Identification of the Human Rhinovirus Serotype 1A Binding Site on the Murine Low-Density Lipoprotein Receptor by Using Human-Mouse Receptor Chimeras

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Human rhinovirus serotype 1A (HRV1A) binds more strongly to the mouse low-density lipoprotein receptor (LDLR) than to the human homologue (M. Reithmayer, A. Reischl, L. Snyers, and D. Blaas, J. Virol. 76:6957-6965, 2002). Here, we used this fact to determine the binding site of HRV1A by replacing selected ligand binding modules of the human receptor with the corresponding ligand binding modules of the mouse receptor. The chimeric proteins were expressed in mouse fibroblasts deficient in endogenous LDLR and LDLR-related protein, both used by minor group HRVs for cell entry. Binding was assessed by virus overlay blots, by immunofluorescence microscopy, and by measuring cell attachment of radiolabeled virus. Replacement of ligand binding repeat 5 of the human LDLR with the corresponding mouse sequence resulted in a substantial increase in HRV1A binding, whereas substitution of repeats 3 and 4 was without effect. Replacement of human receptor repeats 1 and 2 with the murine homologues also increased virus binding. Finally, murine receptor modules 1, 2, and 5 simultaneously introduced into the human receptor resulted in HRV1A binding indistinguishable from mouse wild-type receptor. Thus, repeats 1 and/or 2 and repeat 5 are involved in HRV1A attachment. Changing CDGGEAD present in the acidic cluster of module 5 in the human receptor to CDGEAD present in the mouse receptor led to substantially increased binding of HRV1A, indicating an important role of the glutamate residue in HRV1A recognition.

Human rhinoviruses (HRVs) account for more than 50% of all mild upper respiratory infections known as the common cold. Belonging to the family Picornaviridae, they possess an icosahedral capsid composed of 60 copies each of the proteins VP1, 2, 3, and 4 and a single stranded RNA genome with positive polarity roughly 7,100 nucleotides in length. Upon translation, this RNA gives rise to a polyprotein that is cotranslationally and autocatalytically processed to the capsid proteins and to nonstructural polyproteins responsible for viral replication (for a review, see reference 25). Rhinoviruses use unrelated membrane proteins as receptors for cell entry. The 10 minor group HRVs bind the low-density lipoprotein receptor (LDLR), the very low-density lipoprotein receptor (VLDLR), and LDLR-related protein (LRP) (9, 13). These membrane receptors are strongly conserved in evolution, and minor group HRVs attach to cells of a number of different species (31). The 91 major group HRVs bind human intercellular adhesion molecule 1 (ICAM-1) (3, 29, 30) and do not attach to the mouse homologue of ICAM-1 (19).

Despite entering mouse cells, wild-type HRV serotype 2 (HRV2), a prototype minor group rhinovirus, fails to replicate in these cells. This indicates that the host restriction is intra-cellular. A variant of HRV2 that was adapted to grow in mouse L cells showed changes in the nonstructural protein 2C, suggesting that interaction with a specific host factor was involved (37). Similarly, variants of the major group viruses, HRV39 (11) and HRV16 (5), could replicate in mouse L cells when the viral genomic RNA was transfected or human ICAM-1 was stably expressed in the cells to allow for viral attachment. These variants also had changes in 2C. Based on these results, it was believed that wild-type HRVs only replicate in humans and in some primates despite entering cells of other origin. However, our laboratory recently discovered that wild-type HRV1A grows in mouse fibroblasts almost as efficiently as in human cells without requiring adaptation (20). In the course of these studies, we also found that HRV1A preferentially attached to the mouse homologue of LDLR and only weakly bound to human LDLR. Nevertheless, the presence of LRP on human cells is sufficient for effective infection. For these reasons, we decided to use recombinant human-mouse chimeras of LDLR to unravel the basis of this receptor discrimination and to eventually identify the HRV1A attachment site.

A hallmark of all members of the LDLR family is the presence of different numbers of imperfect ligand binding repeats, each about 40 amino acids in length and stabilized by 3 disulfide bonds and a central Ca\(^{2+}\) ion (2). LDLR has seven contiguous repeats, and VLDLR has eight contiguous repeats. In LRP, the repeats are arranged in clusters of 2, 8, 10, and 11 modules. Adjacent to the N-terminal ligand binding domains are regions with similarity to epidermal growth factor precursor, a YWTD domain with a \(\beta\)-propeller structure, followed by a more or less O-glycosylated membrane proximal domain, a transmembrane sequence, and a cytoplasmic domain with AP2.
adapter binding motives conferring clathrin-dependent endocytosis (28). Most of these receptors are also involved in signal transduction (17).

