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Contrasting Roles for Domain 4 of VCAM-1 in the Regulation of Cell Adhesion and Soluble VCAM-1 Binding to Integrin α₄β₁

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Cell adhesion mediated by the interaction between integrin α₄β₁ and VCAM-1 is important in normal physiologic processes and in inflammatory and autoimmune disease. Numerous studies have mapped the α₄β₁ binding sites in VCAM-1 that mediate cell adhesion; however, little is known about the regions in VCAM-1 important for regulating soluble binding. In the present study, we demonstrate that 6D VCAM-1 (an alternatively spliced isoform of VCAM-1 lacking Ig-like domain 4) binds α₄β₁ with a higher relative affinity than does the full-length form of VCAM-1 containing 7 Ig-like extracellular domains (7D VCAM-1). In indirect binding assays, the EC₅₀ of soluble 6D VCAM-1 binding to α₄β₁ on Jurkat cells (in 1 mM MnCl₂) was 2 × 10⁻⁹ M, compared with 7D VCAM-1 at 11 × 10⁻⁹ M. When used in solution to inhibit α₄β₁ mediated cell adhesion, the IC₅₀ of 6D VCAM-1 was 13 × 10⁻⁹ M, compared with 7D VCAM-1 measured at 150 × 10⁻⁹ M. Removal of Ig-like domains 4, 5, or 6, or simply substituting Asp³²⁸ in domain 4 of 7D VCAM-1 with alanine, caused increased binding of soluble 7D VCAM-1 to α₄β₁. In contrast, cells adhered more avidly to 7D VCAM-1 under shear force, as it induced cell spreading at lower concentrations than did 6D VCAM-1. Finally, soluble 6D VCAM-1 acts as an agonist through α₄β₁ by augmenting cell migration and inducing cell aggregation. These results indicate that the domain 4 of VCAM-1 plays a contrasting role when VCAM-1 is presented in solution or as a cell surface-expressed adhesive substrate. The Journal of Immunology, 2006, 176: 5041–5049.

The type 1 transmembrane glycoprotein VCAM-1 is a member of the Ig gene superfamily (1) and is involved in a number of different physiologic and pathological processes. It can be constitutively expressed or up-regulated on a variety of cell types including endothelial cells (1), fibroblasts from a variety of tissues (2–4), follicular dendritic cells (5), bone marrow stromal cells (6), and thymic epithelium (7). VCAM-1 plays a role in leukocyte transendothelial migration (reviewed in Refs. 8 and 9), leukocyte retention in tissues (10), and cellular activation (11, 12) by interaction with its primary cell surface ligand, the integrin cell adhesion molecule α₄β₁ (13).

In humans, VCAM-1 is comprised of two isoforms (14). The full-length form of VCAM-1 contains 7 Ig-like extracellular domains (7D VCAM-1) and is thought to be the predominant form expressed on the cell surface (14). An isoform of VCAM-1, 6D VCAM-1 (1), is a result of alternative splicing and lacks domain 4 (14). VCAM-1 can be released from the cell surface due to the activity of neutrophil-derived serine proteases such as neutrophil elastase and cathepsin G (15) or metalloproteases such as TNF-α converting enzyme (16). As such, increased concentrations of circulating VCAM-1 have been reported in patients with rheumatoid arthritis (17), multiple sclerosis (18), systemic lupus erythematosus (17), and sickle cell anemia (19). VCAM-1 has been found bound to integrin α₄β₁ on synovial fluid T cells (20) and in solution has been reported to be chemotactic for T cells (21) and monocytes (22) and angiogenic for endothelial cells (23). By binding to integrin α₄β₁, it can also enhance directed cell migration of lymphocytes on the integrin α₄β₁ substrate ICAM-1 (24). It is currently unknown which isoform of VCAM-1 predominates in the circulation in the above disease states.

Although integrin α₄β₁ can bind to domain 1 or domain 4 of VCAM-1 (25–27) to promote cell adhesion, binding is primarily mediated by residues within the NH₂-terminal domains in the intact, full-length 7D VCAM-1 (28, 29). An α₄β₁ binding “footprint” has been characterized within VCAM-1 domains 1 and 2. This is composed of a primary binding site in domain 1 centered around a solvent-exposed loop created by anti-parallel β-strands (the C-D loop, which contains an essential Asp⁴⁰ residue) and a secondary “synergy” site (the C’-E loop-E strand) in domain 2 (30). Although there has been much work focused on the key residues in VCAM-1 required for mediating α₄β₁ integrin-dependent cell adhesion, very little work has focused on the molecular requirements for soluble VCAM-1 binding to α₄β₁.

