Ultrastructure and Function of Dimeric, Soluble Intercellular Adhesion Molecule-1 (ICAM-1)

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ABSTRACT

Previous studies have demonstrated dimerization of intercellular adhesion molecule-1 (ICAM-1) on the cell surface and suggested a role for IgSF domain 5 and/or the transmembrane domain in mediating such dimerization. Crystallization studies suggest that domain 1 may also mediate dimerization. ICAM-1 binds through domain 1 to the I domain of the integrin $\alpha_L\beta_2$ (lymphocyte function associated antigen 1). Soluble C-terminally dimerized ICAM-1 was made by replacing the transmembrane and cytoplasmic domains with an $\alpha$-helical coiled coil. Electron microscopy revealed C-terminal dimers that were straight, slightly bent, and sometimes "U"-shaped. A small number of apparently closed ring-like dimers and "W"-shaped tetramers were found. To capture ICAM-1 dimerized at the crystallographically defined dimer interface in domain 1, cysteines were introduced into this interface. Several of these mutations resulted in formation of soluble disulfide-bonded ICAM-1 dimers (domain 1 dimers). Combining a domain 1 cysteine mutation with the C-terminal dimers (domain 1/C-terminal dimers) resulted in significant amounts of both closed ring-like dimers and "W"-shaped tetramers. Surface plasmon resonance studies showed that all of the dimeric forms of ICAM-1 (domain 1, C-terminal, and domain 1/C-terminal dimers) bound similarly to the integrin $\alpha_L\beta_2$ I-domain, with affinities ~1.5-3-fold greater than that of monomeric ICAM-1. These studies demonstrate that ICAM-1 can form at least three different topologies, and that dimerization at domain 1 does not interfere with binding in domain 1 to $\alpha_L\beta_2$. 
INTRODUCTION

Intercellular adhesion molecule-1 (ICAM-1, CD54) is the most important of a group of related immunoglobulin superfamily (IgSF) molecules that serve as ligands for the integrin $\alpha L\beta 2$ (1). ICAM-1 is basally expressed on the surface of cells important in immune responses. Its expression is further enhanced on these cells, and induced on other cell types, including endothelial, epithelial, and fibroblastic cells, by inflammatory mediators. Increased ICAM-1 expression augments immune responses and leukocyte accumulation in inflamed tissues. ICAM-1 contains a binding site for $\alpha L\beta 2$ in domain 1, and a binding site for the related leukocyte integrin $\alpha M\beta 2$ in domain 3. ICAM-1 is also subverted by the major group of rhinoviruses as a receptor for entry into nasal epithelial cells, and by P. falciparum for sequestration of infected erythrocytes in the peripheral vasculature.

ICAM-1 consists of five extracellular IgSF domains (domains 1-5), a hydrophobic transmembrane domain, and a short cytoplasmic domain (1) (Fig. 1). Electron micrographs of truncated, soluble ICAM-1 (sICAM-1) show that its 5 IgSF domains assume a rod-like shape 18.7 nm in length, with a characteristic bend ~11 nm from the N-terminus between D3 and D4 (2,3). Crystal structures have been determined for domains 1 and 2 of ICAM-1 (4,5). The binding site for $\alpha L\beta 2$ has been identified in ICAM-1 by mutagenesis, and is located near the middle of domain 1 on the edge of the $\beta$-sandwich (2). The binding surface is slightly convex, and the most important residue is Glu-34, located in an edge $\beta$-strand. $\alpha L\beta 2$ must be activated by conformational change or clustering in the membrane to be adhesive for ICAM-1 (6). An inserted (I) domain in the integrin $\alpha L$ subunit appears to directly bind to ICAM-1. The I domain bears a Mg$^{2+}$ ion that is hypothesized to ligate Glu-34 of ICAM-1. Conformational movements in the I domain have been demonstrated to dramatically alter its adhesiveness and affinity for ICAM-1 (7-9).

ICAM-1 appears to exist as a dimer and higher multimers in its native state on the cell surface, as shown by cross-linking studies (10,11); however, the architecture of these
multimers is unknown. Soluble ICAM-1 can be dimerized in fusion proteins, and this increases its avidity for $\alpha_L\beta_2$ and rhinovirus (11-13). However, the function of dimerization in the context of native, cell surface expressed ICAM-1 remains to be determined.

Multiple modes of dimerization have been suggested for ICAM-1. Previous cross-linking studies have suggested that domain 5 and/or the transmembrane domain can mediate dimerization (10,11). In addition, a crystal structure of domains 1 and 2 of ICAM-1 revealed a putative dimerization interface on the face containing $\beta$-strands B, E and D (BED sheet) of domain 1. The $\alpha_L\beta_2$-binding interface is on the opposite side of domain 1 from the dimerization interface, such that the two Glu-34 residues are far from one another and pointing away from the interface. It was hypothesized that two $\alpha_L\beta_2$ molecules could bind simultaneously to the dimer (4). The domain 1 dimerization interface contains at its center hydrophobic residues including leucines 18, 42, 43 and 44 (4). Its hydrophobicity suggests this interface might be biologically relevant (14). However, the size of this interface is insufficient to drive strong dimerization, leaving its physiologic relevance inconclusive. Furthermore, in another crystal study with domains 1 and 2 of ICAM-1 in which three N-linked glycosylation sites were mutated, this dimer interface was not seen (5). Instead, a hydrophilic dimer interface, such as is commonly found in crystal lattice contacts, was seen.

Thus far, it has not been clear whether the C-terminal and domain 1 dimer interfaces are structurally compatible with one another. Could dimerization occur at both interfaces simultaneously? Furthermore, what would be the nature of such dimers? One way to reconcile dimerization in domain 1 with dimerization near domain 5 or the transmembrane domain would be if dimerization at these regions occurred in cis, with formation of a closed, ring-like dimer (4). On the other hand, dimerization might occur in trans, linking different pairs of molecules at each interface. Furthermore, it has not yet been established whether domain 1 can, in fact, support dimerization. Moreover, it is not clear whether and how the maintenance of structural constraints by dimerization via domain 1 or the transmembrane domain would affect binding to $\alpha_L\beta_2$. In this study, we
have investigated these issues.

