

Activated Endothelium Binds Lymphocytes Through a Novel Binding Site in the Alternately Spliced Domain of Vascular Cell Adhesion Molecule-1

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Summary

Vascular cell adhesion molecule-1 (VCAM-1) is induced on endothelial cells by inflammatory cytokines, and binds mononuclear leukocytes through the integrin very late antigen-4 (VLA-4) ($\alpha_4\beta_1$). This adhesion pathway has been implicated in a diverse group of physiological and pathological processes, including B cell development, leukocyte activation and recruitment to sites of inflammation, atherosclerosis, and tumor cell metastasis. The major form of VCAM-1 (VCAM-7D) has seven extracellular immunoglobulin (Ig)-like domains, of which the three NH₂-terminal domains (domains 1–3) are similar in amino acid sequence to domains 4–6. By functional analysis of VCAM-7D relative to VCAM-6D (a minor 6-domain form of VCAM-1 in which domain 4 is deleted because of alternative splicing), and chimeric constructs between VCAM-1 and its structural relative intercellular adhesion molecule-1 (ICAM-1), we show that either the first or the homologous fourth domain of VCAM-1 is required for VLA-4-dependent adhesion. Either of these binding sites can function in the absence of the other. When both are present, cell binding activity is increased relative to monovalent forms of the molecule. The homologous binding regions appear to have originated by internal duplication of a portion of a monovalent ancestral gene, before the mammalian radiation. Thus VCAM-1 exemplifies evolution of a functionally bivalent cell–cell adhesion molecule by intergenic duplication. We have also produced a new class of anti-VCAM-1 monoclonal antibodies that block domain 4-dependent adhesion, and demonstrate that both binding sites participate in the adhesion function of VCAM-1 on endothelial cells *in vitro*. Therefore both sites must be blocked in clinical, animal, or *in vitro* studies depending on the use of anti-VCAM-1 antibodies to inactivate the VCAM-1/VLA-4 adhesion pathway.

Vascular cell adhesion molecule-1 (VCAM-1)¹ (also known as inducible cell adhesion molecule-110 [INCAM-110]) was first identified as an adhesion molecule induced on endothelial cells by inflammatory cytokines (TNF and IL-1) and LPS (1, 2). It binds to cells bearing the integrin very late antigen (VLA-4) ($\alpha_4\beta_1$), including T and B lymphocytes, monocytes, basophils, and eosinophils, but not neutrophils, and participates in recruitment of these cells from the bloodstream to areas of infection and inflammation (3–7). The VCAM-1/VLA-4 adhesion pathway is important in a number of physiological and pathological processes. Although VLA-4 is normally restricted to hematopoietic lineages, it is found on melanoma cell lines, and thus VCAM-1 has been suggested to participate in metastasis of such tumors (1). *In vivo*, VCAM-1 is found on areas of arterial endothelium

representing early atherosclerotic plaques in a rabbit model system (8). It is also found on follicular dendritic cells in normal human lymph nodes (9), and on bone marrow stromal cells in the mouse (10), where it plays a role in B cell development.

VCAM-1 belongs to the Ig superfamily. The major form on endothelial cells, VCAM-7D, has seven Ig homology units or domains. Domains 4–6 are similar in amino acid sequence to domains 1–3, suggesting an intergenic duplication event in the evolutionary history of the gene. There is also a minor 6-domain form (VCAM-6D) generated by alternative splicing, in which the fourth domain is deleted (2, 11–15). Biological significance of this minor form is unknown.

It has been shown previously that the first three domains of VCAM-1 can bind to VLA-4, when fused to an Ig constant region (16). The first three domains of VCAM-1 isolated by proteolytic cleavage, and the first two domains of VCAM-1 produced as a soluble recombinant molecule, also support both VLA-4-dependent adhesion and the binding of blocking mAb 4B9 when bound to plastic (R. B. Pepinsky,

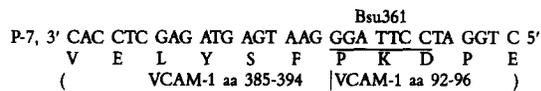
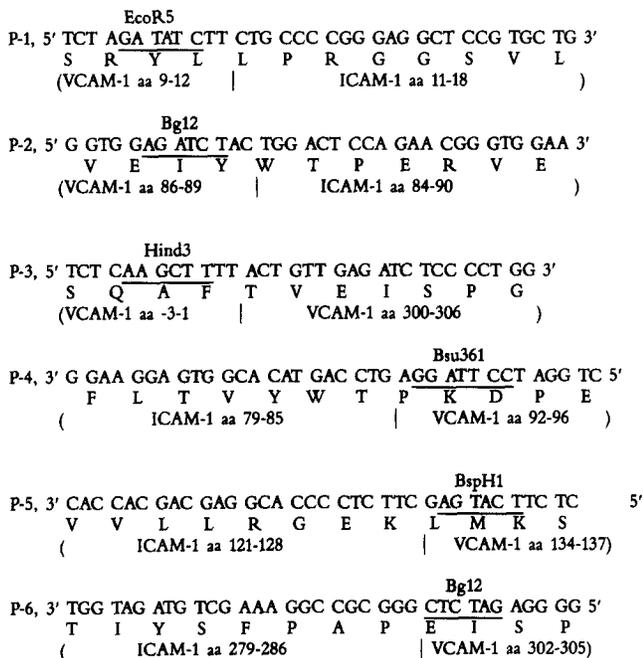
¹ Abbreviations used in this paper: ELAM-1, endothelial leukocyte adhesion molecule-1; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.