Cells bearing integrin α₄β₁ adhere to the NH₂-terminal domains 1 and 2 of VCAM-1 (2D VCAM-1), independent of the intact molecule (30). However, as a soluble ligand, 2D VCAM-1 binds α₄β₁ with a lower apparent affinity relative to full-length 7D VCAM-1 (31). This could be due to a second potential α₄β₁ binding site within domain 4 of 7D VCAM-1 (25–27). To address this,
we generated a number of VCAM-1 constructs, including full-length 7D VCAM-1 and the alternatively spliced 6D VCAM-1, and tested their binding to integrin αβ1. In the present study, we report the unexpected finding that 6D VCAM-1 bound αβ1 with a higher relative affinity than did 7D VCAM-1 and that it was an efficient agonist of αβ1 function. In contrast, as an adhesive substrate, 6D VCAM-1 was less efficient than 7D VCAM-1 in inducing cell spreading and in mediating cell adhesion under increasing shear force. Thus, as a soluble integrin αβ1 agonist, 6D VCAM-1 may play a more important role than previously anticipated.

Materials and Methods

Cell lines
Jurkat clone E6.1 (American Type Culture Collection), the Jurkat cell line selected for loss of expression of the integrin αβ1 subunit (Jurkat αβ1-)(32) (provided by D. Rose, University of California at San Diego, La Jolla, CA). Ramos (American Type Culture Collection), and HPB-ALL were grown in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) at 37°C, 5% CO2, in a humidified atmosphere. Peripheral blood T cells were derived from the Buffy coat (Gulf Coast Regional Blood Center) of healthy donors as previously described (33). To generate activated peripheral blood T cells, cells were incubated in complete medium with 10 ng/ml PMA (Sigma-Aldrich) and 1 mM ionomycin (Sigma-Aldrich) for 12 h and then were maintained in complete medium with 100 U/ml IL-2. Activated cells were typically >90% CD3+ positive as determined by FACS analysis with mAB OKT3 (American Type Culture Collection).

Construction of VCAM-1 mutants

The following primer sets (obtained from Sigma-Genosys or Seqwright (American Type Culture Collection).) were used for Quikchange mutagenesis. The following primer set was used: 7D VCAM-1(Ig) was deleted with the following overlapping sequence GAGATCCAGAA-3’ and 5’-GAAGCAGTACGGTCCTTGAG-3’. To create the alternatively spliced isoform of VCAM-1 (FLAG) construct was deleted with the following overlapping sequence TAGCCCTGAG-3’ and 5’-GTCGAGAGGGCTTCTAGGC-3’. The following primer sets (obtained from Sigma-Genosys or Seqwright (American Type Culture Collection).) were used for Quikchange mutagenesis. The following primer set was used: 7D VCAM-1(Ig) was deleted with the following overlapping sequence GAGATCCAGAA-3’ and 5’-GAAGCAGTACGGTCCTTGAG-3’.

Affinity Soluble 6D VCAM-1 Binding to Integrin αβ1

Statc cell adhesion assays

Goat anti-mouse IgG2a (Southern Research Associates) or anti-FLAG mAb M2 (Sigma-Aldrich) was immobilized (5 μg/ml) overnight at 4°C in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl (TBS) onto Hi-Bind plates (BD Biosciences). After blocking with 1% BSA in TBS for 1 h at RT, 7D VCAM-1(Ig), 6D VCAM-1(Ig), 7D VCAM-1(FLAG), or 6D VCAM-1(FLAG) was captured at indicated concentrations in TBS for 1 h at RT. During this time, Ramos cells (25 × 10⁶) were resuspended in 1 ml of complete medium and incubated for 1 h at 37°C in the presence of 50 μg/ml calcein-AM (Molecular Probes). After labeling, cells were resuspended at a concentration of 5 × 10⁶/ml in TBS supplemented with 1 mM MnCl₂. For experiments involving inhibition of cell adhesion, indicated concentrations of 6D and 7D VCAM-1(FLAG) were preincubated with cells before addition to plates coated with 7D VCAM-1(Ig). Cells (2.5 × 10⁶ in 50 μl) were added to plates and incubated for 30 min at 37°C. After further incubation, plates were washed three times with TBS (pH 7.5). Adherent cells were lysed, and cell adhesion was quantitated on an Ultra384 plate reader (Tecan) using 485 and 535 nm excitation and emission filters after cell lysis in 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, and 5 mM EDTA.

Parallel plate flow assay

Goat anti-mouse IgG2a (20 μg/ml) in 0.1 M NaHCO₃ (pH 9.5) was immobilized overnight at 4°C on 24 × 50-mm slides cut from 150-mm polyester petri dishes. The slides were then washed with PBS, and 7D VCAM-1(Ig) or 6D VCAM-1(Ig) was immobilized as described above for cell adhesion assays in a separate 96-well plate. After immobilization, plates were incubated for 1 h at RT with biotinylated mAb 1.4C3 (Chemicon International) or anti-FLAG mAb M2 (Sigma-Aldrich) was immobilized with 7D VCAM-1 bound cells for 30 min at 4°C. Monoclonal Ab 1.4C3 recognizes both 6D and 7D VCAM-1 to a similar extent (27). Cells were then washed and incubated with FITC-conjugated goat anti-mouse Ab (BioSource International). Bound VCAM-1 was immobilized with FACS analysis. A minimum of 5000 events were analyzed per sample.
Cell spreading assays

