Vascular cell adhesion molecule-1 (VCAM1) is a member of the immunoglobulin (Ig) superfamily which interacts with the integrin very late antigen-4 (VLA4). The VCAM1/VLA4 interaction mediates both adhesion and signal transduction and is thought to play an important role in inflammatory and immune responses in vivo. The major form of human VCAM1 contains seven extracellular Ig-like domains, with domain 1 designated as the most N-terminal. We have examined the relationship between human VCAM1 structure and function using a combination of domain truncation mutants and proteolytic fragmentation of recombinant soluble VCAM1. We have characterized two regions of VCAM1, localized to domains 4 and 5, which are highly sensitive to proteolytic cleavage, localized the epitope of the blocking monoclonal antibody 4B9 to domain 1, and found that domains 1-3 are sufficient for both its adhesive function and its ability to initiate T cell activation.

Vascular cell adhesion molecule-1 (VCAM1) is an adhesion molecule expressed in vitro on cytokine-activated endothelium (1-4) and in vivo on inflamed vascular endothelium, as well as on macrophage-like and dendritic cell types in both normal and inflamed tissue (5, 6). VCAM1 interacts with the β1 integrin VLA4 on mononuclear leukocytes, eosinophils, and basophils (7-12). VCAM1 can also activate human T cells in conjunction with coimmobilized mAbs directed to the T cell receptor (13-15), indicating that the VCAM1/VLA4 pathway mediates signal transduction as well as adhesion. Thus, the VCAM1/VLA4 interaction may play a pathophysiological role in immune responses as well as in leukocyte emigration to sites of inflammation (3, 4, 6).

VCAM1 is a member of the immunoglobulin superfamily (1). Alternative splicing is observed in human umbilical vein endothelial cells, generating two forms of VCAM1 with either six or seven extracellular Ig-like domains, with the longer form predominant (16-18). We have begun to examine the relationship between the structure of seven-domain human VCAM1 and its function by generating truncated soluble secreted forms of VCAM1, as well as proteolytic fragments, and examining their functional activity. We have characterized two regions of VCAM1, localized to domains 4 and 5, which are highly sensitive to proteolytic cleavage, localized the epitope of the blocking monoclonal antibody 4B9 to domain 1, and found that domains 1-3 are sufficient for both its adhesive function and its ability to initiate T cell activation.

**EXPERIMENTAL PROCEDURES**

Cell Lines and Antibodies—Ramos cells were obtained from the ATCC and grown in RPMI 1640 supplemented with 10% fetal bovine serum (RPMI 10), as described (1). Monoclonal antibodies HP2/1 (IgG1) to human VLA4, 4B9 (IgG1) to human VCAM1, and BB1 (IgG2b) to human BLA1, have been described elsewhere (7, 19, 20). A polyclonal antiserum recognizing human VCAM1 was obtained from mice immunized with CHO cells stably expressing VCAM1 (19).

