Arrangement of Domains, and Amino Acid Residues
Required for Binding of Vascular Cell Adhesion
Molecule-1 to Its Counter-Receptor VLA-4 (\(\alpha_4\beta_1\))

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Abstract. Interaction of the vascular cell adhesion molecule (VCAM-1) with its counter-receptor very late antigen-4 (VLA-4) (integrin \(\alpha_4\beta_1\)) is important for a number of developmental pathways and inflammatory functions. We are investigating the molecular mechanism of this binding, in the interest of developing new anti-inflammatory drugs that block it. In a previous report, we showed that the predominant form of VCAM-1 on stimulated endothelial cells, seven-domain VCAM (VCAM-7D), is a functionally bivalent molecule. One binding site requires the first and the other requires the homologous fourth immunoglobulin-like domain. Rotary shadowing and electron microscopy of recombinant soluble VCAM-7D molecules suggest that the seven Ig-like domains are extended in a slightly bent linear array, rather than compactly folded together. We have systematically mutagenized the first domain of VCAM-6D (a monovalent, alternately spliced version missing domain 4) by replacing 3–4 amino acids of the VCAM sequence with corresponding portions of the related ICAM-1 molecule. Specific amino acids important for binding VLA-4 include aspartate 40 (D40), which corresponds to the acidic ICAM-1 residue glutamate 34 (E34) previously reported to be essential for binding of ICAM-1 to its integrin counter-receptor LFA-1. A small region of VCAM including D40, QIDS, can be replaced by the similar ICAM-1 sequence, GIET, without affecting function or epitopes, indicating that this region is part of a general integrin-binding structure rather than a determinant of binding specificity for a particular integrin. The VCAM-1 sequence G65NEH also appears to be involved in binding VLA-4.

The interaction of cells with one another via their membrane-bound adhesion molecules is a dynamic process with multiple physiologic and pathologic consequences. Molecular regulation of cellular adhesion and de-adhesion is responsible for modeling of the tissues and organs during development, and many of the same molecules continue to be important in both repair and normal function in the adult. Since its discovery a few years ago, the vascular cell adhesion molecule-1 (VCAM-1) (also called INCAM-110) (Rice and Bevilacqua, 1989) is an Ig superfamily protein which is synthesized by endothelial cells in response to IL-1, TNF, LPS, or IL-4, and is found constitutively on a few other cell types, such as follicular dendritic cells in lymph nodes (Freedman et al., 1990) and bone marrow stromal cells (Miyake et al., 1991). The first counter-receptor identified for VCAM-1 was a member of the heterodimeric integrin family known as VLA-4 or \(\alpha_4\beta_1\), found on most mononuclear leukocytes to sites of inflammation (Osborn et al., 1989; Elices et al., 1990). It has been implicated in the pathology of disorders affecting a wide range of processes, including metastasis of tumors (Rice and Bevilacqua, 1989), atherosclerosis (Cybulsky and Gimbrone, 1991), and autoimmune encephalitis (Yednock et al., 1992).

VCAM-1 (also called INCAM-110) (Rice and Bevilacqua, 1989) is an Ig superfamily protein which is synthesized by endothelial cells in response to IL-1, TNF, LPS, or IL-4, and is found constitutively on a few other cell types, such as follicular dendritic cells in lymph nodes (Freedman et al., 1990) and bone marrow stromal cells (Miyake et al., 1991). The first counter-receptor identified for VCAM-1 was a member of the heterodimeric integrin family known as VLA-4 or \(\alpha_4\beta_1\), found on most mononuclear leukocytes, but not on neutrophils. VLA-4 can also bind to an alternately spliced site in fibronectin called CS-1 (Wayner et al., 1989; Guan and Hynes, 1990). The \(\alpha_4\) integrin chain in association with a different beta chain, \(\alpha_5\beta_1\) or \(\alpha_5\beta_3\), appears to be capable of binding to both VCAM-1 and fibronectin (Ruegg et al.,...
1992; Chan et al., 1992), as well as to another recently discovered Ig molecule known as mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Briskin et al., 1993; Berlin et al., 1993).