Goat anti-mouse IgG2a (Southern Biotechnology Associates) was immobilized (20 μg/ml in 0.1M NaHCO3 (pH 9.5)) overnight at 4°C onto high-binding 96-well plates (Costar). Plates were washed with PBS, incubated with indicated concentrations of 7D VCAM-1(Ig) or 6D VCAM-1(Ig) for 1 h at RT in PBS, and blocked with 1% (w/v) BSA (2 h at RT). Wells were washed with PBS and 3.0 × 10^5 HPB-ALL T cells in complete medium were added to wells and incubated for 30 min at 37°C. Images of the cells were captured by a VI-470 CCD video camera (Optronics Engineering) using a ×20 objective on a Nikon DIAPHOT-TMD inverted microscope. The images were analyzed using NIH Image 1.58 (available via http://rsweb.nih.gov/pub/nih-image/), and data are presented as the percentage of cells spreading.

Migration assays

Migration assays were performed in 3-μM pore size Transwells (24-well; Costar). Upper chambers were precoated with goat anti-mouse IgG2a (5 μg/ml) in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl (50 μl) overnight at 4°C. Upper chambers were then blocked with 10% BSA. After blocking, ICAM-1(Ig) (5 μg/ml) was incubated for 1 h at RT. After washing, upper chambers were loaded with Jurkat cells (2 × 10^5 cells) in 160 μl of medium (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin). Lower chambers contained 600 μl of medium supplemented with 15 mg/ml stromal cell-derived factor 1α to induce chemotaxis. In control experiments, the α4-specific cyclic hexapeptide TBC772 (34) was preincubated with the cells (15 min at RT) at a concentration of 100 μM. To block binding to ICAM-1, the upper wells of Transwells were incubated with anti-ICAM-1 mAb HA58 (20 μg/ml) for 15 min at RT before the addition of Jurkat cells. In tests with soluble VCAM-1 (FLAG tagged), cells were preincubated with indicated concentrations of VCAM-1 construct and then added to the upper chamber of the ICAM-1-coated Transwells. After a 4-h incubation at 37°C, upper chambers were removed and cells in the lower chamber were collected and counted on a hemocytometer. Results are expressed as the total number of cells migrated.

Homotypic aggregation assay

Jurkat cells or α4-negative Jurkat variant cells were resuspended to 2 × 10^6 cells in complete medium and then added (200 μl) to 96-well tissue culture plates with indicated concentrations of soluble 6D and 7D VCAM-1(FLAG) constructs or indicated VCAM-1(Ig) constructs. Cells were incubated for 2 h at 37°C, 5% CO2. For blocking experiments, TBC772 was used at a concentration of 100 μM and added at the same time as the soluble VCAM-1 constructs. Images of cells were captured on an IMT-2 inverted microscope (Olympus) equipped with a SPOT-cooled CCD camera (Diagnostic Instruments). Original magnification was ×20. Qualitative assessment of cellular aggregation was based on the scale presented in Fig. 7A. Briefly, random images from wells were scored on a scale between 1 and 5, with 1 representing unstimulated cells and 5 representing maximal aggregation.

Results

Alternatively spliced 6D VCAM-1 binds to integrin αβ4 with a higher relative affinity than 7D VCAM-1

Extracellular Ig-like domains 1 and 4 of 7D VCAM-1 can mediate integrin αβ4-dependent cell adhesion (25–27, 29, 30); however, the functional importance of these two domains in regulating soluble VCAM-1 binding to αβ4 has not been examined. To address this, we generated COOH-terminal FLAG-tagged 7D VCAM-1 (7D VCAM-1(FLAG)) and the alternatively spliced 6D VCAM-1 (6D VCAM-1(FLAG)), which lacks domain 4 (14), and tested them for binding to cell surface-expressed αβ4. Unexpectedly, 6D VCAM-1(FLAG) bound with a higher relative affinity to αβ4 on Jurkat cells than did 7D VCAM-1(FLAG) (Fig. 1A). This was true in the presence of 1 mM MnCl2 (Fig. 1A) or when the binding buffer contained 1 mM MgCl2 and 1 mM CaCl2 (data not shown). The EC50 of 6D VCAM-1(FLAG) binding to integrin αβ4 on Jurkat cells in the presence of MnCl2 was 2 × 10^-9 M, whereas the EC50 of 7D VCAM-1(FLAG) binding under similar conditions was 11 × 10^-7 M. Increased binding of 6D VCAM-1(FLAG) was also observed on activated peripheral blood T cells (data not shown). Binding was completely inhibited by the α4 specific antagonist peptide TBC772 (data not shown).