A recent investigation has shown that only a single lysine in the H1 loop of the viral capsid protein VP1 is strictly conserved within all 10 minor group HRVs. This lysine, together with other basic amino acid residues in the vicinity appears to be responsible for attachment to negatively charged residues in the ligand binding domain of the receptors (34). As determined by cryoelectron microscopy image reconstruction of complexes between recombinant soluble human VLDLR and HRV1A, the receptor attaches to these patches of positive surface potential close to the 5 fold axes of the icosahedral viral shell; two consecutive repeats, V2 and V3 of VLDLR, bind two nonequivalent sites present on two neighboring subunits (16). Although the footprint of the receptor on the HRV2 surface was well defined with this technique, its orientation could not be determined due to the small size and the limited resolution of 15 Å.

Whereas the sequences of the ligand binding domain of murine and human VLDLR are very similar (95.1% identity), the two LDLR homologues exhibit only 77% identity (with 226 identical amino acid residues out of 290); these differences must be responsible for their different affinities toward HRV1A. In the present study, we systematically replaced selected ligand binding repeats in the human LDLR with the corresponding ligand binding repeats of the murine homologue and assessed the binding of HRV1A and HRV2 to these chimeric proteins. We demonstrate that LDLR repeat 5 (L5) and L1 and/or L2 provide binding sites for HRV1A. Single repeat in virus binding with a particular role of L5 and L1 and/or L2 in species-specific LDLR attachment of HRV1A.

**MATERIALS AND METHODS**

**Materials.** All chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise specified. Enzymes were from New England Biolabs (Beverly, Mass.). Tissue culture media and supplements were obtained from GibcoBRL (Gaithersburg, Md.). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, Calif.). Tissue culture plates and flasks were from Costar (Cambridge, Mass.). [35S]Methionine/[35S]Cysteine was purchased from Hartmann Analytics (Braunschweig, Germany). Benzamine was from Merck (Darmstadt, Germany).

**Antibodies.** Mouse monoclonal antibodies SFS (2.2 mg/ml) against HRV2 (27), 5P9 (hybridoma supernatant) against HRV1A, and chicken immunoglobulin Y (IgY) against the ligand binding domain of LDLR (25 mg/ml) (12) were prepared in the laboratory. Alexa Fluor-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, Oreg.). Cyanine dye-conjugated secondary antibodies and horseradish peroxidase (HRP)-conjugated goat anti-chicken antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, Pa.).

**Cells and viruses.** Simian virus 40 large-T-antigen-immortalized murine fibroblasts (M1) and cells deficient in LRP and LDLR (M4) (6, 10), were kindly provided by Joachim Herz (Southwestern Medical Center, Dallas, Tex.). HRV2 and HRV1A (American Type Culture Collection, Rockville, Md.) were grown and metabolically labeled with [35S]Methionine/[35S]Cysteine was purchased from Hartmann Analytics (Braunschweig, Germany). Benzamine was from Merck (Darmstadt, Germany).

**Construction of LDLR chimeras, site-directed mutagenesis, and expression in M4 cells.** All chimeras of the LDLR ligand binding domain were generated by PCR from human (36) and mouse (obtained from Orbigen, San Diego, Calif.) LDLR cDNAs used as templates with the primers listed in Table 1 by following standard protocols. Site-directed mutagenesis (8) was carried out by overlap extension PCR with the primers shown in Table 1 by using a human receptor cDNA, already containing mouse repeat 4, as a template. Receptor DNA was inserted into pEFhLDLR806 (20) by using two BstXI restriction sites present in the vector and in the human LDLR cDNA sequence. Note that the sequencing of the novel receptor revealed that V23, G27, K61, P145, K155, A186, and E187 (Swissprot entry) are in fact A23, C27, E61, Q145, N155, G186, and R187, respectively. The latter sequence is identical with that supplied by Orbigen.