**MATERIALS AND METHODS**

**Cell lines and antibodies** - 293T cells (a human renal epithelial transformed cell line) were grown in DMEM (Gibco) supplemented with 10% FBS, non-essential amino acids (Gibco) and 50 µg/ml of gentamicin. CHO-K1 cells were maintained in Ham’s F12K medium, 10% FBS, and 50 µg/ml of penicillin/streptomycin. CHO.Lec 3.2.8.1 cells (a glycosylation-defective variant of CHO-K1 cells) and SKW3 cells were maintained as previously described (13,15). mAbs R6.5 (16), CA-7 (17), CL203 (18), and CBRIC1/11 (19) have been previously described. Within ICAM-1, mAb R6.5 maps to domain 2 (2), mAb CBRIC1/11 maps to domain 3 (19), mAb CL203 maps to domain 4 (2), and mAb CA-7 maps to domain 5 (17).

**cDNA constructions** - The human wild-type ICAM-1 cDNA (20) was subcloned into the Hind III and Not I restriction sites of the pAprM8 vector (21) to generate ICAM-1/pAprM8. For the C-terminal dimer, cDNA encoding domains 1-5 of ICAM-1 was fused to the α-helical coiled coil domain of the yeast transcription factor GCN4 containing a disulfide-promoting cysteine substitution (22, 23) by overlap extension PCR. In the first PCR reaction, using ICAM-1/pAprM8 as template, a 260 bp fragment was generated which included an internal ICAM-1 Bgl II site and codons for the last amino acids of the ICAM-1 ectodomain (SPRYE) fused with the first amino acids (RMKQCLEDKVEELLSDKNYHL) of the GCN4-p1 peptide. In the second PCR reaction, also using ICAM-1/pAprM8 as a template, an ~200 bp fragment was generated, which included a sequence encoding the last C-terminal amino acids (LSKNYHELNEVARLKLKLVG) of the GCN4-p1 peptide, a stop codon and 170 bp non-translated ICAM-1 sequence followed by a vector 3’ Not I site. In the final PCR reaction, the 260 and ~200 bp fragments were used together as overlapping templates (region of overlap indicated by the italicized GCN4-p1 residues shown above) to prepare an ~460 bp product. After digestion with Bgl II and Not I, this product was used to replace the corresponding wild-type sequence of ICAM-1 in the ICAM-1/pAprM8 vector, thus giving rise to the mutant
plasmid sICAM-1_GCN4/pAprM8.

For domain 1 dimers, Protein Data Bank files were made from accession 1ic1 containing the crystallographic symmetry-related dimers of the A or B molecules (4). Residues near the dimer interface that might form disulfide bonds if mutated to cysteine were identified with the program SSBOND (24), or by visual inspection with LOOK (25). A three-round PCR method for single site-specific mutagenesis was performed to introduce cysteine mutations. Briefly, 5’ and 3’ primers were designed to include unique restriction sites which were used in two separate PCR reactions with a pair of mutagenic inner complementary primers. The resulting overlapping products were used as the template for the third PCR reaction, the product of which was digested and ligated into the corresponding pre-digested plasmids.

The cDNA encoding soluble monomeric ICAM-1 (sICAM-1/pAprM8) was described previously (26). To generate stable cell lines, all mutant cDNAs were further subcloned into the BamHI and Not I sites of pEF1/V5_puro vector (27), a modified vector from pEF1/V5_neo. All constructs were verified by DNA sequencing. A schematic representation of the ICAM-1 constructs used in this study is depicted in Fig. 1.

cDNA transfections- Proteins were expressed transiently in 293T or CHO.Lec 3.2.8.1 cells using FuGENETM 6 transfection reagent according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN) (28) (13). Stable cell lines that express soluble monomeric or dimeric ICAM-1 were generated by FuGENETM 6-mediated transfection of 2 µg of various ICAM-1/pEF1/V5_puro constructs into CHO.Lec 3.2.8.1 cells, followed by selection with 10 µg/ml of puromycin beginning at 48h post-transfection. All stable cell lines were maintained in complete medium supplemented with same concentrations of antibiotic.

Radiolabeling and immunoprecipitation- Metabolic labeling and immunoprecipitation were previously described (29). Briefly 5 x 10^6 cells in 4 ml labeling medium (cysteine/methionine-free RPMI containing 15% dialyzed FBS) were
labeled with 0.5 mCi of $^{35}$S]cysteine and methionine (ICN Biochemicals) overnight at 37°C. Labeled cell culture supernatants (500 µl) were then incubated with R6.5 (an ICAM-1 D3-specific mAb) coupled at 3 mg/ml to Sepharose CL-4B beads (50 µl of a 1:1 slurry) for 3 h at 4°C. The immunoprecipitates were analyzed by SDS 10% PAGE (with or without 10 mM DTT) and fluorography.

**Protein purification** - The purification of monomeric and dimeric ICAM-1 was carried out at 4°C. Culture supernatant (2 L) containing ICAM-1 was passed through a CBRC1/11 mAb Sepharose CL-4B affinity column (30 ml at 2 mg/ml), followed by extensive washing with 10 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl. Bound proteins were eluted with 50 mM triethylamine, pH 11.5, containing 0.15 M NaCl and fractions were collected in test tubes containing 1/10 volume 1 M Tris-HCl, pH 6.5, to neutralize the pH. For mono-Q column chromatography, the protein samples and column were equilibrated with 20 mM Tris, pH 8.0 and eluted using a linear gradient of 0 to 1 M NaCl. For size exclusion chromatography, the samples were passed over a Superdex-200 column in PBS.