Construction and Expression of Deletion Mutants—Truncated forms of VCAM1 were constructed from either the full-length seven-domain VCAM1 pCDM8 clone 1E11 (17) or the full-length six-domain VCAM1 pCDM8 clone 41 (1). Seven-domain- (7D) soluble VCAM1 was produced by truncation of full-length 7D VCAM1 at nt 2193 by digestion with AluI and ligation of a stop codon adaptor. This construct encodes the VCAM1 signal sequence and the extracellular first 674 amino acids of VCAM1 (17). Six-domain- (6D) soluble VCAM1 was produced by truncation of full-length 6D VCAM1 at nt 1924 by digestion with AluI and ligation of a stop codon adaptor as above. This construct encodes the VCAM1 signal sequence and the extracellular first 582 amino acids of the 6D VCAM1 sequence (1). Truncated VCAM constructs were obtained by polymerase chain reaction amplification from the appropriate NotI-digested VCAM1- pCDM8 plasmids and a forward-reverse primer pair in which the reverse primer encodes a stop codon. For constructs D1, D1+2, D1+2+3, and D1+5+6, the polymerase chain reaction products were digested with ApoLI, and the appropriate fragments were purified from agarose gels. These were ligated to a pCDM8 VCAM1 vector fragment predominant (16-18). We have begun to examine the relationship between the structure of seven-domain human VCAM1 and its function by generating truncated soluble secreted forms of VCAM1, as well as proteolytic fragments, and examining their functional activity. We have characterized two regions of VCAM1, localized to domains 4 and 5, which are highly sensitive to proteolytic cleavage, localized the epitope of the blocking monoclonal antibody 4B9 to domain 1, and found that domains 1-3 are sufficient for both its adhesive function and its ability to initiate T cell activation.
into 7T5 flasks at about 1.5 x 10^6 cells/flask in Dulbecco's modified Eagle's medium, 10% fetal bovine serum. 48-h post-transfection, monolayers were washed twice with Hank's balanced salt solution, incubated with 5 ml/flask of PBS containing 5 mM EDTA, washed once with Hank's balanced salt solution, and proteins labeled with 5 ml/flask of Cys/Met-RPMI/containing 150 μCi each of [35S]cysteine and methionine (Du Pont-New England Nuclear) for 4 h at 37 °C. Conditioned medium was collected and cells removed by centrifugation. Adherent COS cells were harvested with 5 ml/Flask of PBS containing 5 mM EDTA, washed once with PBS, and by for 40 min on ice with 1 ml of 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.0, containing 2% Triton X-100, 0.1% aprotinin, and 2 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation (Eppendorf G414 microfuge, 5 min) and the resulting cell lysates used at 250 μl/immunoprecipitation (IP), which was performed as follows. To generate a pre-clearing reagent, protein G-Sepharose (Pharmacia LKB Biotechnology Inc.) was coated with anti-ELAM1 mAb BB11 (20) at 2 μg/ml of beads, in 0.5 ml of IP wash buffer (PBS, pH 7.2, containing 0.5% Tween 20, 0.05% SDS, 0.1% bovine serum albumin, and 0.02% sodium azide) for a minimum of 2 h and unbound mAb removed with five washes with IP wash buffer. 0.5 ml of labeled conditioned medium or 0.25 ml of cell lysate was precleared over 10 μl of BB11 (protein G beads for at least 2 h at 4 °C with rocking. Protein G beads were coated either with 4B9 exactly as described for BB11 or with polyclonal anti-VCAM1 murine serum at a dilution of 1:20, and the pre-cleared supernatants were then incubated with 10 μl of beads for at least 2 h at 4 °C with rocking, and then washed 5 times with 1 ml each of IP wash buffer. Beads were boiled for 3 min in 50 μl of SDS-PAGE reducing sample buffer, eluted proteins run on a 4-20% Daiichi gradient gel, and visualized by radioautography.

Adhesion Assays—The ability of truncated soluble forms of VCAM1 to support adhesion was assessed after immunoprecipitation of cell lysates and immunoaffinity chromatography, immobilized on plastic essentially as described (21). Briefly, COS cell-conditioned medium was used as described above and containing truncated soluble forms of VCAM1 was concentrated 10-fold by ultrafiltration and run over a 1-ml 4B9 immunoaffinity resin. After elution at pH 3.0, column fractions were immediately neutralized, diluted 1:5 into a bicarbonate binding buffer, pH 9.2, and incubated in polystyrene 96-well plates overnight. Eluted material was not quantified. After blocking with bovine serum albumin, adhesion of Ramos cells to the plates was assessed as described (21).

Proteolytic Digestion of VCAM1—To analyze VCAM1 sequence, truncated soluble forms of VCAM1 were transiently expressed in COS cells, purified from conditioned medium by immunoaffinity chromatography, immobilized on plastic, and selected fractions assayed for adhesion as described above. Sam- ple-derived products were sequenced using the procedure of Mat-sudaira (23). Samples were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immobilon, Millipore), stained with Coomassie Blue, and sequenced directly.

