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Human-Murine Chimeras of ICAM-1 Identify Amino Acid Residues Critical for Rhinovirus and Antibody Binding

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Human ICAM-1 is the cellular receptor for the major group of human rhinoviruses (HRVs). Previous studies have suggested that the N-terminal domain of ICAM-1 is critical for binding of the major group rhinoviruses. To further define the residues within domain 1 that are involved in virus binding, we constructed an extensive series of ICAM-1 cDNAs containing single and multiple amino acid residue substitutions. In each case, substitutions involved replacement of the human amino acids with those found in murine ICAM-1 to minimize conformational effects. To facilitate the mutagenesis process, a synthetic gene encompassing the first two domains of ICAM-1 was constructed which incorporated 27 additional restriction sites to allow mutagenesis by oligonucleotide replacement. Each of the new constructs was placed into a Rous sarcoma virus vector and expressed in primary chicken embryo fibroblast cells. Binding assays were performed with six major group HRVs, including one high-affinity binding mutant of HRV-14, and two monoclonal antibodies. Results indicated that different serotypes displayed a range of sensitivities to various amino acid substitutions. Amino acid residues of ICAM-1 showing the greatest effect on virus and antibody binding included Pro-28, Lys-29, Leu-30, Leu-37, Lys-40, Ser-67, and Pro-70.

Human rhinoviruses (HRVs) represent a subgroup of picornaviruses and are the major causative agents of the common cold in humans (12). It has been shown in earlier studies that HRVs initiate infection by binding to specific receptors on susceptible cells (5). Ninety-one of the 102 serotypes tested interact with a single cellular receptor and are designated the major group, while 10 of the remaining 11 serotypes competed for a second receptor and compose the minor group (1, 6, 30). One serotype, HRV-87, appears to utilize a receptor distinct from the major and minor group receptors (30).

Previous studies have described the isolation of the major group receptor from HeLa cells by immunoaffinity chromatography (27), and biochemical characterization of the purified receptor protein determined that it was a 90-kDa acidic glycoprotein (29). The identification of the major group receptor as intercellular adhesion molecule-1 (ICAM-1) was first made by Greve et al. (11) and subsequently by others (26, 28). ICAM-1 is a cell surface ligand for the lymphocyte function-associated antigen-1 (LFA-1) adhesion receptor (18) and is a member of the immunoglobulin supergene family (8, 25). The interaction of ICAM-1 and LFA-1 plays an important role in leukocyte adhesion and in the execution of immunological and inflammatory functions mediated by leukocyte adhesion (8).

The prediction that ICAM-1 is structurally related to members of the immunoglobulin supergene family enabled structural alignment of the ICAM-1 molecule with domains of known immunoglobulin structures. As a result of such alignments, ICAM-1 is predicted to have five homologous domains (23, 25) and is structurally related to the CD4 receptor used by the human immunodeficiency virus (HIV) (16) and to the immunoglobulinlike protein identified as the cellular receptor for poliovirus (19). The CD4 receptor is postulated to have four domains, while the poliovirus receptor has three homologous immunoglobulinlike domains (14, 19). Several laboratories have mapped the cellular binding site for HIV gp120 to an external loop on the CD4 receptor that resides within the first 53 amino acids of the N-terminal domain (2, 4, 14, 16, 20, 22, 31). Recent crystallographic studies have visualized the HIV binding site to the C″ loop of CD4 domain 1 (22, 31).

Employing an in vitro transcription-translation system to generate fragments of the ICAM-1 receptor, the binding sites of monoclonal antibodies (MAbs) that block virus binding were previously mapped to the N-terminal 82 amino acid residues of ICAM-1, although no virion binding could be demonstrated with these truncated molecules (17). Using stable cell lines, Staunton et al. (24) expressed several human-murine ICAM-1 chimeras and showed that HRV binding requires the first 168 amino acids (domains 1 and 2) of human ICAM-1 and that the primary binding site is localized in domain 1. In addition, mutagenesis studies indicated that Gln-58 plays a major role in HRV-14 binding, while Glu-34 appears to be involved in LFA-1 binding (24). Because the previous study encountered conformational effects and involved mostly multiple rather than single-residue changes, the present study was performed to further elucidate individual amino acid residues involved in the binding of several HRV serotypes and blocking MAbs.

MATERIALS AND METHODS

Construction of synthetic ICAM-1. The Intelligenetics program Site was utilized to identify 27 potential restriction endonuclease sites that could be encoded within domains 1 and 2 of human ICAM-1 that were not found in the cloning vector pGEM-7Zf (9). Two additional sites, *Apa*I and *Nsi*I, were added to the 5′ and 3′ ends of the synthetic gene as

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cloning sites for the pGEM-7Zf vector. Five positive-strand oligonucleotides (representing nucleotides 1 to 20, 1 to 118, 198 to 325, 406 to 532, and 603 to 723) and four negative-strand oligonucleotides (representing nucleotides 98 to 220, 306 to 426, 511 to 626, and 704 to 723) were synthesized and gel purified.

