The Minor Receptor Group of Human Rhinovirus (HRV) Includes HRV23 and HRV25, but the Presence of a Lysine in the VP1 HI Loop Is Not Sufficient for Receptor Binding

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Like all 10 minor receptor group human rhinoviruses (HRVs), HRV23 and HRV25, previously classified as major group viruses, are neutralized by maltose binding protein (MBP)-V33333 (a soluble recombinant concatemer of five copies of repeat 3 of the very-low-density lipoprotein receptor fused to MBP), bind to low-density lipoprotein receptor in virus overlay blots, and replicate in intercellular adhesion molecule 1 (ICAM-1)-negative COS-7 cells. From phylogenetic analysis of capsid protein VP1-coding sequences, they are also known to cluster together with other minor group serotypes. Therefore, they belong to the minor group; there are now 12 minor group and 87 major group HRV serotypes. Sequence comparison of the VP1 capsid proteins of all HRVs revealed that the lysine in the HI loop, strictly conserved in the 12 minor group HRVs, is also present in 9 major group serotypes that are neutralized by soluble ICAM-1. Despite the presence of this lysine, they are not neutralized by MBP-V33333 and fail to replicate in COS-7 cells and in HeLa cells in the presence of an ICAM-1-blocking antibody. These nine serotypes are therefore “true” major group viruses.

Human rhinoviruses (HRVs), the main causative agents of common cold, were originally classified as acid-sensitive picornaviruses (34). Later, based on competition for cellular binding sites, two different groups of viruses using nonidentical receptors for cell attachment were defined within the genus Rhinovirus (18). Subsequently, 24 (1) and finally 100 serotypes (counting the subtypes HRV1A and HRV1B as one strain) were assigned to the two receptor groups by using cross-competition and inhibition of cell binding by a monoclonal antibody recognizing intercellular adhesion molecule 1 (ICAM-1), the receptor of the major group of HRVs (32, 33, 35). According to these reports, 90 serotypes bind ICAM-1, whereas both subtypes of HRV1 (HRV1A and HRV1B) and 8 other serotypes were categorized as belonging to the minor group; they were later shown to use members of the low-density-lipoprotein receptor (LDLR) family for cell entry (12, 20, 36). HRV87 was noted to be an exception in that it binds a sialylated membrane protein. Based on comparison of nucleotide sequences in several genomic regions, HRV87 was subsequently classified as an acid-sensitive enterovirus and found to be a prime strain of enterovirus 68 that uses decay-accelerating factor as a receptor (2, 27). By phylogenetic analysis of capsid protein VP4/VP2 coding sequences of all HRV prototype strains, 74 HRV serotypes, including all the minor receptor group ones, were classified as HRV species A, while the 25 remaining serotypes form HRV species B (27). In a paper from the Colonno group, primary data on the competition with an ICAM-1-blocking antibody were not explicitly presented for all serotypes, in particular not for HRV23 and HRV25, and the presumed allocation of all HRVs to either group was depicted only in the form of a summarizing figure (35). Despite this, it was generally accepted that 90 serotypes, including HRV23 and HRV25, were major group viruses. Later, Crump and colleagues reported that recombinant soluble ICAM-1 and a chimeric ICAM-1/immunoglobulin A molecule failed to neutralize these two serotypes and concluded that they most probably use a receptor different from the major and minor group receptors (4, 5). This finding remained largely unnoticed by the picornavirus community. As soon as the capsid protein VP1 sequences of all human rhinoviruses were determined (15, 16), we became aware of genetic clustering of HRV23 and HRV25 close to certain minor receptor group serotypes and of the presence of a lysine in the HI loop. This residue is strictly conserved in all minor group viruses and is believed to be essential but not sufficient for attachment to members of the LDLR family (37). A lysine is also present at the equivalent position in HRV8, -18, -24, -40, -54, -56, -58, -85, -95, and -98. However, these latter serotypes had been explicitly shown to be neutralized by soluble recombinant ICAM-1 (5). Therefore, we wondered whether at least HRV23 and HRV25 might use members of the LDLR family as receptors. We also wondered whether the other HRVs containing the conserved lysine might eventually possess dual receptor specificity and attach to ICAM-1 as well as to LDLR.