RESULTS

Studies with Secreted Truncated Forms of VCAM1—The ability of the VCAM1-directed blocking mAb 4B9 (19), and a murine polyclonal anti-VCAM1 serum, to immunoprecipitate secreted truncated forms of VCAM1 was examined (Fig. 1A). The polyclonal anti-VCAM1 antisera was able to immunoprecipitate proteins encoded by constructs containing domains 1 and 2 (D1+2), domains 2 and 3 (D2+3), and domains 1, 2, and 3 (D1+2+3), but not domain 1 alone. The results show that the truncated two- or three-domain forms of VCAM1, but not the single domain construct, were well secreted by transiently transfected COS cells. When mAb 4B9 was used, the proteins encoded by D1+2 and D1+2+3, but not D2+3, were immunoprecipitated (Fig. 1A). Differences in

FIG. 1. Panel A, immunoprecipitation of soluble forms of VCAM1. Truncated soluble forms of VCAM1 were transiently expressed on COS cells, labeled with [35S]cysteine/methionine, and immunoprecipitated either with polyclonal anti-VCAM1 antisera (left) or anti-VCAM1 mAb 4B9 (right). Domain constructs are indicated at the top. Apparent differences in kDa are consistent with differential glycosylation of VCAM1-derived fragments, since potential N-glycosylation sites occur in domains 3, 4, 5 (two sites), and 6 (two sites) in VCAM1 (1, 17). Molecular weight markers (× 10^3) are indicated at the left. Panel B, adhesion of Ramos cells to immobilized soluble forms of VCAM1. Truncated soluble forms of VCAM1 were transiently expressed in COS cells, purified from conditioned medium by 4B9 immunoprecipitation chromatography, immobilized on plastic, and their ability to support Ramos cell adhesion assessed. Ramos cell adhesion to VCAM1 containing seven domains, six domains, D1+2+3, and D1+2, either in the presence or absence of blocking mAb 4B9, is shown.

T Cell Activation—T cell activation assays were performed as described (15). Briefly, mAb OKT3 was incubated in microtiter wells overnight at 4 °C, excess removed, and rsVCAM1 or VCAM1(1-292) then added for 2-3 h at room temperature. Plates were washed, incubated with medium, purified T cells added, and their proliferation measured as 3 days as described (15).

N-terminal Sequence Analysis—Samples were subjected to N-ter-

minal sequence analysis in an Applied Biosystems 470 sequencer. Phenylthiobutyrylaminoo acid acids were analyzed on line in a 120A Phenylthiobutyrylaminoo acid acid analyzer. Samples derived from HPLC fraction columns were loaded on polybrene-treated discs. Other selected products were sequenced using the procedure of Matsudaira (23). Samples were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immobilon, Millipore), stained with Coomassie Blue, and sequenced directly.
the apparent molecular weights by SDS-PAGE of domain constructs of the same length likely reflect the presence of N-glycosylation sites in domains 3 through 6 (Fig. 1A). These results suggested that mAb 4B9 binds to either domain 1 or the junction formed by domains 1 and 2. A construct containing domains 1, 5, and 6 was also examined (Fig. 1A). mAb 4B9 immunoprecipitates the protein encoded by this construct also, supporting the conclusion that the 4B9 epitope lies within the most N-terminal domain of VCAM1, domain 1.