Polymerase chain reaction (PCR) amplification was performed in a Perkin-Elmer DNA Thermal Cycler with 0.1 μM each oligonucleotides except the oligonucleotide representing nucleotides 1 to 20. The reaction mixture was passed through a Worthington mini-Spin column, ethanol precipitated, dried, and then resuspended in H_2O. A second PCR was performed with one-half of the above reaction mixture plus 5 μM each oligonucleotide encoding nucleotides 1 to 20 and 704 to 723, processed as above, and digested with *Apa*I and *Nsi*I.

The 704-bp fragment resulting from the second PCR was loaded on a 1% agarose gel, excised, and purified with Geneclean (Bio 101). The purified fragment was ligated into the *Apa*I-*Nsi*I cloning sites of pGEM-7Zf and transfected into competent *Escherichia coli* DH5α cells. Sequence analysis of positive colonies confirmed the presence of the synthetic cDNA construct, designated pD1D2.

Construction of pRI20. The region encoding ICAM-1 from domain 3 to the C-terminal end was amplified by PCR with two oligonucleotides (5′ CCCCCACAACTAGTCAGCC and 5′ CAGTATCGATAGCTTTATTAACTAAC) and native ICAM-1 cDNA (pSVL-HRVr1) (28) as the template. The PCR oligonucleotides generated an *Spe*I site at amino acid 197 (the same as in pD1D2) and a *Cla*I site in the 3′ untranslated sequence. The resulting PCR fragment and pD1D2 were digested with *Cla*I-*Spe*I, and the products were gel purified. These fragments were then ligated together with the *Cla*I-CiAP-digested Rous sarcoma virus (RSV) expression vector pNPRAV/ClaI (9) and transformed into *E. coli* to generate pRI12. To delete the 3′ *Cla*I site, pRI12 was partially digested with *Cla*I and filled in with Klenow fragment. The linear 17-kb fragment was gel purified, self-ligated, and transformed into competent *E. coli* DH5 cells to create pRI20. Minipreparations were screened with *Cla*I-*Spe*I for 16.3- and 0.7-kb fragments. This construct was preparatively digested with *Cla*I-*Spe*I. The 16.3-kb vector was gel purified and used for cloning and expression of all mutants.

Construction of mutants. pD1D2 was double digested with selected restriction enzymes and gel purified with Geneclean. Complementary oligonucleotides encoding murine ICAM sequences were desalted with a Worthington Spin column, gel purified on an 8% denaturing gel, excised, crushed, and shaken overnight at 25°C in 0.3 M Na acetate (pH 5.2). The eluted oligonucleotides were passed over a Pharmacia NAP 5 column, ethanol precipitated, dried, and resuspended in double-distilled H_2O. Complementary oligonucleotides were annealed at a final concentration of 10 μg/ml in 50 mM Tris (pH 7.5)–10 mM $MgCl_2$ for 2 min at 95°C and then incubated for 30 min at 37°C. Annealed oligonucleotides were then ligated into pD1D2 at a 10:1 molar excess and transformed into competent *E. coli* DH5α. All mutants were confirmed by DNA sequencing. Following digestion with *Cla*I and *Spe*I, the 690-bp insert was gel purified and ligated into the RSV expression vector, pRI20, cleaved at the same sites. The resulting plasmid was transformed into *E. coli*, and positive clones were resequenced in the pRI20 vector.

RSV expression vector and CEF cells. Chimeras inserted into pRI20 were transfected by the Ca_2PO_4 procedure into pathogen-free chicken embryo fibroblast (CEF) cells (SPA FAS, Inc., Norwich, Conn.) and cultured in GIBCO F10-minimal essential medium supplemented with 7.2% fetal calf serum and 1.6% chicken serum (15). Confluent monolayers in 75-cm flasks were split 3 days after transfection and assayed for virus and MAb binding 7 to 9 days after transfection as described below.

Virus, antibodies, and reagents. The acquisition, propagation, and radiolabeling of HRV serotypes with [^{35}S]methionine or [^{3}H]uridine have been described previously (1). Serotype HRV-14-G155, containing a single amino acid substitution of Gly for Pro at residue 155 in VP1, has been detailed previously (7). The methods involved in the isolation, purification, and ^{125}I labeling of MAbs 1A6 and 2C2 are described elsewhere (17).