The ligand binding domain at the N terminus of LDLR that is implicated in binding minor group HRVs is composed of seven imperfect direct repeats of roughly 40 amino acid resi-
dyes in length. Three disulfide bridges and a Ca$^{2+}$ ion within an octagonal cage formed by the carboxylates of three aspartates and one glutamate, together with two backbone oxygens, stabilize this structure (7). The very-low-density lipoprotein receptor (VLDLR) and LDLR-related protein (LRP) possess 8 and 31 such modules, respectively (30). In LRP the modules are not contiguous but are arranged in clusters of 2, 8, 10, and 11.

We have previously shown that a soluble recombinant fusion protein composed of the maltose binding protein (MBP) and an artificial concatemer of five copies of repeat 3 (V3) of VLDLR arranged in tandem (MBP-V33333) exhibits strong virus neutralization capacity toward the 10 minor group HRVs (36). Therefore, by using cell protection assays with this protein, ligand blots, and replication in COS-7 cells that do not express ICAM-1, we investigated the nature of the strains containing a lysine residue in the HI loop and found that HRV23 and HRV25 possess all characteristics of minor group viruses. Examination of the major group serotypes with the lysine in the HI loop as mentioned above showed that none was able to use both ICAM-1 and LDLR for cell binding. Therefore, out of the numbered 99 HRV serotypes, altogether, 12 serotypes (if we count HRV1A and -B separately) belong to the minor receptor group.

**Materials and Methods**

**Viruses.** HRV23 and HRV25 were obtained from the National Institute for Public Health and the Environment, Bilthoven, The Netherlands, but were originally from the American Type Culture Collection (ATCC), and their sequences are practically identical to those published by Ledford et al. (16). HRV89 was from Janssen Pharmaceuticals, and all remaining serotypes used in this study were from the ATCC. Media for tissue culture and additives were from GIBCO-BRL (Gaithersburg, MD). HRVs were grown in HeLa-H1 cells (Flow Laboratories). The identities of HRV23 and -25 were confirmed using type-specific antisera from the ATCC. The two almost identical sets of VP1 sequences published by Ledford and colleagues (16) and Laine and coworkers (15) were retrieved from the picornavirus database (http://www.iah.bbsrc.ac.uk/virus/picornavirus/index.html).

**Virus neutralization and binding tests.** Neutralization tests with antisera and recombinant MBP-V33333 were carried out essentially as described previously (19, 26). Briefly, virus at 10 or 1,000 50% tissue culture infective doses as indicated was mixed with twofold serial dilutions of receptor in minimal essential medium containing 2% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (infection medium), incubated for 30 min at room temperature, and transferred onto subconfluent HeLa-H1 cells grown in 96-well plates. After incubation at 34°C for 3 days, cells remaining attached to the plastic were stained with crystal violet (1% in water), and the lowest concentration of the inhibitor giving recognizable cell protection was scored positive. For virus overlay blots, a plasma membrane fraction was prepared as described previously (16) from immortalized M1 mouse wild-type (wt) fibroblasts used to compare virus binding to the receptor (VLDLR) and LDLR-related protein (LRP) possess five symmetry-related binding sites at the star-shaped plateau around the fivefold axes of icosahedral symmetry (14). It became clear that compared to the prototype minor group virus HRV2 and the major group viruses HRV14 and HRV85, HRV23 and HRV25 were neutralized to a similar extent as HRV2 (Table 1). Conversely, as shown previously (36), the presence of MBP-V33333 at the highest concentration failed to protect the cells against infection with HRV14. HRV85, a serotype that has also been explicitly demonstrated to be neutralized by soluble ICAM-1 (4, 5), we first turned to these for experimental verification of their receptor specificity.

