The formation of complexes between the minor receptor group human rhinovirus HRV2 and two recombinant soluble receptor fragments derived from the human very low density lipoprotein receptor (VLDLR) and containing ligand-binding repeats 1–3 (MBP-VLDLR_1–3) or 1–8 (MBP-VLDLR_1–8) fused to the carboxyl terminus of the maltose-binding protein was analyzed by affinity capillary electrophoresis. At low molar ratios of receptor: virus, the peaks corresponding to substoichiometric complexes were broad indicating heterogeneity. When the receptors were present in molar excess with respect to the virus, the peaks were sharp, suggesting saturation of all binding sites. For the determination of the stoichiometry, constant amounts of receptor were measured and plotted versus total virus concentration. Extrapolation of the linear part of the resulting curve to zero concentration of free receptor enabled quantitation of the molar ratios of the components present in the complex. Using this method, we determined that about 60 molecules of MBP-VLDLR_1–3 but only about 30 molecules of MBP-VLDLR_1–8 were bound per virion.

Human rhinoviruses (HRVs), members of the picornavirus family, are small (~30 nm in diameter) icosahedral particles composed of 60 copies each of the viral capsid proteins VP1 through VP4 and a positive strand RNA genome of about 7200 nucleotides in length (1). The 102 serotypes recognized to date use three different classes of receptors for cell entry. 91 serotypes (the major group) bind to several members of the low density lipoprotein receptor (LDLR) family (5–7), and 1 serotype (HRV87) binds to a glycoprotein of so far unknown function (8). ICAM-1 is a member of the immunoglobulin superfamily with five immunoglobulin-like domains making up its extracellular part. The LDLR family comprises a number of membrane proteins all having various numbers of highly conserved complement type A repeats of about 40 amino acids in length containing six cysteines each, which exhibit extensive disulfide bridging (for review, see Ref. 9). Whereas the structure of ICAM-1 is known at atomic resolution (10, 11), only the structures of single ligand-binding repeats have been determined by NMR (12) and x-ray crystallography (13).

The binding site of ICAM-1 on HRV14 and on HRV16 has been characterized by electron cryo-microscopy followed by image reconstruction techniques (14, 15). This confirmed the earlier prediction of receptor attachment occurring within the canyon, a cleft encircling the 5-fold axes of the viral icosahedral symmetry (16, 17). In an attempt to determine the binding site of LDL receptors on minor group viruses, we had previously expressed soluble fragments of LDLR in Sf9 insect cells using the baculovirus system (18, 19). Although these recombinant minireceptors are extremely potent in protecting HeLa cells against infection with various minor group virus serotypes, it was not possible to demonstrate the formation of virus-receptor complexes in solution.

The earlier observation of comigration of the detergent-solubilized chicken homolog of human VLDLR and HRV2 on sucrose density gradients (6) prompted us to investigate whether minireceptors derived from human VLDLR might be capable of forming stable virus complexes. We demonstrate here that this is indeed the case. Using electron cryo-microscopy, we have discovered that the receptor binding site does not lie in the canyon; its exact location has been determined (20).

An icosahedron exhibits three kinds of symmetry-related sites. 60 equivalent sites result from 2-fold symmetry and 20 sites from 3-fold symmetry, and 12 equivalent sites are present at the 5-fold axes. Therefore, determining the number of receptor molecules attached to the virion allows predictions of the geometric class of the binding site. For example, we have previously shown that about 30 molecules of the virus-neutralizing monoclonal antibody 8F5 can attach to HRV2 (21); this was then confirmed with the finding that the antibody binds bivalently to two epitopes related by 2-fold icosahedral symmetry (22).

Scatchard analysis of data obtained from surface plasmon resonance measurements revealed about 67 binding sites for ICAM-1 on the surface of HRV14 (23), which is close to the theoretical 60 sites and corresponds well with the data obtained by electron cryo-microscopy showing that 60 receptors are attached to all 60 available sites (14). Nevertheless, steric hindrance, low affinity, or other constraints might lead to lack of complete occupancy of all theoretically available sites. Therefore, knowledge of the stoichiometry can indicate whether the number of receptors attached to the virus is compatible with...
the theoretical value or whether the receptor attaches in an unexpected way.

Electrophoretic separation of analytes by capillary electrophoresis (CE) is based on the differences of mobilities in a given buffer solution. With this method, affinity interactions can be studied in solution, and no attachment of any of the components to a solid support is required (which is the case for enzyme-linked immunosorbent assay (ELISA)-type formats or for surface plasmon resonance methodology). As a consequence, all available sites should be equally accessible. Attachment of antibody or receptor molecules to the viral surface results in a change in the molecular mass of the particle and/or in its charge giving rise to an alteration of the electrophoretic mobility (24). The method appears thus ideally suited for the analysis of complex formation not only because of its resolution power, but also because the amounts of material required for analysis are extremely low (the nanogram range).