R. Lobb, and C. Hession, unpublished data). We wished to test for other regions of VCAM-1 involved in cell adhesion. In an effort to avoid structural instability caused by deletions of portions of the protein, we made chimeric constructs, replacing portions of the VCAM-1 molecule with analogous regions of its closest structural relative, intercellular adhesion molecule-1 (ICAM-1), and tested transfectants for expression, cell binding, and antibody binding. We find that binding of VLA-4 to VCAM-1 can occur via two separate sites, one requiring domain 1, and the other requiring domain 4. We have produced a novel group of anti-VCAM-1 mAbs that bind to the alternately spliced domain 4. Two of these antibodies can inhibit domain 4-dependent cell adhesion, and can inhibit cell binding to VCAM-1 on cultured endothelial cells, implying that binding through domain 4 is an important functional component of the VLA-4/VCAM-1 interaction *in vivo*.

Materials and Methods

Antibodies and Cells. Previously described mAbs used were anti-CD18, 60.3 (17); anti-VLA-4, HP1/2 (18); and anti-VCAM-1, 4B9 (19). GE4, GH12, and ED11 are IgG1 mAbs made from mice immunized with recombinant soluble VCAM-7D (20), and will be described in detail elsewhere (C. D. Benjamin and I. Douglas, unpublished data). Polyclonal rabbit anti-VCAM-1 serum was made by inoculating rabbits with recombinant soluble VCAM-7D. Human umbilical vein endothelial cells (HUVECs), COS7, and leukocytic cells were cultured as previously described (2).

Plasmid Constructions. Aliquots of the parent plasmids VCAM-1E11/CDM8 (7 domain) (13) or VCAM41/CDM8 (6 domain) (2, 13) were digested by restriction endonucleases (see Fig. 2) and agarose gel purified. ICAM-1 inserts were made by PCR, using as template an ICAM-1/CDM8 plasmid isolated from the endothelial cDNA library previously described (2), probed with oligonucleotides based on the published sequence of ICAM-1 (21, 22). The PCR primers were designed as follows:



PCR synthesis for inserts for construct V/I-1 was primed with P-1 and P-4; V/I-2, P-1 and P-5; V/I-3, P-2 and P-6; V1/V4, P-3 and P-7. V6D/I-1 and -2 were made using the same insert as V/I-1 and -2, respectively, cloned into VCAM-6D (clone 41) backbones instead of VCAM-7D. After PCR synthesis (performed as described in reference 13), the insert fragments were digested with the appropriate restriction endonucleases and gel purified. Backbone and insert fragments were ligated using New England Biolabs (Beverly, MA) ligase and buffer, ethanol precipitated, and electroporated into bacterial host strain MC1061/p3 using a gene pulser (Bio-Rad Laboratories, Richmond, CA). Clones were screened by PCR and restriction digests, and at least one isolate of each clone was sequenced across cloning junctions and insert using a Sequenase kit (United States Biochemical Corp., Cleveland, OH).

Cell-Cell Adhesion Assays. COS7 cells were transfected by electroporation, as described (2), except 20 μ g of plasmid was used and voltage was set at 300 V instead of 280 V. Cells were plated in 48-well plates for cell adhesion assays, and in 100-mm dishes for FACS[®] analysis (Becton Dickinson & Co., Mountain View, CA). Transfection efficiency ranged from 15 to 25% of surviving cells as estimated by FACS[®] analysis (described below). HUVECs were treated as described (2). Suspension cells (Ramos, Jurkat, HL60, etc.) were labeled for 20 min with BCECF-AM (Molecular Probes, Junction City, OR), as described (23). Antibodies were preincubated with transfected COS7 cells or HUVECs (anti-VCAM), or leukocytic cells (anti-CD18, anti-VLA-4), for 15 min at room temperature just before the adhesion assay. Adhesion assays were performed by adding 0.15 ml of labeled suspension cells at 3 million cells per ml in RPMI/10% FCS to each well of transfected COS7 cells or HUVECs, which had been plated in 48-well plates and preincubated with 0.15 ml antibody at 2 \times final concentration or RPMI/10% FCS alone. The plates were incubated for 15 min at 37°C except where noted, and then washed three or four times with 0.2 ml per well of HBSS with calcium and magnesium, added to the wells with a multichannel pipetter, and removed by inverting the plate and briefly shaking. After washing, cells were lysed with 0.12 ml 1% NP-40, transferred to 96-well plates, and quantified using a Fluoroskan II plate reader (Labsystems, Inc., Shrewsbury, MA).

