Residues Within a Conserved Amino Acid Motif of Domains 1 and 4 of VCAM-1 Are Required for Binding to VLA-4

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Abstract. Vascular cell adhesion molecule 1 (VCAM-1), a member of the Ig superfamily originally identified on activated endothelium, binds to the integrin very late antigen-4 (VLA-4), also known as $\alpha_4\beta_1$ or CD49d/CD29, to support cell–cell adhesion. Studies based on cell adhesion to two alternatively spliced forms of VCAM-1 or to chimeric molecules generated from them and intercellular adhesion molecule-1 (ICAM-1) have demonstrated two VLA-4 binding sites on the predominate form of VCAM-1. Here, we studied VLA-4–dependent adhesion of the lymphoid tumor cell line Ramos to cells expressing wild type and mutant forms of VCAM-1. Results based on domain deletion mutants demonstrated the existence and independence of two VLA-4-binding sites located in the first and fourth domains of VCAM-1. Results based on amino acid substitution mutants demonstrated that residues within a linear sequence of six amino acids found in both domain 1 and 4 were required for VLA-4 binding to either domain. Five of these amino acids represent a conserved motif also found in ICAM domains. We propose that integrin binding to these Ig-like domains depends on residues within this conserved motif. Specificity of integrin binding to Ig-like domains may be regulated by a set of nonconserved residues distinct from the conserved motif.

THE binding of vascular cell adhesion molecule 1 (VCAM-1)[1] to very late antigen-4 (VLA-4) ($\alpha_4\beta_1$) has been implicated in numerous physiologic and pathophysiologic processes involving cell–cell adhesion (51). On activated endothelium, VCAM-1 supports the adhesion of lymphocytes (7, 15, 36, 42, 47), monocytes (6), eosinophils (4, 14, 62), basophils (4), natural killer cells (1), and certain tumor cells, particularly malignant melanoma and osteosarcoma (19, 27, 51, 56). Adhesion of VLA-4-expressing cells to endothelium has been implicated in the pathogenesis of many diseases and disease processes, including rheumatoid arthritis (24, 30, 40, 57), osteoarthritis (24, 30), allogeneic graft rejection (5, 38), graft-versus-host disease (33–35), encephalomyelitis (2, 65), delayed-type hypersensitivity reactions (32), tumor metastasis (19, 27, 41, 56), and atherogenesis (9). VCAM-1 has also been identified on a variety of nonvascular cell types, including follicular dendritic cells (17, 26), bone marrow stromal cells (29, 45, 48), secondary myoblasts (44), some macrophages (43), certain renal epithelial cells (43), fibroblast-like synoviocytes (28, 30), reactive mesothelial cells (23, 43), stimulated astrocytes (22), and stimulated cortical neurons (3). Roles for VCAM-1 in germinal center development (17), lymphopoiesis (29, 45, 48), and myogenesis (44) have been suggested.

Alternative splicing of mRNA from a single VCAM-1 gene can produce two forms of VCAM-1 (8, 21, 39): a form containing seven Ig-like domains (VCAM-7D) and a form containing six Ig-like domains (VCAM-6D). The two forms of VCAM-1 are identical in amino acid sequence except for the absence in VCAM-6D of domain 4 (8, 21, 36, 39). Both forms of VCAM-1 have been isolated as cDNA from libraries prepared from stimulated endothelial cells, and as expressed in transfected cell lines, both support VLA-4–dependent adhesion (21, 59). mRNA for both VCAM-6D and VCAM-7D has been identified in cultured endothelial cells (8, 21, 28), synovial fibroblasts (28), and mesothelial cells (23), although VCAM-7D mRNA predominates particularly in chronically stimulated cells. At the protein level, only VCAM-7D has been identified on endothelial cells and synovial fibroblasts stimulated for 24 h with TNF (8, 28); however, after 2 h of stimulation of synovial fibroblasts, a glycoprotein with an apparent molecular weight 10–15 kD less than VCAM-7D has been clearly identified (28). In the mouse, a three-domain, glycophosphatidylinositol-anchored form of VCAM-1 has been identified in addition to VCAM-7D (66; and Kinashi, T., Y. St. Pierre, C.-H. Huang, and T. A. Springer, manuscript submitted for publication).

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1. *Abbreviations used in this paper*: HUVEC, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated; VCAM, vascular cell adhesion molecule; VLA-4, very late antigen-4.

Recent studies suggest that VLA-4 binds to VCAM-1 via two distinct sites. Based on the ability of anti-VCAM-1 mAb to inhibit lymphocyte adhesion to VCAM-7D versus VCAM-6D transfectants, we have concluded that one VLA-4 binding site is shared between VCAM-7D and VCAM-6D, whereas a second binding site localizes to the alternatively spliced domain 4 (59). The high degree of sequence homology between domain 1 and domain 4 suggested that the shared VLA-4 binding site localizes to domain 1. Based on lymphoid cell adhesion to chimeric constructs generated from VCAM-7D or VCAM-6D and intercellular adhesion molecule-1 (ICAM-1), Osborn et al. (37) has concluded that VLA-4 binds distinctly to domains 1 and 4.