Within the Ig superfamily, VCAM-1 belongs to a growing subfamily of cell surface molecules including ICAM-1, -2, and -3, and MAdCAM-1, that bind leukocyte integrin ligands. Members of this family are distinguished by the presence of four cysteine residues instead of the usual two, in domains important for counter-receptor binding. Here we show by rotary shadowing and electron microscopy that purified recombinant VCAM-1, like ICAM-1 and the immunoglobulins, assumes an extended conformation rather than a more compactly folded tertiary structure.

VCAM-1 exists in two alternately spliced forms on endothelial cells, a major form comprised of seven Ig-like domains (VCAM-7D), and a minor form lacking domain 4 (VCAM-6D) (Fig. 1) (Cubulsky et al., 1991; Hession et al., 1991). Previously we showed that VCAM-7D has two homologous binding sites for VLA-4, one requiring the first (NH2-terminal) Ig-like domain (D1) and the other requiring the fourth (D4) (Vonderheide and Springer, 1992; Osborn et al., 1992). It is probable that the mechanism of binding to VLA-4 is the same for these two domains, as they are very similar in sequence, having only 11 nonconservative amino acid differences within the 90-amino acid domain, and can substitute for each other structurally and functionally in recombinant constructs. To map more finely areas of the protein structure required for binding to VLA-4, we have systematically mutagenized domain 1 and analyzed the mutant constructs for maintenance of functional mAb epitopes and cell binding activity.

Materials and Methods

Rotary Shadowing and Electron Microscopy

Purified rVCAM-7D or equimolar mixtures of rVCAM and antibody were incubated at 4°C for 1 h at 0.5-1 mg/ml in PBS. 1-2 μl samples were rapidly mixed with ice-cold 40-45% glycerol, sprayed onto mica and platinum rotary shadowed at a glancing angle of 6-8 degrees. Micrographs were taken at a magnification of 48,000 and selected images from scanned prints were measured using the program Image 1.45 (NIH). A JEOL 100CXII operating at 80 KV was used. After each electron microscopy session, the microscope was calibrated with negatively stained images of T4 phage tails (4.1 nm repeat period).

Antibodies and Cells

Previously described monoclonal antibodies used were anti-α4, HPI/2 (Polido et al., 1991); and anti-VCAM-1, 4B9 (Carlos et al., 1990). EH8 is an IgG1 mAb made from mice immunized with recombinant soluble VCAM-7D (Lobbo et al., 1991), and will be described in detail elsewhere (Benjamin, C., and I. Dougas, unpublished results). Polyclonal rabbit anti-VCAM-1 serum was made by immunizing rabbits with recombinant soluble VCAM-7D. COS7 and Ramos cells were cultured as previously described (Osborn et al., 1992).

Protein and Nucleic Acid Sequence Analysis

Sequence analysis was carried out using the GCG Package (Genetic Computing Group, Madison, WI).

