Integrins are a family of heterodimeric adhesion receptors that mediate cellular interactions with a range of matrix components and cell surface proteins. Vascular cell adhesion molecule-1 (VCAM-1) is an endothelial cell ligand for two leukocyte integrins (α4β1 and α4β7). A related CAM, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is recognized by α4β7 but is a poor ligand for α4β1. Previous studies have revealed that all α4 integrin-ligand interactions are dependent on a key acidic ligand motif centered on the CAM domain 1 C-D loop region. By generating VCAM-1/MAdCAM-1 chimeras and testing recombinant proteins in cell adhesion assays we have found that α4β1 binds to the MAdCAM-1 adhesion motif when present in VCAM-1, but not when the VCAM-1 motif was present in MAdCAM-1, suggesting that this region does not contain all of the information necessary to determine integrin binding specificity. To characterize integrin-CAM specificity further we measured α4β1 and α4β7 binding to a comprehensive set of mutant VCAM-1 constructs containing amino acid substitutions within the predicted integrin adhesion face. These data revealed the presence of key “regulatory residues” adjacent to integrin contact sites and an important difference in the “footprint” of α4β1 and α4β7 that was associated with an accessory binding site located in VCAM-1 Ig domain 2. The analogous region in MAdCAM-1 is markedly different in size and sequence and when mutated abolishes integrin binding activity.

Under normal conditions, leukocytes exhibit a weakly adhesive phenotype, however, at sites of inflammation or at specialized lymphoid tissues, leukocyte adhesion receptor activity is modulated and cells become capable of interacting with ligands expressed on the luminal surface of the vasculature. Leukocyte integrin receptors and endothelial immunoglobulin superfamily cell adhesion molecules (IgCAMs) make key contributions to the events that facilitate leukocyte emigration into the tissues (1, 2).

Integrins are α/β heterodimeric cell surface adhesion receptors that recognize a wide variety of extracellular ligands (3). For most integrins, the mechanism of ligand recognition appears to be critically dependent upon one of two short acidic peptide motifs: RGD and LDV (4, 5). Several extracellular matrix molecules express the RGD motif in an invariant form and are ligands for a number of integrins (e.g. α5β1, αIIbβ3, αvβ1, αvβ3; Refs. 6–10). In contrast, the LDV motif exhibits sequence variation. For example, the leukocyte integrins α4β1 and α4β7 recognize an LDVP motif in fibronectin (11–15), an IDSP sequence in the N-terminal domain of IgCAM vascular cell adhesion molecule-1 (VCAM-1)1 (16–22), and an LDTS sequence in a second IgCAM, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (23–26).

Integrin-ligand interactions can be perturbed by short peptides based on RGD or LDV. Interestingly LDV-containing peptides (and small peptide mimetics) competitively inhibit α4β1 interactions with both fibronectin and VCAM-1 indicating that the integrin ligand-binding sites for both of these ligands are either overlapping or identical (16, 27, 28).

The x-ray crystal structure of the two N-terminal Ig domains of human VCAM-1 has recently been solved. Both domains adopt a β-β sandwich topology, composed of an anti-parallel array of β-strands (28, 29). As predicted by homology modeling studies the IDSP motif is located on a projected loop between β-strands C and D, atypical of previously characterized Ig domains (16, 30, 31). Other homology modeling and sequence analysis studies have suggested that three β2 integrin IgCAM ligands (intercellular adhesion molecules 1, 2 and -3, ICAM-1, -2, -3) also have an Ig structure analogous to that of VCAM-1 (32–36). Since ICAM-1, -2, and -3 are also dependent on an LDV-like motif for adhesive activity (IETP, LETS, and LETS, respectively) this suggests that integrin-binding IgCAMs have a common active site (33, 37–39). Extensive mutational analysis of IgCAMs has provided invaluable information regarding integrin-CAM interactions, and certain residues that lie outside the LDV motif of VCAM-1 (16, 18, 19, 22), ICAM-1 (33), and ICAM-3 (38, 39) have been shown to play an important role in integrin recognition.

Thus, integrin-IgCAM interactions appear to share several common features: a characteristic Ig-fold and a dominant acidic peptide motif flanked by non-contiguous accessory residues. Key questions that remain are: what are the principal features

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1 The abbreviations used are: VCAM-1, vascular cell adhesion molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; mAb, monoclonal antibody; HBS, HEPES-buffered saline; MES, 4-morpholineethanesulfonic acid.

2 R. Lobb, personal communication.
of IgCAM structures that govern receptor selection and specifically does the dominant LDV-like motif encode integrin receptor specificity or are additional residues required to discriminate between integrin receptors?

Using the VCAM-1 crystal structure (29) as a basis for a mutagenesis study we have generated IgCAM chimeras and investigated the link between IgCAM LDV motif degeneracy and integrin binding. Using this data we have further characterized the interaction of α4β1 and α4β7 with VCAM-1 and have identified additional key binding/regulatory residues both within and outside the C-D loop of Ig domain 1. Our results provide evidence of distinct but overlapping α4β1/α4β7 adhesion footprints that extend over both of the most membrane distal domains of VCAM-1 and provide an insight into the mode of integrin-IgCAM specificity.

EXPERIMENTAL PROCEDURES

Cell Lines and Monoclonal Antibodies—A375-SM cells, a human metastatic melanoma cell line (provided by I. J. Fidler, M. D. Anderson Hospital and University of Texas, Houston, TX) were cultured as described (40) in Eagle's minimal essential medium containing 10% (v/v) fetal calf serum, minimal essential medium vitamins, non-essential amino acids, 1 mM sodium pyruvate, and 2 mM glutamine (all from Life Technologies, Inc., Paisley, United Kingdom). COS-1 cells were cultured in Dulbecco's minimal essential medium, 0.11g/liter of sodium pyruvate, 10% fetal calf serum and 2 mM glutamine. Anti-VCAM-1 monoclonal antibodies (mAbs) 4B2 (anti-domain 1), 1E10 (anti-domain 1), and 19C3 (anti-domain 2) were gifts of J. Clements (British Biotech, Cowley, Oxford, UK).

