Identification of monoclonal antibody epitopes and critical residues for rhinovirus binding in domain 1 of intercellular adhesion molecule 1

(rhinovirus receptor/IgG-like domain/amin acid replacements)

ALAN McCLELLAND*, JOANNA DeBEAR, SUSAN CONNOLLY YOST, ANN M. MEYER, CHRISTOPHER W. MARLOR, AND JEFFREY M. GREVE

Molecular Therapeutics, Inc., Miles Research Center, 400 Morgan Lane, West Haven, CT 06516

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ABSTRACT  Intercellular adhesion molecule 1 (ICAM-1) is the cellular receptor for the major group of human rhinoviruses (HRVs) and the adhesive ligand of lymphocyte function-associated antigen 1. Analysis of a series of chimeric exchanges between human and murine ICAM-1 shows that two distinct epitopes recognized by monoclonal antibodies that block rhinovirus attachment and cell adhesion map to the N-terminal domain of ICAM-1. Furthermore the specificity for HRV binding is entirely contained within the first 88 amino acids. Mutagenesis of the four sites of N-linked glycosylation within the second domain shows that carbohydrate is not involved in virus recognition. Homologue replacement mutagenesis localizes the epitopes for virus-blocking antibodies to two regions of domain 1 predicted to form β strand D and the loop between the F and G strands of an immunoglobulin fold structure. Analysis of virus binding to the mutants predicts a large surface of contact between HRV and ICAM-1 domain 1 but shows that the regions most important for virus binding are coincident with the monoclonal antibody epitopes.

There are more than 100 distinct serotypes of human rhinoviruses (HRVs), the primary causative agent of the common cold (1). Viral entry mediated by binding to a cellular receptor is a critical first step in the infection process and is an important determinant of viral tropism. Ninety percent of HRV serotypes utilize a common cellular receptor to initiate infection (2, 3), which we have recently shown is intercellular adhesion molecule 1, or ICAM-1 (4). ICAM-1 is a member of the IgG supergene family; it interacts with the leukocyte integrin lymphocyte function-associated antigen 1 (LFA-1) (5, 6). The molecule is an integral membrane glycoprotein with an extracellular region of 453 amino acids containing five domains with sequence similarity to the IgG constant regions.

The three-dimensional structure of HRV14, which binds to ICAM-1, and of HRV1A, which binds to the as yet unidentified minor receptor, has been determined (7, 8). Determination of the structure of HRV14 led Rossmann to propose the canyon hypothesis, which suggests that a 20-Å-wide surface depression, which encircles the fivefold axis of symmetry of each icosahedral face of the virus, may contain the receptor binding site. Support for the canyon hypothesis comes from site-directed mutagenesis of canyon residues, which alter the receptor binding properties of HRV14 (9), and from studies with capsid binding drugs, which induce a conformational change in the floor of the canyon and prevent receptor binding (10). The dimensions of the canyon are sufficient to accommodate a single unpaired IgG domain, and it has recently been shown by electron microscopy that ICAM-1 and the related neural cell adhesion molecule (NCAM) have long elongated structures consistent with an end-to-end arrangement of unpaired IgG domains (11, 12). The N-terminal domain of ICAM-1 is therefore likely to project furthest from the cell surface and be most accessible to virus. Furthermore ICAM-1 is heavily glycosylated with the exception of the first domain. Staunton et al. (11) have shown by site-directed mutagenesis and construction of a human–mouse chimera that both the HRV and LFA-1 binding sites are contained within the first two N-terminal domains of ICAM-1. A three-dimensional model of the first domain of ICAM-1 based on alignment with known IgG structures was docked with the HRV canyon and used to predict possible contact residues (13).

Recently, considerable attention has been focused on viral receptors due to their potential as targets for anti-viral therapy and the identification of several receptors at the molecular level (14). These advances have raised the prospect of gaining new understanding of virus–receptor interactions at the molecular level through manipulation of the cloned receptor genes and their products. Furthermore the finding that several of these recently identified viral receptors are members of the IgG superfamily with roles in cell–cell recognition suggests that insights into the normal cellular function of these molecules may be obtained through investigation of their role in viral attachment and entry.

Here we show that the first 88 residues of ICAM-1 contain all of the necessary specificity for HRV binding. We have examined this region of the molecule in detail by construction of a series of mouse–human chimeras in which individual predicted β strands or turns were exchanged with corresponding murine ICAM-1 residues. Analysis of these hybrid molecules and several single point mutations allows the assignment of the epitopes for two monoclonal antibodies (mAbs) that block virus binding and inhibit LFA-1/ICAM-1-dependent cell adhesion. The locations of these epitopes show excellent correlation with regions of domain 1 that are important for virus binding.