Results and Discussion

Visualization of VCAM-1 by Electron Microscopy

Because mAb 4B9, which binds to domain 1, is capable of blocking both domain 1 and domain 4-dependent binding to VLA-4 (Vonderheide and Springer, 1992; Osborn et al., 1992), we had considered that the VCAM-7D molecule might be folded so that the domain 1 and domain 4–depem-
dent binding sites are in close proximity. To determine the general shape of the VCAM-7D molecule, rotary shadowing electron microscopy was performed. Fig. 2 shows an example of a general field of recombinant soluble VCAM-7D. Two classes of images were observed, corresponding to straight and bent rods (see selected views in Fig. 3). The straight rod-shaped structures, representing ~25% of the molecules, are 28.1 ± 4.4 nm in length and 5.7 nm in width (Fig. 3 k). Sometimes these images show an enlargement or small bend at one end of the molecule (Fig. 3 k and l, right molecule). The bent rods, the remainder of the population, display either one or two bends along the long axis of the molecule. In the images with one bend, the bend has an angle of 115° ± 32°, and is located about two thirds down the length of VCAM-7D, dividing it into a long and a short arm (Fig. 3, l–o). The long arm is 18.0 ± 2.6 nm long, and the short arm is 12.4 ± 2.5 nm long. The total length of the one-bend molecules is greater than that of the straight molecules, at 30.4 ± 2.6 nm. In the molecules with two bends, the additional bend is located at variable positions in the short arm (Fig. 3 l, left molecule and Fig. 3 m); the molecule is Z-shaped, and the overall length is 31.4 ± 3.4 nm. It is probable that at least some of the molecules that appear straight in fact represent foreshortened views of molecules with one or two bends. Therefore, we take the measurements of the bent molecules to be a better reflection of the overall length of VCAM-7D. To obtain the actual length of the molecule, the measurements along the long axis were corrected by subtraction of 2.5 nm at each end of the molecule to take into account the increase in size due to the platinum particles deposited during shadowing. The corrected length of the molecule is therefore 25.9 nm. Since the width of VCAM-7D is comparable with the size of two Pt grains, making the same correction for this dimension would give a necessarily inaccurate number. We therefore prefer not to give corrected figures for the width of VCAM-7D. Taking the extended length of the molecule to be 25.9 nm, each Ig domain contributes ~3.7 nm to the overall length of the molecule. This is in good agreement with previous observations on the length of Ig domains (Staunton et al., 1990; Williams and Barclay, 1988).

Figure 2. General view of VCAM-7D visualized after glycerol spray and rotary shadowing. The field shows isolated rod-like molecules. Some molecules are straight and others show one or two bends along the long axis of the molecule. Bar, 40 nm.
To examine the arrangement of the Ig domains in VCAM-7D, we visualized complexes of VCAM-7D with the mAbs 4B9, which maps to domain 1, and EDI1, GE12 and GE4, which map to domain 4. mAb 4B9 always binds to an end of the molecule (Fig. 3, a-e), often cross-linking two molecules (Fig. 3, a, b, and d). The angle of the bend in these complexes molecules is not statistically different from the free molecules. In contrast, each of the antibodies to domain 4 bind on the convex face of the bend (Fig. 3, f-j), again frequently cross-linking two VCAM-7D molecules (Fig. 3, f-h). The angle of the bend is similar to the angle in the bend of free VCAM-7D. Thus it is clear that the Ig domains of

**Figure 3.** Selected images of immuno-complexes formed between VCAM-7D and the monoclonal antibodies 4B9 (specific for an epitope in domain 1) and EDI1, GE4, and GE12 (specific for epitopes in domain 4). a–d show views and e shows a schematic representation with a single point of contact between VCAM-7D and 4B9. f–g, h, and i correspond to typical views of immuno-complexes formed between VCAM-7D and antibodies GE12, EDI1 and GE4, respectively. j is a representation of the binding of any of these antibodies to a site on the convex side of the bend of VCAM-7D. (k–o) are images of straight and bent single VCAM-7D molecules. Bar, 40 nm.

**Figure 4.** Comparison of Ig-like domains involved in integrin binding. From top to bottom: (1) human ICAM-1 domain 3 (Staunton et al., 1990; Diamond et al., 1991); (2) human ICAM-2 domain 1 (Staunton et al., 1989); (3) human ICAM-3 domain 1 (Vazeaux et al., 1992; Fawcett et al., 1992); (4) ICAM-1 domain 1 fl strand predictions (Staunton et al., 1988); (5) conserved residues between human ICAM-1 domain 1 and all VCAM-1 domains below; (6) human ICAM-1 domain 1 (Osbom et al., 1989); (7) human VCAM-1 domain 1 (Polte et al., 1990); (8) mouse VCAM-1 domain 1 (Hession et al., 1992); (9) rat VCAM-1 domain 1 (Hession et al., 1992); (10) conserved residues between VCAM-1 domains that bind human VLA-4; (11) predicted hVCAM-1 domain 1 secondary structure based on computer-generated predictions, below; (12) Chou-Fasman (CF) secondary structure prediction, H and h = helical, T and t = beta turn, B and b = /3 strand (uppercase = strong, lowercase = weak); (13) Garnier-Osguthorpe-Robson (GOR) secondary structure prediction, H = helical, T = turn, B = beta. The sequences were aligned by inspection (the ICAM-1 domain 3 sequence is less homologous than the others, and was only aligned in regions important for binding). Residues underlined have been shown to be required for binding to integrin ligands.