Flow Cytometry. Transfected COS cells were removed from 100-mm tissue culture dishes by incubation in HBSS/5 mM EDTA for 15 min at 37°C, followed by vigorous pipetting. 3–5 \times 10⁵ cell aliquots were incubated individually in a volume of 0.1 ml with 2 μ g/ml mAbs to VCAM-1 or ICAM-1, or with polyclonal antiserum to VCAM-1 diluted 1:500, for 20 min at room temperature in PNF (PBS/0.1% sodium azide/2% fetal bovine serum), washed two times with PNF, and incubated in a volume of 0.1 ml for 20 min at room temperature with 0.2 μ g/ml secondary fluoresceinated Ab (rabbit anti-mouse Ig, or goat anti-rabbit Ig) in PNF. Cells were then washed three times with PBS, suspended in PBS/1% formaldehyde, stored dark at 4°C, and analyzed using a FACStar[®] (Becton Dickinson & Co.). Number of cells (y axis) versus fluorescence (FL1) was plotted, and the number of positive cells in each sample was determined as follows. COS7 cells transfected with CDM8 vector alone were stained in parallel with each mAb, and a marker was set on the FACS[®] histogram (Becton Dickinson & Co.) at the right edge of the peak, to exclude 99% of the negative cells. The number of positive cells (to the right of this marker) was determined for each experimental transfection. Mean fluorescence of these cells was determined, divided by 100, and multiplied by the number of positive cells to give the "expres-

sion index", a number that represents total fluorescence of positive cells in the sample. The expression index therefore reflects both the number of positive cells and the mean fluorescence of those cells, and directly varies with the total number of molecules expressed in each sample.

Results

Ramos Cell Binding to VCAM-6D vs. VCAM-7D. Ramos is a B-lymphoblastoid cell line that expresses VLA-4 and thus binds to VCAM-1, but does not bind to other induced endothelial cell molecules such as ICAM-1 or endothelial leukocyte adhesion molecule-1 (ELAM-1) (2). Thus it is a convenient model line with which to assay VLA-4-dependent binding. Ramos cells bind to both VCAM-6D and -7D when these molecules are expressed on COS cells that have been transfected with cDNA cloned into the vector CDM8. We had previously noted that binding to VCAM-7D was somewhat greater than to VCAM-6D in transient transfection assays of a single preparation of each plasmid (13). To determine if this quantitative difference in binding activity was reproducible, several preparations of each plasmid were made, and electroporated in parallel into COS cells. Repeated transfections reveal that although expression of the two plasmids is similar (Fig. 1 A), VCAM-7D, on average, binds significantly more cells per mm² than does VCAM-6D (Fig. 1 B, no Ab). Binding to both 6 and 7 domain forms can be inhibited by the anti-VLA-4 mAb HP1/2, or by anti-VCAM-1 mAb 4B9 (Fig. 1 B).

Structural Similarities of VCAM-1 and ICAM-1. Although both mouse and rat VCAM-1 cDNAs have been cloned and characterized (24), the use of interspecies chimeras was contraindicated by our finding that both mouse and rat VLA-4 bind to human VCAM-1, suggesting that human VLA-4 might well bind to mouse and/or rat VCAM-1 (L. Osborn, unpublished data, and 24). Thus interspecies chimeras would not allow us to map cell binding regions of the molecule

by loss of function. Several features of ICAM-1 suggested it would be a suitable partner for VCAM-1 in chimeric constructs. Although the genes encoding ICAM-1 and VCAM-1 map to different chromosomes in both mouse and human, and so are not members of a typical closely linked multigene family like the Igs or selectins (25, 26), there are structural similarities between the two proteins that can probably be attributed to similarities in their function. ICAM-1 and VCAM-1 are, along with ICAM-2, the only known Ig superfamily molecules that bind integrin counter-receptors, and might be expected to share structural features based on that common function. Amino acid sequence similarity analysis of VCAM-1 vs. the National Biomedical Research Foundation data base using the ALIGN program (Genetics Computer Group, Madison, WI) gave a higher score (10.1) for ICAM-1 than any other protein (2). Upon alignment and calculation of percent identity, ICAM-1 and VCAM-1 are 20–30% identical in amino acid sequence. When conservative amino acid substitutions are taken into account, their similarity rises to 60–70% (Fig. 2). Finally, ICAM-1 shares with VCAM-1 an unusual structural feature of the domains now known to be most important for cell binding: the first Ig domain of both molecules, and the fourth Ig domain of VCAM-1, have four rather than the usual two cysteine residues, and the position of the extra cysteine residues are conserved among all three domains exhibiting them (ICAM-1 domain 1, VCAM-1 domains 1 and 4) (Fig. 2).

Construction and Expression of VCAM-1/ICAM-1 Chimeric Constructs. To choose suitable exchanges between the two sequences, the first four domains of the ICAM-1 sequence were manually aligned with VCAM-7D (Fig. 2). It should be noted that the details of this alignment are somewhat arbitrary at many positions, and thus other alignments are possible. VCAM-1/ICAM-1 (V/I or V6D-I) constructs were made using unique restriction endonuclease sites in the VCAM-1 plasmids, and PCR-generated fragments of ICAM-1. Of seven V/I and V6D/I constructs made and tested, five were well