The relative differences in affinity between 6D VCAM-1(FLAG) and 7D VCAM-1(FLAG) for αβ4 were further compared in experiments designed to determine their ability to inhibit αβ4-dependent cell adhesion. Soluble 6D and 7D VCAM-1(FLAG) were preincubated with Ramos cells in the presence of MnCl2 before adhesion to captured 7D VCAM-1(Ig). In this system, Ramos cell adhesion to immobilized 7D VCAM-1(Ig) was αβ4-dependent based on function blocking Ab studies using mAb HP2/1 (data not shown). 7D VCAM-1(FLAG) inhibited Ramos cell adhesion with an IC50 of ~50 nM (Fig. 2B). 6D VCAM-1(FLAG) inhibited cell adhesion with an IC50 of ~13 nM (Fig. 1B), indicating a >10-fold increase in relative affinity of 6D VCAM-1 over 7D VCAM-1 for integrin αβ4.

Ig-like domains 4 through 6 and Asp328 negatively regulate soluble VCAM-1 binding to integrin αβ4

To elucidate the residues and domains important for increased soluble 6D VCAM-1 binding to integrin α4β1, a variety of amino acid
substitutions and domain deletions within IgG2a-tagged VCAM-1 were generated (represented schematically in Fig. 2A) and compared for soluble binding to $\alpha_\beta_1$. Protein purity was verified by SDS-PAGE and Coomassie staining (Fig. 2B). Briefly, aspartic acid to alanine substitutions were made in the reported $\alpha_\beta_1$-binding domains (D40 and D328) either singly or dually in full-length VCAM-1 (designated VCAM-1(D40A), VCAM-1(D328A), or VCAM-1(D40,328A)). Deletions of Ig-like domains were also conducted including the second (VCAM-1(D2)), third (VCAM-1(D3)), fourth (referred to here as 6D VCAM-1(Ig)), fifth (VCAM-1(D5)), and sixth (VCAM-1(D6)) domain.

Removal of domains 2 or 3 of full-length VCAM-1 did not affect soluble VCAM-1 binding (Fig. 2C); however, similar to the 6D VCAM-1(FLAG), removal of the 4th Ig-like domain in this IgG2a-tagged construct (referred to as 6D VCAM-1(Ig)) resulted in increased soluble VCAM-1 binding to Jurkat cells over wild-type 7D VCAM-1(Ig). Likewise, removal of domain 5 or 6 gave similar increased binding. A single amino acid substitution within domain 4 (D328A) demonstrated increased $\alpha_\beta_1$-dependent binding. No binding of either the single substitution (D40A), or the double substitution mutant (D40,328A) was detected. All constructs were tested at a concentration of 30 nM in the presence of 1 mM MnCl$_2$. In the presence of 1 mM MgCl$_2$ and CaCl$_2$, removal of domain 4 or substitution of Asp$^{328}$ with Ala resulted in a similar increase in soluble VCAM-1 binding (data not shown). Significant binding of 2D VCAM-1(Ig), a construct containing only the two NH$_2$-terminal domains of VCAM-1 tagged with the Fc region of IgG2a, was only observed in the presence of 1 mM MnCl$_2$ (data not shown). In summary, Asp$^{328}$ is essential for soluble VCAM-1 binding, and Asp$^{328}$ substitution with alanine or removal of the fourth, fifth, or sixth Ig-like domain increases soluble VCAM-1 binding to integrin $\alpha_\beta_1$.

**Increased strength of adhesion to 7D VCAM-1 under conditions of flow**

Adhesion assays were performed on both Ig-tagged and FLAG-tagged VCAM-1 constructs to verify the role played by VCAM-1 domain 4 in static cell adhesion assays. We observed very little difference in the $E_{50}$ of binding to VCAM-1 constructs with or without this domain (Fig. 3). From two experiments, the $E_{50}$ range of cell adhesion for 7D VCAM-1(Ig) was 50 – 56 $	imes$ 10$^{-12}$ M, and the $E_{50}$ of cell adhesion for 6D VCAM-1(Ig) was 50 – 62 $	imes$ 10$^{-12}$ M. Equal capture of VCAM-1 was confirmed by ELISA with biotinylated VCAM-1 mAb 1.4C3 (Fig. 3, dotted line), which recognizes an epitope within domain 2 of VCAM-1 in both 7D and 6D VCAM-1 (27). No significant adhesion was seen to control wells coated with BSA (data not shown). Similar results were obtained when FLAG-tagged 6D and 7D VCAM-1 were captured with anti-FLAG Ab M2, where the range in $E_{50}$ for Ramos cell adhesion to 7D VCAM-1(FLAG) was 100 – 140 $	imes$ 10$^{-12}$ M, and to 6DVCAM-1(FLAG) it was 90 – 110 $	imes$ 10$^{-12}$ M (data not shown).