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Table I. Binding of Monoclonal Antibodies and Ramos Cells to VCAM-6D Domain 1 Mutants

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Percent wild-type binding after normalization for expression as determined by binding to mAb EH8 (to unaffected domain 5) was averaged for 2-7 experiments in each case.

* n = 2, SD = 0.
† n = 4, SD = 5.8.
‡ n = 7, SD = 7.3.
¥ n = 4, SD = 7.0.

VCAM-7D are linearly arranged, like other molecules composed of Ig domains such as ICAM-1 (Kirchhausen et al., 1993) and CD4 (Kirchhausen, T., and S. Harrison, unpublished data). Therefore the ability of mAb 4B9 to block domain 4-dependent binding of VCAM-7D to VLA-4 must have an alternative explanation.

**Design of Mutant Constructs**

Previously we showed that there are two independent binding sites for VLA-4 in VCAM-7D, one requiring domain 1 and the other requiring domain 4. The alternately spliced VCAM-6D, which is missing domain 4, has only one functional VLA-4 binding site, requiring domain 1. To simplify analysis of mutants, VCAM-6D was used as the parent construct from which to derive a series of mutants, each of which has one or more amino acid changes in domain 1. The VCAM-1 sequence was analyzed preliminarily using the program PeptideStructure, which predicts secondary structure according to the algorithms of Chou-Fasman (CF) and Garnier-Osguthorpe-Robson (GOR). Prediction of the position of α strands and loops in VCAM-1 domain 1 was done by making modest use of these predictions, and more extensive use of homology with known Ig domain structures. We chose to mutagenize both predicted loops and strands, rather than attempt to target the loops, where contact residues are generally expected to be found. We and others find that predictions of secondary structure, while useful in the absence of physical data, are often later shown to be somewhat inaccurate compared with physical data generated by NMR or x-ray crystallography (Barclay, 1992). Furthermore, evidence that binding sites are likely to be found in loops rather than strands is based largely on analogy with hypervariable antibody complementarity determining regions. It is possible that more distantly related Ig superfamily members like VCAM-1 might show a different organization of binding sites.

**Construction, Expression, and Assay of Mutant Constructs**

Amino acids to be mutated were exchanged for the analogous amino acids found in ICAM-1 domain 1 (Fig. 4). This was done with the expectation that analogous sequences from a related protein might stabilize the resulting mutant's structure; since we changed several contiguous amino acids in each mutant, the common approach of substituting with alanine or serine residues would have resulted in extremely unnatural proteins.

Mutant constructs were made using gapped-heteroduplex oligonucleotide-directed mutagenesis, as described in Materials and Methods. After transfection into COS cells, expression was assessed by FACs analysis using antibody EH8, which binds between the NH2-terminal portion of domain 5 and the COOH terminus of the protein, in an area unaffected by the mutagenesis. Maintenance of proper folding of each mutant was assessed by analysis of binding to a panel of mAbs (see below). Ability of mutants to bind to VLA-4 was...
Figure 5. Binding of human VCAM-1 domain-1 mutant constructs expressed in cos cells to VLA-4 and mAbs. Alignment of VCAM-1 to ICAM-1 and prediction of β strands is described in Fig. 3 legend. Bolded, underlined letters represent ICAM-1 residues substituted for homologous VCAM-1 residues in each mutant. mAbs: E3 = EH8, Ig = IG11, I4 = 1.4C3, Bb = BBA-6, 4b = 4B9, 6d = 6D9, le = I5. “Block” indicates whether each mAb is able (+) or unable (−) to block binding of VLA-4-bearing cells to VCAM-1 domain 1. “Domain” indicates to which domain each mAb maps (see text for further details). Expression of each construct was quantified by binding to raAb Ramos: + = 50-300%, + = 10-50%, − = 0-10% wild-type binding. 