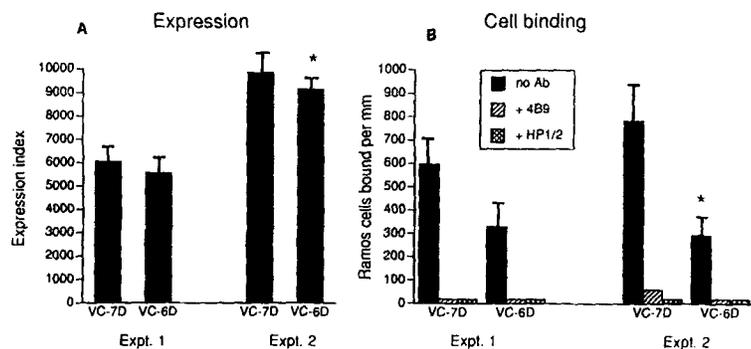


Figure 1. (A) COS7 cells transfected by electroporation with two (*) or three preparations of cDNA encoding VCAM-7D (VC-7D) or VCAM-6D (VC-6D), analyzed by flow cytometry after indirect immunofluorescent staining with anti-VCAM-1 mAb 4B9, which recognizes domain 1 of both VCAM-6D and VCAM-7D. The number of cells with fluorescent intensity (FL1) greater than that shown by 99% of control non-VCAM transfectants was determined and multiplied by the mean fluorescence of these cells, to give the "expression index", which directly correlates with the number of VCAM-1 molecules present. In each experiment, the expression index was averaged for the two or three plasmid preparations tested, and SD is indicated by error bars. Data shown is from two experiments, representative of data obtained in four experiments. Expression index may be compared within but not between experiments, as time between staining and fixing, and FACS[®] analysis varied between experiments. (B) The transfected COS7 cells described in A above were plated in parallel in 48-well plates at 3×10^5 cells per well, grown to confluence (48 h), and assayed for binding to fluorescently labeled Ramos cells, in the absence or presence of blocking mAbs to VCAM-1 (mAb 4B9) or to VLA-4 (mAb HP1/2).

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HindIII      EcoRV                                  BglIII
V 1  F^KIETTPESRYL^AQIGDSVSLTCSTTGCESPFFSWRTQIDSEPLNGKVINE-GTTSTLIMNPFVSGNEHSYL-CTATC-ESRKLEKGIQVEIY^S
I 1  p psxP Sbhf GsSV fTCSTx CapP h IaxPL K Gpppf fS pEpS C ssC aspp Kah fpfY
      QTSVSP-SKVI LPRGGSVLVTCSTS-CDQPKL---LGIETPLPKKELLPGNNRKVYE--LSNVQEDSQPMCYSNCPDGGQSTAKTF-LTVY W

Bsu361                                  BspHI
V 2  F^PKDPEIH-LSGPLEAGKPIITKCSVADVYFFDRLEIDLLKGDH^LMKSQEFLE-EDADRKSLETKSLEVTFPVIEDI-GKVLVCRAKLHI----DEMDSVPTVRQAVKELQVY
I 2  Ppp Ef L s sGK fTfbCpV ss P spLpfsLLbGab hKpp f E Aa p TpsL V pD G shsCRspLpf Eh ps xs fp h
      T PERVELAPLPSWQPFVGNLTLRCQVEGGAPRANLTVVLLRGEK ELKREPAVGEPAEVT---TTVL-----VRRDHHGANFSCRTELDLRPQGLELFENTSAFYQLQTF

V 3  ISPKNTVISVNPSTKL-QE-GGSVTMTCSSEGL-PAPEIFWSKKLDNGNLQHLGSGNATLTL---IAMRM-----EDSG--IYVCEGVNLIQGNRREVELIVQ
I 3  h P p VsPps L E sspThsCS aGL Ps E b Ls Gs QbL N TfTh hs b EDpG hsc sV L GppppEs Vp
      VLPATPPQLVSPRV-L--EVDTQGTVVCSLDGLFPVSEAQV-H-LALGD-QRL--NFTVTYGNDSFSAKASVSVTADEGTQRLTC-AVIL-GNQSQETLQTVTI

BglII
V 4  EKPFV^EISPG-PRIAAQIGDSVMLTCSVMGCESPSFSWRT-QIDSPLSGKVRSEGTNSTLTLSPVSEFENEHSYLCTVTCGHKK-LEKGIQV-ELYS ...
I 4  p s pf s Phfs GppV fpCps p s spLs Rsp xP aN bSo CxsT L b Qs EL s
      YSFPAP NVILTKPEVSE--GTEVTVKCEAH--PRA-KVTLNGVPAQPLGP--RAQ--LLLK-ATP--EDNGRSFSCSATLEVAGQLIHKQNTRELRVLYG ...

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a - acid - D, E
f - aliphatic - L, I, V
h - hydrophobic - L, I, V, M, Y, F
o - aromatic - Y, F, W
p - polar - K, R, H, D, E, Q, N, T, S
s - small - A, G, S, T, V, N, D
b - base - R, R, H
x - hydroxyl - S, T

Figure 2. Alignment of VCAM-1 and ICAM-1 amino acid sequences, domains 1-4. The sequences were aligned by inspection. (V1) VCAM-1 domain 1; (I1) ICAM-1 domain 1; etc. Domain boundaries were assigned based on exon-intron borders reported by Cybulsky et al., (15). Restriction sites of VCAM-1 that were used to make chimeric constructs (see Fig. 3) are indicated at the site where the amino acid sequences of VCAM-1 and ICAM-1 are joined. The restriction sites fall near but not necessarily at the junction codons (see Materials and Methods for details).