**Antibody binding assay** – mAbs R6.5 and CA-7 were separately adsorbed to the wells of a flat-bottom 96-well polystyrene plate (Flow Laboratories, McLean, VA) by incubation overnight at 4°C. Nonspecific binding sites were blocked with 1% heat-treated BSA for 1 h at 37°C. sICAM-1 or sICAM-1_GCN4 (500 ng/ml in PBS) were then added to the wells and incubated for 30 min at 37°C followed by washing 3 X with PBS. Binding of sICAM-1 and sICAM-1_GCN4 was detected by incubation with biotin-conjugated CBRC1/11 mAb followed by washing with PBS and addition of streptavidin-conjugated horseradish peroxidase and 2,2’-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt as substrate. Absorbance at 414 nm was then measured.
Gradient sedimentation and electron microscopy – Proteins were subjected to glycerol gradient sedimentation and sedimentation coefficients were obtained as previously described (30). Rotary-shadowed specimens were prepared directly from gradient fractions and subjected to electron microscopy as described (30).

Determination of binding constants for soluble monomeric or dimeric ICAM-1 by surface plasmon resonance - The binding of a designed mutant (K287C/K294C) of the αLβ2 I domain, which is locked in the high affinity open conformation (open αLβ2 I domain) to ICAM-1 was monitored with a BIAcore 1000 instrument (BIAcore, Piscataway, NJ), as previously described (8). Briefly, open αLβ2 I domain or BSA (control) proteins were covalently immobilized onto sensor chips and ICAM-1 dimers or monomers were flowed over the sensor chips. \( k_{\text{on}} \) and \( k_{\text{off}} \) values were obtained by curve fitting of the association and dissociation phases of sensograms, respectively, using either a 1:1 binding model for monomeric ICAM-1 or a bivalent analyte binding model for dimeric ICAM-1 using BIAevaluation software (BIAcore). \( K_D \) was then calculated from \( k_{\text{on}} \) and \( k_{\text{off}} \) (\( K_D = k_{\text{off}} / k_{\text{on}} \)).

Homotypic aggregation assay – Since the LFA-1/ICAM-3 interaction is slightly less efficient than that of LFA-1/ICAM-1 (15), ICAM-1 is expected to compete efficiently with ICAM-3 for LFA-1 binding. SKW3 cells undergo LFA-1 and ICAM-3 dependent homotypic aggregation (15). The ability of soluble forms of ICAM-1 to bind cell surface LFA-1 can, therefore, be sensitively monitored as inhibition of SKW3 homotypic aggregation. Thus, SKW3 cells were dissociated by washing in Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS and then resuspended in L15 media containing 2.5% FBS (L15/FBS) at 2 \times 10^6 cells/ml. The cells were then combined with 10 \( \mu \)g/ml of the αLβ2 activating mAb CBR-LFA1/2 and 50 \( \mu \)l/well was added to 96-well microtiter plates containing 50 \( \mu \)l of L15/FBS with 0-4 \( \mu \)M of either monomeric ICAM-1 or domain 1 dimeric, C-terminal dimeric or domain 1/C-terminal dimeric ICAM-1 and incubated 30 min at 37°C.
Samples were then visualized via light microscopy and aggregation was scored from 0 to 5 as previously described (31).
RESULTS

ICAM-1 dimerized through a C-terminal GCN4 coiled coil. The transmembrane domain of ICAM-1 has glycine residues that cluster on one side of the predicted transmembrane α-helix, and form a hydrophilic bald patch in the inner leaflet of the bilayer that is postulated to mediate dimerization (Fig. 1B) (10). To mimic dimerization through lateral association of α-helical transmembrane domains, we fused a water-soluble α-helical coiled-coil to the C-terminus of the extracellular domain (sICAM-1_GCN4, Fig. 1). The four heptad repeats of the yeast GCN4 protein were used, which form an α-helical coiled-coil homodimer. A cysteine was used in the fourth position of the first heptad repeat, to further stabilize the homodimer by formation of a disulfide bond (23). sICAM-1_GCN4 was expressed and metabolically labeled in 293T cells, and subjected to immunoprecipitation, SDS-PAGE, and fluorography (Fig. 2A). Disulfide-linked sICAM-1_GCN4 dimers were efficiently made, as confirmed by the presence of an ~180 kDa dimeric band and an ~90 kDa monomeric band in the absence and presence of reduction with DTT, respectively (Fig. 2A). The yield of sICAM-1_GCN4 dimer was similar to that of sICAM-1 monomer, which migrated at ~90 kDa under both reducing and nonreducing conditions (Fig. 2A).

The mAb CA-7 is specific for domain 5 of ICAM-1 (17). It reacts well with ICAM-1 with an artificial glycosylphosphatidylinositol anchor, but poorly with native cell surface ICAM-1, and recognizes domain 5 of ICAM-1 in monomeric but not dimeric ICAM-1 (11). To examine reactivity with CA-7 mAb, sICAM-1_GCN4 and sICAM-1 were expressed in CHO.Lec 3.2.8.1 cells, purified, and compared for binding to CA-7 mAb and R6.5 mAb (a dimerization-independent mAb to domain 2) in a capture ELISA (Fig. 2B). Interestingly, sICAM-1 was recognized equally well by CA-7 and R6.5 mAbs, whereas sICAM-1_GCN4 was recognized poorly by CA-7 compared to R6.5. When similar peptides are fused to the C-terminus that do not result in dimerization, the CA-7 epitope is not shielded (C.D. Jun, C. V. Carman, T. A. Springer, unpublished). Thus the CA-7 epitope is shielded both in sICAM-1_GCN4 dimers and in native ICAM-1 dimers on the cell surface, suggesting that the disposition of domain 5 is similar in both, and that...
sICAM-1_GCN4 dimers may mimic cell surface dimers.

Purified preparations of sICAM-1 and sICAM-1_GCN4 C-terminal dimers were further characterized by glycerol gradient sedimentation, rotary shadowing, and electron microscopy. In good agreement with previous studies (2,3) sICAM-1 appeared as slightly bent rods ~18 nm in length (Fig. 3A). The sICAM-1_GCN4 preparation sedimented on a glycerol gradient with a sedimentation coefficient of 5.5S (Fig. 3B). Rotary-shadowed electron micrographs confirmed that sICAM-1_GCN4 was dimeric. Its contour length was 33-43 nm, about twice that of sICAM-1. Most of the sICAM-1_GCN4 dimers were extended (Fig. 3C, panel I). About 10-20% of the molecules showed a pronounced bend into a symmetrical U shape, and rarely the two ends of the U were in contact, forming a circle (Fig. 3C, panel II).