In a series of articles we have previously demonstrated the separation of rhinoviral particles from contaminants (25), the resolution of native virus and subviral particles (26), and the detection of antibody-virus complexes (24) by CE. Using this technique, we show here that a recombinant soluble very low density lipoprotein receptor fragment, which is fused to the carboxyl terminus of maltose-binding protein and comprises ligand-binding repeats 1 to 3 (MBP-VLDLR1–3) binds HRV2 with a stoichiometry of about 1:60. A recombinant receptor protein containing repeats 1–8 (MBP-VLDLR1–8) attaches to the virus with a stoichiometry of about 1:30. This suggests that the presence of the additional five repeats is either sterically hindering the attachment of other receptor molecules or that this larger molecule possesses additional binding sites for the virus, which are presumably contributed by the extra repeats.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were obtained from Sigma unless specified otherwise. The background electrolyte (BGE) was 100 mM boric acid containing 10 mM SDS; it was adjusted to pH 8.3 with 1 M NaOH. Samples were dissolved in a buffer solution corresponding to half-diluted BGE without SDS added but containing o-phthalic acid (20 µg/ml) as an internal standard. Buffers were filtered through a 0.45-µm cellulose nitrate membrane before use. All solutions were centrifuged for 2 min in a tabletop centrifuge at 5000 × g prior to CE analysis.

**Virus Preparation**—Human rhinovirus serotypes 2 and 14, as originally obtained from the American Type Culture Collection, were produced and purified from infected cells as described previously (25, 27). The concentration was determined spectrophotometrically using an extinction coefficient of 77 at 260 nm (A260) for a 1% w/v solution (28) and taking into account any contaminants with absorption at 260 nm as identified by CE (24). Purified virus was suspended in 50 mM Tris-HCl, pH 7.4, at an approximate concentration of 3 mg/ml and kept at −80 °C until use.

**Receptor Preparation**—The cDNAs encoding repeats 1–3 or 1–8, respectively, were amplified from pVLDLR (29) by polymerase chain reaction, cloned into pMalc2b (New England BioLabs) and expressed as a carboxyl-terminal appended hexa-His tag the proteins were enriched from the low speed supernatant finally obtained from the American Type Culture Collection, were produced and purified from infected cells as described previously (25, 27). The concentration was determined spectrophotometrically using an extinction coefficient of 77 at 260 nm (A260) for a 1% w/v solution (28) and taking into account any contaminants with absorption at 260 nm as identified by CE (24). Purified virus was suspended in 50 mM Tris-HCl, pH 7.4, at an approximate concentration of 3 mg/ml and kept at −80 °C until use.

**CE Separations**—Appropriately diluted virus (10 µl) was mixed with 10 µl of purified receptor resulting in the molar ratios indicated in the figures. Incubation was for 60 min at room temperature.

**RESULTS**

**Analysis of Complexes between HRV2 and MBP-VLDLR1–3 by Capillary Electrophoresis**—To assess whether stable complexes between HRV2 and MBP-VLDLR1–3 were formed in solution, a constant amount of HRV2 and increasing amounts of MBP-VLDLR1–3 were mixed, incubated at room temperature, and analyzed by CE. As depicted in Fig. 1, addition of the receptor to HRV2 led to a decrease in the virus peak. The peak first became broader, suggesting the presence of a heterogeneous population of virus-receptor complexes, but upon further addition of receptor, the complex peak gradually shifted toward longer migration times and became sharper and more symmetric again with a concomitant appearance of a peak corresponding to free receptor. Obviously, under conditions of excess MBP-VLDLR1–3 the fully saturated complex is formed (Fig. 1, *bottom trace*). Spectral analysis of the peak assigned as virus-receptor complex revealed a significant increase of the ratio of the signals measured at 205 and 260 nm as compared with the ratio of these values for virus alone. Absorbance at 205 nm is nonspecific, whereas absorbance at 260 nm indicates the presence of nucleic acid. For pure HRV2, the ratio of A205/A260 was found to be about 6 (as determined for the virus peak in Fig. 1, *top trace*) whereas it had increased to a value of about 10 in the peak corresponding to the complex (as determined from Fig. 1, *bottom trace*). This clearly supports the assumption of an increase in protein content in the complex with respect to the virus. As expected, there is still an absorption maximum at 260

![FIG. 1. Formation of complexes between MBP-VLDLR1–3 and HRV2.](http://www.jbc.org/content/early/2018/01/24/jbc.M118032203/F1.large.jpg)
were mixed, incubated, and analyzed by CE. The ratios of the components present in the complex between HRV2 and MBP-VLDLR1–3 were determined by CE analysis of HRV14 (0.03 μM); lower trace, HRV14 (10 μl at 0.05 μM) and MBP-VLDLR1–3 (10 μl at 1.8 μM) were mixed, incubated, and analyzed by CE. The ratios of the components in the mixtures are indicated on the right. IS, internal standard, 30 nm, indicating the presence of the genomic RNA (spectra not shown).