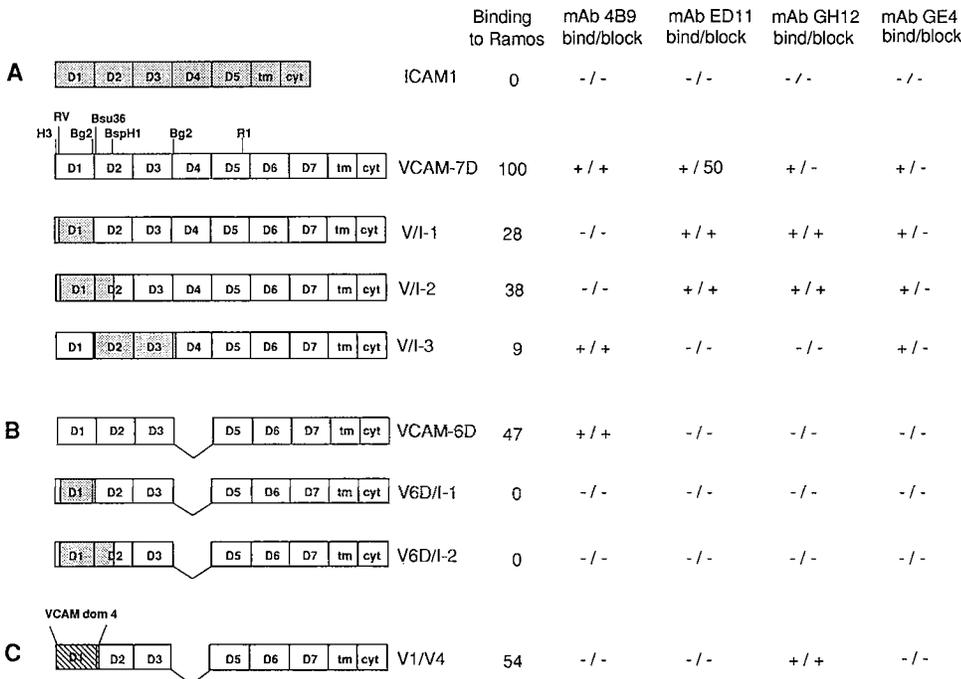


Figure 3. Schematic diagram of parental and chimeric constructs, adhesion to Ramos cells, and binding and blocking of adhesion by anti-VCAM-1 mAbs. (A) ICAM-1, VCAM-7D (clone 1E11, (13), and chimeric VCAM/ICAM-1 constructs V/I-1, -2, and -3. (Open boxes) VCAM-1 derived sequences are indicated. (Stippled boxes) ICAM-1 derived sequences. CDM8 vector sequences are not shown. Restriction endonuclease sites: HindIII, H3; EcoRV, RV; BglII, Bg2; EcoRI, R1; Bsu36I, Bsu36; BspHI, BspHI. (B) VCAM-6D (VCAM/CDM8, (2) and chimeric constructs V6D/I-1 and -2. (C) Construct V1/V4, in which VCAM-6D domain 1 is replaced by VCAM-7D domain 4 (hatched box). At right, binding of each construct to Ramos cells, as a percentage relative to VCAM-7D, is indicated. Results are averages of triplicate determinations from one experiment, representative of at least three experiments. Two independently isolated clones of each construct except V/I-2 and V1/V4 were tested, and found to be the same in expression and cell binding. Expression of each construct was determined in every experiment by FACS[®] using polyclonal anti-VCAM serum as primary antibody, as described in legend to Fig. 1 and Materials and Methods, and differences in expression were found to be negligible, as correction for expression changed raw data by <3%. Ability of mAbs 4B9, ED11, GH12, and GE4 to bind to each construct (assessed by FACS[®]), and to block Ramos cell adhesion is summarized as plus or minus, as results were essentially either positive or negative in all cases, except mAb ED11 blocking of Ramos binding to VCAM-7D, which was partial (about 50%) at the single concentration tested (5 μg/ml).

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expressed (schematically diagrammed in Fig. 3, A and B) when transfected into COS cells, assessed by FACS[®] analysis after staining with polyclonal antiserum to VCAM-1. In constructs V/I-1 and V/I-2, most of domain 1, or most of domain 1 and the first half of domain 2, are replaced by ICAM-1 sequence. In construct V/I-3, domains 2 and 3, and small portions of domains 1 and 4, are replaced by ICAM-1 sequence. Two constructs, in which the regions between the Bsu361 and BspH1, and the BspH1 and EcoR1 sites were replaced, were not well expressed, and were not analyzed further.