Experiments were then performed to measure the rates of attachment and strength of adhesion of Jurkat cells to 7D VCAM-1(Ig) and 6D VCAM-1(Ig). In Fig. 4, VCAM-1 constructs were captured onto plastic immobilized goat anti-mouse IgG2a. Jurkat cells were perfused over the substrates in a parallel plate flow chamber at a flow rate of 0.3 ml/min (2.8 dynes/cm$^2$), and cell adhesion was quantified from images captured every 20 s. Although the rate of adhesion to 6D VCAM-1(Ig) was consistently

**FIGURE 2.** Structure/function analysis of soluble VCAM-1 binding to integrin $\alpha_\beta_1$. A, Schematic representation of purified VCAM-1 constructs generated. Constructs contain either a FLAG tag or mouse IgG2a Fc region on the C terminus. Deleted domains are shaded. B, SDS-PAGE analysis of purified VCAM-1 constructs. Baculovirus-expressed IgG2a-tagged constructs were purified on protein-G Sepharose columns, and FLAG-tagged VCAM-1 constructs were purified on protein-G Sepharose columns, and FLAG-tagged VCAM-1 constructs were purified on protein-G Sepharose columns, and FLAG-tagged VCAM-1 constructs were purified on protein-G Sepharose columns. Proteins were separated by SDS-PAGE (10%) under reducing conditions and visualized with Coomassie blue staining. C, soluble VCAM-1 binding to Jurkat cells. Jurkat cell were incubated with various Ig-tagged soluble VCAM-1 constructs at 30 nM in the presence of 1 mM MnCl$_2$. After washing, bound VCAM-1 was detected with FITC-conjugated goat anti-mouse IgG2a. For blocking experiments, mAb HP2/1 (IgG1 isotype) was preincubated with Jurkat cells before the addition of soluble VCAM-1. Data are expressed as the average MFI ± SD from three separate experiments. MFIs were calculated from at least 5000 events.

**FIGURE 3.** Static Ramos cell adhesion to VCAM-1. A, Cell adhesion assays were performed as described in Materials and Methods in the presence of 1 mM MnCl$_2$. 7D VCAM-1(Ig) and 6D VCAM-1(Ig) were captured at indicated concentrations. In a parallel 96-well plate that was coated with similar concentrations of VCAM-1, an ELISA was performed to measure the amount of captured VCAM-1. Primary anti-VCAM-1 mAb used was biotinylated 1.4C3. Ab binding was detected with streptavidin-HRP. Data are expressed as average ± SD from triplicate determinations.
higher than to 7D VCAM-1, this difference did not appear significant. The cells immediately adhered to the 6D and 7D VCAM-1 substrates, as no cell rolling was detected. No adhesion was observed to immobilized BSA in control chambers (data not shown). In Fig. 4B, Jurkat cells were perfused over substrate, then flow was stopped and cells were allowed to adhere for 10 min. Flow was then initiated, and cell adhesion was quantified as shear stress was increased. Under these conditions, cells adhered more avidly to full-length 7D VCAM-1(Ig) than to 6D VCAM-1(Ig) (Fig. 4B). Cells also adhered more avidly to 7D VCAM-1(Ig) than to VCAM-1(D328A) immobilized at similar concentrations (data not shown).

**Increased cell spreading on 7D VCAM-1**

Im mobilized 7D VCAM-1(Ig) was also more potent than immobilized 6D VCAM-1(Ig) in inducing cell spreading. HPB-ALL cells were used in these assays because $\alpha_4\beta_1$-dependent shape changes are more readily quantified with this cell type (35). Very little spreading occurred on 6D VCAM-1(Ig) that was captured at a concentration of 0.5 nM, whereas cell spreading is clearly seen on 7D VCAM-1(Ig) when coated at an identical concentration. 6D VCAM-1(Ig) could induce cell spreading at coating concentrations greater than 1 nM; however, significantly less 7D VCAM-1(Ig) was required to induce cell spreading of HPB-ALL cells (Fig. 5B). Thus, even though there is very little difference in static cell adhesion of cells to the different VCAM-1 constructs, the strength of adhesion of cells at high shear forces and the ability to support cell spreading is greater with 7D VCAM-1(Ig) than with 6D VCAM-1(Ig).