The circles and U’s suggest that noncovalent domain 1-domain 1 interactions might occur by bending a GCN4 dimer to bring its ends into contact. If this interaction were stable it should produce circles, but the U-shaped molecules suggest that most circles are disrupted and the ends separated somewhat. We believe this disruption happens as the molecules are deposited on the mica. There are at least two precedents for non-covalent protein-protein bonds being disrupted in this fashion. Rotary shadowed dimeric factor XIIIa appeared as two variably separated subunits, only rarely in contact, suggesting that the subunits separated and moved apart after being deposited on the mica (32). A recent study of cell adhesion molecule L1 provided evidence that it was folded into a horseshoe conformation in solution, but after deposition on mica the horseshoe unfolded into an elongated conformation (33). The small number of W-shaped molecules suggest that domain 1 association can also occur between two GCN4 dimers (Fig. 3C, panel III). These results encouraged us to design additional constructs to further probe the relationship of domain 1 and the segment following domain 5 to ICAM-1 dimerization and function.

Cysteines introduced into the putative dimerization interface in domain 1 can form disulfide-linked sICAM-1 dimers  sICAM-1 has previously been characterized as
monomeric (11). The hydrophobic interface visualized in domain 1 in crystals might be sufficient to drive dimerization of native molecules on the cell surface (4) but is not sufficient to form stable sICAM-1 dimers in solution (11). We rationalized that transient dimers formed in solution might be “captured” if cysteines were introduced into the dimerization interface in positions where disulfide bond formation was compatible with an interface stabilized by noncovalent interactions. We measured the distances separating β-carbons (Cβ atoms) of pairs of residues in the interface between domain 1 visualized in the crystal structure of domains 1 and 2 of ICAM-1 (4) (Table 1). There are two independent molecules in the asymmetric unit of these crystals, termed molecules A and B. Hydrophobic dimer interfaces lie between two-fold symmetry-related A molecules, and between two-fold symmetry-related B molecules. There are small differences in the orientation at these two interfaces, and thus we measured distances at both (Table 1). Symmetry related L42 and L43 residues had C-β atoms that were within the distance of 3.41 to 4.25 Å optimal for disulfide formation (24), and symmetry related L18 and L44 residues had C-β atoms that were somewhat farther apart. Each of these residues was accessible on the surface of ICAM-1 monomers (Table 1). Thus when mutated to cysteine, all residues were predicted to be accessible for disulfide bond formation.

Each of these four residues was mutated to cysteine, and sICAM-1 variants containing the L18C, L42C, L43C, and L44C mutations were expressed in CHO.Lec 3.2.8.1 cells. CHO.Lec 3.2.8.1 cells are mutant for complex carbohydrate processing enzymes, and therefore the molecular masses of monomeric and dimeric ICAM-1 species were lower than when produced in 293T cells. The sICAM-1 L42C and L43C mutants formed disulfide-linked dimers, as shown by the presence of a ~115 kDa band in SDS-PAGE (Fig. 4). This band was completely converted to the monomeric size of ~58 kDa after reduction (data not shown). By contrast, L18C and L44C sICAM-1 molecules failed to form stable dimers (Fig.4). This correlated with longer Cβ-Cβ distances, but not with exposure on the monomer surface (Table 1).

Dimeric L43C sICAM-1 was purified by immunoaffinity and gel filtration chromatography (Fig. 5A). Glycerol gradient sedimentation yielded a coefficient of 4.4S
Electron micrographs of the dimers purified by sedimentation revealed rods 30-36 nm in length exhibiting extended or "V"-shaped topologies with a bend in the middle (Fig. 5C). Thus, stable disulfide-linked domain 1 dimers of ICAM-1 were formed, and these appeared to be guided by noncovalent contacts in the crystal-defined domain 1 interface, because dimerization correlated with Cβ atom proximity in this interface.

**Expression and ultrastructure of sICAM-1 with covalent stabilization of both C-terminal and domain 1 dimerization motifs**

We tested whether the U-like and ring-like dimers and the W-shaped tetramers observed with sICAM-1_GCN4 (Fig. 3C, panels II and III) could be stabilized. The disulfide-forming L43C cysteine substitution was introduced into the domain 1 dimerization interface together with C-terminal dimerization through the GCN4 coiled-coil. The sICAM-1_GCN4(L43C) and sICAM-1_GCN4 constructs were expressed in CHO.Lec 3.2.8.1 cells and radiolabeled material was subjected to immunoprecipitation and SDS-PAGE (Fig. 6A). sICAM-1_GCN4 yielded both monomeric and dimeric material as shown above, with molecular masses when produced by CHO.Lec 3.2.8.1 cells of 58 kDa and 112 kDa, respectively (Fig.6A). sICAM-1_GCN4(L43C) also showed monomeric and dimeric bands at 58 and 112 kDa, respectively (Fig.6A). However, it also exhibited a third band migrating at ~180 kDa (determined below to represent ring-like dimers) and a lesser fourth band migrating at ~200 kDa (determined below to represent “W”-shaped tetramers) (Fig.6A).

To characterize topology, sICAM-1_GCN4(L43C) was expressed in CHO.Lec 3.2.8.1 cells and immunoaffinity purified. In subsequent ion-exchange chromatography (Fig. 6B left), distinct forms of sICAM-1_GCN4(L43C) were enriched in different fractions as shown by nonreducing SDS-PAGE (Fig. 6B right). Fraction #15 consisted almost entirely of the ~180 kDa form, whereas fraction #16 contained ~10%, ~40% and ~50% of ~58 kDa, ~112 kDa and ~180 kDa forms, respectively (Fig.6B). Fraction #21 contained all four forms (~58, ~112, ~180 and ~200 kDa) in significant amounts (Fig.6B). Each of these fractions (#15, #16 and #21) was then subjected to glycerol
gradient sedimentation. This separated the larger proteins from the monomer, and resulted in fractions enriched in one, two, or three of the larger forms (Fig. 7A, C, E).