VLDLR does not bind major receptor group HRVs (8). To assess whether complex formation was specific, a member of the major group, HRV14, was incubated with MBP-VLDLR1–3 and analyzed by CE under conditions identical to those used for analysis of HRV2. As depicted in Fig. 2, the peak of free receptor was seen to be clearly separated from the peak corresponding to free HRV14. When compared with the control, no change of migration time or shape of the virus peak was noted upon incubation with receptor. This unambiguously indicates the absence of binding of this recombinant receptor to HRV14 and proves the specificity of the interaction with HRV2.

**Determination of Binding Stoichiometry**—To determine the stoichiometry of the components present in the complex between MBP-VLDLR1–3 and HRV2, a constant amount of receptor (10 μl at 5.3 μM) was incubated with increasing amounts of virus (10 μl from 0 to 0.076 μl), and analysis of complexes formed was carried out by CE (electropherograms not shown). The peak area of free MBP-VLDLR1–3, normalized to that of the internal standard, was then plotted versus the total concentration of HRV2 present in the mixture. The resulting curve was linear up to 0.01 μl HRV2 (which corresponds to a molar ratio of 1:145). At lower ratios the slope decreases; this is indicative of saturation of binding (Fig. 3). Extrapolation of the linear part of the curve to zero concentration of receptor (intercept with the x axis) enables quantitation of the amount of virus required to bind all receptor molecules present in the mixture. The binding stoichiometry, as derived from the ratio of these two values was found to be 61±2:1 for the complex MBP-VLDLR1–3-HRV2. The error of the ratio is expressed as the S.D. obtained from the estimate of the intercept by linear fit.

To ensure that the data used for extrapolation are derived under conditions where the majority of receptor binding sites are occupied, we plotted the effective mobility of the complex versus the ratio of receptor and virus in the incubation mix (Fig. 4). The curve was then fitted using the rectangular hyperbolic equation, which allowed determination of mobility at 100% saturation. From this, it becomes clear that the linear part of the curve (Fig. 3), which was used for the extrapolation, represents data points within at least 95% saturation (at a virus concentration of 0.1 μM under the conditions given in Fig. 3).

**Assessment of Complex Formation between HRV2 and MBP-VLDLR1–3 by CE**—In earlier experiments, we have shown that repeats 3–5 of LDLR were sufficient to protect HeLa cells against infection with HRV2, whereas minireceptors with only two repeats failed to protect the cells but were still able to recognize virus in ligand blots (19). Using the same assay, we have further shown that bacterially expressed MBP-VLDLR fusion proteins with repeats 1–3 and with repeats 1–8 are active in virus binding, whereas a minireceptor with repeats 4–6 is inactive (30). This raised the question of whether the entire ligand binding domain encompassing eight repeats might have only one single binding site for the virus or whether a second binding site, presumably residing within the carboxyl-terminal repeats, was present in this receptor. In this latter case, the receptor might allow for cross-linking of multiple virions or might attach bivalently to any of the symmetry-related receptor binding sites present on the viral surface. We thus asked whether the stoichiometry of the components in the complex between HRV2 and MBP-VLDLR1–3 was different from the complex of HRV2 with the smaller receptor.

MBP-VLDLR1–8 was incubated with HRV2 under the same conditions as used for MBP-VLDLR1–3 (see above and Fig. 1) with the exception that the molar ratios between virus and receptor were somewhat different (Fig. 5). Again, the complex forming at low ratios appeared as a broad peak, which became sharper upon increasing the receptor concentration. As mentioned above, this might indicate the transition from a heterogeneous population of complexes with various degrees of receptor saturation to homogenous complexes with all virions carrying the same number of receptor molecules. Nevertheless, comparison of the HRV2-MBP-VLDLR1–3 complexes (Fig. 1) reveals some differences. The peaks of the complexes of HRV2 with the larger receptor are broader at all ratios and appear more heterogeneous. Furthermore, already at a molar ratio of 1:8 between virus and MBP-VLDLR1–8, a substantial change in the migration is apparent. Such an obvious difference cannot be explained exclusively by the different molecular masses of the receptor fragments (~59 kDa for MBP-VLDLR1–3 versus 81 kDa for MBP-VLDLR1–8) as the contribution of the difference in the receptor molecular masses to the total mass of the complex is negligible (e.g. only 2% in this particular case). It might therefore rather reflect higher affinity of the virus for MBP-VLDLR1–8 as compared with MBP-VLDLR1–3 resulting in a larger number of receptor molecules bound per virion under equilibrium conditions.