CAM-Fc: Cloning and Expression—Truncated CAM-Fc chimeras consisting of Ig domains 1 and 2 of human VCAM-1 or murine MadCAM-1 fused to the hinge and Fc region of human IgG1 were constructed as follows. pcDM8-VCAM (a gift of J. Clements) and pcDM8-MadCAM (a gift of D. Kehry; Kehry/Reinherz Laboratory, German Cancer Research Center, Heidelberg, Germany) were used as templates for the polymerase chain reaction amplification of DNA encoding 5′ and 3′ restriction sites, the 5′ untranslated region, Ig domains 1 and 2 of VCAM-1 or MadCAM-1 and a 3′ splice donor consensus sequence (35). Amplified DNA was cloned into the phagemid vector pUC118 (VCAM) or pUC119 (MadCAM) and sequenced. CAM cDNA constructs were then subcloned using EcoRI (VCAM) and XhoI (MadCAM) into the plg mammalian expression vector (a gift of D. Simmons, Institute of Molecular Medicine, Oxford, UK) encoding a consensus splice acceptor sequence and the hinge and Fc portion of human IgG1 downstream of the multiple cloning site.

Mutations were introduced into CAM cDNAs by the method of Kunkel et al. (41) using the Mutagen phagemid mutagenesis kit (Bio-Rad, Hemel Hempstead, Herts, UK). Mutants were confirmed by sequencing. A list of mutagenic primers (size range 20–74-mer) is available on request.

COS-1 cells in exponential phase were trypsinized and washed twice with divalent cation-free phosphate-buffered saline and resuspended in cation-free phosphate-buffered saline to a concentration of 6–8 × 106 cells/50-μl aliquot. Triplicate aliquots were mixed with 10–20 μl of 10 mg/ml heat-denatured bovine serum albumin. DNA diluted with HBS and blocked for the degree of spreading using phase-contrast microscopy as described (43). Each data point was obtained by counting at least 300 cells/well from a number of randomly selected fields. No cell spreading was observed on wells coated only with heat-denatured bovine serum albumin.

Cell Attachment Assay—96-well plates were coated for 60 min at room temperature with 50 μl of CAM-Fc diluted with HBS. Wells were blocked for 60 min at room temperature with 100 μl of 10 mg/ml heat-denatured bovine serum albumin (42). A375-SM cells were detached with 0.05% (w/v) trypsin, 0.02% (w/v) EDTA, resuspended to 1 × 106/ml in Dulbecco's minimal essential medium, 25 mM HEPES and allowed to recover for 10 min at 37 °C. 100-μl aliquots of cells were added to each well and plates incubated in a humidified atmosphere of 7% (v/v) CO2, 60 min at 37 °C. Cells were fixed with the addition of 20 μl of 50% (v/v) glutaraldehyde and monitored for the degree of spreading using phase-contrast microscopy as described (43). Each data point was obtained by counting at least 300 cells/well from a number of randomly selected fields. No cell spreading was observed on wells coated only with heat-denatured bovine serum albumin.

Cell Spreading Assay—96-well plates were coated for 60 min at room temperature with 50 μl of CAM-Fc diluted with HBS and blocked with heat-denatured bovine serum albumin (see above). JY cells were centrifuged at 1400 × g for 3 min, washed in Dulbecco's minimal essential medium, 25 mM HEPES, resuspended at 1 × 106/ml in assay medium and allowed to recover at 37 °C for 10 min. 50-μl aliquots of cells were added to CAM-Fc-coated wells containing 50 μl of HBS, 0.2 mM MnCl2 and incubated in a humidified atmosphere of 7% (v/v) CO2, 20 min at 37 °C. Cells attached to untreated wells were fixed directly with 20 μl of 50% (v/v) glutaraldehyde to enable quantitation of cell number. Nonspecifically attached cells were aspirated from experimental wells and the residual cells fixed with 5% (v/v) glutaraldehyde. Fixed cells were then washed three times with 0.1 M HEPES and stained for 60 min with 0.1% (w/v) crystal violet in 0.2 M MES, pH 6. Excess stain was removed with three water washes before cells were lysed with 10% (v/v) acetic acid and the released stain measured spectrophotometrically at 570 nm.

RESULTS

The predicted C-D loop sequences of VCAM-1, MadCAM-1, and ICAMs 1–3 have all been implicated as sites for integrin binding and, without exception, possess an acidic residue essential for receptor interaction. Alignment of these sequences (Fig. 1) reveals a consensus integrin-binding motif of the form (R/G)XX(G/S)XXG (22). Despite only subtle variations within this consensus and the use of a relatively conserved Ig domain structure, integrins are capable of discriminating between IgCAM ligands. Although the two related integrins, α4β1 and α4β7, can both bind VCAM-1 and MadCAM-1, there is a ligand preference between the two integrins; α4β1 is primarily a receptor for VCAM-1 whereas α4β7 is primarily a receptor for MadCAM-1. The C-D loops of VCAM-1 and MadCAM-1 differ in two major respects: (i) the
VCAM-1 amino acids that form β-strand secondary structure are numbered. VCAM-1 fragment containing the two N-terminal Ig domains were synthesized as fusion proteins joined to the Fc region of human IgG1. Expression of IgCAM protein in a soluble form provides a major advantage over cell-surface expressed molecules since it is possible to examine the dose dependence of adhesiveness in a quantitative manner and determine relative activities. Since VCAM-1 and MadCAM-1 Ig domain 1 C-D loops are predicted to differ in size, two VCAM-1/MadCAM-1 chimeras were constructed, each chimera differing with respect to their parent molecule and C-D loop size (see Fig. 2). CAM-Fc fusion proteins were purified and tested for their ability to support α4β1-dependent or α4β7-dependent cell adhesion and structural integrity monitored using a panel of mAbs (Fig. 3, Table I). VCAM-Fc supported α4β1-dependent cell spreading, but MadCAM-1 was a poor ligand and failing to support cell adhesion. Similarly α4β7-dependent assays revealed that both MadCAM-Fc chimeras demonstrated very low adhesive activities. However, again VCAM-Fc chimera B supported increased α4β7 binding compared with native VCAM-Fc (Fig. 3). Although local structural perturbations may be responsible for the reduced binding activities of some of the chimeras, as a whole these data suggest that the VCAM-1 C-D loop is not entirely responsible for preferential recognition of VCAM-1 by α4β1 compared with MadCAM-1. However, interestingly, they do suggest that the MadCAM-1 C-D adhesion loop represents a more ideal α4 integrin binding structure.