Electron microscopy of rotary-shadowed preparations showed that each of the three higher molecular mass bands seen in SDS-PAGE corresponded to a distinct topologic form of ICAM-1 (Fig. 7). Strikingly, the molecules in fraction #15, which contained only the ~180 kDa form (Fig. 7A), were all ring-like, oval-shaped structures with a contour length (i.e. circumference) of 32-44 nm. This topomer is a dimer based on its length. Its ring-like shape is consistent with a “closed” conformation in which both domain 1 and C-terminal dimerization have been covalently stabilized. This topomer migrated anomalously in SDS-PAGE, since the C-terminal and domain 1 dimers migrated at 112 kDa. SDS-denatured proteins normally assume long, rod-like shapes. In contrast, the ~180 kDa form would be constrained by disulfide bonds near its N- and C-termini to be approximately circular, which might alter the way it is sieved or oriented during electrophoresis through a polyacrylamide gel, and give rise to anomalous migration in SDS-PAGE.

Glycerol gradient-purified fraction #16 contained both the ~180 and ~112 kDa bands (Fig. 7C), and showed two distinct topomers in electron micrographs (Fig. 7D). Some molecules were closed rings, identical to those found in fraction #15 (Fig. 7D, upper row), and others were extended linear molecules (Fig. 7D, lower two rows). The extended dimers had lengths of ~36-40 nm, and sometimes formed U-shapes (Fig. 7D, center row). The closed rings were identical to those seen in fraction #15, and thus correspond to the 180 kDa form in SDS-PAGE. The linear molecules were seen in fraction #16 and not in fraction #15, and thus were identified as the ~112 kDa band. These molecules are the same mass and length as both the C-terminal dimers and the domain 1 dimers, and the linear molecules in #16 appear to be a mixture of these two forms.

Finally, three molecular species were observed in fraction #21 of ~200, ~180, and ~112 kDa in SDS-PAGE (Fig. 7E). Ring-like dimers ~32-38 nm in length, as well as "U"-shaped dimers ~36-40 nm in length, were observed in micrographs (Fig. 7F, middle and lower rows, respectively). These appear to correspond to the topomers described
above that migrate at ~180 and ~112 kDa in SDS-PAGE, respectively. In addition, fraction #21 contained significant amounts of long extended molecules with irregular bends, sometimes forming "W"-shaped structures (Fig. 7F, top row). These correspond to the additional ~200 kDa form seen in this fraction and not in fractions 15 or 16. Based on the length of ~73-77 nm and the molecular mass of ~200 kDa, these "W"-shaped structures represent tetramers, in which domain 1 and C-terminal dimerization occur in trans, i.e. between different pairs of molecules.

**Ligand binding of domain 1, C-terminal, and domain 1/C-terminal ICAM-1 dimers** – To determine whether the domain 1 dimers, C-terminal dimers, and the ring-like, closed domain 1/C-terminal dimers retained the ability to bind ligand, we used a BIAcore to measure binding to the αL I domain. We used a recently described mutant αL I domain with two cysteine substitutions that form a disulfide bond that locks the I domain into the open, high affinity conformation (7-9). The high affinity mutant αLβ2 I domain was immobilized on the surface of a BIAcore sensor chip. Initial experiments demonstrated specificity, in that sICAM-1 bound in a Mg2+-dependent manner to chips with immobilized αLβ2 I domain, and did not bind to chips with immobilized BSA (data not shown). The domain 1 dimer, C-terminal dimer, and ring-like domain 1/C-terminal dimer from fraction #15 all retained specific ligand binding for the αL I domain (Table 2). Moreover, the association rate constants (k_{on}) (ranging from 28,000 to 41,100 M^{-1}s^{-1}) and dissociation rate constants (k_{off}) (ranging from 1.6 x 10^{-3} to 3.16 x 10^{-3} s^{-1}) for all dimeric ICAM-1 proteins (domain 1, C-terminal, and domain 1/C-terminal dimers) were quite similar (Table 2). Accordingly, the equilibrium constants (K_D) ranged from 56-109 nM for the ICAM-1 dimers, ~1.5-3-fold lower than that of sICAM-1 (169 nM) (Table 2). Thus, there was no impairment whatsoever compared to monomer in binding of any of the dimeric topomers to the αL I domain. The slower k_{on} and k_{off} of the dimers compared to the monomers is a reflection of their larger size; such effects have been well
As a second measure of domain 1, C-terminal, and domain 1/C-terminal dimer function we used them to inhibit homotypic aggregation by SKW3 cells (data not shown). SKW3 cells were induced to form $\alpha_L\beta_2$ and ICAM-3 dependent homotypic cell aggregates (15) by activation of $\alpha_L\beta_2$ with the mAb CBR-LFA1/2. Aggregation performed in the presence of increasing amounts of sICAM-1 revealed effective inhibition of aggregation with an IC$_{50}$ of ~1600 nM. Experiments with ICAM-1 dimers revealed that domain 1, C-terminal, and domain 1/C-terminal (ring-like conformers; fraction #15) dimers all inhibited aggregation with similar IC$_{50}$s of ~800 nM. Thus, in these assays, as with the BIAcore studies, the ICAM-1 dimers bound to $\alpha_L\beta_2$ as effectively as monomeric ICAM-1.
DISCUSSION

To function effectively, ICAM-1 must be displayed with an appropriate orientation, valency, and distribution on the cell surface so it can interact with $\alpha L\beta 2$ on the surface of an opposing cell. Furthermore, since $\alpha L\beta 2$ and ICAM-1 are often coexpressed on leukocytes, there must be mechanisms that favor interactions between molecules on opposite cells over interactions between molecules on the same cell.

Crosslinking of ICAM-1 on the cell surface has shown a predominance of dimers over monomers, and also the existence of a substantial proportion of higher order oligomers of $> 200$ kDa (10,11). The mAb CA7 specific for domain 5 of ICAM-1 was found to bind poorly to native cell surface ICAM-1 or ICAM-1 with all but two residues of the cytoplasmic domain deleted, but to bind well to ICAM-1 with a glycosylphosphatidyl inositol anchor substituted for its native transmembrane domain. Furthermore, CA7 bound well to soluble monomeric ICAM-1 but not dimeric ICAM-1 (11). These findings suggested that ICAM-1 dimerized through its transmembrane domain, in agreement with the presence of a glycine patch in helical wheel displays that favors dimerization to avoid exposure of polar carbonyl and amide backbone groups in the membrane (Fig. 1) (10,11).