<table>
<thead>
<tr>
<th>Mutant</th>
<th>M1</th>
<th>M2</th>
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It is important to distinguish mutants that have lost VLA-4 binding activity due to large scale perturbations in structure from those that show effects limited to contact residues. To identify mutants exhibiting improper folding of domains 1 and 2, we stained with a panel of mAbs representative of every known epitope of VCAM-1 within these domains. mAbs that map to domain 1 include blocking (i.e., able to block binding of VCAM-1 to VLA-4) mAbs 4B9 (Carlos et al., 1990) and IG11 and non-blocking mAb 6D9. Domain 2 mAbs include blocking mAbs BBA-6 and IG11, and non-blocking mAb 1.4C3. All of these mAbs bind to different epitopes, distinguished by cross-blocking in competitive binding assays or by functional ability to block binding of VCAM-1 to VLA-4. Analysis of mutants allows us to divide these mAbs into two classes: those whose epitopes are perturbed by several mutations, to linearly distant areas of domain 1 (designated “sensitive” epitopes), and those that are usually or always unaffected by such mutations (“insensitive” epitopes). Most but not all mutants that have lost more than one sensitive epitope (those recognized by domain 1 mAbs 4B9, 6D9, IG5 and domain 2 mAb BBA-6) have also lost the ability to bind to Ramos cells, probably due to improper folding. The exceptions, M11 and 12, retain partial binding to cells. Of mutants that retain sensitive epitopes, most bind well to VLA-4, indicating that the substituted ICAM-1 amino acid residues can support both proper folding and ligand binding.
G64NEH. Residue D40 of VCAM-1 is analogous to E34 of ICAM-1, which was shown by Staunton and coworkers (Staunton et al., 1990) to be required for binding of ICAM-1 to its integrin counter-receptor LFA-1. Both E34 of ICAM-1 and D40 of VCAM-1 are located in the predicted loop between β strands c and d ("c-d loop"). When VCAM-1 D40 is replaced by alanine (M25), binding to VLA-4 is abolished. Interestingly, our original mutant in this area, which substituted the ICAM-1 sequence G32IET for the analogous VCAM-1 Q8IIDS (M9), resulted in complete retention of all epitopes, and of binding to VLA-4 that was specifically inhibited by anti-α4 mAb HP1/2. This ability of ICAM-1 sequence to functionally substitute for VCAM-1 residues in binding to VLA-4 indicates that these residues do not determine specificity for a particular integrin ligand, but are probably part of a general integrin binding structure that may include important contact residues, yet requires other contacts for its specificity. Comparison of the predicted c-d loop among other sequences known to bind human VLA-4, LFA-1, or Mac-1 (Fig. 2) reveal in each case a sequence with the common feature "αp-L," where α is an acidic residue (D or E), p is a polar residue (usually a hydroxyl derivative S or T, but an amino derivative Q in the Mac-1 binding site), and L is a leucyl residue. This small region has been shown experimentally to be an important component of the integrin binding site in ICAM-1 domains 1 and 3, human VCAM-1 domains 1 and 4, murine VCAM-1 domain 1 and rat VCAM-1 domain 1 (Staunton et al., 1990; Diamond et al., 1991; Moy et al., 1993; this work; Osborn, L., and B. Browning, unpublished data).