**Soluble 6D VCAM-1 induces cell migration on the $\alpha_4\beta_2$ substrate ICAM-1**

7D VCAM-1 signaling through integrin $\alpha_4\beta_1$ can induce Jurkat cell locomotion on the $\alpha_4\beta_2$ substrate ICAM-1 (24). To determine whether soluble 6D VCAM-1 could promote $\alpha_4\beta_2$-dependent migration, cell migration assays on ICAM-1-coated Transwells were performed. As ICAM-1 was captured through its IgG2a COOH-terminal tag, FLAG-tagged VCAM-1 constructs were used to prevent their binding to the capture Ab. Stromal cell-derived factor 1 $\alpha$ was used in the lower chambers of the Transwells to induce Jurkat chemotaxis, and 6D VCAM-1(FLAG) (100 nM) was added to the upper chamber of Jurkat cells ($2 \times 10^5$ cells). As seen in Fig. 6, 6D VCAM-1(FLAG) augmented Jurkat chemotaxis on the $\alpha_4\beta_2$ substrate ICAM-1. The $\alpha_4\beta_2$ antagonist peptide TBC772 (34) did not affect basal migration of cells on ICAM-1; however, it did inhibit 6D VCAM-1(FLAG)-induced migration. Monoclonal Ab HAS8, a function-blocking anti-ICAM-1 mAb (36), consistently inhibited Jurkat cell migration to basal levels (Fig. 6), which was similar to that seen on BSA-coated Transwells (data not shown). Thus, 6D VCAM-1 can augment $\alpha_4\beta_2$-dependent cellular locomotion on ICAM-1.
FIGURE 6. 6D VCAM-1-induced migration of Jurkat cells on αβ1 substrate ICAM-1. Jurkat cells were either unstimulated or stimulated with soluble 6D VCAM-1(FLAG) (100 nM) before placement in the upper chamber of ICAM-1-coated Transwells. In control experiments, a cyclic peptide antagonist of αβ1 (TBC772) was preincubated with Jurkat cells for 15 min at RT before the addition of 6D VCAM-1(FLAG) and placement into the upper chambers. For anti-ICAM-1 treatment, upper Transwell chambers were preincubated with mAb HAS8 (20 μg/ml) for 15 min before the addition of Jurkat cells. Cells migrating through the Transwells into the lower chamber were collected and enumerated with a hemocytometer. Results are presented as the mean ± SE of the total number of migrated cells from duplicate Transwells. Data are representative of three experiments performed.

Soluble VCAM-1 induces cellular homotypic aggregation

When performing migration assays, cellular aggregation was observed in soluble VCAM-1-treated cells that were incubated on control Transwells coated with BSA (data not shown). Further analysis demonstrated that dose-dependent aggregation of Jurkat cells occurred upon the addition of 6D VCAM-1(FLAG), with maximal aggregation occurring at 30 nM (Fig. 7B). 7D VCAM-1(FLAG) also induced homotypic aggregation of Jurkat cells (Fig. 7B). VCAM-1-induced aggregation was αβ1 dependent, as TBC772 completely inhibited aggregation (Fig. 7C). Likewise, Jurkat cells mutagenized and selected for loss of integrin αβ1 expression (32) did not aggregate upon the addition of soluble 6D VCAM-1(FLAG) (Fig. 7D). Analysis of the dimeric Ig-tagged wild-type and mutant VCAM-1 constructs demonstrated a pattern of induction of homotypic aggregation (Fig. 7E) that was similar to their extent of measured binding in soluble VCAM-1 binding assays (Fig. 2). Thus, both 6D and 7D VCAM-1, in monomeric or dimeric form, can induce αβ1-dependent cellular homotypic aggregation.

Discussion

Although VCAM-1 can support αβ1-dependent cell adhesion through two of its Ig-like domains, domains 1 and 4 (25–27, 29, 30), the role of these domains in the regulation of soluble VCAM-1 binding to integrin αβ1 has not been addressed. In the present study, we report that the alternatively spliced isoform of VCAM-1, which is generated by the removal of domain 4 (termed 6D VCAM-1), results in an unexpected increase in the apparent affinity of soluble VCAM-1 for αβ1. This was observed in ligand binding assays and in the inhibition of αβ1-dependent cell adhesion. Soluble 6D VCAM-1 was also an efficient agonist of αβ1-dependent functions such as induced cellular migration and homotypic aggregation. When acting as an adhesive substrate, however, domain 4 of full-length VCAM-1 (7D VCAM-1) was required to promote cell spreading and increased resistance to shear force. These results suggest that 6D VCAM-1 and 7D VCAM-1 may play different roles in vivo when present either in solution or as a cell surface-expressed adhesive substrate.

As measured by flow cytometry, the integrin αβ1 binds 7D VCAM-1 with an EC50 of ~11 × 10−9 M in the presence of 1 mM MnCl2. This is similar to that previously reported for high-affinity interactions between αβ1 and 7D VCAM-1 (EC50 of ~50 nM (31)). Under similar conditions, however, 6D VCAM-1-bound integrin αβ1 with a >5-fold increase in apparent affinity (EC50 of 2 × 10−6 M) compared with 7D VCAM-1. These results were not due to the use of monomeric FLAG-tagged VCAM-1 constructs, as increased binding of dimeric Ig-tagged 6D VCAM-1 was also observed (Fig. 2C). It is not known whether in vivo circulating VCAM-1 is a monomer or dimer. Although other cell adhesion receptors of the Ig family that bind integrins, such as the αβ2 ligand ICAM-1 and αβ5 ligand mucosal addressin cell adhesion molecule-1, can be found on the cell surface or in solution as dimers or oligomers (37, 38), recent studies indicate that in vitro-expressed 7D VCAM-1 is monomeric (39). Regardless, we demonstrate here that either as a monomer or as an Ig-tagged dimer, 6D VCAM-1 binds integrin αβ1 with a higher relative affinity than does 7D VCAM-1. Furthermore, this increased binding was observed on a variety of different lymphocyte cell lines and on activated peripheral blood T cells.