**TABLE 1. Primers used in the construction of chimeras and mutants by PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>mLDR.F</td>
<td>TAGCTCAATTGCACTGAGTAGGACACCAGGGATCTGATGCCAGTCCCTGGCACTGCTCACCTGATGGGCTCATCAGTCAT</td>
</tr>
<tr>
<td>hLDR.F</td>
<td>CAATGCGTACTGCTCACCTGATGGGCTCATCAGTCAT</td>
</tr>
<tr>
<td>mLDR.R</td>
<td>CAGTCAGTCGAGTACCCACTGATGGGCTCATCAGTCAT</td>
</tr>
<tr>
<td>hLDR.R</td>
<td>TGCACTCAACTACACAGTCAGTTGGAACACTTCAAC</td>
</tr>
<tr>
<td>mLR2.S</td>
<td>CCAGAAGCAGTGTCCTCCAGG</td>
</tr>
<tr>
<td>hL2.R.S</td>
<td>TCCCTGGGACAGCTTCTTG</td>
</tr>
<tr>
<td>mLR3.S</td>
<td>TCCCTGGCGGTTGAACCTGCTCGCCGGCC</td>
</tr>
<tr>
<td>hL3.R.S</td>
<td>GCCCAAAAGGGTGACCGGCAGGACCTGCTCACCTGATGGGCTCATCAGTCAT</td>
</tr>
<tr>
<td>mLR4.S</td>
<td>GCCCTGCAAGGGTCCGAGACCAGGTGGCAAACCTGATGGGCTCATCAGTCAT</td>
</tr>
<tr>
<td>hL4.R.S</td>
<td>GGCCTGTCTCCAGACCCCTACAGGCGTTCGAGGACCTGATGGGCTCATCAGTCAT</td>
</tr>
<tr>
<td>mLR5.S</td>
<td>CAGGGTTGCCACCGGCCAGTTC</td>
</tr>
<tr>
<td>hL5.R.S</td>
<td>CACTGCGCGGTTGACCGACCTGCTCGCCGAGT CATCAGTCAT</td>
</tr>
<tr>
<td>mLR7.S</td>
<td>ATCAAGGAGTGCGGAGCACAGAATGCA</td>
</tr>
<tr>
<td>hL7.R.S</td>
<td>TGCAGTCGTTCCGACCTCCTCGTTAGTGGGCTCATCAGTCAT</td>
</tr>
</tbody>
</table>

* Human LDLR sequences are shown in boldface type; mouse sequences are underlined. Sequences identical in both species are shown in boldface type and underlined. hLDR.F is complementary to the 5’ region flanking the LDLR cDNA in pEFhLDLR806. F, forward; R, reverse; S, sense; AS, antisense. Primers given in the lower section were used for site-directed mutagenesis, e.g., SSWR->RSWV AS is the reverse primer used to change SSWR to RSWV.
LDLR chimeras were stably transfected into M4 cells by using Lipofectamine 2000. After selection in puromycin (2 μg/ml), individual clones were isolated and screened for LDLR expression by immunofluorescence microscopy.

**Total cell extracts.** Cells (1.2 × 10^8) stably expressing the LDLR chimeras were seeded in 6-cm-diameter culture dishes and attached during 1 h of incubation at 37°C. After washing with phosphate-buffered saline (PBS), 250 μl of nonreducing Laemmli sample buffer containing 62 U of benzamidine was added. Cells were lysed for 1 h at room temperature under agitation. Ten microliters of the lysates was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

**Western blots.** Total cellular proteins were separated under nonreducing conditions on SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.). The membranes were blocked with PBS containing 2% Tween 20 (blocking buffer) for 1 h and incubated with an IgY preparation from a chicken immunized with recombinant human LDLR, diluted 1:5,000 in PBS-0.1% Tween 20 (incubation buffer), for 1 h. The membranes were washed three times with incubation buffer and incubated for 1 h with HRP-conjugated goat anti-chicken antibody (1:5,000). HRP was detected by using the ECL kit (Pierce, Rockford, Ill.) and Kodak autoradiography films.

**Virus overlay blots.** HRV2 and HRV1A were metabolically labeled with [35S]methionine/[35S]cysteine (15). Membranes were blocked with Tris-buffered saline containing 2 mM CaCl2 and 2% Tween 20, probed with an IgY preparation from a chicken immunized with HRV2 and HRV2/m1 in 10 ml of Tris-buffered saline containing 2 mM CaCl2 and 0.1% Tween 20 for 1.5 h, and washed three times for 10 min with incubation buffer. The membranes were air dried and autoradiographed for 24 h with Kodak MR films.