Here we have characterized VLA-4 binding sites in VCAM-1 based on domain deletion and amino acid substitution mutants, similar to the strategy previously used to identify binding sites in ICAM-1 for its integrin receptors LFA-1 (52) and Mac-1 (13). In a first series of experiments, domain 1 deletion mutants of both VCAM-7D and VCAM-6D were analyzed for expression and lymphoid cell binding, and compared to wild-type forms. The domain specificities of several anti-VCAM-1 mAb were determined and compared to the ability of the mAb to inhibit cell binding. In a second series of experiments, amino acid substitution mutations were targeted to domains 1 and 4, and analyzed for binding to VLA-4–expressing cells. Our results not only demonstrate independent VLA-4 binding sites in domain 1 and domain 4 of VCAM-7D, but also demonstrate a critical binding function for residues within a six–amino acid sequence found in domain 1 and domain 4. Five of these represent a conserved motif also found in ICAM domains.

Materials and Methods

Cell Culture

The VLA-4–expressing human B lymphoblastoid cell line Ramos and SV-40 transformed African green monkey kidney cells (COS) were maintained in RPMI 1640 with 10% FCS, 5 mM glutamine, and 50 μg/ml gentamicin. Human umbilical vein endothelial cells (HUVEC) were maintained as previously described (59).

Antibodies

The mouse anti-VCAM-1 mAbs HAE-2a, HAE-2b, HAE-2c, HAE-2d, HAE-2j, and HAE-2f were generated following immunization of female BALB/c mice with HUVEC stimulated for 6 h with TNF, according to previously described methods (50). Hybridomas were screened by testing tissue culture supernatant for reactivity with purified VCAM-7D. Other mouse anti-VCAM-1 mAb used were 1.4C3 (63) and 4B9 (7). All mAb used were isotype IgG_1. mAb were used as neat tissue culture supernatant (HAE-2b, HAE-2c, HAE-2d, HAE-2j, and HAE-2f), as a 1:500 dilution of ascites (HAE-2a), or as 20 μg/ml of purified protein (1.4C3 and 4B9). The mouse anti-human VLA-4 mAb HP2/1 (46) was used as 40 μg/ml of purified protein (AMAC Corp., Westbrook, ME).

Generation of VCAM-1 Mutants

Oligonucleotide-directed mutagenesis was used to generate VCAM-1 domain deletion and amino acid substitution mutants, according to methods previously described (52). Domain deletion mutations were made using single strand templates containing the VCAM 7D cDNA clone 1E11 or the VCAM-6D cDNA clone 41 in the expression vector CDM8. Domain deletions were guided by previous assignment of VCAM-1 domain boundaries (36). Amino acid substitution mutants were made using single strand templates of VCAM-6D or VCAM 7D/D1$^-$, a domain 1 deletion mutant of VCAM-7D. Following second-strand synthesis and transformation in *Escherichia coli*, mutants were isolated by screening for unique restriction sites conferred by mutant oligonucleotides. DNA sequencing confirmed each mutation.

Generation of a VCAM-1 Domain 4/Human IgG Chimeric Molecule

A chimera containing VCAM-1 domain 4 fused to human IgG_1 hinge, CH_2, and CH_3 was generated according to methods previously described (55). Using VCAM-7D cDNA and the oligonucleotide primers 5′TTTACTGTTGAGATCTCCCCT and 5′TTTCTCGAGTCTCACCTGAGTAGAGCTCCACCTGGAT, a polymerase chain reaction product containing domain 4 sequence was generated, digested with restriction enzymes HindIII and XhoI, and then ligated to the expression vector pCDG1 (55). This construct, pCDVC-D4/IgG, was transfected into COS cells, and a soluble chimera containing a dimer of VCAM-1 domain 4 fused to hinge, CH_2, and CH_3 of IgG1 (VC-D4/IgG) was purified from tissue culture supernatant by protein A–Sepharose chromatography.

Transfected COS Cell Adhesion Assay

COS cells were transfected with purified plasmid and prepared for assay three days later, as previously described (59). Transfected COS cells were washed three times with 1% FBS/RPMI at 25°C, and in some cases, preincubated for 15 min with anti-VCAM-1 mAb. Ramos cells were labeled as described (59) with the carboxyfluorescein compound BCECF-AM (Bioprobes, Eugene, OR), resuspended in 1% FBS/RPMI, and in some cases, preincubated with the anti-VLA-4 mAb HP2/1 for 15 min at 25°C. Ramos cells were added to 60 mm × 15 mm tissue culture dishes (4 × 10^6/dish) for 10 min at 25°C. Nonadherent cells were removed by five washes using 1% FBS/RPMI, and dishes were fixed with 2% paraformaldehyde in PBS.