We next attempted to identify those residues of the VCAM-1 C-D loop that were responsible for α4β1 binding activity and thus provide a molecular explanation for the super-adhesive activity of the MadCAM-1 adhesion loop. The x-ray crystal structure of VCAM-1 domain 1 C-D loop (29) was analyzed and those residues that were both conserved between species (human, mouse, and rat) and were solvent-accessible were targeted for mutagenesis. Residues Arg, at position 36 of the mature protein (Arg36), Gln38, Ile39, Asp40, Pro42, and Leu43 met these criteria and VCAM-Fc proteins, mutated at each of these positions, were expressed, purified, and tested for their ability to support α4β1/α4β7-dependent cell adhesion and for structural integrity (Figs. 4-6, Table I). The D40A mutation (alanyl substituted for aspartate at position 40) has been previously shown to abolish VCAM-1-α4β1 binding and was used as a control (16, 18, 19, 21, 22). In contrast to published data (19), substitution of glutamate for aspartate (D40E) at this position markedly reduced α4β1-dependent cell spreading, but did not abolish it (Fig. 4). Mutation R36A was found to have no effect on α4β1-dependent cell adhesion, however, R36E markedly reduced activity. Mutations at position 38 that intro-

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**Fig. 2. Generation of VCAM-1/MadCAM-1 C-D loop chimeras.** VCAM-1 amino acids that form β-strand secondary structure are numbered. VCAM-1 fragment containing the two N-terminal Ig domains were synthesized as fusion proteins joined to the Fc region of human IgG1. Expression of IgCAM protein in a soluble form provides a major advantage over cell-surface expressed molecules since it is possible to examine the dose dependence of adhesiveness in a quantitative manner and determine relative activities. Since VCAM-1 and MadCAM-1 Ig domain 1 C-D loops are predicted to differ in size, two VCAM-1/MadCAM-1 chimeras were constructed, each chimera differing with respect to their parent molecule and C-D loop size (see Fig. 2). CAM-Fc fusion proteins were purified and tested for their ability to support α4β1-dependent or α4β7-dependent cell adhesion and structural integrity monitored using a panel of mAbs (Fig. 3, Table I). VCAM-Fc supported α4β1-dependent cell spreading, but MadCAM-1 was a poor ligand and failing to support cell adhesion. Similarly α4β7-dependent assays revealed that both MadCAM-Fc chimeras demonstrated very low adhesive activities. However, again VCAM-Fc chimera B supported increased α4β7 binding compared with native VCAM-Fc (Fig. 3). Although local structural perturbations may be responsible for the reduced binding activities of some of the chimeras, as a whole these data suggest that the VCAM-1 C-D loop is not entirely responsible for preferential recognition of VCAM-1 by α4β1 compared with MadCAM-1. However, interestingly, they do suggest that the MadCAM-1 C-D adhesion loop represents a more ideal α4 integrin binding structure.

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A panel of three mAbs were used, 4B2 and 1E10, both anti-VCAM-1 Ig domain 1 and 19C3 an anti-VCAM-1 Ig domain 2. Data are recorded as percentages of native VCAM-Fc response, numbers in italics are standard deviation. Data were obtained from two separate experiments, where $n = 3$ in each experiment. VCAM A = VCAM-Fc chimera A. VCAM B = VCAM-Fc chimera B. G iET = VCAM Q38IDS/GEIT; VII1CD = VCAM - R36QIDS/LGIGET (VCAM-1 C-D loop mutated to predicted ICAM-1 domain 1 C-D loop) and VIIICD - VCAM-1 R36QIDS/KIALETS (VCAM-1 C-D loop mutated to predicted ICAM-3 domain 1 C-D loop). See text for further explanation.