T4 DNA ligase, Klenow fragment, and all restriction endonucleases were purchased from New England BioLabs except *Sst*I and *Sst*II (Bethesda Research Laboratories), *Esp*I (Anglian), *Eco*81I (U.S. Biochemicals), and *Spl*I (Amersham). DH5α competent cells were purchased from Bethesda Research Laboratories. All mutant oligonucleotides were made by Midland Certified Reagent Company. The vector pGEM-7Zf(+) was purchased from Promega.

Binding assays. Medium was removed from transfected monolayers in 48-well cluster plates and replaced with 0.1 ml of F10-minimal essential medium containing 5 mM $MgCl_2$. Control wells were preincubated for 30 min at 25°C with 10 μl of a 1/10 dilution of rabbit anti-ICAM-1 polyclonal antiserum (29) to standardize expression levels or 10 μg of either MAb 1A6 or 2C2 to measure specific virus binding. Following incubation, the polyclonal antiserum was removed, cells washed twice with medium, and 0.1 ml of fresh medium was added. The unlabeled MAbs were not removed from control wells. Radiolabeled HRVs or MAbs (10 μl) were added to both MAb-treated and untreated wells containing 0.1 ml of medium, and 10 μl of ^{125}I-labeled protein A was added to the polyclonal antibody-treated cells. Following incubation at 34°C for 90 min, medium was removed to counting vials, monolayers were washed with 0.2 ml of phosphate-buffered saline (PBS), and the PBS wash was combined with the removed medium to represent unbound sample. Monolayers were then incubated with 0.2 ml of 0.1% sodium dodecyl sulfate for 10 min and transferred to a separate counting vial and combined with a similar 0.2-ml PBS wash to generate the bound sample. All assays (n = 1 to 3) were performed in duplicate, and the specific binding of each individual virus and antibody sample was determined by subtracting the binding obtained in the presence of blocking MAb (usually 1 to 2%). Average control binding values (n = 15) were 34% (HRV-3), 23% (HRV-14), 32% (HRV-14-G155), 42% (HRV-15), 50% (HRV-36), 47% (HRV-41), 23% (polyclonal antiserum), 24% (MAb-1A6), and 17% (MAb-2C2).

Molecular modeling. Molecular graphics and visualization were accomplished by using QUANTA version 3.0 (Polygen Corp., Waltham, Mass.) operating on a Silicon Graphics IRIS 40/70GT. A three-dimensional model of ICAM-1 domain 1 was prepared by refinement of model coordinates previously described (10). Energy minimization using the CHARMM (3) force field allowed for the formation of two putative disulfide bonds between Cys pairs at positions 21 with 65 and 25 with 69. The phi and psi angles of residue 78 were altered to align the C-terminal segment of domain 1 with corresponding positions in crystal structures of immunoglobulin domains.

CHARMM molecular dynamics simulation studies were used to investigate the conformational preferences of the

ICAM-1 domain 1 loops. These calculations were performed on isolated domain 1 in vacuo with a distance-dependent dielectric constant to allow for undamped movement of residue positions while mimicking the effects of solvent. A 1-fs time step was used for integration, and coordinates were saved for analysis every 0.5 ps. The molecular dynamics protocol used for the simulation involved a 10-ps segment of slow heating from 0 to 300 K, 50 ps of scaled-velocity equilibration at 300 K, and a 100-ps segment of production dynamics at 300 ± 15 K.

RESULTS

Previous studies have implicated the N-terminal domain of human ICAM-1 as the primary receptor site of major group HRVs (17, 24). The murine homolog of ICAM-1 shares 50% homology at the amino acid level but is incapable of binding the major group of HRVs (13, 30). To identify the critical amino acid residues in human ICAM-1 involved in virus binding, we utilized a strategy of systematically replacing amino acid residues in the human gene with those found in the murine species. A similar strategy was previously used to map critical residues involved in HIV binding to CD4 (4). The human and murine molecules are likely to have similar structural characteristics since the murine homolog binds human LFA-1 (24). The proposed amino acid changes should therefore minimize structural alterations in the expressed protein and allow the identification of those amino acids directly involved in virus and MAb binding.

To facilitate the generation of mutants, we replaced the first two domains of the native human ICAM-1 cDNA with a synthetic gene encoding the same amino acid sequence, but incorporating 27 unique restriction sites (Fig. 1), using a series of oligonucleotides and the PCR methodology described in Materials and Methods. This approach enables mutants to then be generated by double-stranded oligonucleotide exchange. To bypass the need to generate stable cell lines for each of the chimeras constructed, we used an RSV vector in which the portion of the RSV genome encoding the *scr* gene was replaced with the synthetic ICAM-1 cDNA (9) to transfect ICAM-1 negative CEF cells. The advantage of this expression system is its ability to quickly integrate the target sequence into all cells of the monolayer by establishing a persistent viral infection following transfection of the viral expression plasmid. The net result, as measured by immunofluorescence, was high-level expression of the inserted gene by virtually all cells within 5 days (data not shown). DNA transfections of RSV plasmids encoding the synthetic and native ICAM-1 cDNAs were compared, and the results (Table 1) show that both cDNAs are expressed equally well and at a higher level than normally found on HeLa R-19 cells.