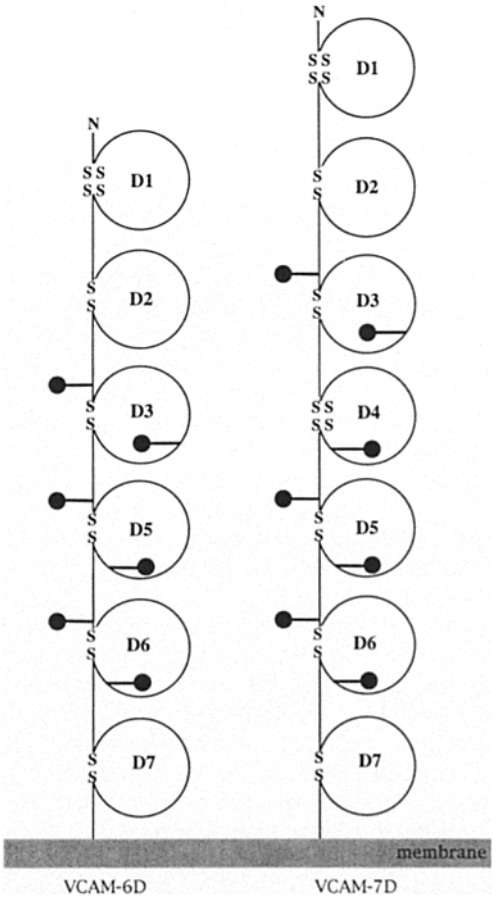

Figure 1. Schematic representation of VCAM-6D and VCAM-7D. Immunoglobulin-like domains are numbered D1–D7 with D1 located amino-terminal and most distal from membrane. VCAM-6D primary structure differs from VCAM-7D by the absence of D4. Sites of potential N-linked glycosylation are indicated by ●.

Table I. Flow Cytometric Analysis of COS Cells Expressing Wild-type or Mutant VCAM-1

	1.4C3	HAE-2f	HAE-2a	HAE-2b	HAE-2c	4B9	HAE-2d	HAE-2j
A. Wild-type and domain 1 deletion mutants*								
	Mean linear fluorescence (% 1.4C3 binding to VCAM-7D ± SE, N = 2–7)							
7D	100	103 ± 5	81 ± 9	86 ± 13	99 ± 18	102 ± 11	62 ± 12	83 ± 11
6D	92 ± 15	78 ± 17	60 ± 2	62 ± 6	74 ± 13	58 ± 6	6 ± 5	28 ± 7
7D/D1⁻	136 ± 14	112 ± 29	8 ± 3	2 ± 1	3 ± 3	1 ± 1	133 ± 24	89 ± 4
6D/D1⁻	33 ± 6	36 ± 9	3 ± 2	2 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
B. Domain 1 amino acid substitution mutants of VCAM-6D								
	Mean linear fluorescence (% 1.4C3 binding to VCAM-7D ± SE, N = 2–7)							
E29/A	63 ± 10	51 ± 6	61 ± 1	61 ± 1	64 ± 10	66 ± 10	0 ± 0	5 ± 4
R36/G	50 ± 10	90 ± 4	38 ± 14	50 ± 3	48 ± 6	53 ± 14	8 ± 8	20 ± 10
Q38/S	105 ± 19	77 ± 1	64 ± 20	95 ± 19	82 ± 14	80 ± 8	4 ± 4	21 ± 9
D40/A	52 ± 1	57 ± 2	55 ± 4	62 ± 2	57 ± 0	ND	0 ± 0	0 ± 0
L43N/AD	66 ± 10	69 ± 13	42 ± 14	58 ± 12	55 ± 13	9 ± 8	13 ± 7	22 ± 9
E76/A	67 ± 8	64 ± 14	46 ± 12	66 ± 12	59 ± 7	57 ± 9	9 ± 7	22 ± 9
R78K/QA	59 ± 4	69 ± 2	78 ± 16	0 ± 0	53 ± 1	22 ± 4	2 ± 1	1 ± 1
50% VCAM-6D‡			30	31	37	29		
C. Domain 4 amino acid substitution mutants of VCAM-7D/D1⁻								
	Mean linear fluorescence (% 1.4C3 binding to VCAM-7D ± SE, N = 1–3)							
E317/A	146 ± 2	106 ± 23	4 ± 3	12 ± 1	14 ± 2	4 ± 3	84 ± 28	168 ± 25
D328/A	106 ± 17	98 ± 24	0 ± 0	9 ± 4	10 ± 1	0	180 ± 17	157 ± 33
L331/A	101 ± 3	109 ± 17	1 ± 1	1 ± 1	3 ± 1	5 ± 4	92 ± 24	111 ± 18
K366K/QA	132 ± 24	138 ± 14	3 ± 2	5 ± 4	11 ± 0	3 ± 3	97 ± 25	80 ± 23
50% VCAM-7D/D1⁻‡							67	45

* In *A* boxes indicate fluorescence values that are less than 50% of that obtained using 1.4C3 and the corresponding VCAM construct.
‡ In *B* and *C* boxes indicate fluorescence values less than 50% of that obtained using the same mAb and COS-expressing VCAM-6D(*B*) or VCAM-7D/D1⁻(*C*).