The ability of soluble truncated forms of VCAM1 to support cell adhesion was next examined (Fig. 1B). The medium conditioned by COS cells transfected with constructs for full-length soluble seven-domain, six-domain, D1+2+3, and D1+2 forms of VCAM1 was passed over a 4B9 immunoaffinity resin (see "Experimental Procedures"), and the eluate immobilized on plastic and examined for its ability to support adhesion. Both the seven- and six-domain forms of VCAM1 support Ramos cell adhesion, which is completely blocked by mAb HP2/1 and 4B9, consistent with the behavior of both forms expressed as membrane proteins in COS cells (17, 24). We found that both D1+2+3 and D1+2 also supported Ramos cell adhesion (Fig. 1B). Interestingly, mAb 4B9 blocked only the three-domain form of VCAM1. In separate experiments, the anti-VLA4 mAb HP2/1 blocked Ramos cell adhesion to all the truncated forms completely (not shown). The data show that the first two N-terminal domains of VCAM1 are sufficient to support VLA4-dependent cell adhesion. However, since no attempt was made to quantitate amounts of truncated forms eluted from the affinity resin, quantitative conclusions regarding comparative adhesive ability could not be drawn.

**Proteolytic Fragmentation of VCAM1**—We have recently described the purification and functional characterization of a soluble form of VCAM1 containing seven extracellular Ig-like domains (rsVCAM1), stably secreted from CHO cells (21). CHO cell-derived 7D material (Fig. 1B) was examined for its ability to support adhesion. For example, endoprotease Glu-C cleaves rsVCAM1 into major fragments of about 80 and 70 kDa (Fig. 2, lane b); chymotrypsin cleaves rsVCAM1 first into fragments of 50 and 40 kDa (lane f) then to 50 and 35 kDa (lane g); and papain cleaves rsVCAM1 first into fragments of 50 and 40 kDa (lane i) then 50 and 35 kDa (lane j). These data suggest that rsVCAM1 contains some particularly protease-sensitive regions.

To determine cleavage sites, the proteolysis of rsVCAM1 with endoprotease Glu-C was examined in more detail. First, the 50- and 35-kDa fragments observed by SDS-PAGE (Fig. 2) were sequenced directly after Western blotting. The 50-kDa fragment gave the same N-terminal sequence as rsVCAM1 itself, while the 35-kDa fragment gave two sequences, indicating that it consisted in fact of two fragments of similar mass. One sequence was that of the N-terminal, while the 35-kDa fragment gave the same N-terminal sequence as rs-VCAM1, while the 35-kDa fragment gave two sequences, indicating that it consisted in fact of two fragments of similar mass.

Two highly conserved cysteines (Cys-400 and Cys-459) found in domain 5 of VCAM1 and characteristic of the majority of Ig-superfamily domains (25, 26). The 50- and 35-kDa fragments were observed upon SDS-PAGE whether reducing or non-reducing sample buffer was used (not shown), suggesting that these two cysteines are not disulfide-linked (see below).

The N-terminal 50- and 35-kDa fragments were purified by immunoaffinity chromatography and gel filtration (Fig. 3).
Another endoproteinase Glu-C digest containing the 50- and 35-kDa fragments was passed over a 4B9 immunoaffinity resin. As expected from the specificity of 4B9 for domain 1, as defined above, and the sequence information obtained on the fragments, the 4B9 resin bound 50- and 35-kDa fragments (Fig. 3, lanes f–h), which both gave the expected N-terminal sequence. Material not binding to the 4B9 resin contained a diffuse 35-kDa band which we presume to be the C-terminal fragment (Fig. 3, lanes c and d). The two N-terminal fragments could be readily separated by gel filtration chromatography (Fig. 3) and isolated in highly purified form for further analysis. The isolation by 4B9 affinity chromatography of the 35-kDa C-terminal fragment, following cleavage at Glu-443 not only confirms that the 2 cysteines in domain 5 are not disulfide-linked but also shows that the two fragments do not remain associated following cleavage.