Our initial strategy was to systematically replace short segments in domain 1 of the human ICAM-1 molecule with the corresponding murine segment. To monitor the effect on virus binding and better control for gross conformational effects, we performed binding assays with six major group viruses (HRV-3, HRV-14, HRV-14-G155, HRV-15, HRV-36, and HRV-41) and two MAbs (MAb-1A6 and MAb-2C2) that bind to distinct epitopes and specifically block virus attachment (17). One of the viruses utilized, HRV-14-G155, is a mutant of HRV-14 and contains a single amino acid change in VP1 (Pro-155 to Gly), previously shown to significantly enhance virus binding (7). To ensure that the inherent expression level of each of the constructs was not a variable when decreased binding was observed, we also used rabbit polyclonal antiserum prepared against human ICAM-1 (29) in each series of binding studies to determine relative expression levels.

In these studies, changes that reduced or increased binding by a factor of 2 were considered significant and beyond the normal variation encountered in such assays, while reduction to <40% of control levels was considered a major phenotype change. Results from the initial round (Table 2) of multiple-exchange chimeras indicated that only the V8 (exchanging 5 of the 9 amino acid residues at positions 6 to 14) and V6 (exchanging 5 of the 10 amino acid residues at positions 76 to 85) chimeras failed to affect virus and antibody binding significantly. Surprisingly, amino acid changes in the other seven chimeras had differential effects on the various antigenically distinct serotypes used, despite the fact that all the serotypes tested bind and compete for the same receptor (6). HRV-14, in particular, appeared to show the greatest sensitivity to amino acid substitutions, suggesting a more complex binding site than other HRV serotypes. To further define the amino acid residues that define the binding sites of each of the viruses and MAbs tested, we pursued single-amino-acid substitutions for each segment yielding altered binding results.

A summary of the binding results is presented in Table 2. The vast majority of amino acid changes failed to affect virus or MAb binding, and all the chimeras containing single-amino-acid changes were expressed at levels within 20% of the control transfections as measured by the binding of polyclonal antiserum. In most cases, decreased binding of a particular serotype or antibody found in the multiple-exchange chimeras could be accounted for by individual changes in subsequent single-amino-acid chimeras. As with the multiple-exchange chimeras noted above, different viruses displayed unique sensitivities to individual amino acid changes. The binding profiles of chimeras containing the 10 individual amino acid changes that appear to play a major role in virus or MAb binding are summarized in Table 3.

HRV-3, HRV-14, and the HRV-14-G155 mutant displayed similar profiles in that substitution at Leu-30, Ser-67, and Pro-70 virtually eliminated virus binding, while changes at amino acids Lys-40 and Tyr-66 reduced binding. Interestingly, replacement of Pro-28 with Asn enhanced the binding of HRV-3 and HRV-14-G155 while reducing the binding of wild-type HRV-14. This result implies that the Pro-155 site of VP1 (HRV-14) interacts with the Pro-28 region of ICAM-1 and that any structural alteration resulting from the Pro-to-Gly substitution in the HRV-14-G155 mutant closely mimicks that normally found in HRV-3.

HRV-15 appears to have a sensitivity profile that is unique among the serotypes assayed. HRV-15 binding was eliminated following substitution at amino acid residues Pro-28, Lys-29, and Ser-67. In addition, a change at Pro-70 drastically reduced binding. None of the other changes significantly affected HRV-15 binding. Serotypes HRV-36 and HRV-41 were most resistant to changes in ICAM-1. In fact, only the substitutions of an Asp for Pro at residue 28 and a Glu for Ser at residue 67 significantly decreased binding (Tables 2 and 3).

To visualize the relative vicinity of key amino acid residues involved in HRV binding to human ICAM-1, we prepared a three-dimensional model as described in Materials and Methods. Based on limited sequence homology with C2-like domains, domain 1 is predicted to exist as a seven-stranded, anti-parallel beta-barrel as shown in Fig. 2A. The locations of the key amino acid residues affecting binding of major group HRVs or blocking MAbs are indicated. This