For each experiment, bound Ramos cells were quantified using a fluorescence microscope to score the number of cells in three to five 100× fields. Phase contrast was used to count the number of COS cells in the same fields. COS cell transfectants were also labeled with a panel of anti-VCAM-1 mAb for flow cytometry as described (12). Transfection efficiency was determined as the percentage of transfected cells labeled by the anti-VCAM-1 mAb 1.4C3 subtracted by the percentage of mock transfected cells labeled by 1.4C3. Binding of Ramos cells was quantified as cells bound/transfected COS cells = ([cells bound to VCAM-1-transfectants/mm^2] − [cells bound to mock transfectants/mm^2])/([COS cells/mm^2] × [VCAM-1 transfection efficiency]) and expressed as percentage of binding to wild-type VCAM-7D.

Results

Generation and Expression of Domain 1 Deletion VCAM-1 Mutants

The amino-terminal Ig-like domains (*D1*) of VCAM-6D and VCAM-7D (Fig. 1) were deleted using oligonucleotide-directed mutagenesis. Expression of each mutant and wild-type form in COS cells was determined using eight anti-VCAM-1 mAb. Two mAb, 1.4C3 and HAE-2f, recognized each of the wild-type and domain 1 deletion mutants. As indicated by the mean linear fluorescence intensity of cells labeled by these mAb (Table I *A*), expression of VCAM-7D, VCAM-6D, and the VCAM-7D domain 1 deletion mutant, VCAM-7D/D1⁻, was comparable. Expression of the VCAM-6D domain 1 deletion mutant, VCAM-6D/D1⁻, was about 65% less than that of VCAM-7D.

Four anti-VCAM-1 mAb (HAE-2a, HAE-2b, HAE-2c, and 4B9) recognized VCAM-7D and VCAM-6D transfectants with a mean linear fluorescence comparable to that of mAb 1.4C3 and HAE-2f (Table I *A*). None of these mAb recognized VCAM-6D/D1⁻ or VCAM-7D/D1⁻ transfectants, and therefore represent a group of anti-VCAM-1 mAb whose epitopes depend on domain 1.

Two other anti-VCAM-1 mAb (HAE-2d and HAE-2j) recognized VCAM-7D and VCAM-7D/D1⁻ transfectants, but failed to bind (HAE-2d) or weakly bound (HAE-2j) VCAM-6D transfectants (Table I *A*). Neither HAE-2d or HAE-2j recognized VCAM-6D/D1⁻ transfectants, and thus these mAb represent a group of mAb whose epitopes depend primarily on domain 4.

Anti-VCAM-1 mAb Reactivity for the Chimera VC-D4/IgG

Based on ELISA, three of eight anti-VCAM-1 mAb (HAE-2b, HAE-2d, and HAE-2j) were found to react with VC-D4/IgG, a chimeric protein consisting of VCAM-1 domain 4 fused to hinge and constant regions of human IgG (Fig. 2). For HAE-2d and HAE-2j, this result was consistent with their labeling of VCAM-7D and VCAM-7D/D1⁻ transfec-

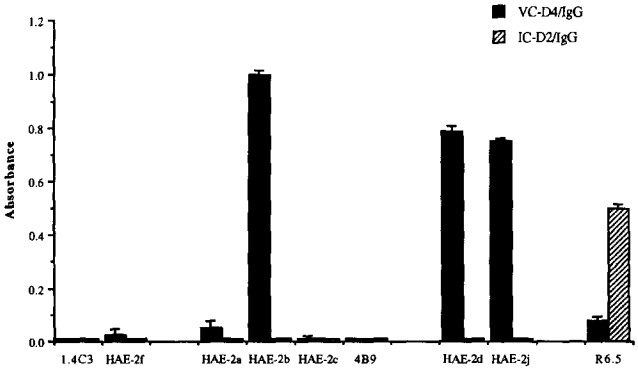

Figure 2. Binding of eight anti-VCAM-1 mAb and the anti-ICAM-1 mAb R6.5 to VC-D4/IgG (*solid bars*) or IC-D2/IgG (*hatched bars*). Binding is shown as absorbance based on ELISA from a representative of three experiments. Error bars represent 1 SD for duplicate results.

tants (Table I *A*). HAE-2b reacted with VC-D4/IgG but failed to recognize domain 4 as expressed by VCAM-7D/D1⁻ transfectants. As a control, none of the anti-VCAM-1 mAb were shown to react with the ICAM-1 chimera IC-D2/IgG (55), although the anti-ICAM-1 mAb R6.5 bound well to IC-D2/IgG.