The other VCAM domain 1 sequence implicated in binding is located in the putative e-f loop. When the sequence G64NEH is changed to VQED (M15), all epitopes are maintained except for partial loss of mAb 6D9 binding, but binding to Ramos cells is greatly reduced. Attempts to define a specific amino acid residue responsible for loss of binding have met with only partial success. The mutation N65 to A (M29) results in a profile of epitopes similar to M15, but loss of Ramos binding is less severe than that of M15. H67 to A (M30) results in full maintenance of epitopes and cell binding, while G64 to A (M28) causes loss of sensitive epitopes and cell binding activity, indicating deleterious effects on protein folding (mutants M28-30, Fig. 5). Studies currently in progress may resolve this issue. In ICAM-1, the analogous region of domain 1 (putative e-f loop) was not found to be involved in binding to LFA-1. However, a mutation in this region of ICAM-1 did affect binding to both human rhinovirus (HRV) and to anti-ICAM-1 mAb RR1/1, which blocks binding to both LFA-1 and to HRV (Staunton et al., 1990). Binding of Mac-1 to ICAM-1 via domain 3 was reduced 70% by mutagenesis of the predicted e-f loop (ICAM E254DE to KEK, where ICAM E254 corresponds to VCAM E66) (Diamond et al., 1991). Thus there is considerable functional as well as structural homolog among the various integrin-binding Ig-like domains.

Several lines of evidence suggest that domain 2 is important both for maintaining proper folding of domain 1, and perhaps for providing contact residues that form part of the binding pocket for VLA-4. As shown in Fig. 6, the c-d and e-f loops are expected to be close to each other in the classically folded Ig-like domain. Both predicted loops are at the surface of domain 1 that is expected to be closest to domain 2. It has been shown that a fragment consisting of VCAM-1 domains 1 and 2 can support binding of VLA-4-bearing cells (Pepinsky et al., 1992), while generation of fragments representing either domain 1 or 2 alone has not been achieved, presumably because they are unstable. A construct in which domain 2 of VCAM-1 has been replaced by domain 2 of ICAM-1 retains the sensitive epitopes in domain 1 recognized by mAb 4B9 and 1E5, but shows greatly diminished binding to VLA-4, suggesting that more stabilization of domain 1 may be insufficient to allow full adhesion (Osborn et al., 1992). Both ICAM-1 and VCAM-1 give rise to blocking mAbs that map within domain 2, and several residues in ICAM-1 domain 2 were found to affect LFA-1 adhesion modestly, though none had as dramatic effect as those in domain 1, described above (Staunton et al., 1990). The current evidence is fully consistent with a model in which domain 2 of VCAM-1 provides necessary contact residues, which depend upon domain 1 for both completion of the VLA-4 binding "pocket," and for conformational stabilization.

The marked functional and structural similarity of VCAM-1 domains 1 and 4 suggest that the mechanism of VLA-4 binding to domains 1 + 2 unit will also account for binding to
the domain 4 + 5 unit. In recombinant constructs domain 4 can fully substitute for domain 1 in cell binding activity, whether in conjunction with domain 2 or with its natural partner domain 5 (Osborn et al., 1992). Reciprocally, domain 1 can substitute for domain 4 (Pepinsky et al., 1992). The high sequence similarity of domain 1 and 4, in human and in other species, has made it difficult to choose probable binding sites by distinguishing conserved from divergent residues. Domains 2 and 5 are not as similar, evincing patches of homology which are good targets for mutagenesis (Fig. 6, indicated residues in Domain 2). Intriguingly, there are conserved sequences in domains 2 and 5 that bear better homology to the VLA-4 binding site in fibronectin (LDV) (Komoriya et al., 1991; Wayner and Kovach, 1992) than does any sequence in domain 1 and 4. Mutational analysis of domain 2, currently underway, may provide answers to the questions we have raised here.

In conclusion, we believe that by comparing members of the integrin-binding Ig sub-family composed thus far of ICAM-1, -2, -3, VCAM-1, and MAdCAM-1, we may derive these adhesion pathways. We have demonstrated that the domains 1 and 2 are often lost concurrently, providing additional evidence that in VCAM-1, domains 1 and 2 (or their homologues 4 and 5) are both required for full VLA-4 binding activity.

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