**Fluorescence microscopy.** Cells were grown on coverslips until 70% confluent and infected with HRV2 or HRV1A at 35 PFU/cell in minimal essential medium supplemented with 2% FCS, 30 mM MgCl2, 2 mM L-glutamine, and antibiotics (infection medium) for 20 min at 34°C. They were washed once with PBS, fixed in 3% paraformaldehyde in PBS for 15 min at room temperature, and quenched for 10 min in 50 mM NH4Cl in PBS. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed again with PBS, and blocked in 5% FCS in PBS for 10 min. Incubation with monoclonal antibody 5F9 (against HRV2, diluted 1:250 in PBS containing 1% FCS) or monoclonal antibody 5F9 (against HRV1A, hybridoma supernatant) was performed for 45 min at room temperature. For simultaneous detection of LDLR expression, IgY anti-LDLR (diluted 1:1,000 in PBS containing 1% FCS) was used. Cells were washed with PBS and incubated with goat anti-mouse Alexa 488 (1:1,000) and goat anti-chicken Cy3 fluorophore-conjugated (1:250) secondary antibodies for 1 h at room temperature. The coverslips were washed three times with PBS, rinsed briefly in double-distilled H2O, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, Calif.). Samples were viewed under a Leica TCS NT confocal microscope (Heidelberg, Germany). Images were processed by using Adobe Photoshop software with the same parameters for all images.

**Quantification of receptor expression and of virus binding and internalization.** Cells grown in 24-well plates until ~80% confluence were washed twice with PBS, fixed with 3% paraformaldehyde in PBS for 40 min at room temperature, and quenched for 10 min in 50 mM NH4Cl. After washing with PBS, the cells were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h, incubated with anti-LDLR IgY (1:1,000) in PBS containing 0.1% BSA for 1 h, washed three times with 0.1% BSA in PBS, and incubated with HRP-conjugated anti-chicken antibody (1:5,000) for 1 h. After washing four times with PBS, HRP was revealed with 300 μl of 3,3′,5,5′-tetramethyl benzidine (100 μg/ml) and 0.03% H2O2 in 100 mM Na acetate (pH 5.6) for 10 min. The reaction was halted with 1/2 volume of 1 M H2SO4, and absorbance was determined on a microplate reader at 450 nm. For determination of virus binding, about 10,000 cpm of [35S]methionine-labeled HRVs (15) in 200 μl of infection medium was added to subconfluent cells grown in 24-well plates. After incubation for 30 min at 34°C, the cells were washed with PBS, and radioactivity in the supernatants plus wash and in the cells recovered by trypsinization was determined by liquid scintillation counting as described previously (20).

**Model building.** L5 sequences from the species indicated were submitted to automatic modeling with SwissModel (4, 18, 24). The models obtained were superimposed onto the known structure of hL5 by using Magic Fit in SwissPdbViewer, version 3.7, and the surface potentials were calculated and visualized with colors set to −5.0 (red), −3.0 (white), and +1.0 (blue). The superimposed models were arranged identically as to turn the face with the strongest negative charge in L5 towards the viewer; the models are centered on the tryptophan that, in V3, has been shown to be directly involved in interaction with the aliphatic side chain of the conserved lysine in the BC loop of HRV2 (33). The validity of the approach was tested by modeling hL5 based on nuclear magnetic resonance (NMR) structures of hL6 (18zA.pdb and 1djA.pdb) and of LRP Cβ8 (1lr8A.pdb) but omitting the X-ray structures of hL5 (1n7A.pdb and 1ajy.pdb).