VLA-4-dependent Adhesion to Domain 1 Deletion Mutants

To characterize the contributions of domain 1 and domain 4 in the binding of VCAM-1 to VLA-4, adhesion of the B lymphoblastoid cell line Ramos to COS cells expressing either mutant or wild-type forms of VCAM-1 was assessed. Binding was quantified relative to Ramos cell binding to VCAM-7D transfectants (Fig. 3). Ramos cells bound well to COS cells expressing VCAM-7D or VCAM-6D, forming rosettes of 10–20 Ramos cells on average per transfected COS cell. Ramos cells also bound to COS cells transfected with VCAM-7D/D1⁻ but only ~60% as well as they bound to VCAM-7D. In contrast, there was no binding of Ramos cells to VCAM-6D/D1⁻. These findings suggest that domains 1 and 4 of VCAM-1 independently bind to VLA-4.

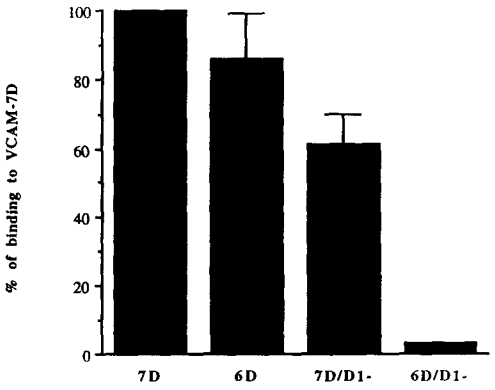

Figure 3. Ramos cell binding to COS cells expressing VCAM-7D, VCAM-6D, VCAM-7D/D1⁻, or VCAM-6D/D1⁻. Binding was quantified relative to the binding to VCAM-7D. Error bars represent 1 SD for three to five experiments.

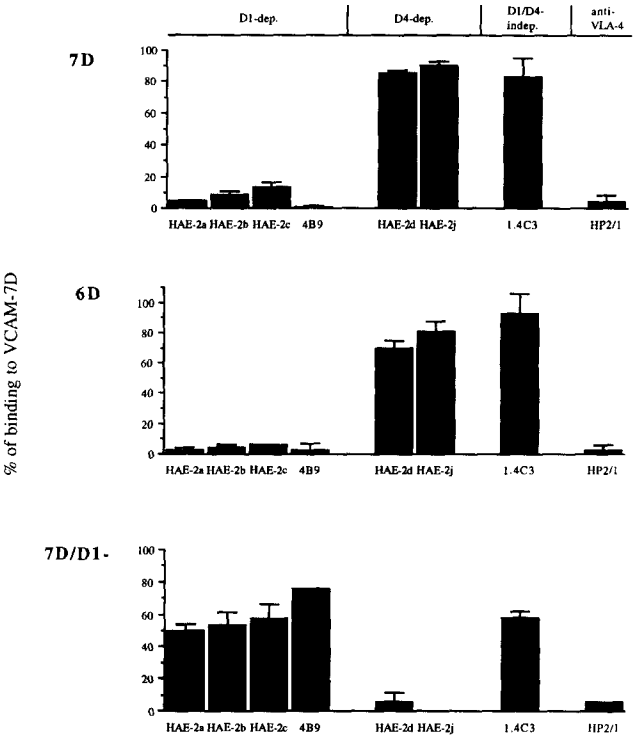

Figure 4. Ramos cell binding to COS cells expressing VCAM-7D (*7D*), VCAM-6D (*6D*), or VCAM-7D/D1⁻ (*7D/D1⁻*) in the presence of anti-VCAM-1 mAb or the anti-VLA-4 mAb HP2/1. Binding was quantified relative to the binding to VCAM-7D in the presence of control medium. Error bars represent 1 SD for two to three experiments. Experiments with VCAM-7D/D1⁻ using mAb 4B9 and HP2/1 were done once.

Seven anti-VCAM-1 mAb were examined for their abilities to inhibit Ramos cell binding to VCAM-7D, VCAM-6D, or VCAM-7D/D1⁻ COS cell transfectants (Fig. 4). The four domain 1-dependent mAb completely blocked Ramos cell binding to both VCAM-7D and VCAM-6D, whereas none of mAb bound well to COS cells expressing VCAM-7D/D1⁻ (Table I *A*), and consequently, none inhibited Ramos cell binding to VCAM-7D/D1⁻. Two domain 4-dependent mAb blocked Ramos cell binding to VCAM-7D/D1⁻ but did not block binding to VCAM-7D or VCAM-6D. mAb 1.4C3, whose epitope was independent of both domains 1 and 4, failed to inhibit binding to VCAM-7D, VCAM-6D, or VCAM-7D/D1⁻. The anti-VCAM-1 mAb HAE-2f was not tested. As a control, the anti-VLA-4 mAb HP2/1 was shown to block Ramos cell binding to any VCAM-1-expressing transfectant. The domain specificity and function-blocking patterns of these mAb provide further evidence for independent VLA-4 binding sites in domain 1 and domain 4 of VCAM-1.

