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Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus

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ABSTRACT **A protein binding to a minor-group human rhinovirus (HRV2) was purified from HeLa cell culture supernatant. The amino acid sequences of tryptic peptides showed identity with the human low density lipoprotein (LDL) receptor (LDLR). LDL and HRV2 mutually competed for binding sites on human fibroblasts. Cells down-regulated for LDLR expression yielded much less HRV2 upon infection than cells with up-regulated LDLR. Virus also bound to the large subunit of the α_2-macroglobulin receptor/LDLR-related protein (α_2MR/LRP). LDLR-deficient fibroblasts yielded considerably less virus in the presence of receptor-associated protein (RAP), providing evidence that α_2MR/LRP also acts as a minor group HRV receptor.**

Common colds most frequently arise through infection with human rhinoviruses (HRVs). The 102 antigenically distinct serotypes are divided into two groups based on receptor specificity (1, 2). The major group binds to the intercellular adhesion molecule 1 (ICAM-1) (3–5), and the minor group has been shown to attach to a membrane protein with a relative molecular mass of about 120 kDa (6, 7). ICAM-1 and the poliovirus receptor (8) are members of the immunoglobulin superfamily. As the three-dimensional structures of representative HRVs from the two different receptor groups (9, 10) and of poliovirus (11) show considerable similarity, it might have been expected that the minor group receptor would also belong to this family. However, in this communication we present evidence that minor-group HRVs gain access to the cell via members of the low density lipoprotein (LDL) receptor (LDLR) family (12, 13).

MATERIALS AND METHODS

Purification of HRV2-Binding Protein. Two hundred liters of HeLa cell culture supernatant were concentrated ten times by ultrafiltration, dialyzed against 250 liters of H_2O containing 0.02% NaN_3, and adjusted to contain 20 mM *N*-methylpiperazine hydrochloride (pH 4.5). Precipitated material was removed, and the filtered supernatant was applied to a 0.5-liter Macroprep 50 Q column (Bio-Rad). Bound material was eluted with the same buffer containing 0.5 M NaCl. After adjustment to pH 7.2 with 1 M Tris·HCl (pH 8), the material was loaded onto a 100-ml *Lens culinaris* lectin column (Pharmacia), and bound protein was eluted with phosphate-buffered saline (PBS) containing 0.5 M α-D-methyl glucopyranoside and precipitated with $(NH_4)_2SO_4$ at 50% saturation. The precipitate was dissolved in 200 ml of PBS, the solution was passed over a 40-ml *Jacalin* agarose column (Vector Laboratories), and bound protein was eluted with 120 ml of 0.1 M α-D-methyl galactopyranoside in PBS and precipitated with $(NH_4)_2SO_4$ as above. The precipitate was dissolved in 20 mM *N*-methylpiperazine hydrochloride (pH 4.5) and desalted on a PD-10 column (Pharmacia). Protein was applied onto a Mono Q HR 5/5 column (Pharmacia) and eluted with a gradient of 0–0.5 M NaCl in the same buffer. The binding activity was monitored throughout the purification procedure on ligand blots (7). Active fractions were concentrated to 1.5 ml with a Centricon-30 microconcentrator (Amicon), and the proteins were resolved on a SDS/7.5% polyacrylamide gel under nonreducing conditions. The band corresponding to the binding activity was localized with 0.3 M $CuCl_2$, and the protein was electrophoretically eluted in 50 mM *N*-ethylmorpholine acetate (pH 8.5) containing 0.1% SDS. To remove any contaminants having the same mobility under nonreducing conditions, the protein was then boiled in sample buffer containing 2-mercaptoethanol, run again on a 7.5% polyacrylamide gel, and eluted as described before, omitting SDS in the elution buffer.

Preparation of Tryptic Peptides, Separation, and Sequence Analysis. Twenty micrograms of HRV2-binding protein obtained from the preparative gel electrophoresis was lyophilized and dissolved in 30 μl of 6 M guanidine hydrochloride/0.4 M NH_4HCO_3, pH 7.6; dithiothreitol was added to 4.5 mM. Incubation was at 56°C for 15 min; after cooling, iodoacetamide was added to 8 mM, and the sample was incubated for 15 min at room temperature. Digestion with trypsin (Promega, 800 ng) was for 18 hr at 37°C in 100 mM NH_4HCO_3 (pH 7.6; final volume 200 μl). The peptides were separated on a Waters μBondapak C_{18} column with a linear gradient of 0.06% CF_3COOH in water to 0.052% CF_3COOH/80% CH_3CN/20% H_2O. Some of the peptides were rechromatographed on a Merck Supersphere C_{18} column under identical conditions. Selected peptides were subjected to Edman N-terminal sequencing on an Applied Biosystems model 477A gas-phase sequenator.