Shielding of an epitope in domain 5 is consistent with its proximity to a dimerization interface in the transmembrane domain, or with an additional role for domain 5 in dimerization. In a crystal structure of domains 1 and 2 of ICAM-1, a dimerization interface was revealed in domain 1 (4) that presented another possible way in which ICAM-1 could dimerize. Residues important in binding ICAM-1 to $\alpha L\beta 2$ are on the side of domain 1 opposite to this dimerization interface; whether dimerization in domain 1 would interfere with binding to ICAM-1 was one of the issues investigated in this study.

Monomeric soluble ICAM-1 is a bent rod (2,3); therefore, one way in which dimerization at both the C-terminus and in domain 1 could be accommodated would be by formation of a ring-like dimer (4,36).

In this study, we have for the first time investigated the architecture of ICAM-1 dimers in the electron microscope, and have obtained evidence for several different ICAM-1 topologies (Fig. 8) and their functional activity. In our initial experiments, we
examined ICAM-1 dimers that were covalently linked through a disulfide-bonded C-terminal α-helical coiled coil. We found that C-terminal dimers had the potential for domain 1-mediated dimerization leading to ring-like dimeric and “W”-shaped tetrameric topologies. “U”-shaped molecules were also observed, which may represent circles in which noncovalent dimerization through domain 1 was disrupted by contact with the mica (32,33).

Previous studies suggested that in solution, dimerization through domain 1 alone was too unstable to form significant amounts of observable dimers (11). We tested whether introduction of cysteine residues into appropriate positions in the domain 1 dimer interface visualized in the crystal study would lead to the formation of disulfide-linked dimers. Indeed, cysteine mutations at several sites in domain 1 allowed formation and isolation of disulfide-linked ICAM-1 dimers. Furthermore, disulfide bonds could be formed by substitution to cysteine of L42 or L43, but not L18 or L44. In the crystal-defined dimer interface, the Cβ-Cβ distances for the former but not the latter residues were within the range optimal for disulfide bond formation of 3.41-4.25 Å (24). The latter residues were solvent exposed, and could have formed disulfides with a small shift in the monomer-monomer interface. Therefore, the specific disulfide bond formation by L42 and L43 suggests that disulfide bond formation was a consequence of favorable noncovalent interactions at the crystal-defined interface. These data provide evidence for domain 1-mediated dimerization of ICAM-1 in solution in a manner that is consistent with that previously observed in crystal studies (4).

We tested whether dimerization in the domain 1 interface would result in an architecture that would be compatible with simultaneous dimerization C-terminal to domain 5. Indeed, domain 1/C-terminal dimers could readily form the predicted ring-like topology. On the cell surface, an equilibrium may exist between closed ring-like and open C-terminal dimers (Fig. 8A, B).

In addition to the ring-like domain 1/C-terminal dimers, we also observed significant amounts of “W”-shaped tetrameric molecules in which domain 1 and C-terminal dimerization occurs between different pairs of molecules (Fig. 8C). Maintenance
of the same domain 1-domain 1 and domain 5-domain 5 dimer interfaces in the ring-like dimer and in the "W"-shaped tetramer requires rotation at the domain 1-domain 2, domain 2-domain 3, domain 3-domain 4, or domain 4-domain 5 boundaries totaling approximately 180° (for simplicity shown as a rotation about the domain 4-domain 5 boundary, with hinge-like motion at both ends of domain 4 in Fig. 8). A bend has previously been visualized between domains 3 and 4 in ICAM-1 (2,3), and most of the rotation may occur at this junction. Rotational or hinge-like movements between domains are consistent with the variation in shapes of the dimeric and tetrameric molecules visualized in this study. Interdomain movement has been visualized at the interface between domains 1 and 2 of ICAM-1 (4), and similar movements may occur at other domain interfaces.

It has previously been observed that the domain 1 dimer interface is on the opposite side of domain 1 from the \( \alpha_L\beta_2 \)-binding face (4). Thus, it is predicted that in both the ring-like and the "W"-shaped structures, the \( \alpha_L\beta_2 \) binding surface in domain 1 would be oriented away from the cell surface, available for integrin engagement (Fig. 8). However, the compatibility of dimerization in domain 1 with ligand binding had not been previously tested. We have measured the affinity of different ICAM-1 topomers for the \( \alpha_L\beta_2 \) I domain. Our results show that dimerization in domain 1, C-terminal to domain 5, or at both sites did not impair binding to ICAM-1. Indeed, when corrected for the presence of 2-fold more binding sites, the affinities of the dimers for the I domain are within 1.5-fold of monomeric sICAM-1.

The topologies we have defined have important implications for the function of ICAM-1 in cell adhesion. Interactions through domain 1 in the ring-like dimer and "W"-shaped tetramer will provide constraints on orientation on the cell surface (Fig. 8). Additional constraints may be provided by the putative dimerization interface in domain 5 that masks the CA-7 epitope. Because of symmetry considerations, it is reasonable to conclude that the 2-fold rotational symmetry axis within the domain 1 dimer interface will be oriented perpendicular to the membrane, as shown in Fig. 8. This will present the binding site in domain 1 optimally for binding to \( \alpha_L\beta_2 \) on an opposing cell, either in the
ring-like dimer or in the tetramer configuration (Fig. 8). We predict that the binding site for αMβ2 in domain 3 of ICAM-1 (37) is on the same face of ICAM-1 and would also be well exposed in both dimers (T.A. Springer, C. Jun, and J. Wang, unpublished). However, the height above the membrane and orientation of these binding interfaces could differ among the ring-like dimer, open dimer, and “W”-tetramer, and thus interconversion among these topomers could have important consequences for regulating cell adhesion. For example, binding of αLβ2 to domain 1 and αMβ2 to domain 3 might be affected differently. Moreover, the W tetramers are available for further multimerization through domain 1, and thus long strings of ICAM-1 molecules could be built up that could have important implications for avidity regulation of cell adhesion.
**Figure Legends**

**Figure 1.** Schematic representation of C-terminally mutated ICAM-1 constructs and a putative transmembrane domain dimerization motif.  A. Wild-type ICAM-1, ICAM-1 truncated prior to the transmembrane domain (sICAM-1), and ICAM-1 with a GCN4 α-helical coiled coil dimerization motif and a cysteine to covalently link the coiled coils (sICAM-1_GCN4). IgSF domains 1 to 5 (D1-D5) are schematized as loops closed by intradomain disulfide (S-S) bonds. Lollipops represent N-linked glycosylation sites. TM: transmembrane domain; Cyto, cytoplasmic domain.  B. Helical wheel representation of the last portion of the putative α-helical ICAM-1 transmembrane domain.