Characterization of the endo-Glu-C cleavage site which generates the 35-kDa N-terminal fragment required determination of the sequence of its C terminus. To do this, the purified 35-kDa fragment was digested with trypsin and the resultant mixture of peptides subjected to anhydrotrypsin affinity chromatography. Peptides other than the C-terminal peptide contain a C-terminal arginine or lysine and are retained by the column. In contrast, since the most C-terminal peptide is derived from an endo-Glu-C digest and has a C-terminal Glu, it will drop through the column. Following anhydrotrypsin affinity chromatography, a single peptide was purified by reversed-phase HPLC (Fig. 4). The peptide (about 150 pmol) was subjected to N-terminal sequence analysis. Its sequence (EVELIVQEKPFVTE) identifies it as Glu-279 to Glu-292 of the mature sequence of VCAM1. Thus, the second cleavage site for endoproteinase Glu-C is Glu-292, six amino acids from the D3/D4 boundary, allowed us to determine this sensitivity is limited to 7D VCAM1, remains to be determined.

The 50-kDa N-terminal fragment could also be readily purified (Fig. 3). We examined whether the purified 50-kDa fragment, when incubated with endo-Glu-C, could be cleaved at Glu-292 to generate the 35-kDa fragment. In fact, under conditions where intact rsVCAM1 was readily cleaved to 50- and 35-kDa fragments, the purified 50-kDa fragment was resistant to cleavage (Fig. 5). This observation argues that the cleavages at the two sites occur independently and that cleavage at Glu-443 results in a conformational change which renders the Glu-292/Ile-293 bond less susceptible to cleavage.

We have also examined the N-terminal sequences of papain-derived VCAM1 fragments. The 35-kDa fragment (Fig. 2), was sequenced following SDS-PAGE and Western blotting, and found to be derived from the N-terminal of rsVCAM1. Fragments of about 28 kDa, derived from further cleavage observed in DMF (22), were derived from this N-terminal 35-kDa fragment (not shown). These fragments had N termini of either Ala-73 or Gly-83, indicating that they contained domains 2 and 3, but had lost most of domain 1. The analyses of the proteolysis of rsVCAM1 are consistent with the presence of two protease-sensitive regions of amino acid sequence in VCAM1, one connecting domains 3 and 4, and the other in domain 5.

Functional Studies with Proteolytic Fragments—The cleavage of rsVCAM1 with endoproteinase Glu-C at Glu-292, only six amino acids from the D3/D4 boundary, allowed us to proteolytically generate convenient quantities of a 292-amino-acid fragment containing D1+2+3 for further functional analysis. To this end, the 35-kDa N-terminal fragment was
purified to homogeneity as shown above (Fig. 3) and examined for function. Consistent with the results of Fig. 1B, when immobilized on plastic VCAM1(1-292) supports VLA4-dependent adhesive function (Fig. 6A). The papain-derived N-terminal fragments were also examined. The 28-kDa fragments containing D2 and D3 could neither bind mAb 4B9 nor support adhesion of Ramos cells, while the 35-kDa fragment containing D1 through D3 did both (not shown).

We have recently shown that 7D rsVCAM1 can stimulate T cell proliferation when coimmobilized with T cell receptor-directed mAbs (15). When VCAM1(1-292) was examined, we found that it also could stimulate T cell proliferation as well as full-length rsVCAM1, and this was blocked by mAb 4B9 (Fig. 6B). The results show that the first three domains of VCAM1 (and probably only the first two, see Fig. 1B) are sufficient for both of the functions of VCAM1 described to date, namely VLA4-dependent adhesion and activation.

Fig. 7 summarizes the fragments of VCAM1 that were generated through both molecular biological and biochemical techniques, and their ability to support VLA4-dependent adhesion and/or activation, and to bind mAb 4B9.