HRV23 and HRV25 are neutralized by MBP-V33333. We have previously shown that all 10 minor group HRVs so far assigned are neutralized by a soluble recombinant fusion protein composed of MBP and a concatemer of five copies of module 3 of VLDLR (36). This artificial receptor has very high avidity for HRV2, most probably because more than one module, if not all five modules, can attach simultaneously to the fivefold axes of icosahedral symmetry (14). It became clear that compared to the prototype minor group virus HRV2 and the major group viruses HRV14 and HRV85, HRV23 and HRV25 were neutralized to a similar extent as HRV2 (Table 1). Conversely, as shown previously (36), the presence of MBP-V33333 at the highest concentration failed to protect the cells against infection with HRV14. HRV85, a serotype that has also been explicitly demonstrated to be neutralized by soluble ICAM-1 (5), was included in the experiment as well.

**Phylogenetic analysis.** Deduced VP1 amino acid sequences were aligned by using the Clustal X (version 1.83) multiple-sequence alignment program (31), which was also used for constructing a phylogenetic tree (excluding the positions with the gaps) by the neighbor-joining method followed by bootstrapping analysis of 1000 replicates.

**Results**

Comparison of the VP1 sequences of all HRVs within the regions equivalent to the site of binding of module 3 of VLDLR to HRV2 (36) revealed the presence of a lysine in the HI loop in the serotypes listed in Fig. 1. This lysine is considered necessary but not sufficient for attachment of minor group HRVs to members of the LDLR family (37). The X-ray structure of a complex between HRV2 and a recombinant receptor fragment composed of repeats V2 and V3, arranged in tandem (V23), shows that tryptophan 132 (W132 [numbering of the precursor protein, SwissProt entry P98155; i.e., W22 of V3]) interacts with the side chain of the lysine in the HI loop of VP1. This lysine, in turn, also establishes electrostatic interactions with the negative-charge cluster around the Ca$^{2+}$ ion in V3 (36). We thus believe that in the absence of the lysine, no interaction between receptor and virus is possible. Exchanging it for other residues by site-directed mutagenesis resulted in a lack of production of progeny virus upon transfection of the mutated viral RNA into HeLa cells (6). On these grounds, we reasoned that HRVs possessing this particular lysine had the potential to bind receptors of the LDLR family. As seen in Fig. 1, out of the 99 HRV serotypes (16), all minor group viruses but also HRV8, -18, -23, -24, -25, -40, -54, -56, -58, -85, -95, and -98 possess a lysine at the equivalent position. However, no obvious conservation of the other residues shown for HRV2 to be in contact with the receptor is apparent within the minor group. Since all these serotypes, with the exception of HRV23 and HRV25, had been shown to be neutralized by soluble ICAM-1 (4, 5), we first turned to these for experimental verification of their receptor specificity.

HRV23 and HRV25 bind LDLR. Virus overlay blots have been used to detect (21, 22) and to finally identify the minor
group receptors as LDLR, VLDLR, and LRP (8, 12, 20). We therefore used a similar approach to assess the specificity of these serotypes for the LDL receptor family. Virus was metabolically radiolabeled with $[^{35}S]$cysteine-methionine (23). In order to increase the sensitivity of the assay, immortalized mouse fibroblasts (M4), deficient in endogenous LDLR and LRP (13, 39), that had been stably transfected to express a truncated version of human LDLR were used. The expressed protein lacks 33 amino acid residues at the cytoplasmic C terminus, including the clathrin localization signal, which results in strong expression at the cell surface (10, 28). Membrane proteins were separated on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel under nonreducing conditions and electrotransferred to a polyvinylidene difluoride membrane. Strips of the blot were incubated with radiolabeled HRV1A, HRV2, HRV23, HRV25, and HRV62, and eventually bound virus was detected by autoradiography. As seen in Fig. 2, HRV23 bound LDLR to a similar extent as the authentic minor group viruses HRV2 and HRV62; HRV25 produced a visible but weaker band. Since HRV1A binds only very weakly to human LDLR (25), no band was seen with this serotype. Therefore, the same experiments were also carried out with the wt mouse fibroblast line M1. Interestingly, HRV25 behaved similarly to HRV1A in that it bound more strongly to the endogenous mouse LDLR than to human LDLR. Major group HRVs do not bind to ICAM-1 or any other protein in