**Figure 2.** Generation and characterization of C-terminally dimerized ICAM-1 (sICAM-1_GCN4).  A. 293T cells transfected with the indicated constructs were labeled with [35S]methionine and cysteine and secreted material was immunoprecipitated with R6.5 mAb. Samples were subjected to SDS 10% PAGE under non-reducing (-DTT) or reducing conditions (+DTT) and fluorography.  B. sICAM-1 (monomer) or sICAM-1_GCN4 (C-terminal dimer) were tested at 500 ng/ml for binding to immobilized R6.5 (a dimerization-independent ICAM-1 mAb) or CA7 (a monomer-specific mAb). Binding was determined by addition of biotin-conjugated CBRIC1/11 mAb followed by ELISA and measurement of absorbance at 414 nm. Values represent the mean ± s.e.m. for at least three separate experiments.

**Figure 3.** Purification and visualization of C-terminal dimers.  A. Representative electron micrographs of purified and rotary shadowed sICAM-1. Bar = 50 nm.  B. Purified sICAM-1_GCN4 C-terminal dimer was sedimented through a glycerol gradient and fractions were subjected to SDS 10% PAGE and staining. The positions of standards sedimented in a parallel gradient are shown above the gel; fraction numbers are shown below the gel.  C. Gradient-purified C-terminal dimers were subjected to rotary shadowing and electron microscopy. Representative extended dimers (panel I), “U”
shaped and ring-like dimers (panel II) or "W"-shaped tetramers (panel III) are depicted.

Figure 4. Generation of covalently dimerized sICAM-1 by formation of a disulfide bond in domain 1. Secreted material from $^{35}\text{S}$-labeled CHO.Lec 3.2.8.1 cells expressing the indicated wild-type or cysteine-substitution mutants of sICAM-1 were assessed for disulfide formation by immunoprecipitation with R6.5 mAb, SDS 10% PAGE under non-reducing conditions, and fluorography. Molecular masses x 10$^{-3}$ of standards are shown on the left and positions of dimer (D) and monomer (M) bands are shown on the right.

Figure 5. Characterization of domain 1 dimers. A. Gel filtration and SDS-PAGE. sICAM-1(L43C) expressed in CHO.Lec 3.2.8.1 cells was purified by CBRIC1/11 mAb affinity column chromatography and subjected to gel filtration on a 2.5 x 50 cm Superdex 200 column (left). Pooled fractions representing peaks 1, 2, and 3 were concentrated and visualized by SDS 10% PAGE under non-reducing conditions (right). Fractions from peak 2 were used for further studies including sedimentation (B), electron microscopy (C), and Biacore analysis (see Table 2). B. Sedimentation. Purified sICAM-1(L43C) domain 1 dimers were sedimented through a glycerol gradient and fractions were subjected to SDS 10% PAGE and Coomassie blue staining. The positions of standards sedimented in a parallel gradient are indicated above the gel; gradient fraction numbers are indicated below the gel. C. Electron microscopy. Gradient-purified domain 1 dimers were subjected to rotary shadowing and electron microscopy. Representative images are depicted. Bar = 50 nm.

Figure 6. Covalently stabilized domain 1/C-terminal dimers. The domain 1 cysteine mutation L43C was introduced into the sICAM-1_GCN4 C-terminal dimer and the resulting domain 1/C-terminal constructs were expressed in CHO.Lec 3.2.8.1 cells. A. $^{35}\text{S}$-labeled secreted material was immunoprecipitated with R6.5 mAb, subjected to either non-reducing (-DTT) or reducing (+DTT) SDS 8% PAGE and visualized by
fluorography. B. Affinity purified sICAM-1_GCN4(L43C) was bound to a mono-Q column and eluted with a linear gradient (0 to 1 M) of NaCl (left). Fractions 15-21 were subjected to SDS 8% PAGE and Coomassie blue staining, revealing major bands of 110, 180, and 200 kDa (right). Molecular weight standards are shown on the right.

Figure 7. Glycerol gradient sedimentation and visualization of domain 1/C-terminal dimers. Fractions #15, #16 and #21 from Fig.6 B were separately subjected to glycerol gradient sedimentation. Glycerol gradient fractions that were enriched in higher molecular weight species as shown by non-reducing SDS 10% PAGE (A,C,E) were subjected to rotary staining and electron microscopy (B, D, F). Representative images are shown for each fraction. A and B, fraction #15; C and D, fraction #16; E and F, fraction #21. Bar = 50 nm. B. Fraction #15 contained ring-like, oval-shaped dimers (all three rows). D. Fraction #16 contained ring-like dimers (row 1), "U"-shaped dimers (row 2) and extended dimers (row 3). E. Fraction #21 contained "W"-shaped and extended tetramers (row 1), ring-like dimers (row 2) and extended and "U"-shaped dimers (row 3).