| Mutant | 4B2 | 1E10 | 19C3 |
|--------|-----|------|------|
| E29A   | 103.80 | 7.09 | 80.86 | 13.14 | 105.65 | 7.12 |
| E39A   | 93.42 | 5.05 | 91.82 | 9.12 | 85.99 | 9.38 |
| R26A   | 126.39 | 15.27 | 121.61 | 10.36 | 119.94 | 17.40 |
| R36E   | 98.52 | 11.17 | 93.19 | 7.29 | 93.58 | 11.83 |
| Q35E   | 74.19 | 11.83 | 71.19 | 5.94 | 86.11 | 8.81 |
| Q33G   | 86.70 | 6.80 | 89.18 | 13.50 | 79.66 | 8.33 |
| Q26L   | 64.86 | 5.78 | 59.67 | 3.53 | 52.29 | 11.92 |
| Q25F   | 109.19 | 4.15 | 115.95 | 15.87 | 104.85 | 9.37 |
| L80K   | 97.11 | 15.96 | 86.66 | 9.68 | 65.46 | 16.03 |
| I38A   | 89.85 | 13.65 | 90.15 | 4.59 | 95.02 | 3.98 |
| I39K   | 88.58 | 5.24 | 78.57 | 12.87 | 86.04 | 14.03 |
| I39R   | 85.16 | 6.22 | 83.80 | 9.09 | 75.15 | 16.06 |
| I39V   | 91.19 | 11.19 | 111.76 | 12.41 | 80.87 | 17.22 |
| D40A   | 92.39 | 6.79 | 101.81 | 5.37 | 91.55 | 13.96 |
| D40E   | 100.87 | 15.08 | 108.95 | 7.59 | 99.40 | 9.14 |
| S14G   | 118.71 | 10.19 | 102.36 | 5.15 | 103.46 | 7.80 |
| D40L   | 173.61 | 5.9 | 119.25 | 11.14 | 119.11 | 11.15 |
| L43A   | 105.82 | 5.81 | 87.20 | 23.21 | 105.63 | 13.42 |
| L43K   | 32.37 | 8.90 | 21.73 | 4.17 | 228.27 | 11.56 |
| K46A   | 86.45 | 13.28 | 78.08 | 3.22 | 146.81 | 9.08 |
| K47A   | 92.19 | 13.00 | 86.48 | 6.57 | 196.31 | 17.16 |
| V47A   | 25.51 | 8.51 | 65.47 | 3.61 | 245.72 | 5.98 |
| S65A   | 55.52 | 7.66 | 58.03 | 6.2 | 67.79 | 8.48 |
| L70A   | 77.03 | 4.39 | 95.90 | 18.35 | 130.23 | 17.33 |
| L70K   | 123.61 | 11.39 | 127.80 | 14.93 | 135.64 | 14.49 |
| T72A   | 137.84 | 16.38 | 135.89 | 9.54 | 87.04 | 8.04 |
| T74A   | 114.57 | 17.08 | 119.59 | 9.94 | 127.25 | 8.57 |
| S77A   | 137.39 | 19.19 | 119.59 | 9.94 | 119.59 | 4.69 |
| K79Q   | 70.20 | 14.35 | 71.10 | 14.08 | 66.12 | 7.8 |
| L80A   | 131.40 | 12.04 | 126.21 | 8.19 | 164.97 | 15.25 |
| L80K   | 118.28 | 10.14 | 104.12 | 6.92 | 150.12 | 12.25 |
| E51A   | 117.33 | 5.06 | 123.62 | 8.90 | 214.18 | 9.66 |
| E57A   | 64.59 | 4.64 | 58.53 | 12.10 | 56.89 | 14.18 |
| E57K   | 50.68 | 16.66 | 71.76 | 7.78 | 56.15 | 14.41 |
| D142A  | 52.70 | 5.53 | 75.37 | 4.75 | 79.49 | 6.58 |
| K147A  | 115.51 | 18.04 | 110.66 | 4.93 | 135.92 | 8.28 |
| S14A   | 68.97 | 9.78 | 74.76 | 9.13 | 84.92 | 9.80 |
| E15A   | 87.27 | 10.85 | 85.83 | 8.03 | 87.28 | 7.97 |
| T15A   | 117.37 | 10.85 | 116.40 | 12.05 | 132.13 | 5.63 |
| K152A  | 111.36 | 8.58 | 94.60 | 12.82 | 90.41 | 6.22 |
| S15A   | 75.21 | 11.58 | 80.90 | 7.53 | 68.58 | 14.47 |
| E155A  | 68.97 | 12.19 | 82.85 | 6.66 | 72.07 | 5.28 |
| T157A  | 84.89 | 13.58 | 88.95 | 5.86 | 109.61 | 9.16 |
| G117T  | 97.28 | 21.02 | 114.23 | 10.73 | 132.01 | 21.66 |
| VII1CD | 10.64 | 1.46 | 20.93 | 4.19 | 86.75 | 4.90 |
| VII3CD | 30.74 | 3.87 | 18.28 | 3.43 | 191.22 | 11.48 |
| VCAM A | 0.67 | 1.08 | 0.00 | 0.00 | 121.55 | 27.03 |
| VCAM B | 145.70 | 22.50 | 128.80 | 8.26 | 141.29 | 12.36 |

For clarity and visual presentation the effects of mutations were classified according to the maximal level of adhesion attained and the concentration required to reach half-maximum adhesion. “Severe” effects produced <50% of the maximal level of spreading on native VCAM-Fc and the protein concentration required for half-maximal spreading was either not reached or was >7-fold higher than native VCAM-Fc. “Marked” effects were classified as those that reached 60–70% maximum native adhesion with a 3–4-fold increase in concentration required for half-maximal adhesion. “Slight” effects produced 80–100% of maximum spreading with only a 2-fold increase in concentration required for half-maximal adhesion. In addition, if adhesion was augmented (maximum adhesion un-

\[\text{VCAM}^B \text{A} \text{N} \text{C} \text{D} \text{L} \text{O} \text{P} \text{Q} \text{R} \text{S} \text{T} \text{U} \text{V} \text{W} \text{X} \text{Y} \text{Z} \]
and Glu150), markedly affected adhesion. One of the main differences between mutagenesis strategy, we have analyzed the molecular basis of Integrin-IgCAM interactions are dependent on a relatively changed but protein concentration required for half-maximum adhesion decreased two-fold or more) then a mutant VCAM-Fc was described as “super-adhesive” (see Fig. 11).

Only two residues outside the C-D loop, when mutated, were found to affect α4β1-VCAM-Fc binding severely, Leu179 and Glu140. Several residues, however (Thr72, Glu81, Asp143, Ser148, Glu155, and Thr157), markedly affected α4β1 interactions. Mutations of amino acids at six further positions resulted in a slight perturbation of α4β1-dependent adhesion (Lys152, Val157, Ser158, Ser159, Glu155, and Thr157). Interestingly, three amino acids outside the C-D loop (Thr74, Leu80, and Lys147), when mutated to alanine, conferred a super-adhesive phenotype on VCAM-Fc. In contrast, α4β7 appeared to differ in its sensitivity to mutation of residues outside the C-D loop region. Only one residue in Ig domain 1 severely affected α4β7 binding (Glu140) and mutations of Leu179 had no effect on α4β7 binding (Fig. 10). Furthermore, Ig domain 1 mutations that markedly affected α4β1 binding (Thr72 and Glu81) had no effect on α4β7-mediated cell attachment. One of the main differences between α4β1- and α4β7-VCAM-1 interactions appeared to lie in Ig domain 2; mutation of three residues at positions 143, 148, and 150 severely affected α4β7-dependent cell adhesion. In addition, residues that markedly affected α4β7 binding were also located in domain 2 (Lys152 and Glu155). A further difference was the super-adhesive activity of T151A for α4β7. The key integrin-binding residues identified in VCAM-1 domain 2 correspond to the C-E loop region, a region in MadCAM-1 domain 2 highlighted to be of potential functional importance (29).