**RESULTS**

**HRV1A and HRV2 bind with different efficiencies to chimeric human and mouse LDLR in virus overlay blots.** HRV1A binds well to mouse LDLR but only very weakly attaches to the human receptor homologue. To identify the receptor module(s) responsible, ligand binding repeats of the human receptor were systematically replaced by the corresponding mouse modules and binding of the two virus serotypes to these chimeric proteins was assessed. The plasmid pEFLDLR806 (28), encoding human LDLR that lacks 33 amino acids including the coated pit targeting signal at its C terminus, was used as a starting point for the construction of human-mouse LDLR chimeras. Upon stable transfection into mouse M4 cells that are deficient in LDLR and LRP expression as a consequence of gene disruption (10, 35), the truncated receptor was produced at a 25- to 50-fold-higher level than the full-length receptor (28). This is most probably due to reduced internalization and lysosomal degradation. Human-mouse LDLR chimeras, as summarized in Fig. 1, were then constructed and stably transfected into M4 cells. One clone was selected from each construct based on high expression level. The amounts of the recombinant LDLR chimeras present in the cells were compared on Western blots by using antibodies recognizing both human and mouse LDLR (14). As seen in the Western blots in Fig. 2A, the expression levels of the human receptor, the chimera carrying the entire binding domain of the murine receptor (m1-7), and the various other chimeric receptors were similar but not identical; this was confirmed by using an enzyme-linked immunosorbent assay (ELISA)-type assay (Table 2). As expected, for nontransfected M4 cells, no LDLR was seen in the Western blot (Fig. 2A) and the ELISA revealed only background binding (Table 2). Sometimes a second and a third band migrating in front of the main band were observed at various intensities; these polypeptides presumably correspond to misfolded, inactive forms of the recombinant proteins, since they did not bind the HRVs in virus overlay blots (Fig. 2). HRV2 (Fig. 2B) bound to all receptor variants. In accord with earlier results (20), attachment of HRV1A to the human receptor was not detectable (Fig. 2C). Binding to m3-4 and to m4 was also below the detection limit. However, HRV1A bound to all constructs containing m5 alone or m5 together with other mouse repeats. In a separate experiment, HRV binding to chimeras containing mouse repeats m1 and m2 was also assessed (right panels). HRV2 as well as HRV1A bound to m1-7, m1-2, and m1-2 and m5. These results suggest that, in accord with earlier results (20), attachment of HRV1A to the human receptor was not detectable (Fig. 2C). Binding to m3-4 and to m4 was also below the detection limit. However, HRV1A bound to all constructs containing m5 alone or m5 together with other mouse repeats. In a separate experiment, HRV1A bound to all constructs containing m5 alone or m5 together with other mouse repeats. In a separate experiment, HRV1A bound to all constructs containing m5 alone or m5 together with other mouse repeats. In a separate experiment, HRV1A bound to all constructs containing m5 alone or m5 together with other mouse repeats.
20 min at 34°C to allow for virus binding and internalization (28). The cells were fixed and stained with anti-LDLR antibodies, verifying the expression of the chimeric proteins (Fig. 3, upper and lower panels). HRV binding was assessed with monoclonal antibodies specific for the respective viral serotypes followed by Alexa 488-labeled secondary antibodies. HRV2 bound similarly to all chimeras (Fig. 3), whereas HRV1A bound well to all receptor constructs except those containing murine repeats 3-4 or 4; likewise, it did not bind to the human receptor. This, again, strongly suggests a predominant role of repeat 5 and repeats 1 and/or 2 in the interaction between HRV1A and LDLR.

**Amino acid residues involved in virus binding.** Since murine repeat 5 alone in the context of the human receptor was able to confer HRV1A binding, we decided to analyze this repeat in more detail. Sequence comparison of L5 from mice and humans reveals that, of the 39 amino acid residues (Swissprot annotation), 30 are identical (Fig. 4). These include the four most conserved aspartic and glutamic acids involved in chelating the central Ca²⁺ ion (2) and the six cysteines. To further cut down the number of amino acid residues potentially responsible for receptor discrimination, we compared various species whose LDLR sequences are known, with respect to discriminative binding. Lysates from HeLa cells, mouse fibroblasts (M1), rat embryo fibroblasts (REF), Chinese hamster ovary cells (CHO), and rabbit kidney cells (RK13) were analyzed in parallel by virus overlay blotting with radiolabeled HRV2 and HRV1A (data not shown). Whereas all LDLR homologues bound HRV2 to a similar extent, HRV1A showed strong binding to the mouse and to the rabbit protein only, and it did not bind to human protein and only weakly bound to rat and hamster LDLR (Fig. 4). The difference in HRV1A binding

FIG. 1. Scheme of the chimeric LDLR used. In human LDLR, carrying a deletion of part of the C-terminal cytoplasmic domain including the clathrin localization signal, ligand binding repeats (white boxes) were exchanged for the corresponding murine repeats (gray boxes) and the cDNA plasmids were stably transfected into receptor-negative M4 cells. The scheme is not drawn to scale, and the transmembrane region and cytoplasmic tail are indicated by triple angle brackets. EGF, epidermal growth factor.
between rat and mouse LDLR was unexpected, since only 2 residues in L5 are not identical. It is thus likely that these residues are directly involved in virus binding.

Based on the strong basic surface potential of the receptor binding site in all minor group HRVs (34) and the known involvement of negative charges of the LDLR in ligand recognition (1), we assumed that a negative surface potential is also responsible for HRV recognition. This led us to suggest that Glu199 in the mouse protein (Fig. 4) was involved in the interaction with HRV1A, as it is replaced by uncharged residues in the weakly binding human, rat, and hamster homologues. Furthermore, it is the only residue different between mouse and rat L5 within the negative charge cluster surrounding the central Ca\(^{2+}\) ion.