![FIG. 1. Alignment of VP1 sequences of all human rhinovirus serotypes possessing a lysine at the position equivalent to the one implicated in receptor recognition of HRV2 (36). Only regions corresponding to the BC, DE, and HI surface loops are depicted. Serotypes, except HRV23 and HRV25, originally classified as major group (35) are boxed. Residues identified in HRV2 as contacting the receptor are in reverse type. The lysine strictly conserved in all minor group HRVs is in italics and highlighted by the caret symbol. Minor group HRVs are arranged in clusters as revealed by phylogenetic analysis (16) (Fig. 4). HRV8 and -95 are bracketed to indicate their antigenic cross-reactivity; they might be considered a single serotype (16). Note that all HRVs shown belong to genus A (16, 27). Parts of the alignment of the entire VP1 sequences made with Clustal W (http://www.ebi.ac.uk/clustalw/index.html#) are shown.](http://jvi.asm.org/)

![Table 1. HRV23 and HRV25 are neutralized by MBP-V33333](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Virus neutralization concn (nmol/liter)</th>
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<tbody>
<tr>
<td>HRV2</td>
<td>0.15</td>
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<tr>
<td>HRV23</td>
<td>0.6</td>
</tr>
<tr>
<td>HRV25</td>
<td>0.15</td>
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<tr>
<td>HRV14†</td>
<td>—</td>
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<td>HRV58‡</td>
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</table>

† HeLa cells grown in 96-well plates were challenged with HRVs (at 1,000 50% tissue culture infective doses) that had been mixed with serial twofold dilutions of a preparation of MBP-V33333, starting with ~1 μg/ml (~75 nmol/liter), in a final volume of 200 μl. After incubation at 34°C for 3 days, cells were stained with crystal violet.
‡ Lowest concentration of receptor resulting in noticeable cell protection under the specified conditions.
§ Major group HRV used as control.
$—$, no neutralization at 75 nmol/liter.
this type of assay, most probably because of the presence of SDS (24). No signal was seen when the blot was incubated with the HRVs in the absence of Ca\(^{2+}\) but in the presence of 10 mM EDTA, indicating the specificity of the assay (data not shown).

**HRV23 and HRV25 infect ICAM-1 negative cells.** COS-7 cells have been used to demonstrate that the major group receptor was ICAM-1. These cells do not express this protein and are refractive to infection with ICAM-1-binding HRVs. Transfection with an ICAM-1-encoding plasmid restored virus binding (29). We have previously used COS-7 cells to demonstrate that a variant of HRV89 adapted to grow in Hep-2 cells (which express only very low levels of ICAM-1) had acquired a new receptor specificity and replicated in COS-7 cells but at the same time retained its affinity for ICAM-1 (24). COS-7 cells were lysed upon infection with HRV2, HRV23, and HRV25 (Fig. 3). From this it is clear that they are not dependent on ICAM-1 for cell entry. HRV14, a major group prototype species B, and HRV85, a species A major group virus possessing the lysine in the VP1 HI loop (“K-type” virus), were again included in the study and showed no cell lysis. Since HRV85 is not neutralized by MBP-V33333 (Table 2), it is unlikely that it binds to the closely related LDLR and LRP. However, because cell entry does not necessarily result in lysis, as replication might be blocked intracellularly in some cell types (9, 17, 40), this does not prove that HRV85 cannot bind ICAM-1-negative cells.

**Major group “K-type” HRVs do not replicate in cells without functional ICAM-1.** Although HRV85 possesses a lysine at a position equivalent to that implicated in receptor interaction with HRV2, it failed to grow in COS-7 cells. To investigate whether the same applies to the other “K-type” HRVs listed in Fig. 1, COS-7 cells were challenged with these serotypes. Furthermore, we also infected HeLa cells in the presence of monoclonal antibody R6.5 (which blocks ICAM-1 [29]), in the presence of MBP-V33333, or in the presence of both and

![Image](http://jvi.asm.org/)

**FIG. 2.** HRV23 and HRV25 bind LDLR in virus overlay blots. Cell membranes were prepared from M4-LDLR806 cells overexpressing truncated human LDLR (28) and from wt M1 cells. Aliquots corresponding to 10^6 cells were separated on sodium dodecyl sulfate-10% polyacrylamide gels under nonreducing conditions. The proteins were electrotransferred to a polyvinylidene difluoride membrane that was stained with crystal violet and photographed. No infection was seen with the major group viruses HRV14, HRV16, and HRV85.