Construction of pSVL-LDLr$^+$ and pSVL-LDLr$^-$. The expression plasmids pSVL-LDLr$^+$ and pSVL-LDLr$^-$ were constructed by ligating the 2.6-kb *Hin*dIII fragment (containing the entire coding sequence of the LDLR) from pTZ-1, a derivative of pLDLR-2 (12), with *Xba* I-digested pSVL (Pharmacia). Both fragments were partially filled in with Klenow fragment before ligation. The orientation of the insert was determined by restriction analysis. Transfection was done with the Lipofectin transfection system (GIBCO).

RESULTS AND DISCUSSION

We have previously shown that a protein with binding activity for minor receptor group HRVs was released from HeLa cells upon incubation with buffer at 37°C (14). This

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Abbreviations: HRV, human rhinoviruses; LDL, low density lipoprotein; LDLR, LDL receptor; α_2MR/LRP, α_2-macroglobulin receptor/LDLR-related protein; ICAM-1, intercellular cell adhesion molecule 1; RAP, 39-kDa receptor-associated protein; pfu, plaque-forming unit(s); FH, familial hypercholesterolemia.

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protein was also shed into the medium upon growing the cells under normal tissue culture conditions (not shown). It was purified to homogeneity from HeLa cell culture supernatants by column chromatography and preparative SDS/polyacrylamide gel electrophoresis. The material eluted from the gel migrated as a single band of 160 kDa under reducing conditions and of 120 kDa under nonreducing conditions (Fig. 1*a*). The molecular mass of the soluble binding protein has previously been determined as 84 kDa (14); a protein with this molecular mass recognized by HRV2 on virus overlay blots was also evident as a minor component in the material from the cell culture supernatant. Therefore, we believe the 84-kDa band to be a degradation product of the 120-kDa binding protein. In accordance with previous results (7), the nonreduced protein was specifically recognized by HRV2 on ligand blots (Fig. 1*b*), since it was competitively blocked by the minor-group virus HRV49 but not by the major-group virus HRV89 (see ref. 6). The material run under reducing conditions did not attach any virus. Moreover, when a similar ligand blot was incubated with the major-group virus HRV14, no binding was seen under any conditions (7). The protein eluted from the preparative gel was digested with trypsin, the digest was resolved by reversed-phase HPLC, and selected peptides were subjected to N-terminal protein sequencing. A comparison of the peptide sequences thus obtained with the SwissProt data bank showed them to correspond to regions of the human LDLR (ref. 12; Table 1). Further confirmation was obtained by recognition of this protein by the monoclonal antibody IgG-C7 that binds the LDLR of human and bovine origin (ref. 15; Fig. 1*b*). However, the presence of a valine at position 592 (peptide F) unequivocally identified the isolated protein as a fragment of the human LDLR, since the bovine protein contains an isoleucine at this position (16).

Attachment and internalization of [^{35}S]methionine-labeled HRV2 (^{35}S-HRV2) was then determined with normal human fibroblasts and fibroblasts from a patient with familial hypercholesterolemia (FH, cells deficient in LDLR synthesis). Normal cells grown under conditions that suppress LDLR expression (17) internalized only 8% of HRV2 compared with cells with upregulated receptors (Fig. 2*A*). The amount of HRV2 associated with FH cells was even lower but was independent of the growth conditions. ^{35}S-labeled HRV14 (^{35}S-HRV14) (a major-group virus) was internalized equally well into both cell types regardless of the growth conditions (not shown). The specificity of the attachment of HRV2 to the LDLR was further demonstrated by competition of HRV2 and LDL for the binding sites on the cell surface (Fig. 2*B*). When unlabeled purified HRV2 was present during the incubation of the cells with ^{125}I-LDL (18), binding was reduced to about one-third. Likewise, unlabeled LDL dramatically decreased the binding of ^{35}S-HRV2. Similar to the results of Beisiegel *et al.* (19), who found only partial inhibition of LDL binding by IgG-C7, incubation of the cells with 100 μg of this particular antibody per ml prior to challenge with HRV2 reduced virus binding to about 50% (data not shown). Further proof that the LDLR can bind minor group HRVs came from transfection experiments. COS-7 cells transfected with the eukaryotic expression vector pSVL carrying the entire coding sequence of the human LDLR in the sense (pSVL-LDLr$^+$) or in the antisense (pSVL-LDLr$^-$) orientation were tested for binding. Under conditions that suppress production of endogenous LDLRs, the cells carrying the sense construction bound about twice as much HRV2 as those with the antisense construction or untransfected cells (Fig. 2*C*). Virus binding to the cells closely followed the amount of LDLR present, as demonstrated with an ELISA carried out directly on the cell monolayer using IgG-C7 (data not shown).