The mechanism by which soluble 6D VCAM-1 binds integrin αβ1 with higher relative affinity than does 7D VCAM-1 is undetermined. We demonstrate here that Asp40 within domain 1 of VCAM-1 is essential for soluble VCAM-1 binding. Also, removal of Ig-like domains COOH-terminal to domain 3 or simply substituting Asp328 with Ala increases the relative affinity of 7D VCAM-1 for αβ1. Previous functional and structural studies of the two NH2-terminal domains of VCAM-1 (domains 1 and 2) have demonstrated that the primary αβ1 binding site in domain 1 resides in a solvent-exposed loop (the C-D loop, which contains the essential residue Asp40) (25, 27, 29, 40, 41). A secondary “synergy” site, the C’-E loop-E strand, is located in the upper face of domain 2 (30), spatially close to the C-D loop of domain 1 (40, 41). This mechanism of αβ1 binding VCAM-1 domains 1 and 2 may be similar to integrin αβ1 binding fibronectin. In this case, αβ1 binds to a primary “RGD” loop in the fibronectin type III repeat 10 and to a synergy site centered around the “PHSRN” loop in fibronectin type III repeat 9 (42). By introducing interdomain disulfide bonds at the interface between fibronectin type III repeats 9 and 10, Altroff et al. (43) have recently shown that maintaining a 28° angle of tilt between these domains results in higher-affinity interactions with integrin αβ1. The crystal structures of VCAM-1 domains 1 and 2 demonstrate that the relative orientation of the two domains is not fixed (40, 41) and that there is a high degree of flexibility (ranging from 7.3–39.9° from five different crystal monomers) in the two-dimensional angle of tilt between the two domains (44). Perhaps removal of Ig-like domains COOH-terminal to domain 3 or merely substituting Asp328 with Ala results in a subtle conformational change that maintains the angle between domains 1 and 2 such that the domain 1 C-D loop and domain 2 C’-E loop-E strand synergy site present an optimal integrin αβ1 binding topology. Indeed, the overall structure of 7D VCAM-1 is dependent on COOH-terminal regions. For example, an endoproteinase Glu-C cleavage site between domain 3 and domain 4 of 7D VCAM-1 is no longer sensitive to proteolysis upon removal of domains 5–7 (45). Furthermore, deletion of domain 4 in 7D VCAM-1 decreases binding of the domain 1-specific mAb 4B9 (27). Mutational studies, including substitution of residues within the domain 2 synergy site (28) in 6D VCAM-1, will begin to address the mechanism of increased 6D VCAM-1 affinity for integrin αβ1. Also, it will be interesting to determine whether 6D VCAM-1 has a higher relative affinity for other VCAM-1 binding
integrins such as \( \alpha_4 \beta_1 \) and \( \alpha_6 \beta_1 \), as domain 2 of VCAM-1 has been implicated in integrin selectivity (30).

Even though 6D VCAM-1 has a higher relative affinity for \( \alpha_4 \beta_1 \) in solution, the strength of adhesion of \( \alpha_4 \beta_1 \)-bearing cells to 7D VCAM-1 is greater under shear, as seen in parallel plate flow chamber experiments. Increased resistance to shear when cells are adherent to 7D VCAM-1 is likely due to postreceptor occupancy events such as changes in cellular morphology, as cells plated on 7D VCAM-1 flattened out and spread to a greater extent than those plated on equivalent concentrations of 6D VCAM-1 (Fig. 5A). However, when 6D VCAM-1 was immobilized at higher densities (e.g., >1 nM coating concentration), cell spreading occurred (Fig 5B) and there was little difference in the strength of cell adhesion (data not shown). This demonstrated that 6D VCAM-1 was capable of inducing cell spreading, and that domain 4 of 7D VCAM-1 was not required. Under these conditions, cell shape change was likely a function of ligand density, as the combination of Ig domains 1 and 4 of 7D VCAM-1 renders it bivalent and promotes cell spreading at lower coating concentrations compared with monovalent 6D VCAM-1.