We therefore directly tested the role of this region of MadCAM-1 in α4 integrin binding by engineering a deletion mutant in which the central acidic region (143–150) of the highly extended Ig domain 2 C-E loop had been deleted. The resultant mutant (MadCAM-ΔC-E-Fc) was then assayed for α4β1 and α4β7 binding activity (Fig. 12). In both experiments, MadCAM-ΔC-E-Fc failed to support α4β1 or α4β7-mediated cell adhesion, confirming the importance of this region for both α4 integrin interactions.

**DISCUSSION**

Using IgCAM chimeras and a structure-guided site-directed mutagenesis strategy, we have analyzed the molecular basis of α4 integrin binding to the IgSF members VCAM-1 and MadCAM-1. All VCAM-1 and MadCAM-1 mutants were produced as recombinant IgG fusion proteins permitting quantitative analysis of purified mutant binding properties.

The main findings reported here are that the C-D loops of these IgCAMs are only partially responsible for integrin specificity, and that α4β1/α4β7 integrin binding footprints on VCAM-1 differ in their relative dependence on residues within VCAM-1 Ig domains 1 and 2. In addition, we report that several amino acids within VCAM-1 modulate integrin interactions with residues at predicted integrin contact sites. These findings for the first time demonstrate a direct role for VCAM-1 Ig domain 2 and suggest a mechanism for integrin selection by IgCAMs.

Integrin-IgCAM interactions are dependent on a relatively conserved acidic peptide motif presented as a surface-exposed structure supported on a conserved Ig-domain scaffold. Despite only slight variations in this binding motif, integrin-CAM specificities are exquisite. This suggests two possibilities, either slight variations within this motif are sufficient to discriminate integrin receptors or this motif might represent a general binding structure and integrin selection may be governed by distal sites. We investigated the role of IgCAM C-D loop motifs in regulating integrin specificity by making chimeras between

![Fig. 4. A375-SM spreading on VCAM-Fc C-D loop mutants](image)

![Fig. 5. A375-SM spreading on VCAM-Fc C-D loop mutants](image)
VCAM-1 and MAdCAM-1 (the former is primarily a ligand for α4β1, the latter for α4β7). α4β1-dependent cell spreading assays revealed that MAdCAM-1 chimeras with the VCAM-1 C-D adhesion loop supported reduced levels of cell attachment whereas the super-adhesive VCAM-Fc chimera B again demonstrated a slight increase in the ability to bind integrin relative to native VCAM-Fc. These data reinforce the importance of CAM C-D loops in integrin binding and suggest that α4 integrins have a distinct preference for the C-D loop structure presented by MAdCAM-1 Ig domain 1. Furthermore, since α4β1 has a greater affinity for VCAM-1 compared with MAdCAM-1 this would suggest that α4β1-VCAM-1 binding is enhanced (or MAdCAM-1 binding is attenuated) by sites outside the C-D loop.

Despite several studies on α4β1-VCAM-1 interactions, α4β1 contact sites within the VCAM-1 C-D loop are not fully understood. In an effort to enhance our understanding of integrin-IgCAM interactions we made a detailed analysis of the VCAM-1 and MAdCAM-1 C-D loops. To aid our study we analyzed the x-ray crystal structure of VCAM-1 (29) and identified those C-D loop residues that were both conserved between species (mouse, rat, and human) and surface-exposed, reasoning that functionally important residues would be conserved and accessible to integrin.

*α4* integrin-dependent adhesion data obtained with purified mutant C-D loop VCAM-Fc fusion proteins has extended our understanding of *α4* integrin binding requirements. Mutation of Arg36 to alanine had no effect, while replacement with a glutamate markedly affected α4β1 interactions. This indicates that the physical characteristics of the arginine side chain are not absolutely required for integrin-VCAM-1 engagement, however, a negative charge in this position (glutamate) appears...
to adversely affect the recognition of adjacent residues. Analysis of the x-ray crystal structure of VCAM-1 in this region reveals that Arg36 is in close proximity to the acidic side chain of Asp40 (see Fig. 8) and therefore might feasibly influence the $pK_a$ of the crucial Asp40 carboxyl group and consequently integrin active site engagement. Alternatively, alterations at position 36 might affect the conformation of neighboring side chains within the C-D loop. Mutagenesis data on amino acid Gln38 suggest an important role in $\alpha_4\beta_1$-VCAM-1 binding, since changing the glutamine to a charged residue (glutamate or lysine) severely affected $\alpha_4\beta_1$ binding. However, removal of the glutamine side chain (Q38G) was found to convert VCAM-1 into a super-adhesive $\alpha_4\beta_1$ ligand and replacement of glutamine with an aliphatic residue (leucine) resulted in a further enhancement of integrin binding activity. When a phenylalanine was substituted into this position a slight decrease in $\alpha_4\beta_1$ binding capacity was observed. At least two explanations can be drawn from these data: first, part of the integrin active site might comprise a restricted pocket with hydrophobic characteristics and thus have a preference for interacting with hydrophobic residues. Alternatively, residues in position 38 might not be directly recognized by the $\alpha_4\beta_1$ active site but might confer subtle structural alterations on the remainder of the C-D loop, thus modifying integrin binding. It is notable that the MAdCAM-1 C-D loop possesses a glycine in a homologous position to Gln38, providing an explanation for the super-adhesive phenotype of VCAM-Fc chimera B.