Generation of VCAM-1 Amino Acid Substitution Mutants

VLA-4 binding sites were further characterized based on amino acid substitution mutants of VCAM-6D and VCAM-7D/D1⁻ generated by oligonucleotide-directed mutagenesis. Based on the functional as well as predicted structural similarities between domain 1 of ICAM-1 and domains 1 and

4 of VCAM-1, we targeted for substitution several amino acids in VCAM-1 analogous to amino acids in ICAM-1 found to be critical for binding to LFA-1 (Fig. 5; see reference 52). Domain 1 mutations were made in VCAM-6D, and domain 4 mutations were made in VCAM-7D/D1$^-$. Additional mutations were made in domain 1 of VCAM-6D in a region of four amino acids (W-R-T-Q) that lacks a structural analog in ICAM-1 (Fig. 5). Notation for the mutations uses the one-letter code for the wild-type sequence followed by a slash and the one-letter code for the corresponding mutant sequence; the position of the first amino acid is indicated relative to the predicted NH_2-terminal phenylalanine residue of VCAM-7D.

Expression and VLA-4–dependent Adhesion to Domain 1 Amino Acid Substitution Mutants

Seven substitution mutants of one to two amino acids were made in domain 1 of VCAM-6D. Expression of each mutant in COS cells was comparable to that of the wild-type VCAM-6D, as determined by flow cytometry using two mAb, 1.4C3 and HAE-2f, for which the epitopes did not depend on domains 1 or 4 (Table I *B*). The conformational integrity of domain 1 was confirmed for seven mutants by the binding of at least two out of four domain 1–dependent mAb. mAb were considered to bind a mutant well if the mean linear fluorescence of the mAb labeling COS cells expressing the mutant was not less than 50% of the mean linear fluorescence of the same mAb labeling COS cells expressing VCAM-6D. The mAb 4B9 poorly recognized mutants L43N/AD and R78K/QA. The mAb HAE-2a marginally recognized mutant R36/G. Mutant R78K/QA was not recognized by mAb HAE-2b (Table I *B*).

Three domain 1 mutants—Q38/S, D40/A, and L43N/AD—failed to support Ramos cell adhesion despite good expression and intact conformation of domain 1 (Fig. 6 *A*). Binding to four other domain 1 mutants—E29/A, R36/G, E76/A, and R78K/QA—was comparable to that observed to wild-type VCAM-6D.

Expression and VLA-4–dependent Adhesion to Domain 4 Amino Acid Substitution Mutants

Substitution mutants analogous to four of those generated in domain 1 of VCAM-6D were made in domain 4 of VCAM-7D/D1$^-$. Expression after transfection was shown using four anti-VCAM-1 mAb that recognized VCAM-7D/D1$^-$, and conformational integrity was confirmed by the binding of two mAb whose epitopes depended primarily on domain 4 (Table I *C*). None of these mutants were recognized by mAb whose epitopes depended on domain 1.

Ramos cells failed to bind to the mutants D328/A and L331/A (Fig. 6 *B*) but did bind to mutants E317/A and K366K/QA as well as they did to VCAM-7D/D1$^-$. Thus, amino acids homologous to those found to be critical in the binding of domain 1 to VLA-4 were also found to be critical in the binding of domain 4 to VLA-4.

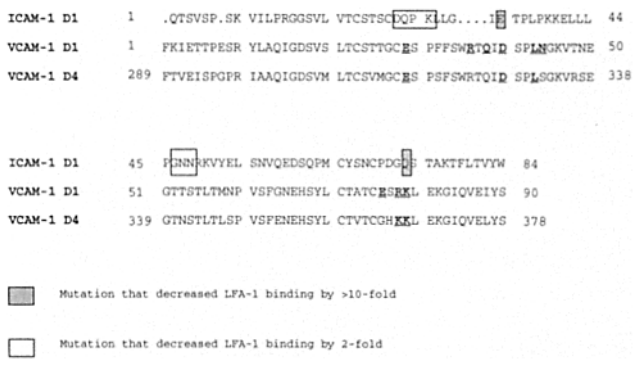

Figure 5. Amino acid sequence alignment of ICAM-1 domain 1, VCAM-1 domain 1, and VCAM-1 domain 4. Mutation of single or groups of amino acids in ICAM-1 domain 1 that significantly decreased LFA-1 binding (52) are boxed. Amino acids in domains 1 and 4 of VCAM-1 that were targeted for mutation in these experiments are underlined.

Discussion

The binding of the integrin VLA-4 ($\alpha_4\beta_1$) to VCAM-1, a member of the Ig superfamily with two alternatively spliced forms, plays an important role in mediating chronic inflammation and numerous other physiologic processes involving cell–cell adhesion. In the present study, we examined cell adhesion to mutant forms of VCAM-1 in order to determine the molecular basis of VLA-4 binding to VCAM-1. Our findings not only demonstrate two independent VLA-4 binding sites in domains 1 and 4 but also identify a critical binding function for amino acids within the linear sequence Q-I-D-S-P-L, which is identically expressed in the two domains.