MATERIALS AND METHODS

Construction of ICAM-1 Chimeras. To construct the human–mouse chimeras, 10 cycles of PCR amplification (15) were performed by using cloned human and mouse ICAM-1 cDNAs as templates. The murine ICAM-1 cDNA was kindly provided by A. Brian (University of California at San Diego). The PCR primers were used to create novel restriction sites at the boundaries between domains in order to facilitate the

Abbreviations: HRV, human rhinovirus; ICAM-1, intercellular adhesion molecule 1; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting; LFA-1, lymphocyte function-associated antigen 1.

To whom reprint requests should be addressed.
gene fusions. Amplified human and mouse fragments were digested with the appropriate restriction enzymes, gel purified, and ligated into the expression vector CDM8 (16). Appropriate clones were identified by restriction mapping and verified by DNA sequencing.

Site-Directed Mutagenesis. ICAM-1 mutants were constructed by using single-stranded template DNA of a full-length ICAM-1 cDNA, pHRR12-19, in the CDM8 expression vector (17) and a Muta-Gen kit (Bio-Rad). In most cases codon degeneracy was exploited to introduce a novel restriction site to allow for rapid and easy identification of mutant plasmids. All mutations were verified by DNA sequencing. The domain 2 glycosylation mutant was made by simultaneous priming with four mutagenic oligonucleotides designed to change asparagine to glutamine at each of the sites.

COS Cell Transfections and Antibody and Virus Binding. COS cell monolayers at 40% confluence in 150-cm² flasks were transfected with 5 μg of plasmid miniprep DNA by the DEAE-dextran method (18). Cells were analyzed 48 hr after transfection by flow cytometry on a FACScan (Becton Dickinson) by using mAbs c78.4A, c78.5A, and CL203 as described (19). Purified CL203 antibody was provided by S. Feronne (New York Medical College). The percentage of ICAM-1-positive COS cells in these experiments varied from 11% to 48%. The murine L-cell line FT16.11 transfected with the mouse ICAM-1 cDNA and anti-mouse ICAM-1 antibody

RESULTS

We have previously described the isolation of a panel of mAbs that protect HeLa cells from infection by HRVs of the major receptor group by binding to ICAM-1 (4). By measuring the binding of 125I-labeled mAb to HeLa cells in the presence of an excess of unlabeled competitor mAb, we determined that these antibodies could be assigned to two groups that recognize distinct epitopes. The epitope recognized by c78.4A is shared by six of the seven mAbs; c78.5A is the sole representative of its group. Both mAbs recognize conformation-dependent epitopes since they immunoprecipitate native ICAM-1 but fail to react with the denatured protein on Western blots. In addition to blocking HRV

Fig. 1. ICAM-1 chimeras. The human–mouse chimeras hm88, hm185, and hm286 consist of human ICAM-1 domain 1, domains 1 and 2, and domains 1, 2, and 3, respectively (boxed), linked to the corresponding C-terminal fragment of mouse ICAM-1.

Fig. 2. ICAM-1 mutants based on alignment of human and murine ICAM-1 domain 1 sequences. Only mouse residues that differ from the human sequence are indicated. A single gap denoted by the star has been introduced in the mouse sequence to allow the best alignment. Dashed lines below the alignment indicate the predicted positions of β strands A–G from the model proposed by Giranda et al. (13). The lower part of the figure shows the mutations that were introduced within domain 1. In addition to the murine substitutions, several single changes were also made based on alignment with the first domain of ICAM-2. A dash in the mutant sequence indicates the position of a conserved residue, which was not altered.

Table 1. Binding of mAbs and HRV to ICAM-1 chimeras

<table>
<thead>
<tr>
<th>ICAM-1 construct</th>
<th>c78.4A</th>
<th>c78.5A</th>
<th>HRV3</th>
</tr>
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<tbody>
<tr>
<td>Human ICAM-1</td>
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<td>50.6</td>
<td>100</td>
</tr>
<tr>
<td>hm88</td>
<td>50.8</td>
<td>50.8</td>
<td>100</td>
</tr>
<tr>
<td>hm185</td>
<td>29.1</td>
<td>27.2</td>
<td>90</td>
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<tr>
<td>hm286</td>
<td>35.6</td>
<td>37.7</td>
<td>74</td>
</tr>
<tr>
<td>Mouse ICAM-1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Deglycosylated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>domain 2</td>
<td>38.5</td>
<td>38.1</td>
<td>78</td>
</tr>
</tbody>
</table>