Blocking mAb 4B9 Binds to Domain 1. Blocking antibodies have often proved useful for defining binding sites of adhesion molecules, as the epitope for the blocking mAb usually maps physically near, though not exactly to, the amino acids involved in binding (27, 28). Anti-VCAM-1 mAb 4B9 blocks binding of VLA-4-bearing cells to VCAM-6D or -7D. As mentioned above, recombinant soluble domains 1+2 of VCAM-1 can bind both cells and mAb 4B9 (C. Hession and R. Lobb, unpublished data). Chimeric constructs V/I-1 and -2 are not stained by mAb 4B9, indicating that the epitope to which 4B9 binds requires domain 1 for recognition (Fig. 3). 4B9 does stain construct V/I-3, confirming that domain 1 contains the 4B9 epitope.

Ramos Cell Binding to VCAM-1/ICAM-1 Chimeric Constructs. Although constructs V/I-1 and V/I-2 do not bind mAb 4B9, both bind Ramos cells at about 30% of the level seen with intact VCAM-7D (Fig. 3). This adhesion is VLA-4/VCAM-1 mediated, as anti-VLA-4 mAb HP1/2 completely inhibited the interaction, while a blocking antibody to the ICAM-1 pathway (mAb 60.3) did not effect cell binding (data not shown). Construct V/I-3 binds Ramos cells weakly but measurably, at about 10% of the level seen with intact VCAM-7D. mAb 4B9, against domain 1 of VCAM-1, and anti-VLA-4 mAb HP1/2 inhibits Ramos cell adhesion to V/I-3 completely, while mAb 60.3 has no effect (data not shown).

Ramos Cells Bind to Two Separate Regions of VCAM-1, Involving Domains 1 and 4. To determine if cell binding to constructs V/I-1 and V/I-2 was due to residual portions of the domain 1-dependent binding site, or to domain 4, which is structurally similar to domain 1, constructs analogous to 1 and 2 were made, using VCAM-6D instead of -7D as the parent plasmid (Fig. 3 B). These constructs were well expressed in COS cells as measured by polyclonal antiserum staining, but were unable to bind Ramos cells (Fig. 3). Thus the binding of Ramos cells to constructs 1 and 2 requires the presence of domain 4.

To confirm this interpretation, construct V1/V4, in which domain 4 of VCAM-7D was substituted for domain 1 of VCAM-6D (Fig. 3 C), was made. V1/V4 binds Ramos cells as well as does the parent VCAM-6D plasmid (Fig. 3), indicating that domain 4 can indeed bind cells, in the absence of domain 1.

How can this result be reconciled with the fact that mAb 4B9, which binds to domain 1 but not to domain 4, can completely inhibit binding of Ramos cells to VCAM-7D? Steric hindrance of domain 4 activity is one possible explanation (see below). It is also possible that binding of 4B9 to

domain 1 perturbs the structure of the molecule so that domain 4 is no longer in the proper conformation to bind cells.

mAbs that Block Domain 4-dependent Cell Binding. BALB/c mice were immunized with recombinant soluble VCAM-1 (7D) and used to make hybridomas. Three mAbs were produced that recognize VCAM-7D but not VCAM-6D, indicating that they recognize a domain 4-dependent epitope (C. D. Benjamin and I. Douglas, unpublished data, and Fig. 3). These antibodies, ED11, GH12, and GE4, were tested for ability to stain and to block Ramos cell binding to constructs exhibiting domain 4-dependent binding (Fig. 3). mAb GE4 stains V/I-1, -2, and -3, does not stain V1/V4, and does not block Ramos cell binding to any of these constructs. mAb ED11 stains and blocks V/I-1 and -2, but not -3 or V1/V4. This antibody also partially blocks Ramos cell binding to VCAM-7D. mAb GH12 stains and blocks V/I-1 and -2, and V1/V4, but not V/I-3. It did not block Ramos cell binding to VCAM-7D at the single concentration tested. Each antibody recognizes a different subset of constructs, indicating that each of the three antibodies recognizes distinct epitopes, and two are capable of blocking domain 4-dependent adhesion of VLA-4 to VCAM-1. This new class of anti-VCAM-1 blocking antibodies provides unique reagents for ensuring complete inhibition of VCAM-1-mediated adhesion.

Inhibition of Ramos Cell Binding to Cytokine-activated Endothelial Cells by Domains 1- and 4-dependent mAbs. To determine if domain 4-dependent mAbs ED11 or GH12 were capable of blocking adhesion to VCAM-1 on endothelial cells (which is predominantly VCAM-7D), HUVECs were treated with 20 ng/ml human recombinant TNF for 4, 24, 48, or 72 h, and binding of Ramos cells and ability of antibodies to block binding were measured (Fig. 4 A-D). mAb GH12 did not block binding reliably at the single concentration tested (10 µg/ml), but mAb ED11 blocked more than 80% of Ramos cell binding to induced endothelial cells at all time points tested. Jurkat (a T cell leukemia line) binding was tested in parallel at 24 h after induction. This line binds more avidly, yielding more than four times as many cells bound per mm² than Ramos (data not shown). In contrast to Ramos, mAb ED11 blocked Jurkat by only about 20%, although, as we previously reported, 4B9 blocked binding of both cell lines by >90% (data not shown). This result, coupled with the inability of ED11 to completely block binding of Ramos to VCAM-7D expressed on COS cells, suggests that the effect of ED11 on domain 1-dependent binding is variable, and may depend on the density of receptor and counter-receptor, and perhaps the activation state of the VLA-4-bearing cell (see below).