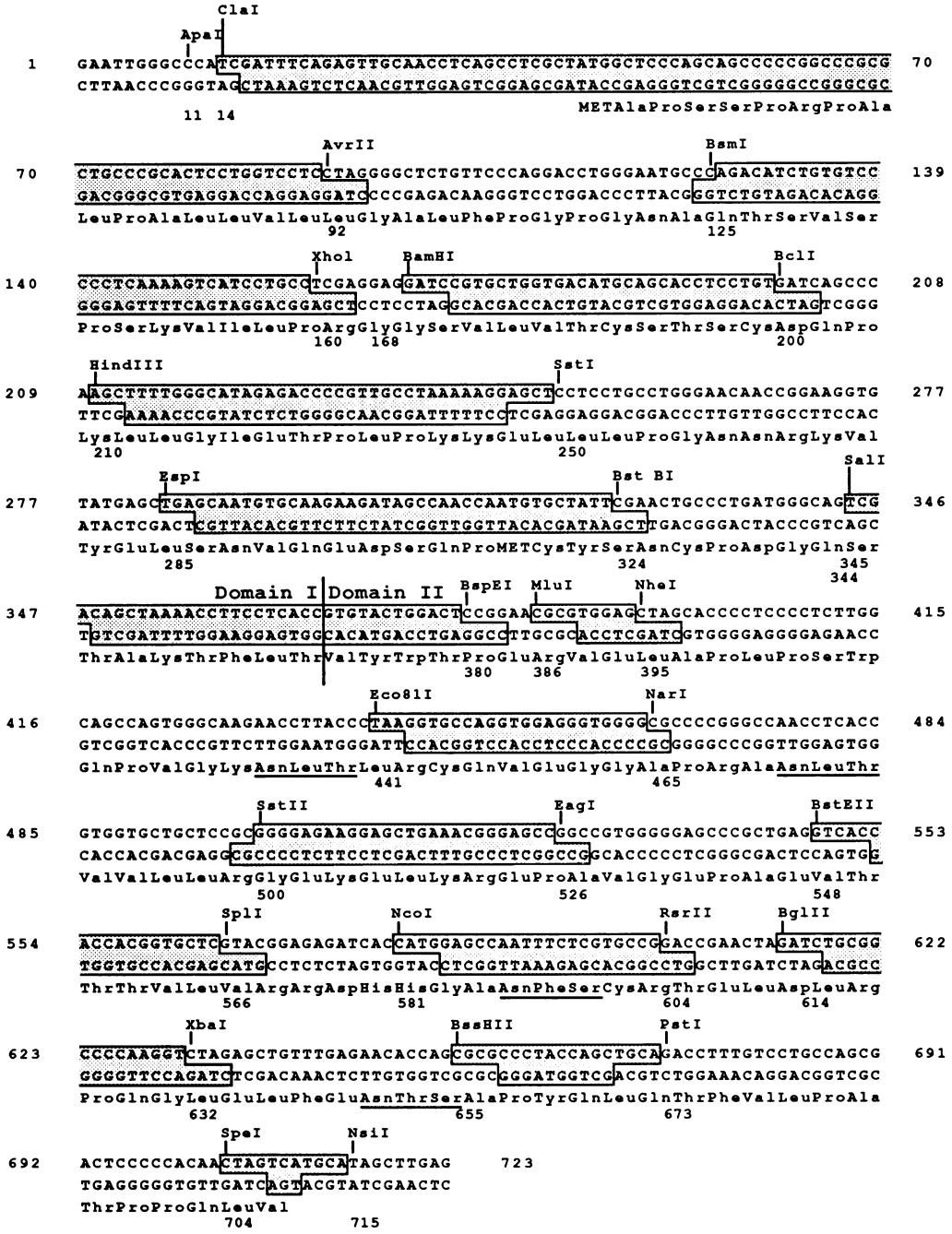

FIG. 1. DNA sequence of ICAM-1 synthetic cDNA. The double-stranded DNA and amino acid sequence it encodes are indicated. Numbers on either side represent nucleotide positions, while internal numbers indicate amino acid residues. Locations of each restriction endonuclease cleavage site are shown, and the alternating shaded and open sequences indicate restriction fragments replaced by double-stranded oligonucleotides. The *Apa*I and *Nsi*I sites were used for cloning into pGEM-7Zf(+), and the *Cla*I and *Spe*I sites were used to shuttle the mutants from pGEM-7Zf(+) to the RSV vector pRI20. The putative boundaries of domains 1 and 2 are separated by a vertical line, and glycosylation sites in domain 2 are underlined.

type of visualization localizes two distinct regions. The first, at the N-terminal end or left part of the molecule, is crucial for binding all the HRVs tested and MAb 2C2. The second important region is located at the top portion of domain 1 and contains the binding site for MAb 1A6, which is as effective as MAb 2C2 in blocking HRV attachment. Figure 2B represents an attempt to dock the N-terminal portion of domain 1 into the HRV-14 canyon (21) so as to maximize the interactions between key amino acid residues in ICAM-1 and those found at the floor of the canyon that were previously shown to affect virus binding (7). While such models are highly speculative, Fig. 2B does indicate that interactions between the highlighted amino acids are possible and that only the N-terminal portion of domain 1 is able to fit into the canyon itself.

