, and R. B. Bankert. 1986. Membrane-associated glycoprotein (gp160) identified on human lung tumor by a monoclonal antibody. *Cancer Res.* 46:6446–6451.