Consistent with previous observations (37, 59), the existence and independence of two VLA-4 binding sites in domains 1 and 4 were clearly demonstrated by Ramos cell binding to domain 1 deletion mutants of VCAM-1. Ramos cells bound well to VCAM-7D, VCAM-6D, and VCAM-7D/D1$^-$ but not to VCAM-6D/D1$^-$. Neither domain 1-depen-

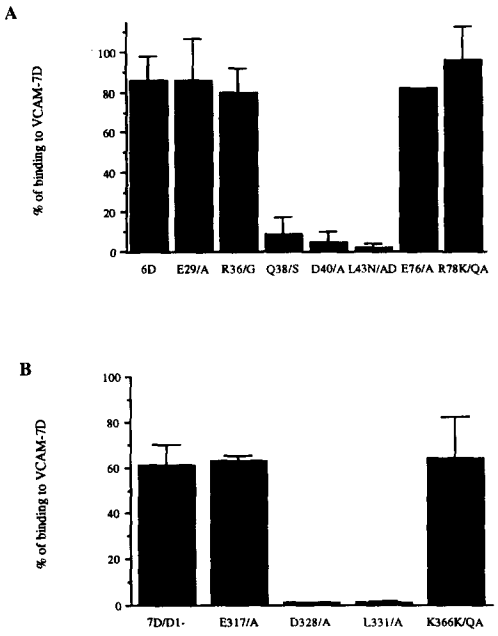

Figure 6. Ramos cell binding to COS cells expressing VCAM-1 amino acid substitution mutants targeted to (*A*) domain 1 of VCAM-6D or (*B*) domain 4 of VCAM-7D/D1$^-$ is shown relative to Ramos cell binding to VCAM-7D. Error bars represent 1 SD for two to three experiments.

dent adhesion (i.e., to VCAM-6D) required the simultaneous expression of domain 4, nor did domain 4-dependent adhesion (to VCAM-7D/D1$^-$) require expression of domain 1. In addition, the ability of anti-VCAM-1 mAb to inhibit domain 1- or domain 4-dependent adhesion correlated with their domain specificities: mAb whose epitopes depended on domain 1 blocked Ramos cell binding to VCAM-6D but not VCAM-7D/D1$^-$, and mAb whose epitopes depended primarily on domain 4 blocked Ramos cell binding to VCAM-7D/D1$^-$ but not VCAM-6D. The domain 1–dependent mAb tested here also completely blocked binding to VCAM-7D.

The biological significance of two VLA-4 binding sites on VCAM-7D versus one VLA-4 binding site on VCAM-6D remains unclear. Although under most (but not all [28]) experimental systems, VCAM-7D expression appears to dominate VCAM-6D expression, a three-domain, glycophosphatidylinositol-anchored form of murine VCAM-1 has been identified in the mouse, apparently with one VLA-4 binding site (66; and Kinashi, T., Y. St. Pierre, C.-H. Huang, and T. A. Springer, manuscript submitted for publication). If it is possible for two VLA-4 molecules to bind simultaneously to VCAM-7D (as size considerations suggest it should be) (51), then bivalent versus monovalent binding may help regulate strength of adhesiveness as well as differential signals transmitted to VLA-4–expressing cells upon ligation.

Amino acid substitution mutants of VCAM-6D and VCAM-7D/D1$^-$ defined binding sites in domains 1 and 4, respectively. Three mutations in domain 1 eliminated binding: Q38/S, D40/A, and L43N/AD. In domain 4 of VCAM-7D/D1$^-$, two mutations were made in amino acids analogous to those required for VLA-4 binding to domain 1—D328/A and L331/A—and both eliminated binding of VLA-4 to domain 4. Thus, VLA-4-binding sites in domains 1 and 4 minimally involve amino acids within a linear sequence of six amino acids that is identical in both domains: Q_{38}-I_{39}-D_{40}-S_{41}-P_{42}-L_{43} in domain 1 and Q_{326}-I_{327}-D_{328}-S_{329}-P_{330}-L_{331} in domain 4.

The first domain of human ICAM-1 conservatively expresses the five COOH-terminal amino acids of this linear sequence; namely, I_{33}-E_{34}-T_{35}-P_{36}-L_{37} (Fig. 7). Furthermore, mutation of E_{34} of ICAM-1, which is homologous to D_{40} of domain 1 of VCAM-1 and to D_{328} of domain 4 (Fig. 7), eliminates binding of ICAM-1 to its integrin receptor LFA-1 (52). In addition, human ICAM-2 and ICAM-3, which also bind integrins (11, 53), express five amino acid motifs homologous to the VCAM-1 and ICAM-1 motif except for a serine residue substituted for proline at the fourth position. Murine ICAM-1, ICAM-2, and VCAM-1 express a motif homologous to the human VCAM-1 and ICAM-1 motif except for one or two other nonconserved substitutions (Fig. 7). For each human and murine Ig domain noted in Fig. 7, the motif is predicted to be found on the turn between β sheets C and D.