**DISCUSSION**

VCAM1 is a member of the immunoglobulin superfamily which serves as an adhesion molecule and signal transducer to leukocytes expressing its counter-receptor VLA4 (3, 4, 13–15). As part of our efforts to probe the relationship between VCAM1 structure and function, as well as to define other putative functions of the molecule, we have begun to examine VCAM1 fragments for functional activity. Examination of both truncated recombinant soluble forms of VCAM1 and protolytic fragments shows that the first three domains of VCAM1 are sufficient for both VLA4-dependent adhesion and T cell activation. In addition, immune precipitation studies of truncated VCAM1 constructs show that the blocking mAb 4B9 (19) binds to domain 1 of VCAM1. Recent studies with human VCAM1/ICAM1 chimeric proteins expressed in COS cells (24), and human/murine VCAM1 chimeric proteins expressed on phage confirm these results. Finally, studies with D1+2 suggest that the first two domains are sufficient for adhesion. In the course of these studies, both Damle and Arufo (13) and Taichmann et al. (27) have generated VCAM1-Ig fusion proteins containing the first three domains of VCAM1. These fusion proteins, which are bifunctional homodimers, can support VLA4-dependent adhesion and, in one instance (13), stimulate CD4+ T cells. Our results confirm and extend these data since they show that monomeric VCAM1 fragments are sufficient for VLA4-dependent adhesion and activation.

The structure of seven-domain VCAM1 consists of two internal repeat units of three Ig-like units, domains 1 and 4, 2 and 5, and 3 and 6, respectively, being highly homologous, and suggesting an intergenic duplication event in the evolutionary history of the gene (17, 18). As the first three domains of VCAM1 are alone sufficient for VLA4-dependent adhesion, the role of the remainder of the molecule remains to be fully defined. We have recently cloned cDNAs for both murine and rat VCAM1 (28). Both cDNAs encode seven-domain forms of VCAM1, which are both about 76% identical to human VCAM1, this degree of identity being maintained throughout all seven of the extracellular Ig-like domains. By way of comparison, the adhesion molecule ICAM1, an Ig superfamily member which shares many similarities with VCAM1 (29), shows about 50% identity between murine and human sequences (30). The very high degree of sequence homology across species and throughout VCAM1 strongly argues that the remainder of VCAM1 plays a critical functional role. The proteolysis experiments described above suggest that the more C-terminal Ig-like domains of VCAM1 have unusual structural features, which may be relevant to VCAM1 function. First, VCAM1 possesses two regions which are very protease sensitive, one between domains 3 and 4, and one in domain 5. The reasons for this are unclear, but release of membrane-associated adhesion molecules from the cell surface, including L-selectin and ICAM1, is well established (31, 32). It is possible that proteolytic degradation of VCAM1 occurs in vivo and that this may play a role in its regulation. Second, the protease-sensitive region in domain 5 occurs between the highly conserved cysteines in this domain, arguing that there is no disulfide bond in domain 5. Although Ig domains without a disulfide bond have been described (25), they are unusual. Third, cleavage within domain 5 generates an N-terminal fragment of about 50 kDa which is no longer sensitive to cleavage between domains 3 and 4. The data from rsVCAM1 argue that the conformation of the intact molecule is strongly dependent upon its C-terminal half, and we can now examine the proteolytic sensitivity of cell-associated full-length VCAM1 to confirm and extend these results.

Recent results also illustrate the importance of domains other than the most N-terminal three domains of VCAM1. A comparison of COS cells expressing the same amounts of either the six- or seven-domain forms of VCAM1, both of which contain D1 through D3, shows that the seven-domain form binds about twice as many VLA4-expressing cells (17, 24). To examine the implications of this result, Osborn et al. (24) generated a series of VCAM1/ICAM1 chimeric con-
FIG. 7. Summary of VCAM1 fragments examined in this study and their ability to support VLA4-dependent Ramos cell adhesion, human T cell activation, and 4B9 binding. Fragments examined are designated on the left, amino acid numbers defining domain boundaries (18) are given above full-length rsVCAM1 along with the amino acid numbers of N and/or C termini of proteolytic fragments, where known. Ability to support adhesion, T cell activation, or 4B9 binding is indicated on the right by a plus or minus. ND, not determined; V8, endo-Glu-C digestion fragment; PA, papain digestion fragment.

Because we could not quantitate the amount of immobilized VCAM1 fragments used in our functional assays (e.g. in Figs. 1 and 6, see “Results”), the fact that D1–3 are sufficient to support VCAM1 function is not in conflict with these conclusions.
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