![Image](http://jvi.asm.org/)

**FIG. 3.** HRV23 and HRV25 infect ICAM-1-deficient COS-7 cells. COS-7 cells grown in 96-well plates until 90% confluent were challenged with HRVs at a multiplicity of infection of 10. After incubation for 3 days at 34°C, cells were stained with crystal violet and photographed. The cytopathic effect resulted in detachment of the cells and lack of coloration. No infection was seen with the major group viruses HRV14, HRV16, and HRV85.

<table>
<thead>
<tr>
<th>HRV serotype</th>
<th>HeLa cells with:</th>
<th>Inhibition of CPE by sICAM (EC_{50})</th>
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<tr>
<td></td>
<td>No addition (control)</td>
<td>Anti-ICAM R6.5</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
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<tr>
<td>29</td>
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<td>24</td>
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<tr>
<td>58</td>
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<td>40</td>
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<tr>
<td>54</td>
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<tr>
<td>98</td>
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<td>(16)</td>
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<td>(8915(^a)</td>
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\(^{a}\) HeLa cells grown in 48-well plates were challenged with the serotypes listed at a multiplicity of infection of 10 without addition (control) or in the presence of indicated additions and incubated for 3 days at 34°C. COS-7 cells were infected without addition of inhibitors. Viable cells remaining attached to the wells were stained with crystal violet. Clear wells indicating cell lysis were scored positive.

\(^{b}\) Boldface, minor group HRVs; HRVs in parentheses lack the lysine in their HI loop.

\(^{c}\) From reference 5.

\(^{d}\) ND, not determined.

\(^{e}\) Hep2-cell adapted mutant HRV89\(_{15}\) using proteoglycan receptors (24, 38).

\(^{f}\) Variant HRV89\(_{15}\) was more strongly neutralized by sICAM than the wt (24).

**TABLE 2.** ICAM-1 is essential for replication of all major group HRVs possessing a lysine in the VP1 HI loop.
HRV23 and -25 cluster with established minor group serotypes (gray boxes numbered 1, 2, and 3), but other HI loop lysine-containing major group serotypes (arrows) do not. The neighbor-joining tree is based on amino acid sequences of entire VP1 capsid proteins of all genus A HRVs; HRV14, which is genus B, is included for comparison. Data are from reference 15. Bootstrapping values are indicated.
observed the cells for the presence of cytopathic effect. As summarized in Table 2, the four minor group HRVs (including HRV23 and HRV25) tested were inhibited by MBP-V33333 but not by R6.5. Conversely, all major group HRVs destroyed the cells in the presence of MBP-V33333, but the cells were protected against infection by R6.5. Whereas COS-7 cells became infected with the minor group HRVs, resulting in a clearly discernible cytopathic effect, the cells remained healthy upon infection with all major group HRVs listed in Table 2. A mutant of HRV89 that had been adapted to use a proteoglycan for infection (38) was also included as a control. HRV89, which infected COS-7 cells and HeLa cells regardless of the simultaneous presence of R6.5 and MBP-V33333, indicating its independence from ICAM-1 and LDL receptors for entry.