These differences can be better appreciated from the plot of effective mobilities of both complexes versus the ratios of receptors and virus in the incubation mix (Fig. 4). From this representation, the steep increase in the mobility is clearly visible for the larger receptor. This might be taken to indicate higher affinity (see “Discussion”). Disregarding the region of very low molar ratios of receptor/virus, both curves have the form of a classical binding isotherm attaining a plateau at seemingly full saturation and can be fitted using a rectangular hyperbolic function. The deviation from the hyperbolic appearance might indicate that binding sites on the viral surface exhibit somewhat different affinities for the receptors.

**Different Stability of the Complexes between HRV2 and MBP-VLDLR1–3 versus MBP-VLDLR1–8**—In previous work...
(25), we discovered that the presence of low concentrations of SDS is required for a reliable separation of virus from contaminants or from virus-antibody complexes. Fortunately, the virus-receptor complexes turned out to be stable in SDS at the concentrations used. This is reminiscent of the finding that the receptors bind virus in ligand blots made after polyacrylamide gel electrophoresis in the presence of SDS, provided that no reducing agent is added. Assuming a correlation between attachment mode and stability of the complexes in the presence of SDS, the two different receptor fragments were incubated with HRV2 under identical conditions. Dissociation of preformed complexes by the addition of SDS was then investigated by CE. When preformed MBP-VLDLR$_{1-3}$HRV2 complexes were incubated at room temperature in 10 mM SDS, dissociation was clearly evident at about 20 min, whereas the MBP-VLDLR$_{1-8}$HRV2 complexes failed to dissociate even when incubated for several hours in the presence of 20 mM SDS (data not shown). This is taken to indicate that the latter receptor binds more strongly to the virus under these particular conditions.

**Stoichiometry of the Reaction between HRV2 and MBP-VLDLR$_{1-8}$**—The stoichiometry of the components in the complex between HRV2 and MBP-VLDLR$_{1-8}$ was determined as described for the binding of MBP-VLDLR$_{1-3}$ to HRV2. A constant amount of receptor was incubated with an increasing amount of HRV2, and each sample was analyzed by CE (electropherograms not shown). The peak area of free MBP-VLDLR$_{1-8}$ was then plotted versus the total virus concentration in the mixture (Fig. 6). This resulted in a hyperbolic relationship with an initial linear range up to ~0.013 µM HRV2 (which corresponds to a ratio of receptor to virus of 140:1). Again, the data points in the linear part of the curve correspond to at least 95% saturation of virus with receptor as determined in the same way as for MBP-VLDLR$_{1-3}$ (see Fig. 5). When this
concentration of HRV2 present in the mixtures versus 4.5%). The molar ratios of receptor/virus are indicated for each point. The peak area corresponding to free receptor is shown. Each point represents the mean of two independent experiments (typical relative span 4.5%). The molar ratios between HRV2 and MBP-VLDLR are indicated on the right. IS, internal standard.

![Figure 5](https://example.com/figure5.png)

**FIG. 5.** Formation of complexes between MBP-VLDLR and HRV2. A constant amount of HRV2 (10 μl at 0.026 μM) was incubated with increasing amounts of MBP-VLDLR (10 μl ranging from 0 to 3.6 μM) and were analyzed by CE. The molar ratios between HRV2 and MBP-VLDLR are indicated on the right. IS, internal standard.

![Figure 6](https://example.com/figure6.png)

**FIG. 6.** Determination of the stoichiometry of HRV2 and MBP-VLDLR in the saturated complex. MBP-VLDLR (10 μl at 3.6 μM) was incubated with 10 μl of HRV2 at concentrations ranging from 0 μM to 0.1 μM. The mixtures were then analyzed by CE. The total concentration of HRV2 present in the mixtures versus the normalized peak area corresponding to free receptor is shown. Each point represents the mean of two independent experiments (typical relative span 4.5%). The molar ratios of receptor/virus are indicated for each point.

linear part of the curve was extrapolated to zero concentration of free receptor (intercept with x axis), the amount of virus needed to completely bind the given amount of receptor is obtained. The stoichiometry for the complex MBP-VLDLR-HRV2 as derived from these data was 29 ± 2:1. This is about half of the value seen for MBP-VLDLR (see above).