COS cells were transfected with cDNA constructs and analyzed by FACS 48 hr after transfection. Analysis of mouse ICAM-1 used a stable L-cell transfectant, FT16.11, and surface expression was confirmed with the anti-murine ICAM-1 mAb 3E5. mAb binding is expressed as the percentage of cells staining above background, which shows some variation since this value is affected by the transfection efficiency. HRV binding is expressed as a percentage relative to wild-type ICAM-1 in the same transfection experiment, which was 27% of input virus. Standard error values for the virus binding data are indicated in parentheses.

3E5 were provided by A. Brian (University of California at San Diego).

For virus binding assays, cells were detached with trypsin 24 hr after transfection and replated in 24-well microtiter plates at ~5 × 10⁵ cells per well. HRV binding assays were done 48 hr after transfection as described (2). For each mutant, 3 wells were preincubated for 60 min at 37°C with virus-blocking mAb c78.4A or c78.5A at 1 μg/ml prior to addition of virus, and 3 wells received no mAb. Specific virus binding was determined by subtracting cell-associated radioactivity in the presence of mAb from the values obtained in the absence of mAb and ranged from 16% to 41% of the input. Each virus binding experiment included control binding to cells transfected with wild-type ICAM-1 and the CDM8 vector.

RESULTS

We have previously described the isolation of a panel of mAbs that protect HeLa cells from infection by HRVs of the major receptor group by binding to ICAM-1 (4). By measuring the binding of 125I-labeled mAb to HeLa cells in the presence of an excess of unlabeled competitor mAb, we determined that these antibodies could be assigned to two groups that recognize distinct epitopes. The epitope recognized by c78.4A is shared by six of the seven mAbs; c78.5A is the sole representative of its group. Both mAbs recognize conformation-dependent epitopes since they immunoprecipitate native ICAM-1 but fail to react with the denatured protein on Western blots. In addition to blocking HRV
attachment, both mAbs inhibit LFA-1-dependent adhesion of JY cells to purified ICAM-1 (J.M.G., unpublished results).

A series of human–mouse chimeras were constructed to localize mAb and HRV binding determinants on the ICAM-1 molecule. Chimeric ICAM-1 molecules were constructed in which human domain 1 (residues 1–88), domains 1 and 2 (residues 1–185), and domains 1, 2, and 3 (residues 1–286) were fused to mouse ICAM-1 (20, 21) at the corresponding location in the mouse sequence (Fig. 1). The chimeric molecules were expressed in COS cells and analyzed by fluorescence-activated cell sorting (FACS) with mAbs c78.4A and c78.5A (Table 1). Since neither mAb reacts with mouse ICAM-1, the binding of both antibodies to the hm88 chimera localizes the epitopes for c78.4A and c78.5A to domain 1 of ICAM-1. HRV binding to transfected COS cell monolayers was tested in parallel with FACS analysis. Virus binding to each of the three chimeric molecules was comparable to wild-type human ICAM-1 (Table 1), whereas cells expressing mouse ICAM-1 showed no specific binding. This result indicates that the region of human ICAM-1 that confers specificity for HRV binding lies within the first 88 amino acids.

ICAM-1 contains eight N-linked oligosaccharide chains, four of which are in the second domain, whereas domain 1 is unglycosylated (5). To determine whether carbohydrate structures in domain 2 are involved in HRV binding, we constructed a mutant ICAM-1 molecule in which each of the four glycosylated asparagine residues at positions 103, 118, 156, and 175 was changed to glutamine. In vitro transcription and translation of this mutant in the presence of dog pancreas microsomal membranes directs the synthesis of a core glycosylated polypeptide of 63 kDa, which is consistent with the absence of four sites of N-linked glycosylation (data not shown). The partially deglycosylated ICAM-1 molecule was expressed in COS cells, which bound HRV at levels comparable to the wild-type molecule (Table 1).