Residues at VCAM-1 positions 39, 40, and 43 are critical for $\alpha_4\beta_1$ binding. Several mutations of Ile39 were tested: replacing isoleucine with a charged residue or an alanine residue severely affected $\alpha_4\beta_1$ binding ability, however, replacement with valine only resulted in a slight decrease in activity. These data are again consistent with a hydrophobic pocket at or near the integrin-ligand-binding site. Charged residues or side chains too small to fill this pocket result in compromised integrin interactions, while longer, branched side chains such as valine appear to be sufficient to permit essentially unperturbed $\alpha_4\beta_1$ binding. Previous reports in which Asp 40 was replaced with alanine, asparagine, or lysine resulted in abolition of VCAM-1 adhesive activity (16, 18, 19), however, mutation of Asp40 to glutamate was reported to have no detectable affect on $\alpha_4\beta_1$ binding (19). Here, we observed a marked effect on $\alpha_4\beta_1$-VCAM-1 binding when Asp 40 was mutated to glutamate. In addition VCAM-1 mutants R36A and P42G have been reported previously to perturb $\alpha_4\beta_1$ binding (19, 22), an explanation for these discrepancies is unclear. Previous mutagenesis studies coupled with sequence alignment data have identified the $\alpha_4\beta_1$ binding motif within VCAM-1 as IDSP; however, we report here that Leu43 is crucial for integrin-VCAM-1 binding and therefore propose that the adhesion motif should be extended to include this residue: i.e. IDSPL.

Analysis of the effects of residues in the C-D loop region on $\alpha_4\beta_7$-VCAM-1 binding revealed essentially the same results as $\alpha_4\beta_1$ binding, however, there were several key differences: (i) the Q38G mutation slightly reduced $\alpha_4\beta_7$ interactions, while Q38L only slightly enhanced $\alpha_4\beta_7$ binding, (ii) mutant I39V had markedly reduced $\alpha_4\beta_7$ binding activity, and (iii) D40E abolished $\alpha_4\beta_7$-VCAM-1 interactions. These data suggest sub-

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**Fig. 8.** VCAM-1 Ig domains 1 and 2 x-ray crystal structure (29). A, MOLSCRIPT (55) representation of VCAM-1 Ig domains 1–2. \( \beta \)-Strands are represented by pointed ribbons indicating the direction of primary sequence (N- to C-terminal) and are organized into two opposing sheets (colored red and blue) in each Ig domain. B, space-fill representation of VCAM-1 Ig domains 1–2. All atoms are represented as spheres proportional to predicted Van der Waal’s radii. Atoms are colored according to type: gray, carbon; red, oxygen; blue, nitrogen; and yellow, sulfur. Atoms colored cyan are from residues that are conserved between species of VCAM-1 (human, mouse, and rat), predicted to be solvent-accessible according to x-ray crystal structure data, and located on the Ig domain 1 C-D loop face. C as B except the structure has been rotated anti-clockwise by 90°.
tle differences between the ligand-binding sites of α4β1 and α4β7 and imply that α4β1 may possess a slightly deeper pocket to accommodate the crucial acidic group at position 40.

Mutation of analogous C-D loop residues within MAdCAM-1 confirmed that both IgCAM C-D loops have essentially identical integrin binding characteristics. Again point mutations provided an explanation for results obtained with VCAM-1/MAdCAM-1 chimeras: amino acid Gly39 (analogous to VCAM-1 Gln38) when mutated to glutamine almost abolished α4β7 binding. Interestingly the native VCAM-(Gln38)-Fc and MAdCAM-
1-Fc chimera A possessed α4β7 binding activity, suggesting that the increased loop size of this construct comprised a more ideal adhesion structure than MAdCAM-G39Q-Fc. The importance of this region of MAdCAM-1 has recently been confirmed by mutagenesis and peptide inhibition studies: MAdCAM-1 residues Leu40, Asp41, Thr42, and Leu44 were found to be required for full α4β7 binding activity (45).

These data demonstrate for the first time the effects of performing full IgCAM C-D loop swaps. However, partial swaps have been investigated previously; the central portion of the ICAM-1 Ig domain 1 C-D loop has been used to replace the analogous region of VCAM-1 and was found to have no negative effect on α4β1 binding (18). Our data with the same mutant in our VCAM-Fc construct (Q38IDS/GIET) demonstrated identical integrin binding activity (data not shown). Analysis of the effects of point mutations described above now provides an explanation for these observations: the overall phenotype of the Q38IDS/GIET mutation is a composite effect of Q38G (superadhesive) and D40E (marked negative effect), thus the overall effect produces a native phenotype. We further investigated the ability of the ICAM-1 (and ICAM-3) C-D loop sequences to substitute for that of VCAM-1 by performing whole loop swaps (8 VCAM-1 C-D loop amino acids replaced with 8 ICAM-1 (or ICAM-3) C-D loop amino acids, see Fig. 1). The resulting constructs failed to bind two anti-VCAM-1 mAbs (4B2 and 1E10, see Table I) and failed to bind α4β1 (data not shown). These data suggest that the ICAM-1 and ICAM-3 C-D loops, although similar to that of VCAM-1 in terms of sequence and function, may adopt markedly different conformations.

Taken together, these data provide an insight into the struc-
tural requirements of α4 integrins for the C-D loops of VCAM-1 and MaDCAM-1 and also identify mutant forms of VCAM-1 capable of conferring a super-adhesive phenotype. In an effort to identify those sites outside the C-D loop that differentially affect α4β1/α4β7 binding, we performed an extensive survey of the contribution of VCAM-1 Ig domain 1 and 2 amino acids. We targeted residues that were conserved between species, surface-exposed, and confined to the C-D loop face of the molecule according to the VCAM-1 x-ray crystal structure (29, Fig. 8). When purified mutant VCAM-Fc proteins were tested for their ability to bind α4β1 and α4β7 in cell-based assays, striking differences between α4β1 and α4β7 binding were observed. Although both integrins had an acute requirement for residues within the C-D loop, their respective adhesion footprints over VCAM-1 Ig domains 1 and 2 differed in two major respects. First, key Ig domain 1 residues required for α4β1-VCAM-1 interactions outside the C-D loop included Leu⁷⁰, Thr⁷², Glu⁸¹, and Glu⁸⁷ but only Glu⁸⁷ appeared to be involved in α4β7-VCAM-1 binding. Second, mutation of amino acids at three positions in Ig domain 2 (Asp¹⁴³, Ser¹⁴⁸, and Glu¹⁵⁰) had a marked, but not severe effect, on α4β1 binding whereas these residues have a much more pronounced affect on α4β7 binding (Fig. 11). Furthermore, mutation of two residues, Lys¹⁵² and Glu¹⁵⁵, had a marked effect on α4β7 binding reinforcing the importance of this region for α4β7-VCAM-1 binding. In addition, several residues were found to augment integrin binding with effects analogous to the Q38G/Q38L mutants. In all instances augmentation sites (Thr⁷⁴, Leu⁸⁰, Lys¹⁴⁷ (both α4β1 and α4β7), and Thr¹⁵¹ (α4β7 only)) lie spatially close to residues that affect integrin interactions (Fig. 11). The identification of Thr¹⁵¹ as an augmentation site in the case of α4β7-VCAM-1 binding further implies that residues in this region of VCAM-1 Ig domain 2 have a greater role for α4β7 interactions compared with α4β1. Although the mechanism of integrin-binding augmentation is unclear, it is likely that changes at these positions confer slight structural changes which lead to enhanced integrin recognition of nearby contact sites. The main evidence to support this hypothesis comes from the finding that replacement of the native residue with amino acids of very different physical characteristics are capable of producing the same result, e.g. L80A, L80K (Fig. 9).