**Site-directed mutagenesis of hL5.** To assess whether Glu199 in mouse L5 was indeed required for binding of HRV1A and to verify that no other residues were involved, selected amino acid pairs in hL5 were exchanged for those present in the mouse (Fig. 4). M4 cells were stably transfected with the plasmids encoding human LDLR with L5 carrying the mutations indicated in Fig. 4. Clones showing high-level expression of the foreign protein, as judged by HRV2 binding by immunofluorescence microscopy, were selected. Discriminative virus binding was then assessed by immunofluorescence as in Fig. 3.

### TABLE 2. LDLR expression of and HRV binding to cell lines stably transfected with human-mouse LDLR chimeras\(^c\)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>LDLR expression(^a)</th>
<th>% of virus bound(^b) HRV2</th>
<th>HRV1A</th>
<th>HRV1A/HRV2 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4</td>
<td>0.23 ± 0.032</td>
<td>2.9 ± 0.85</td>
<td>5.6 ± 1.82</td>
<td>1.930</td>
</tr>
<tr>
<td>M4-h</td>
<td>0.85 ± 0.075</td>
<td>34.6 ± 3.08</td>
<td>8.9 ± 4.38</td>
<td>0.258</td>
</tr>
<tr>
<td>M4-m1-7</td>
<td>0.61 ± 0.026</td>
<td>23.2 ± 3.73</td>
<td>27.6 ± 5.84</td>
<td>1.191</td>
</tr>
<tr>
<td>M4-m1-5</td>
<td>0.61 ± 0.082</td>
<td>30.4 ± 6.82</td>
<td>36.3 ± 4.69</td>
<td>1.195</td>
</tr>
<tr>
<td>M4-m3-5</td>
<td>0.97 ± 0.059</td>
<td>22.1 ± 2.85</td>
<td>25.5 ± 6.13</td>
<td>1.150</td>
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<tr>
<td>M4-m3-4</td>
<td>0.99 ± 0.003</td>
<td>23.6 ± 4.54</td>
<td>28.3 ± 10.47</td>
<td>1.197</td>
</tr>
<tr>
<td>M4-m4</td>
<td>0.93 ± 0.039</td>
<td>44.8 ± 10.39</td>
<td>5.7 ± 1.98</td>
<td>0.127</td>
</tr>
<tr>
<td>M4-m5</td>
<td>0.76 ± 0.044</td>
<td>23.2 ± 4.88</td>
<td>6.9 ± 3.58</td>
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<tr>
<td>M4-m1-2</td>
<td>0.75 ± 0.034</td>
<td>15.1 ± 2.68</td>
<td>9.1 ± 1.16</td>
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<tr>
<td>M4-m1-2</td>
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<td>M4-m4-5</td>
<td>0.48 ± 0.040</td>
<td>21.8 ± 7.23</td>
<td>30.0 ± 2.54</td>
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<tr>
<td>AF&gt;SL</td>
<td>0.68 ± 0.055</td>
<td>19.1 ± 3.31</td>
<td>6.5 ± 2.73</td>
<td>0.339</td>
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<tr>
<td>LSG&gt;GSS</td>
<td>1.08 ± 0.047</td>
<td>52.3 ± 11.11</td>
<td>7.1 ± 4.48</td>
<td>0.135</td>
</tr>
<tr>
<td>SSWR&gt;RSWV</td>
<td>0.96 ± 0.045</td>
<td>31.8 ± 4.91</td>
<td>6.0 ± 2.48</td>
<td>0.190</td>
</tr>
<tr>
<td>GP&gt;EA</td>
<td>0.67 ± 0.066</td>
<td>16.2 ± 4.76</td>
<td>23.8 ± 6.82</td>
<td>1.475</td>
</tr>
</tbody>
</table>

\(^a\) LDLR expressed on the cell surface was determined by ELISA. The A\(_{450}\) of the HRP substrate was determined on a microplate reader.

\(^b\) \(^{35}\)S-labeled HRVs were incubated with the cells, and bound radioactivity is given as the percentage of total radioactivity present in the assay.

\(^c\) Results for LDLR expression and virus binding are means of the results from 3 to 5 independent experiments ± standard deviations.