TABLE 1. HRV-36 binding to cell monolayers

Cell	Transfection	MAb 1A6	% Binding
HeLa	None	−	26
HeLa	None	+	2
CEF	None	−	2
CEF	Native ICAM	−	40
CEF	Native ICAM	+	3
CEF	Synthetic ICAM	−	43
CEF	Synthetic ICAM	+	2

DISCUSSION

Similar to other animal viruses, HRVs initiate infection by interacting with specific cellular receptors present on susceptible cells. The major group of HRVs requires the presence of ICAM-1, an immunoglobulin G-like receptor related in structure to two other viral receptors; the CD4 receptor of HIV and the receptor for poliovirus. Recent crystallographic studies of the CD4 receptor have solved the atomic structure of the first two domains of the CD4 receptor and allowed the structural localization of amino acid residues known to be involved in HIV gp120 interaction (22, 31). These studies have confirmed several molecular and biochemical studies that amino acids between positions 41 and 59 play a major role in HIV gp120 binding to CD4 receptors (2, 4, 14, 16, 20, 22, 31). More specifically, crystallographic studies have localized the HIV-binding site to the vicinity of the C″ strand, encompassing amino acid residues 43 to 46 (22, 31).

Initial mapping studies on ICAM-1 have also localized the N-terminal domain as the domain involved in HRV binding. These studies include the construction of human-murine chimeras to determine the domains that are required for HRV binding (24), mutagenesis studies to identify residues involved in virus and LFA-1 binding (24), and in vitro mapping studies to localize the binding epitope of MAbs capable of blocking major group HRV attachment (17). The previous chimera studies indicated that HRV binding requires the first 168 amino acids (domain 1 and all but 17 residues of domain 2) of the human sequence (24).

A series of ICAM-1 mutants has been previously constructed with both multiple- and single-amino-acid changes (24). Of the single-amino-acid substitutions, 12 were at sites conserved between the human and murine ICAM-1 molecules and only the substitution of Glu-34 to Ala eliminated LFA binding while having a minimal effect on HRV-14 binding. Nine of the single-site mutants changed amino acids at the same sites as those described in Table 2 of this study. Analysis of the data from both studies indicates that changes at seven of these sites, Ser-5, Gln-27, Lys-40, Tyr-52, Met-64, Asp-71, and Thr-75, had no significant effect on HRV-14 binding in either study.

Mutations at the remaining two sites yielded conflicting data. Replacement of Gln-58 with His virtually eliminated HRV-14 binding in the earlier study (24), while the presence of a Gly at this position only reduced binding to 83% of control levels in the present study (Table 2). Likewise, substitution of Tyr-66 with Thr failed to affect HRV-14 binding in the former study, while a change to Phe reduced HRV-14 binding to 28% of control levels in the present study. The reasons for these differences are unclear at present and may reflect the amino acid substitutions made. For Gln-58 to His, the difference could be due to the ability of the His imidazole ring to be protonated, thus changing the electrostatic nature of the domain and compromising its ability to bind. In the second mutation, the Tyr-66-to-Phe mutation removed the hydroxyl group found in wild-type ICAM-1 while being maintained in the Thr mutant, possibly explaining the decrease in binding of the former and not the latter mutant.

In the present set of experiments, we took advantage of the finding that murine cells, which express an ICAM-1 homolog, fail to bind major group HRVs. The fact that these receptor homologs are nearly identical in size and play a similar functional role suggests that they both have similar structural features. This strategy appears to have worked since none of the single-site chimeras displayed gross conformational abnormalities that simultaneously eliminated the binding of all of the HRVs and MAbs tested. With the exception of chimera V16, even the multiple-residue replacements were well tolerated as indicated by the binding of most viruses and MAbs (Table 2).

The results showed that the six HRVs utilized could be divided into three distinct groups based on their sensitivity to single-amino-acid substitutions. The first contains HRV-3,