To map the mAb and HRV binding determinants within domain 1, we systematically substituted human residues for corresponding mouse residues throughout this region of the protein (Fig. 2). The mutated cDNAs were transfected into COS cells, and expression was analyzed by FACS using mAbs c78.4A, c78.5A, and CL203; the latter recognizes a site in the membrane proximal part of the molecule (11). All of the mutants were expressed on the cell surface, with 10–40% positive cells in each experiment. Staining with c78.4A or c78.5A is expressed relative to the percentage of CL203-positive cells in the same transfection (Fig. 3 A and B). The only mutation that affected the binding of both mAbs was M64CYS/LCFE (see the legend to Fig. 3 for an explanation of the mutant designations), which reduced c78.4A binding significantly and virtually eliminated c78.5A binding. This mutation introduces a negative charge within the F strand at residue 67, which is predicted to have its side chain pointing inward and therefore probably alters the overall conformation of domain 1. In support of this interpretation, the mutant M64CYS/LCFE had no effect on the binding of either mAb. Since none of the other mutations disrupted both epitopes, effects on antibody or virus binding are likely to be indicative of contacts in the area of the mutation rather than global conformational alteration of the domain. These could be due either to local conformational changes or to direct involvement of the altered residues.

Mutations that specifically affect c78.4A binding are exclusively within the region predicted to comprise β strand D. Thus either K40/D, L43/E, or the double mutation K40/DLL/DELE completely eliminates c78.4A binding but react normally with c78.5A. Residue glutamic acid-41, which is conserved in the mouse protein, does not appear to be involved in c78.4A recognition, since the change to valine had no effect. Mutations that affect the binding of c78.5A are located in the predicted loop between β strands F and G at the N-terminal end of the domain. Single and double mutations at aspartic acid-71 and glycine-72 reduce c78.5A binding significantly, while reactivity is completely eliminated by the triple mutation P70DG/GTV. A modest but reproducible effect on c78.5A binding is also seen in the mutation D260PK/KEFL, which is predicted from the alignment to form the loop between β strands B and C. The IgG fold structure places the B to C and F to G loops in relatively close proximity at the N-terminal end of the molecule (Fig. 4), consistent with an antibody contact to both. The epitopes for c78.4A and c78.5A thus map to the D strand and the F to G loop, respectively, with a small contribution to the c78.5A epitope from residues in the B to C loop. These mutations define a minimum area of potential contact between the

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**Fig. 3.** mAb and HRV binding to ICAM-1 mutants. The mutants are named according to the wild-type residues followed by a slash and the corresponding residues in the mutant. The first residue of the wild-type sequence is followed by its position number. Mutants were transfected into COS cells and analyzed by FACS with mAbs c78.4A, c78.5A, and CL203. The binding of c78.4A (A) and c78.5A (B), expressed relative to control staining of the same cell population with CL203. (C) Relative virus binding is expressed as the percent binding to mutant versus wild-type ICAM-1 in the same transfection experiment. Standard errors (HRV binding) were determined from at least two independent experiments. No adjustment was made for variations in expression since the level of ICAM-1 expressed in each experiment was sufficient to allow maximal virus binding.
antibody molecules and domain 1, since conserved residues and tolerated replacements at residues involved in mAb binding would not be identified.

The results of 35S-labeled HRV3 binding to COS cell monolayers transfected with each of the mutants are summarized in Fig. 3C. Strikingly, the mutations that result in the greatest reduction in HRV binding correspond to mutations that also affect the binding of mAbs c78.4A and c78.5A. The mutations K40/D and L43/E in the c78.4A binding site produce a 3-fold and 2-fold reduction, respectively, whereas the double mutation exhibits a 10-fold reduction. Mutations in the c78.5A binding site show reduced virus binding that correlates with the reduction in reactivity with the mAb. The single mutation D71/G reduces virus binding 5-fold; the double mutation D71G/TV reduces binding 10-fold; and the mutation P70D/GTV, which eliminates c78.5A binding, abolishes HRV attachment. Mutation of residues 26–29, which are also implicated in c78.5A binding, produces a 4-fold reduction in virus binding. The mutation M64CYS/LCFE also completely eliminates virus binding, but as discussed above, probably induces a major conformational change, which disrupts the virus binding site. Of the mutations that reduce virus binding by 50% or more but have no effect on mAb binding, several involve regions of the domain that are likely to be in close proximity to if not part of the mAb combining sites. These are L30LGIL/SLGL in the C strand, M64C/LCF in the F strand, and T75AK/SAS at the N-terminal end of the G strand. Only two mutations that reduce virus binding are predicted to lie at the C-terminal end of the domain, in the loops between the A and B strands (R13/Q) and between the E and F strands (K56VQEDSQ/EIGKEDSS).