Overall, the data strongly suggest that although the two cell binding sites of VCAM-1 (domains 1- and 4-dependent) can be separated and remain functional, antibodies to each can interfere with cell binding to the other. This is so even though the antibodies do not cross-react with each other's binding sites (shown by the differential antibody binding data to constructs, Fig. 3, and conventional cross-blocking studies with labeled antibodies, C. D. Benjamin and I. Douglas, unpublished data). Steric hindrance might account for this, as

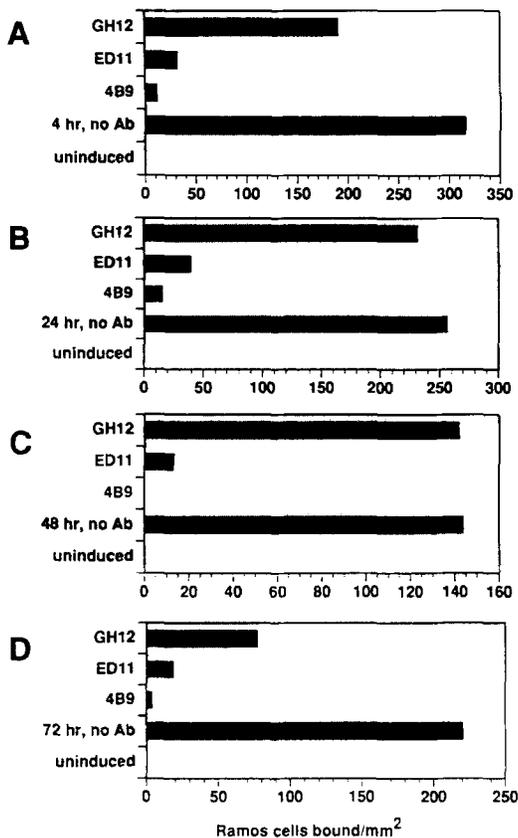


Figure 4. Ability of anti-VCAM-1 mAbs 4B9, GH12, and ED11 to block Ramos cell adhesion to TNF-activated endothelial cells. 20 ng/ml recombinant human TNF was added to confluent HUVECs in 48-well clusters at the indicated time before adhesion assay: (A) 4 h; (B) 24 h; (C) 48 h; and (D) 72 h; note that x axes are not equivalent. mAbs were added to the binding assay to a final concentration of 10 μ g/ml, as described in Materials and Methods. Data is from a single experiment, representative of two.

we suggested for the similarly surprising ability of 4B9 to inhibit completely binding to VCAM-7D. The arrangement of VCAM-1 domains has not yet been determined by electron microscopy. However, even if domains 1 and 4 are maximally distant from each other, an antibody molecule or Fab fragment is large enough to span the distance between the two, so that an antibody bound to domain 1 could overhang and thus sterically hinder access to domain 4, provided its binding orientation was suitable. Similarly, an antibody bound to domain 4 in the correct orientation could overhang domain 1. We might then expect to find four types of VCAM-1 blocking antibodies: (a) domain 1 binders that block only domain 1; (b) domain 4 binders that block only domain 4; (c) domain 1 binders that block both domains 1 and 4; and (d) domain 4 binders that block both domains 4 and 1. Several partially blocking anti-VCAM-1 mAbs are found in the literature, and may belong to class 1 or 2 above (e.g., mAb 2G6 [29], and mAb E1/6 [1]). The completely blocking mAb 4B9 clearly belongs to class 3, while the case for ED11 is still unclear; perhaps it is a weak member of class 4. Attractive as this simple model of steric hindrance may be, it is

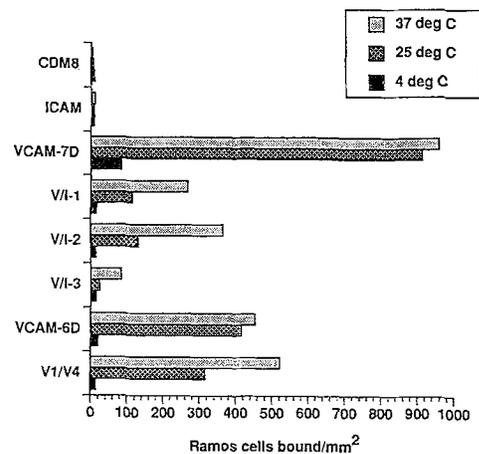


Figure 5. Temperature sensitivity of VCAM-1/VLA-4 binding. Ramos cell adhesion to COS7 cell transfectants was assayed at 4, 25, and 37°C.

also possible that binding of the antibody to one domain can perturb the structure of another domain of the molecule. It is clear that considerable caution must be used in drawing conclusions based on antibody blocking data in this system.