**DISCUSSION**

In this report we demonstrate that HRV23 and HRV25 are minor group HRVs. This was accomplished by showing that they (i) are neutralized by MBP-V33333, (ii) bind LDLR, and (iii) infect ICAM-1-deficient COS-7 cells and HeLa cells in the presence of an ICAM-1-blocking monoclonal antibody. This result extends the number of minor group HRVs to 12 (counting HRV1A and HRV1B as separate entities) and reduces the number of major group HRVs to 87, taking into account the antigenic identities of HRV Hanks and HRV21 and of HRV8 and HRV95 (Fig. 4). Based on the VP1 sequences, Ledford and colleagues already noted that only 13 amino acid residues in VP1 were different between HRV25 and HRV62 (16). Out of these, four lay in the region we have proposed previously to be involved in receptor discrimination (37). The authors hypothesized that very few changes might be required to acquire specificity for LDL receptors. Our work now demonstrates that no such changes are necessary and that the clustering of HRV23 and HRV25 with other minor group serotypes (Fig. 4), as noted previously (15, 16), is not without reason. The clusters now include HRV1A and 1B (cluster 1); HRV2, -23, -30, and -49 (cluster 2); and HRV25, -29, -31, -44, -47, and -62 (cluster 3) (Fig. 4). Interestingly, this grouping is related to the number of deletions within the BC loop with respect to HRV1A and HRV1B. In cluster 2 there are two deletions, and in cluster 3 there are four deletions (Fig. 1). When a parsimony tree was calculated with Clustal X (11, 31) by using only the residues assumed to be in contact with the receptor (based on the X-ray structure of HRV2 complexed with V3, i.e., DTDRKKS for HRV1A, DNDRKEF for HRV1B, etc. [Fig. 1] [36]), the same serotypes were found together in the same clusters, although the bootstrapping values were substantially lower for some serotypes (data not shown). This demonstrates that the three sets and the distinction of minor and major groups result from characteristic amino acid residues and not from the gaps within the BC loop of VP1.

There are nine more serotypes, taking into account the virtual identity of HRV8 and HRV95, possessing a lysine at the position equivalent to that involved in HRV2 receptor binding (36) that have been convincingly demonstrated to belong to the major group by being neutralized by soluble ICAM-1 (5). None of them induced cytopathic effects in COS-7 cells, and they infected HeLa cells in the presence of MBP-V33333. In a phylogenetic tree based on VP1 sequences, they did not cluster together or with any of the minor group clusters (Fig. 4).

For control purposes we also included HRV89 in the experiments. This variant was isolated upon adaptation of HRV89 to grow in Hep-2 cells that express low levels of ICAM-1 and can replicate in COS-7 cells (24) by using heparan sulfate proteoglycan for cell attachment (38). As expected from these properties, it was the only serotype strain that replicated in HeLa cells in the presence of both R6.5 and MBP-V33333. To definitely exclude binding to the minor (LDLR) or major (ICAM-1) group receptors, HeLa cells were preincubated with R6.5 to block ICAM-1, and MBP-V33333 was added to block LDLR-specific binding sites on the virus. Under these conditions, none of the wild-type serotypes achieved lysis of the cells. So, except from the lysine in the HI loop, what determines the specificity for LDL receptors? We have previously examined the surface properties of three-dimensional models of those HRVs whose sequences were available and found the presence of a somewhat more positive electrostatic potential compared to the equivalent position in major group HRVs (37). Based on the now-available three-dimensional X-ray structure of the HRV2-V23 complex, this view has become somewhat questionable, since except for the lysine, none of the contact residues is strictly conserved and a phylogenetic tree failed to demonstrate separation of the two receptor groups (Fig. 4). The sequence alignment within the BC, DE, and HI loops also shows that major and minor group HRVs are very similar with respect to sequence variation and conservation within the loops. Whereas the DE and HI loops do not contain any insertions and the alignment is unambiguous, this is not the case for the BC loop, with the deletions making the alignment somewhat problematic (Fig. 1). However, in this region there tend to be more hydrophilic and negatively charged residues in major group HRVs, whereas the residues are highly variable in the case of minor group HRVs (not shown). Thus, this loop might play a predominant role in determination of the receptor specificity.

Blockade of ICAM-1 by a monoclonal antibody completely prevented HRV14 infection even at a very high multiplicity of infection, showing the virtual absence of variants being able to use another receptor (3). However, HRV14 belongs to genus B (27), and all HRVs considered in the present work are genus A. It might thus be easier to switch receptor specificity for serotypes belonging to the same genus. Based on the results of our present work, experiments aimed at changing receptor specificity of the “K-type” major group HRVs are in progress.

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