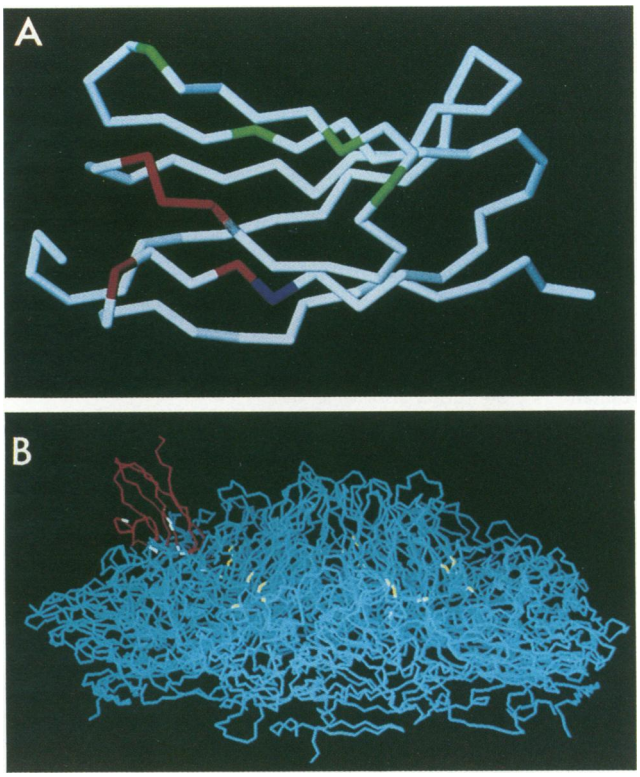

FIG. 2. Three-dimensional models for domain 1 of ICAM-1 and domain 1 docked in HRV-14. (A) C-alpha trace of domain 1 and the seven-stranded beta-barrel motif. In this view, the N-terminal segment is found at the left, while the C-terminal end, and hence domains 2 to 5, extends outward to the right. Residues shown to be important for virus (red), MAb 1A6 (green), and MAb 2C2 (red and blue) binding (see Table 3) are highlighted. (B) Same C-alpha trace docked into the canyon of HRV-14. Domain 1 of ICAM-1 is shown in red, with residues important for virus binding highlighted in white. The HRV-14 pentamer (21) is drawn in blue, with amino acid residues previously shown to affect HRV binding (7) highlighted in yellow.

TABLE 2. Binding data for human-mouse chimeras of ICAM-1

Chimera	Amino acids[a]	% of control binding								
		Rhinovirus						Antibody		
		3	14	14G	15	36	41	Polyclonal	1A6	2C2
Human	1 QTSVS P 6									
V7	*V*IH *	103	42	88	109	104	106	101	90	102
V50	*V*****	106	45	97	93	93	85	72	106	109
Human	6 PSKVILPRG 14									
V8	*REAF**Q*	80	80	78	100	104	114	123	100	106
Human	15 GSVLVTCST 23									
V9	***Q*N**S	72	30	59	69	90	82	110	25	74
V48	***Q*****	121	105	97	111	110	105	87	90	117
V49	*****N***	96	66	78	103	123	109	95	55	102
V23	********S	111	107	112	111	106	102	96	112	87
Human	24 SCDQPKL 30									
V16	**KEDLS	58	3	32	2	0	16	84	64	77
V18	**K****	91	97	104	144	119	119	120	106	110
V19	***E***	137	135	90	50	97	94	103	95	90
V20	****D**	167	55	143	0	32	35	103	93	47
V21	*****L*	96	82	64	2	107	84	116	118	57
V22	******S	6	0	6	135	109	116	128	118	44
Human	31 LGIETPLPKK 40									
V12	**L**QWL*D	126	134	101	93	103	93	89	2	96
V24	**L*******	113	128	128	99	92	112	100	102	103
V25	*****Q****	114	77	84	101	103	105	89	113	102
V26	******W***	147	120	125	77	87	104	88	0	100
V27	*******L**	96	84	114	106	111	101	101	104	91
V30	*********D	63	46	75	94	117	94	102	0	105
Human	41 E L LLPQNNRKVYE 53									
V13	* -[b]*ES*P*W*LF*	151	32	88	112	60	68	84	0	108
V31	* * *E*********	113	70	94	105	122	99	93	92	101
V32	* * **S********	121	138	143	112	98	112	85	31	106
V33	* * ****P******	95	118	131	92	102	96	95	100	101
V34	* * ******W****	87	108	91	123	108	102	97	111	101
V35	* * ********L**	110	72	104	109	93	87	93	37	105
V36	* * *********F*	104	103	128	108	110	109	95	102	101
Human	54 LSNVQEDSQPM 64									
V14	**EIG***S*L	38	33	43	45	77	57	97	108	77
V37	**E********	107	93	112	106	98	128	103	105	93
V38	***I*******	78	87	67	117	81	88	99	99	55
V39	****G******	96	83	106	103	125	99	99	114	109
V40	********S**	104	102	101	110	97	100	108	104	103
V41	**********L	107	116	108	125	114	112	103	102	95
Human	65 CYSNCPDGQST 75									
V15	*FE**GTV***	2	0	0	2	79	1	110	117	0
V42	*F*********	62	28	49	95	98	92	118	98	66
V43	**E********	0	0	0	1	43	2	109	99	0
V44	*****G*****	1	1	1	25	96	115	94	93	4
V45	******T****	68	66	69	112	116	108	92	100	59
V46	*******V***	74	59	89	90	101	66	114	103	125
V47	**********S	89	63	87	105	102	109	101	97	87
Human	76 AKTFLTVYWT 85									
V6	*SATI****S	98	64	66	96	100	110	108	102	90

[a] *, amino acid identity.
[b] -, no amino acid at this position→deletion in murine sequence.