DISCUSSION

We have exploited the fact that the murine homologue of ICAM-1 does not bind HRV to map regions of the protein important for viral attachment. Since murine ICAM-1 is capable of binding to human LFA-1 (22), we have not examined the binding of LFA-1 to the mouse–human chimeras described here. By transferring intact domains of human ICAM-1 to the mouse molecule, we have shown that the epitopes for both virus-blocking mAbs c78.4A and c78.5A reside within the first domain and that human domain 1 alone is capable of converting murine ICAM-1 so that it binds HRV. This result demonstrates that HRV binding specificity is imparted by the first 88 residues of human ICAM-1, although it does not rule out the involvement of other domains in virus binding.

The first domain of ICAM-1 contains no glycosylation sites, but there are four sites of N-linked glycosylation in domain 2. We therefore addressed the potential role of glycosylation of ICAM-1 in HRV binding by mutating each of these sites. The partially deglycosylated receptor binds virus indistinguishably from the wild-type molecule. This result argues against a direct role of N-linked glycosylation in HRV binding. Lineberger et al. (23) concluded that glycosylation was involved in virus binding based on the lack of virus binding to receptor made in the presence of tunicamycin. A possible explanation for these apparently conflicting results is that glycosylation is required for the proper folding and transport of ICAM-1 to the cell surface. In support of this interpretation, we have observed that a soluble form of domains 1 and 2 fails to be secreted when all four glycosylation sites are deleted (S.C.Y. and A.M., unpublished results).

We have examined domain 1 in greater detail by replacing predicted B strands or turns with corresponding murine sequence. This largely avoided the introduction of changes that alter the conformation of the domain. This conclusion is based on the finding that only one of 25 mutations simultaneously affected the binding of two independent conformation-dependent mAbs. This mutation introduced a negatively charged residue predicted to be oriented towards the hydrophobic interior of the domain. Mutations that specifically eliminated binding of c78.4A were limited to residues 40 and 43, which are predicted to lie in B strand D on the four-strand side of the B sandwich (13, 24). If residues 40 and 43 are part of a B structure, their spacing dictates that only one of the two residues is oriented to the exterior of the domain. It is quite plausible that mutation of an inward-pointing residue could influence the local conformation in the region of antibody
contact. The N-terminal loop between strands F and G forms a major part of the c78.5A epitope, which to a lesser extent appears to involve the adjacent B to C loop. Since the interface between antibody and protein antigen can be 25–30 Å in diameter (25), the mutations that affect binding localize key residues of the epitopes rather than defining the entire contact surface.

The effect of the antibodies on virus attachment could be due to direct masking of virus contact points on the ICAM-1 molecule, to steric effects preventing the binding site residues from penetrating the canyon, or a combination of both. However, the virus binding results suggest that the antibodies recognize groups of residues in domain 1 that are directly involved in contact with the virus, since the greatest effects on binding were caused by mutations in these areas (Fig. 4). These results indicate that an extensive area of the surface of ICAM-1 domain 1 is involved in contact with HRV, including the N-terminal end of the molecule and E strands D and G on the four- and three-strand faces of the domain, respectively (Fig. 4). Our mutagenesis data provide support for the model of ICAM-1/HRV interaction proposed by Giranda et al. (13), in that the areas of proposed interaction are those that we find are most important for virus binding.

Our results agree in part with a previous mutational analysis (11), but there are significant differences. Although both studies have shown that the N-terminal domain 1 of ICAM-1 is required for virus binding, they differ in the specific regions of the domain identified as important. We have found that residues in β strands D and G and in the F to G loop form a key part of the virus binding site, whereas the former study reported minimal effects in these areas. Furthermore it was concluded that glutamine-58 was the single most important residue for virus binding, based on a mutation to histidine, which completely eliminated binding (11). We find that the identical mutation has no effect and that a mutation that includes the change glutamine-58 to glycine retains considerable binding activity. Residue glutamine-58 is predicted from sequence alignments to be in a loop at the C-terminal end of domain 1, close to the interface with domain 2. Taken together with our evidence that virus interaction involves residues at the N-terminal end and on the four- and three-strand sides of the domain, it is unlikely that this part of the molecule would contain the most important residue for HRV binding. Other differences between the results reported here and those in ref. 11 may reflect the different mutagenesis strategies used. Our approach ensured examination of most of the domain and largely eliminated effects due to conformational changes. The fact that both virus and blocking mAb binding identified many of the same residues supports the conclusion that we have mapped regions of domain 1 that are important for HRV attachment.

Note. We have recently obtained data for HRV14 binding to several of the mutants described in this report, including the Q58/H mutation. The results are in excellent agreement with those obtained using HRV3.

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