![](https://example.com/fig3.png)

**FIG. 3.** Virus binding assessed by indirect immunofluorescence microscopy of M4 cells stably transfected with the human-mouse chimeric LDLR as indicated. Cells grown on coverslips were incubated with \(10^6\) TCID\(_{50}\)/ml (MOI, \(\sim35\)) of the respective HRV serotypes for 20 min at 34°C, washed, and fixed, and eventually bound virus was detected with monoclonal antibody 8F5 (HRV2) or 5F9 (HRV1A) followed by Alexa 488-conjugated secondary antibody. Receptor expression was monitored with IgY directed against LDLR followed by Cy3-conjugated anti-chicken IgY antibody. Cells were viewed under a confocal fluorescence microscope, and all samples were photographed with the same parameter settings. Note that the two panels on the right (m1-2 and m1-2m5) are from a different experiment.
Except for the exchange of GlyPro198-199 for GluAla (GP→EA), all mutations were without effect on HRV1A binding (Fig. 5). Only the latter mutation resulted in substantially increased HRV1A binding. These results underscore the importance of the glutamic acid residue at this position for the recognition of HRV1A. Since the alanine next to the glutamic acid residue is also present at the equivalent position in the rat receptor, which only binds weakly, we believe that this residue is not important. Nevertheless, in the absence of additional mutational data, we cannot exclude that Pro200 also contributes by modifying the conformation of Glu199. This result again strongly supports the contention that the acidic cluster is involved in direct interaction with the virus. We thus believe that this face of the molecule contacts the HI and the BC loop of VP1 when complexed to the viral capsid.

Quantification of receptor expression and virus binding. The validity of the attachment tests employed was further verified by using more quantitative assays. All cell clones expressing the various receptor chimeras and mutants were grown in parallel in 24-well plates, and surface expression of the receptors was determined by an ELISA. As seen in Table 2, all receptors were expressed but not to identical levels. This is not unexpected, since integration of the receptor cDNA into the genome occurs largely at random, and positional effects govern the expression level. The cells were then challenged with radiolabeled HRV1A and HRV2, respectively, and incu-
attachment almost identical to that of the entire mouse receptor. This was confirmed by measuring the cell binding of radiolabeled HRVs (Table 2 and Fig. 6). The results are similar to findings by Esser and colleagues (1), who have shown by deletion analysis that L5 was essential for binding of β-VLDL but that other repeats also contributed. The increase in binding upon replacement of hL1 and hL2 for mouse L1 and L2 is in line with the possible involvement of L2 in LDL binding. A variant of LDLR lacking the first two repeats leads to familial hypercholesterolemia (23), but L1 is dispensable for lipoprotein binding (32).

Concentrating on L5, we then analyzed the LDLR of several species for discrimination between HRV1A and HRV2 and found that, in addition to the mouse receptor, the rabbit receptor bound HRV1A strongly, whereas like the human receptor, the rat and hamster receptors displayed only marginal binding. This is particularly interesting, since mouse and rat L5 share all but two residues (Fig. 4). Based on these data, we exchanged selected amino acid residues in L5 of the human receptor (containing mouse L4) for those present in the mouse and found that the change (in italics) from CDGGPD to CDGE4D in the acidic cluster conferred HRV1A binding activity to the human receptor. Since the alanine is also present in the rat, we believe that the exchange of glycine to glutamate is sufficient to acquire specific binding of HRV1A. All of these findings were confirmed by a more quantitative binding assay with radiolabeled HRVs (Fig. 6).

Based on alignments of L4 and L5 with V5 and V6 (repeats 5 and 6 of VLDLR) of various species and by using the X-ray structure of the entire LDLR at low pH, it was proposed (22) that these repeats interact with the β-propeller in the low-pH environment present in endosomal compartments. This latter structure is believed to intramolecularly compete for the binding site with the ligands, resulting in their dissociation from LDLR. According to this model, among other residues, W193, D196, and D200 in L5 (numbering from the first amino acid in the mature protein) establish contacts with E581 and K582 of the β-propeller. W193 is exposed to solvent and interacts with the aliphatic part of the side chains while the acidic groups create a strong negative-charge cluster, establishing interactions with the ε-amino group of the lysine. On these grounds, we believe that the lysine (K224 in VP1 of HRV2), which is conserved in all minor group HRVs, interacts with W193, D196, and D200. Indeed, the recently solved X-ray structure of a complex between HRV2 and V23, a tandem of repeats 2 and 3 of VLDLR (33), shows contacts of the aliphatic side chain of K224 with the tryptophan and of its ε-amino group with the glutamic acid of V3 (compare to Fig. 4). Since the aspartates contribute with their carboxylates to the Ca\(^{2+}\) complex, they are not available for ionic interactions but nevertheless provide a negative electrostatic potential (Fig. 7). Glycine 198 in L5 contributes with its amide oxygen to Ca\(^{2+}\) chelation (2); a glutamic acid at its position might thus have its carboxylate free to make a salt bridge with the lysine in the H1 loop of VP1 on the viral surface.