HRV-14, and the mutant HRV-14-G155, whose binding was eliminated with changes at amino acids Leu-30, Ser-67, and Pro-70 of ICAM-1. HRV-15 is the sole representative of the second group and appears to have a strong requirement for Pro-28, Lys-29, and Ser-67. The third group, composed of HRV-36 and HRV-41, was the most resilient to the changes made, with the only significant replacements affecting binding located at Pro-28 and Ser-67. Substitution at the Ser-67

TABLE 3. Chimeras showing a major effect on HRV and MAb binding

Amino acid substitution	% of control binding							
	HRV						MAb	
	3	14	14G	15	36	41	1A6	2C2
Pro-28 to Asp	167	55	143	0	32	35	93	47
Lys-29 to Leu	96	82	64	2	107	84	118	57
Leu-30 to Ser	6	0	6	135	109	116	118	44
Leu-37 to Trp	147	120	125	77	87	104	0	100
Lys-40 to Asp	63	46	75	94	117	94	0	105
Pro-45 to Ser	121	138	143	112	98	112	31	106
Val-51 to Leu	110	72	104	109	93	87	37	105
Tyr-66 to Phe	62	28	49	95	98	92	98	66
Ser-67 to Glu	0	0	0	1	43	2	99	0
Pro-70 to Gly	1	1	1	25	96	115	93	4

residue with Glu showed the greatest effect on HRV binding since it eliminated the binding of five of the six viruses tested and reduced the binding of the sixth virus (HRV-36) to 36% of control binding (Tables 2 and 3). These binding patterns represent the first definitive data that indicate some structural diversity within the putative virion canyon attachment site.

The binding sites of two MAbs that block major group binding with equal efficiency were mapped to two distinct epitopes. MAb 1A6 binding to ICAM-1 was eliminated by substitutions at Lys-40 or Trp-37 and was significantly inhibited by changes at Pro-45 and Val-51 (Tables 2 and 3). MAb 2C2 displayed a completely different profile that more closely mimicked that seen with HRV-3, HRV-14, and HRV-15, in which the primary binding site was centered around Ser-67 and Pro-70 and some impairment of binding was detected when substitutions were made in the cluster of Pro-28, Lys-29, and Leu-30. Similar to studies which mapped the binding site of MAbs that block HIV binding on CD4 close to the gp120 attachment site, the current MAb mapping data also support the conclusion that the primary binding site for HRV binding resides within the N-terminal portion of domain 1.

The MAb binding data also support the anti-parallel, beta-barrel model for domain 1 (Fig. 2A). In this model, the amino acid residues important for MAb-1A6 binding are localized to one face of the domain, while those amino acids involved in interaction with MAb-2C2 and HRVs cluster at a different region of the beta barrel. In fact, this model suggests that other amino acid residues in ICAM are also important for antibody binding. This alignment of key amino acid residues also implies that the N-terminal portion of domain 1 is the region of human ICAM-1 that interacts with the virion canyon. Spatial considerations appear to support this hypothesis, since docking of domain 1 structure appears to involve approximately one-half of domain 1 (Fig. 2B). The docked model presented in Fig. 2B takes into account several pieces of experimental data. First, it adequately accounts for those ICAM mutations shown to affect virus binding by positioning the important amino acids near the bottom of the virus canyon, while placing those not involved with binding outside the canyon region. Second, the VP1 amino acid sites Lys-103, His-220, and Ser-223, located at the base of the canyon and shown to be important for activity (7), are located in good proximity to domain 1 of ICAM-1. Last, the position of domain 1 within the canyon itself has been adjusted to lean toward the side of the canyon which contains amino acid residues located within the rim that appear to be important for virus binding (5a).

The present study focused on identifying amino acid residues of ICAM-1 that are involved in HRV attachment and did not address the consequences of these changes on viral infection. Since CEF cells expressing ICAM-1 do not support HRV infection (unpublished data), we were unable to determine whether ICAM-1 molecules displaying low levels of binding could support HRV infection. Future studies in which these ICAM-1 mutants are transfected into susceptible cells, such as Vero cells, may answer this question directly. Clearly, much more additional work, including crystallization of a virus-receptor complex, needs to be done to further define the specific interactions between HRVs and ICAM-1 and the role, if any, of ICAM-1 in viral eclipse.

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