Previous studies have demonstrated that triggering the integrin \( \alpha_4 \beta_1 \) with soluble 7D VCAM-1 can induce cell locomotion (24). In the present study, we show that domain 4 in 7D VCAM-1 is not necessary for this response and that monomeric 6D VCAM-1 can also induce cell migration on ICAM-1. Furthermore, we now demonstrate that soluble VCAM-1 binding to integrin \( \alpha_4 \beta_1 \) on Jurkat cells induces cellular homotypic aggregation. To our knowledge, this is the first demonstration of aggregation induced by a naturally occurring \( \alpha_4 \beta_1 \) ligand, as previous studies have relied on the use of Abs specific for certain epitopes on the integrin \( \alpha_4 \) or \( \beta_1 \) chain (46–48). Interestingly, TBC772, an \( \alpha_4 \)-specific cyclic peptide antagonist (34) thought to mimic the C-D loop from domain 1 of VCAM-1 (49), did not induce aggregation or promote cellular locomotion. This suggests that other regions outside the C-D loop of domain 1 in VCAM-1, did not induce aggregation or promote cellular locomotion. This suggests that other regions outside the C-D loop of domain 1 in VCAM-1 can mediate this effect. Thus, by binding soluble 6D or 7D VCAM-1, \( \alpha_4 \beta_1 \) can act as an agonist receptor regulating cellular functions such as cell migration and homotypic aggregation, which could have important physiologic consequences.

In diseases involving inflammation, it is unclear whether soluble VCAM-1 participates in disease progression, down-regulates the severity of disease, or is simply a byproduct of increased proteolytic activity in inflammatory sites. Circulating VCAM-1 in rheumatoid arthritis (17), multiple sclerosis (18), or systemic lupus erythematosus (17) may facilitate leukocyte transendothelial migration by stimulating cell migration on the \( \alpha_4 \beta_2 \) integrin ligand ICAM-1, or it may act as a chemotactic agent (22). By inducing cellular aggregation, soluble VCAM-1 could also retain inflammatory cells at specific sites, for instance, B16 melanoma cells

**FIGURE 7.** Homotypic cellular aggregation induced by soluble VCAM-1. A, Jurkat cells were treated with various concentrations of VCAM-1 and scored for homotypic aggregation. The aggregation scale ranged from 1 (untreated, basal aggregation of cells as demonstrated in the left panel of A) to a maximal aggregation of 5 (A, right panel). B, Various doses of soluble VCAM-1 (6D and 7D-FLAG) were tested for their induction of homotypic aggregation. Results are from one representative experiment of three performed. C, VCAM-1(FLAG)-induced (100 nM) homotypic aggregation was inhibited by the \( \alpha_4 \beta_1 \) cyclic peptide antagonist TBC772 (100 \( \mu \)M). Results are representative of three experiments performed. D, \( \alpha_4 \) null (\( \alpha_4^\text{null} \)) Jurkat cells were incubated with 6D VCAM-1(FLAG) (30 nM) and homotypic aggregation was scored as in A. Results are representative of two experiments performed. E, Indicated Ig-tagged VCAM-1 constructs (all used at 100 nM) were tested for their ability to induce homotypic aggregation in Jurkat cells. Aggregation was scored as in A. Results are representative of three experiments performed.
overexpressing $\alpha_\beta_1$ aggregate in an $\alpha_\beta_1$-dependent manner, which prevents spontaneous metastasis after s.c. injection (50). In contrast with inflammation-promoting effects, high concentrations of soluble VCAM-1 could be anti-inflammatory by functioning as a competitive antagonist of $\alpha_\beta_1$. It will be important to determine the role of soluble VCAM-1 in inflammation, as it could have different effects on disease progression or resolution depending on the stage of inflammation and the cellular source from which it is generated. It will also be of interest to determine the relative levels of 7D VCAM-1 and 6D VCAM-1 that can be found in the circulation in different disease states.

Soluble VCAM-1 may also play a more systemic role in diseases such as sickle cell anemia. For instance, sickle RBCs and premature erythrocytes (reticulocytes) express integrin $\alpha_\beta_1$ (51, 52), which may play a role in vaso-occlusive events (53, 54) by binding VCAM-1 on endothelial cells (51, 55). Enhanced levels of circulating VCAM-1 have been reported in this disease, with peak concentrations correlating with vaso-occlusive crisis (19). It will be interesting to determine whether soluble VCAM-1 can induce aggregation of sickle RBCs or reticulocytes, as this could be a contributing factor in vaso-occlusion.

In the present study, we provide evidence that the domain 4 of 7D VCAM-1 plays contrasting roles in VCAM-1 function. In solution, domain 4 acts as a negative regulator of soluble VCAM-1 binding to the integrin $\alpha_\beta_1$, as 6D VCAM-1 binds $\alpha_\beta_1$ with a higher relative affinity than does 7D VCAM-1. As an immobilized ligand, however, 6D VCAM-1 is not as effective in inducing cell spreading and resisting shear force as 7D VCAM-1. These results suggest that the relative in vivo importance of 6D VCAM-1 may lie in its role as a soluble agonist rather than an expressed substrate referred to the cell surface. Given the potential role for soluble VCAM-1 in a number of diseases, it will be important to determine whether there is tissue-specific alternative splicing of VCAM-1, the ratio of circulating 6D VCAM-1 compared with 7D VCAM-1 under different disease conditions, and the structural mechanisms underlying the higher relative affinity of soluble 6D VCAM-1 for integrin $\alpha_\beta_1$.

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References