Overall these data suggest an important shift in the “binding footprint” between α4β1 and α4β7. α4β1 primarily binds to VCAM-1 Ig domain 1 via a group of residues comprised of the

![Fig. 11. Space-fill representation of VCAM-1 Ig domains 1–2 with target residues (see Fig. 8) color coded according to the effects of mutation on adhesion. All atoms are represented as spheres proportional to predicted Van der Waal’s radii. All other residues are “ghosted.” Color code for effect of mutations on integrin binding: green, no effect; yellow, slight; orange, marked; red, severe; purple, augmentation (see text for effect classification). A, effects of mutations on α4β1 binding; and B, effects of mutations α4β7 binding.](image-url)
Since consecutive glutamate residues (human MAdCAM-1, Ref. 51), glutamate residues (murine MAdCAM-1, Ref. 24) or five conserved acidic and contains a sequence of three consecutive glutamate residues in the Ig domain 2, this suggests a key role for this region in MAdCAM-1 interactions with VCAM-1 Ig domain 2, this suggests a key role for this region in MAdCAM-1 (29). The C-E loop interacts with the extracellular matrix protein fibronectin including the RGD motif, located in the F-G loop (FnIII(7–10)) has recently been solved and reveals a tandem array of fibronectin type III repeats; each composed of an anti-parallel array of β-strands analogous to Ig domains (53). The RGD motif lies in the F-G loop of FnIII(10) and the synergy sequence is found in the C-E loop and part of E β-strand. Comparison of the arrangement of active sites in fibronectin with that of VCAM-1 highlights three similarities: (i) both sets of adhesion motifs are on surface-exposed loops, (ii) both molecules display active sites on the same face of adjacent domains, and (iii) the functional groups of the key amino acids in each motif are approximately 30–40 Å apart. Thus, two different integrin ligands appear to share a broadly similar “binding topology” composed of a dominant adhesion motif (IDSPL, VCAM-1, and RGD, fibronectin) coupled to second “synergy site” that may regulate integrin specificity (Ig domain 2 C-E loop-E strand, VCAM-1 and III(9) C-E loop and part of E β-strand, fibronectin).

Historically, peptide-based approaches have dominated the identification of integrin ligand active sites, and this has led to the perception that integrins principally recognize discrete peptide motifs such as RGD and LDV. However, data, largely provided by site-directed mutagenesis studies, is now accumulating to suggest that integrin recognition sites on ligands are more complex and are comprised of a dominant acidic peptide flanked by additional contact residues. In the future it will be of interest to assess the relative contribution of functionally important residues in providing the free-energy of interaction between integrin and ligand, an approach successfully used to identify residues responsible for human growth hormone-human growth hormone receptor interactions (54). In turn these findings, together with the use of reporter mAbs (such as those that recognize integrin neo-epitopes arising as a result of ligand engagement), might provide clues as to the connection between ligand engagement by integrin and the conformational responses that occur prior to signal transduction across the plasma membrane.