Comparisons of repeat L5 with repeats CR3, CR7, and CR8 from LRP indicated a very similar overall conformation despite substantial sequence diversity (26). Therefore, we believe that automatic modeling of the three-dimensional structure of repeats with so far unknown structure based on the available

**DISCUSSION**

LDLR is strongly conserved throughout evolution, and the cysteines forming disulfide bonds together with the residues complexed with the Ca\(^{2+}\) ion are at identical positions in most of the ligand binding repeats. Nevertheless, HRV1A was found to bind more strongly to the mouse homologue than to the human version of the receptor (20). To assess whether a particular repeat was responsible for this specificity, we systematically replaced human repeats for mouse repeats (Fig. 1) and expressed the chimeric receptors in a mouse cell line deficient in LDLR and LRP expression. This avoids problems with misfolding that might occur in in vitro binding assays employing bacterially expressed proteins (21). Virus overlay blots from cell extracts (Fig. 2) and immunofluorescence microscopy (Fig. 3) demonstrated that the presence of murine L5 in the context of the human receptor was sufficient for HRV1A binding. The exchange of repeats 1 and 2 with the corresponding murine repeats had a similar effect, and the presence of all of these three murine repeats (m1, m2, and m5) resulted in HRV1A binding almost identical to that of the entire mouse receptor. This was confirmed by measuring the cell binding of radiolabeled HRVs (Table 2 and Fig. 6). The results are similar to findings by Esser and colleagues (1), who have shown by deletion analysis that L5 was essential for binding of β-VLDL but that other repeats also contributed. The increase in binding upon replacement of hL1 and hL2 for mouse L1 and L2 is in line with the possible involvement of L2 in LDL binding. A variant of LDLR lacking the first two repeats leads to familial hypercholesterolemia (23), but L1 is dispensable for lipoprotein binding (32).

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**FIG. 6.** Ratio between HRV1A and HRV2 binding and internalization. M4 cells expressing the various chimeric receptors were grown in 24-well plates and challenged with about 10,000 cpm of radiolabeled HRV1A and HRV2, respectively, for 30 min at 34°C and washed, and cell-associated radioactivity divided by total radioactivity in the assay was determined by liquid scintillation counting. Data are from Table 2.
X-ray and NMR coordinates of related repeats (2, 22) might be rather accurate. First, we assessed the adequacy of the approach by building a model of hL5 with SwissModel (4, 18, 24) by using the NMR structures of hL6 and human LRP CR8 as templates but omitting the two available X-ray structures of hL5 present in the data bank (no other structures were selected as templates by automatic modeling). Despite the sequence identity between hL5 and hL6 and CR8 being only 48 and 64%, respectively, the root mean square deviation between the X-ray structure and the model obtained (including side chains) was 1.24 Å. Considering the much higher identities between hL5 and its homologues (between 70% for rabbit and 79% for rat), models built by inclusion of the two X-ray structures of hL5 are certainly of much higher quality. We thus calculated three-dimensional models of L5 of the different species, previously tested for virus binding (Fig. 4), and examined their surface potentials for the presence of prominent charge patterns (Fig. 7). The electrostatic potential map indicates, as expected, that Glu199 in the mouse substantially increases the negative surface potential in the CDXEXDC sequence (with the 2 D’s contributing their carboxylates to Ca²⁺ chelation) compared to the human homologue. This is similar for the rabbit sequence CDGDADC, containing an aspartate at the same position and for the mutant human protein in which GlyPro was exchanged for GluAla (Fig. 4). The absence of an acidic residue at the equivalent position in the rat and hamster leads to a reduction in the overall negative potential.

Determination of the structure of a complex between HRV2 and various recombinant VLDLR fragments by cryoelectron microscopy revealed that V3 was bound to the HI and BC loops in VP1; however, V2 was also bound, although weakly, to a nonequivalent site on the HI loop of the neighboring subunit (7, 16). Therefore, it is likely that two or even more repeats unequally contribute to the overall binding strength. As indicated by the stronger binding of chimeras containing more than two mouse repeats, in particular m1 and m2 plus m5, repeats other than L5 are able to attach. As L1 has been shown to be dispensable for binding LDL and VLDL (32), most probably only L2 is involved. As shown here for HRV1A, single amino acid differences in the modules have a strong influence on receptor attachment and might result in a loss of binding. Nevertheless, infection is still possible because the virus always finds a suitable binding partner in the large selection of receptor modules present within the members of the LDLR family.
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