**DISCUSSION**

Analysis of the binding stoichiometry between a prototype virus of the minor receptor group of human rhinoviruses (HRV2) and a member of the low density lipoprotein receptor family (VLDLR) was analyzed by CE. Previous attempts at isolating defined complexes between various recombinant minireceptors derived from LDLR and expressed in the baculovi-

rus system failed possibly because of extensive aggregation (18) or too low affinity (19). Based on the observation of comigration of the chicken ovarian homolog of this receptor with HRV2 on sucrose density gradients (6) and that of a strong reaction of a VLDL receptor fragment shed from HeLa cells with HRV2 in ligand blots (7, 32), we reasoned that this receptor might exhibit higher affinity toward minor group HRVs than the LDL receptor. Making use of two different VLDL receptor fragments, one encompassing repeats 1–3 and the other the whole ligand binding domain with repeats 1–8 expressed as maltose-binding protein fusions in bacteria (30), we analyzed the binding stoichiometry by capillary electrophoresis. We thus obtained circumstantial evidence for the longer receptor binding more strongly to HRV2 and found that about 60 MBP-VLDLR molecules were accommodated on the viral surface, but only about 30 molecules of MBP-VLDLR bound to HRV2. This leaves us with the question of whether the additional repeats inhibit binding of 60 copies of the receptor (as seen for MBP-VLDLR) because of steric hindrance or whether they are able to simultaneously attach to another site related by icosahedral symmetry. There are some arguments in favor of the latter interpretation. (i) The interaction between MBP-VLDLR and HRV2 is clearly stronger than that of MBP-VLDLR as shown by the lower concentration required to appreciably shift the viral peak. (ii) The complete absence of aggregation indicates that MBP-VLDLR is not able to bind to two virions simultaneously, and (iii) a receptor fragment containing only repeats 4–6 does not bind HRV2 (30) and might act as an inert spacer between repeats 1–3 and repeats 6–8. Furthermore, there are nine additional amino acids between repeats 5 and 6 that have been proposed to impart some flexibility to the molecule allowing the amino terminus and the carboxyl terminus of the molecule to approach each other (33). Indeed, a recent model of the LDL receptor, as obtained by electron cryo-microscopy image reconstruction, suggests that the ligand-binding repeats are not arranged linearly, but there might be a kink between repeats 3 and 4 (34). This could result in a conformation of the molecule, which allows for the simultaneous attachment of amino-terminal and carboxyl-terminal repeats to two symmetry-related viral binding sites. The recent structure determination of a concatamer of ligand-binding repeats 1 and 2 of human LDLR by NMR technology (35) revealed that these two repeats could move freely with respect to each other, and it is thus likely that the whole molecule can adopt various conformations. This might be one of the reasons for its potential to interact with a number of structurally unrelated ligands. Based on an approximate length of 3 nm for one repeat, the total length of the ligand binding domain can be estimated to be about 24 nm. If only two repeats might be available to function as spacers (with the remaining six being engaged in interactions with the virus) the distance between the viral 5-fold axes (about 16 nm) might not permit binding over the 2-fold axes of viral icosahedral symmetry. However, other modes of attachment have also been observed. In the case of rabbit hemorrhagic disease, on virus-like particles, which exhibit 180 equivalent monoclonal antibody binding sites, steric hindrance prevents the simultaneous occupation of 2-fold symmetry-related sites, and the antibodies bind bivalently over local 3-fold axes resulting in 50% occupation (36). If MBP-VLDLR indeed adopts a conformation with a pseudo 2-fold symmetry and binds bivalently, it will be of great interest to determine the nature of the interactions between the viral symmetry-related sites and the two clusters of ligand-binding repeats located at the termini of the receptor molecule.

Recent data of cryo-electron microscopy image reconstruction of complexes between HRV2 and MBP-VLDLR point to
a binding site different from the canyon floor with five receptor molecules attaching very close to the 5-fold axes of symmetry (20). Structure determination of complexes between HRV2 and the receptor containing all eight ligand binding repeats will finally clarify whether the lower stoichiometry of the larger receptor results from bivalent binding or only from steric effects.

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VLDL Receptor Fragments of Different Lengths Bind to Human Rhinovirus HRV2 with Different Stoichiometry: AN ANALYSIS OF VIRUS-RECEPTOR COMPLEXES BY CAPILLARY ELECTROPHORESIS

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