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References

1. Butcher, E. C. (1992) Adv. Exp. Med. Biol. 323, 181–194
2. Springer, T. A. (1994) Cell 76, 301–314
3. Hynes, R. O. (1992) Cell 69, 11–25
4. Humphries, M. J. (1990) J. Cell Biol. 117, 179–189
5. Newham, P., and Humphries, M. J. (1991) J. Cell Biol. 110, 2185–2193
6. Clements, J. M., Newham, P., Shepherd, M., Gilbert, R., Dougas, I., and Carter, W. G. (1989) J. Cell Biol. 110, 1321–1330
7. Mould, A. P., Wheldon, L. A., Komoriya, A., Wayner, E. A., Yamada, K. M., and Humphries, M. J. (1993) J. Biol. Chem. 268, 4020–4024
8. Komoriya, A., Green, L. J., Mervic, M., Yamada, K. M., and Humphries, M. J. (1993) J. Biol. Chem. 268, 15075–15079
9. Chan, B. M., Elies, J., Murphy, E., and Hemler, M. E. (1992) J. Biol. Chem. 267, 8366–8370
10. Ruegg, C., Postigo, A. A., Sikorski, E. E., Butcher, E. C., Pytela, R., and Erle, D. J. (1992) J. Cell Biol. 117, 179–189
11. Clements, J. M., Newham, P., Shepherd, M., Gilbert, R., Dougas, I., Needham, L. A., Edwards, R. M., Berry, L., Brass, A., and Humphries, M. J. (1994) J. Cell Sci. 107, 2127–2133
12. Osborn, L., Vassallo, C., and Benjamin, C. D. (1992) J. Exp. Med. 176, 99–107
13. Osborn, L., Vassallo, C., Browning, B. G., Tizard, R., Haskard, D. O., Benjamin, C. D., Douglas, I., and Kirchhausen, T. (1994) J. Cell Biol. 124, 601–608
14. Renz, M. E., Chiu, H. H., Jones, S., Fox, J., Kim, K. J., Presta, L. G., and Fong,
S. (1994) J. Cell Biol. 125, 1395–1406
20. Vonderheide, R. H., and Springer, T. A. (1992) J. Exp. Med. 175, 1403–1442
21. Vonderheide, R. H., Tedder, T. F., Springer, T. A., and Staunton, D. E. (1994) J. Cell Biol. 125, 215–222
22. Chiu, H. H., Crowe, D. T., Renz, M. E., Presta, L. G., Jones, S., Weissman, I. L., and Fong, S. (1995) J. Immunol. 155, 5257–5267
23. Berlin, C., Berg, E. L., Briskin, M. J., Andrew, D. P., Kilshaw, P. J., Holzmann, B., Weissman, I. L., Hamann, A., and Butler, E. C. (1993) Cell 74, 185–195
24. Briskin, M. J., McEvoy, L. M., and Butler, E. C. (1993) Nature 363, 61–64
25. Vonderheide, R. H., Tedder, T. F., Springer, T. A., and Staunton, D. E. (1994) J. Cell Biol. 125, 215–222
26. Chiu, H. H., Crowe, D. T., Renz, M. E., Presta, L. G., Jones, S., Weissman, I. L., and Fong, S. (1995) J. Immunol. 155, 5257–5267
27. Makarem, R., Newham, P., Askari, J. A., Green, L. J., Clements, J., Edwards, M., Humphries, M. J., and Mould, A. P. (1992) J. Biol. Chem. 269, 4005–4011
28. Wang, J.-H., Pepinsky, R. B., Stehle, T., Liu, J.-H., Karpuusas, M., Browning, B., and Osborn, L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5714–5718
29. Jones, E. Y., Harlos, K., Bottomley, M. J., Robinson, R. C., Driscoll, P. C., Edwards, R. M., Clements, J. M., Dudgeon, T. J., and Stuart, D. I. (1995) Nature 373, 539–544
30. Newham, P., Tuckwell, D. S., Brass, A., and Humphries, M. J. (1992) Biochem. Soc. Trans. 21, 3398
31. Harpaz, Y., and Chothia, C. (1994) J. Mol. Biol. 238, 528–530
32. Staunton, D. E., Dustin, M. L., and Springer, T. A. (1990) Nature 339, 61–64
33. Staunton, D. E., Dustin, M. L., Erickson, H. P., and Springer, T. A. (1990) Cell 61, 243–254
34. Berendt, A. R., McDowall, A., Craig, A. G., Bates, P. A., Sternberg, M. J., Marsh, K., Newbold, C. I., and Hogg, N. (1992) Cell 68, 71–81
35. Fawcett, J., Holness, C. L., Needham, I., Turley, H., Gatter, K. C., Mason, D. Y., and Sims, C. (1992) J. Cell Biol. 114, 581–584
36. Vazeux, R., Hoffman, P. A., Temita, J. K., Dickinson, E. S., Jasman, R. L., St John, T., and Gallatin, W. M. (1992) Nature 360, 485–488
37. Li, R., Nortamo, P., Valmu, L., Tolvanen, M., Huuskonen, J., Kantor, C., and Gahrberg, C. G. (1993) J. Biol. Chem. 268, 17513–17518
38. Holness, C. L., Bates, P. A., Littler, A. J., Buckley, C. D., McDowall, A., Bossoy, D., Hogg, N., and Simmons, D. L. (1995) J. Biol. Chem. 270, 877–884
39. Klickstein, L. B., York, M. R., de Fougerolles, A., and Springer, T. A. (1996) J. Biol. Chem. 271, 23920–23927
40. Kozlowski, J. M., Hart, R., Feidler, I. J., and Hanna, N. (1984) J. Nat. Cancer Inst. 72, 913–917
41. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
42. Humphries, M. J., Akiyama, S. K., Komoriya, A., Olden, K., and Yamada, K. M. (1986) J. Cell Biol. 103, 2475–2478
43. Holness, C. L., Bates, P. A., Littler, A. J., Buckley, C. D., McDowall, A., Bossy, D., Hogg, N., and Simmons, D. L. (1995) J. Biol. Chem. 270, 877–884
44. Strauch, U. G., Lifka, A., Gasior, U., Klakshw, P. J., Clements, J., and Holzmann, B. (1994) Int. Immunol. 6, 263–275
45. Skyvan, A. M., Bertagnolli, M., Kenney, C. J., and Briskin, M. J. (1996) J. Immunol. 156, 2851–2857
46. Aota, S., Nagai, T., and Yamada, K. M. (1991) J. Biol. Chem. 266, 15938–15943
47. Bowditch, R. D., Halloran, C. E., Aota, S., Obara, M., Plow, E. F., Yamada, K. M., and Ginsberg, M. H. (1991) J. Biol. Chem. 266, 23225–23232
48. Bowditch, R. D., Hariharan, M., Teminna, E. F., Smith, J. W., Yamada, K. M., Getzoff, E. D., and Ginsberg, M. H. (1994) J. Biol. Chem. 269, 10856–10863
49. Nagai, T., Yamakawa, N., Aota, S., Yamada, S. S., Akiyama, S. K., Olden, K., and Yamada, K. M. (1991) J. Cell Biol. 114, 1295–1305
50. Aota, S., Nomizu, M., and Yamada, K. M. (1994) J. Biol. Chem. 269, 24764–24761
51. Mardon, H. J., and Grant, K. E. (1994) FEBS Lett. 340, 197–201
52. Danen, E. H., Aota, S., van Kraats, A. A., Yamada, K. M., Ruiter, D. J., and van Muijen, G. N. (1995) J. Biol. Chem. 270, 21612–21618
53. Leahy, D. J., Aukhil, I., and Erickson, H. P. (1996) Cell 84, 155–164
54. Clackson, T., and Wells, J. A. (1995) Science 267, 383–386
55. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950

α4β1 and α4β7-IgCAM Binding Footprints