Both Domains 1- and 4-dependent Binding of VCAM-1 to Cells Is Temperature Sensitive. Recently, reports from other laboratories have described assays of VCAM-1-dependent binding under conditions of reduced temperature, in rabbit (15) and in mouse (10). This was puzzling, as previously we noted that binding to VCAM-1 of the VLA-4-bearing cell lines we have tested (Ramos, Jurkat, U937, THP1, and HL60) is temperature sensitive; chilling cells to 4°C just before and during adhesion assay completely abolished VCAM-1-dependent binding, although ELAM-1-transfected controls bind HL60 cells equally well at 4, 25, or 37°C. Because many of our early assays on transfected cells were performed using VCAM-6D, we tested VCAM-6D, -7D, and our chimeric constructs, as well as induced endothelial cells, for adhesion to various VLA-4-bearing leukocytic cell lines at reduced temperature, to determine if cold has differential effects on binding mediated by the two sites. Ramos cell binding to domains 1-, 4-, and 1- and 4-dependent constructs is abolished at 4°C (Fig. 5). VCAM-1-dependent binding of Ramos, Jurkat, THP1, and HL60 cells to endothelial cells at 4, 24, 48, or 72 h after TNF induction is also abolished at 4°C (data not shown). Aside from the rather unlikely possibility of species variation, the apparent contrast of our results with those cited above might be caused by a difference in VLA-4 conformation or activation state on the surface of the leukocytic cells tested. A number of published reports suggest that different affinity states of VLA-4 exist (29–31). Perhaps the temperature-sensitive cell lines all carry a low affinity form of VLA-4, which contrasts to a relatively temperature insensitive, high affinity form produced by posttranslational charges in the conformation of VLA-4, similar to that reported for the β_2 integrin LFA-1 (32, 33). Future experiments should be able to resolve this issue.

Evidence for the Evolutionary Origin and Functional Utility of Bivalent VCAM-7D. It is immediately apparent that the

homology of domains 1–3 to domains 4–6 most likely is the result of an intergenic duplication event during the evolutionary history of the gene (Fig. 6; 2, 11–15). Duplication of the adhesive portion of the molecule might well result in a molecule with potential for bivalent interaction with its counter-receptor, if the functional region was completely duplicated and if the proximal site was not sterically blocked from access to the counter-receptor by the new portion of the molecule. However, if the function of one or the other binding sites were not of any utility in the function of the molecule, one binding site would be expected to lose function during evolution, as it diverged because of random mutation. This is clearly not the case for VCAM-1. Although the intergenic duplication must have occurred before the mammalian radiation (about 200 million years ago), as it is also found in the rat and mouse genes (24), we have shown that both sites are still fully functional, in the human species. A closer examination of the amino acid similarity between the duplicated domains reveals that the domains most crucial to binding function, 1 and 4, are 73% similar, while domains 2 and 5, and 3 and 6, are each only 59% similar to each other (Fig. 6). This strongly suggests that there is increased selective demand for maintenance of the structures of domains 1 and 4, compared with the less functionally pressured domains 2, 3, 5, and 6. It seems likely that the bivalence of VCAM-7D provides increased avidity for its counter-receptor (as suggested by Fig. 1), and that this is advantageous to the physiology of the organism. The additional domain may of course also play some further role in adhesion, endothelial transmigration of mononuclear leukocytes, or B cell development. The reagents described here should provide valuable tools for determining additional functions of the two binding sites.

Discussion

Other adhesion molecules have been shown to have more than one binding site for distinct counter-receptors, or non-homologous binding sites for a single counter-receptor. For example, ICAM-1 has separate binding sites for LFA-1 and Mac-1 (34), fibronectin has separate binding sites for VLA-5

and for VLA-4 (35), and fibrinogen has two dissimilar sites that bind the integrin IIb/IIIa. VCAM-1 is the first molecule found to have two homologous binding sites for a single receptor, VLA-4, generated by an intergenic duplication. Possible arenas for functional interplay include signaling and binding. For example, VCAM-1 can cause proliferation of T cells through domain 1–3 in the presence of a second signal (36, 37). It is not yet known whether domain 4–6 also has this capacity.

Recent experiments from Vonderheide and Springer (38) also have suggested the existence of dual binding sites in VCAM-1, based on the properties of mAb E1/6, which can completely inhibit binding of Ramos cells to our VCAM-6D construct, but only partially to VCAM-7D. These authors also suggest that an additional α_4 integrin counter-receptor is present on stimulated endothelium, based on inability of mAb 4B9 to inhibit completely binding of two cell lines to HUVEC, although the anti- α_4 mAb HP2/1 can inhibit completely. It will be interesting to follow future experiments aimed at defining this novel receptor.

VCAM-6D, a minor form of VCAM-1 lacking domain 4 because of alternate splicing, remains mysterious with regard to biological significance. It may be caused simply by an occasional error in splicing resulting from skipping of the exon encoding domain 4. However, if differential distribution or regulation of these two forms is found, VCAM-6D could provide clues as to the physiological significance of the two binding domains.

In summary, VCAM-1 has been shown to have two functional binding sites for VLA-4, and antibodies that block this site have been described. All physiological and pathological processes involving VCAM-1, including B cell development, leukocyte activation and recruitment, atherosclerosis, and metastasis, are potentially affected by the additional site, and it should be considered when using anti-VCAM-1 antibodies to block this adhesion pathway. The discovery of the new site, and blocking mAbs to domain 4 of VCAM-1, will provide valuable new tools for dissecting the mechanism and biology of the VCAM-1/VLA-4 adhesion and signaling pathway, and for implementing therapeutic strategies based on stimulation or suppression of this pathway.

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