Figure 8. Model of ICAM-1 topomers on the cell surface. ICAM-1 is drawn as five linked ovals representing domains 1-5 expressed on the cell surface (two planes representing the membrane bilayer). The \( \alpha_L \beta_2 \) binding site has previously been localized to the face of domain 1 opposite the dimerization interface (4), whereas the \( \alpha_M \beta_2 \) binding site is located in domain 3 (37), and is predicted to lie on the same side of the ICAM-1 molecule as the \( \alpha_L \beta_2 \) binding site (T. A. Springer, C. Jun, and J. Wang, unpublished). To maintain the same dimerization interfaces in domains 1 and 5 upon conversion from the ring-like open dimer (A) to the "W"-shaped tetramer (C), a rotation of approximately 180° and some hinge-like motions must occur in one or more of the interdomain linkages located between domains 1 and 5. In the figure, this is symbolized by rotation at the domain 4-domain 5 linkage (circular arrows) and a hinge-like motion at both ends of domain 4.
Table 1. Cβ-Cβ distances and exposure of symmetry-related residues in the domain 1 dimer interface seen in a crystal structure of domains 1 and 2 of ICAM-1

<table>
<thead>
<tr>
<th>Residue</th>
<th>Dimer Cβ-Cβ distance (Å)</th>
<th>Surface Exposure (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A dimer</td>
<td>B dimer</td>
</tr>
<tr>
<td>Leu-18</td>
<td>4.50</td>
<td>6.03</td>
</tr>
<tr>
<td>Leu-42</td>
<td>3.41</td>
<td>4.40</td>
</tr>
<tr>
<td>Leu-43</td>
<td>3.61</td>
<td>2.48</td>
</tr>
<tr>
<td>Leu-44</td>
<td>10.8</td>
<td>4.40</td>
</tr>
</tbody>
</table>

The putative dimerization interface in domain 1 was deduced from symmetry-related contacts seen between pairs of A molecules (A dimer) and B molecules (B dimer) in a crystal structure of ICAM-1 domains 1 and 2 (4). The distance separating the Cβ atoms of identical residues in the interface was measured. Surface accessibility of each of these residues in the absence of the interface, i.e. in monomer molecules, was measured as exposed surface area (Å²) using the program DSSP (38).
Table 2. BIAcore measurements of the kinetics of dimeric and monomeric ICAM-1 binding to $\alpha_L\beta_2$ I domain.

<table>
<thead>
<tr>
<th>ICAM-1 topology</th>
<th>(k_{on} \text{ (M}^{-1}\text{s}^{-1} \times 10^{-4}))</th>
<th>(k_{off} \text{ (s}^{-1} \times 10^{3})</th>
<th>(K_D \text{ (nM)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-terminal dimer</td>
<td>4.11±0.05</td>
<td>2.40±0.06</td>
<td>58.4±1.6</td>
</tr>
<tr>
<td>domain 1/C-terminal dimer</td>
<td>2.94±0.31</td>
<td>3.16±0.12</td>
<td>109.1±15.6</td>
</tr>
<tr>
<td>domain 1 dimer</td>
<td>2.80±0.09</td>
<td>1.60±0.04</td>
<td>56.1±2.1</td>
</tr>
<tr>
<td>sICAM-1 (monomer)</td>
<td>13.30±0.60</td>
<td>22.60±1.60</td>
<td>168.7±5.5</td>
</tr>
</tbody>
</table>

The purified mutant open, high affinity $\alpha_L\beta_2$ I domain was immobilized on a BIAcore sensor chip surface and binding of ICAM-1 preparations was measured under a constant flow of 10-60 $\mu$L/min in TBS containing 1 mM MgCl$_2$. The $K_D$ values are expressed in moles of dimer or monomer; for expression in terms of binding sites the $K_D$ values of the dimers should be multiplied by 2. Curve fitting of the association and dissociation phases with BIAevaluation 3.1 software was used to calculate $k_{on}$, $k_{off}$ and $K_D$ values. All values are expressed as mean ± s.e.m. for three separate experiments, with the exception of the domain 1/C-terminal dimer for which only enough material for two experiments was generated.
REFERENCES


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A.

**ICAM-1**

N

\[ \begin{array}{cccccc}
\text{S-S} & \text{S-S} & \text{S-S} & \text{S-S} & \text{S-S} & \text{TM} \\
\text{D1} & \text{D2} & \text{D3} & \text{D4} & \text{D5} & \text{Cyto} \\
\end{array} \]

\[ \text{LFA-1} \rightarrow \text{Mac-1} \]

**sICAM-1**

N

\[ \begin{array}{cccccc}
\text{S-S} & \text{S-S} & \text{S-S} & \text{S-S} & \text{S-S} & \text{C} \\
\text{D1} & \text{D2} & \text{D3} & \text{D4} & \text{D5} & \text{453} \\
\end{array} \]

**sICAM-1\_GCN4**

N

\[ \begin{array}{cccccc}
\text{S-S} & \text{S-S} & \text{S-S} & \text{S-S} & \text{S-S} & \text{GCN4} \\
\text{D1} & \text{D2} & \text{D3} & \text{D4} & \text{D5} & \text{Cys} \\
\end{array} \]

B.

ICAM-1 Transmembrane dimerization motif:

\[ I(465)MG\text{TAGLS(472)} \]

\[ \begin{array}{cccccc}
\text{T468} & \text{L471} & \text{S472} & \text{I465} & \text{A469} & \text{M466} \\
\text{I465} & \text{G467}^* & \text{G470}^* & \text{G467} & \text{M466} & \text{A469} \\
\text{S472} & \text{I465} & \text{T468} & \text{L471} & \text{M466} & \text{A469} \\
\end{array} \]

Fig. 1. Jun et al.
A. Mock sICAM-1 sICAM-1_GCN4

IP: Anti-ICAM-1 mAb (R6.5)

- DTT + DTT

B. Absorbance at 414 nm

- sICAM-1 (monomer)
- sICAM-1_GCN4 (C-term. dimer)

R6.5 CA-7

ig. 2. Jun et al.
A.

B. 11.3 S  4.6S  3.5S

C.

C-term. dimer

Fraction #

ig. 3. Jun et al.
sICAM-1 +:

Mock
WT
L18C
L42C
L43C
L44C

D
M

ig. 4. Jun et al.
Fig. 5. Jun et al.
ig. 6. Jun et al.
ig. 7. Jun et al.
Fig. 8. Jun et al.
Ultrastructure and function of dimeric, soluble intercellular adhesion molecule-1 (ICAM-1)
Chang-Duk Jun, Christopher V. Carman, Sambra D. Redick, Motomu Shimaoka, Harold P. Erickson and Timothy A. Springer

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