Based on these functional and predicted structural similarities of VCAM-1 and ICAM domains, we propose that residues with the conserved motif I(L)-D(E)-S(T)-P(or S)-L are necessary but insufficient for integrin binding. A set of nonconserved amino acids may primarily regulate integrin specificity for these Ig-like domains. In the case of ICAM-1, several residues distinct from the conserved I(L)-D(E)-S(T)-P(or S)-L motif are important for LFA-1 binding (52), but no residues in VCAM-1 analogous to these in ICAM-1 were found to be important for VLA-4 binding. Similarly, the residue Q_{38} in domain 1 of VCAM-1 was required for VLA-4 binding, but a residue analogous to Q_{38} is not expressed in ICAM-1 (Fig. 5). Most likely there are other nonconserved residues of VCAM-1 that confer specificity for binding to VLA-4.

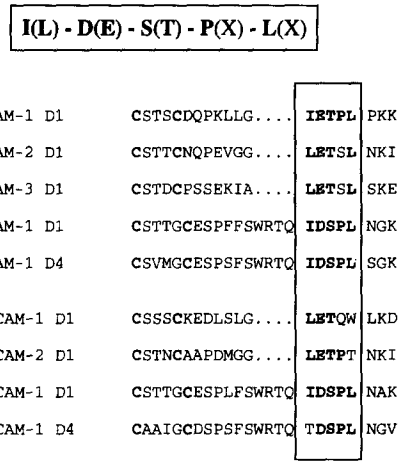

Figure 7. Alignment of residues from human ICAM-1, ICAM-2, ICAM-3, and VCAM-1 and murine ICAM-1, ICAM-2, and VCAM-1 (8, 10, 16, 20, 21, 36, 39, 49, 53, 54, 58, 64). A conserved amino acid motif is boxed. Residues within this motif are proposed to be involved in the binding of these domains to integrins.

There is good precedence for conserved amino acid motifs dictating integrin binding. The motif R-G-D has long been recognized as contributing to integrin binding, particularly to extracellular matrix proteins such as fibronectin (51). Neither VCAM-1 nor ICAM-1 includes the R-G-D (or R-G-E) motif. Fibronectin binds to VLA-5 via an R-G-D site, but VLA-4 binds to fibronectin via the CS-1 peptide (18, 31, 60). The minimal essential sequence for CS-1 binding to VLA-4, L-D-V (25, 61), is related to the I-D-S-P-L sequence of VCAM-1 in that both express I(L)-D. The tripeptide L-E-V, however, does not support VLA-4 binding (25), whereas either an aspartate or glutamate residue is functional in the I(L)-D(E)-S(T)-P-L sequence within human VCAM-1 and ICAM-1.

It is not clear how the domain 1–dependent mAb tested here blocked VLA-4–dependent adhesion to domain 4 as expressed on VCAM-7D, although this phenomenon is clearly not the case for all domain 1–dependent mAb that have been examined (37). It has been suggested (37) that certain mAb bind to domain 1 on VCAM-7D in such a configuration as to hinder domain 4 activity sterically, or, that upon binding of these mAb, the conformation of domain 4 is perturbed, rendering it incapable of interacting with VLA-4. Another explanation would be that these mAb have a high affinity for a domain 1 epitope and a low affinity for a domain 4 epitope. The 73% amino acid identity between domains 1 and 4 favors cross-reactions between them. High-affinity binding of one Fab of anti-VCAM-1 IgG to domain 1 would greatly increase the local concentration of the other Fab, favoring its binding to domain 4, thus inhibiting domain 4 binding to VLA-4. In the absence of domain 1 expression, the affinity for domain 4 would ordinarily be too low for mAb binding to be detected by flow cytometry. The use of Fab fragments

of these domain 1-dependent mAb could help elucidate these possibilities. In the present study, one mAb, HAE-2b, did not bind to VCAM-7D/D1$^-$, which contained one copy of domain 4, but did bind to VC-D4/IgG, which contained two copies, perhaps because bivalent binding of HAE-2b was facilitated. It is also possible that HAE-2b binds VC-D4/IgG and not VCAM-7D/D1$^-$ because domains 2 and 3 on VCAM-7D/D1$^-$ mask its epitope in domain 4.

In summary, we have demonstrated that VLA-4 binds independently to domains 1 and 4 of VCAM-1, involving amino acids within the linear sequence Q-I-D-S-P-L that is identically expressed in each domain. Five of these amino acids represent the conserved motif I(L)-D(E)-S(T)-P(or S)-L, also found in human ICAM domains. We propose that this conserved motif provides a necessary but insufficient contribution to each VLA-4-binding site and that additional nonconserved residues confer specificity for integrin binding.

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