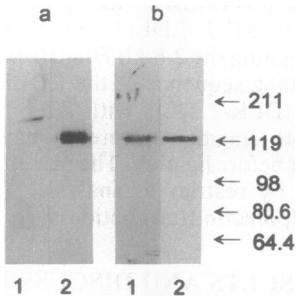

FIG. 1. Analysis of purified HRV2-binding protein. (*a*) Fifty nanograms of purified HRV2-binding protein was electrophoresed on a SDS/7.5% polyacrylamide gel under reducing (lane 1) or nonreducing conditions (lane 2) and visualized by silver staining. (*b*) Twenty-five nanograms of purified HRV2 binding protein was electrophoresed under nonreducing conditions as in *a* and blotted onto nitrocellulose. Lanes: 1, developed with ^{35}S-HRV2 essentially as described (7); 2, incubated with IgG-C7 and visualized with the ECL (enhanced chemiluminescence) detection system (Amersham). Molecular mass markers in kDa are shown at right.

Table 1. Sequences in single-letter code of the tryptic peptides derived from the purified virus binding protein

Peptide	Position*	Sequence†
A	165	XLYVFQGDSSPXXAFEFXXLXXXXI
B	373	XVGSIAYLFFTN
C	420	XYWSDLSQR
D	451	DIQAPXGLAVXXIXSNIYXXXXVL
E	500	XIVVXPVHGFMYXTXXGTPAK
F	584	XAHPFSLAVFEXK

*Numbers are the corresponding amino acid position in the human LDLR (12).
†The amino acid that is different in the bovine LDLR is underlined; "X" is an unidentified amino acid.

From these experiments it was expected that cells deficient in the LDLR would not allow viral entry and therefore would be refractory to infection. Nevertheless, both normal and FH fibroblasts could be infected with HRV2. Therefore, virus yields obtained from both cell types grown under conditions of up- and downregulation of LDLR expression were compared by plaque tests. Normal cells grown in LDL-free medium produced about 100 times more HRV2 than those grown under conditions of suppressed LDLR expression. Only a marginal difference was seen for HRV14 (Fig. 3). Virus progeny obtained from FH cells was slightly lower with both serotypes in the presence of the sterols, probably due to the reduced metabolic activity under these conditions. Surprisingly, however, FH cells that do not express LDLRs at all yielded significantly more HRV2 than did normal fibroblasts with suppressed LDLR expression. This result and the ability of the mutant cells to internalize a small amount of virus (Fig. 2*A*) indicate that there must be additional receptor(s) for these viruses; such receptor(s) may compensate for the lack of the LDLR in FH cells. Differences in the genetic background between wild type and the FH cell line employed may also contribute, as these cells are not isogenic. The recently discovered sterol-insensitive α_2-macroglobulin receptor/LDLR-related protein (α_2MR/LRP) (13) or the Heymann nephritis antigen gp330 (20) are obvious candidates for additional minor-group rhinovirus receptors, as they are structurally related to the LDLR. Membrane proteins from normal fibroblasts and FH cells were therefore separated on a gradient polyacrylamide gel (4–12%) and blotted onto nitrocellulose. This system resolves high molecular mass polypeptides that normally do not enter 7.5% gels. The results of probing such blots with ^{35}S-HRV2 or with an antiserum recognizing the 515-kDa and 84-kDa subunits of α_2MR/LRP and the 39-kDa receptor-associated protein (RAP; ref. 21), revealed that HRV2 indeed binds to a polypeptide comigrating with the large subunit of α_2MR/LRP in

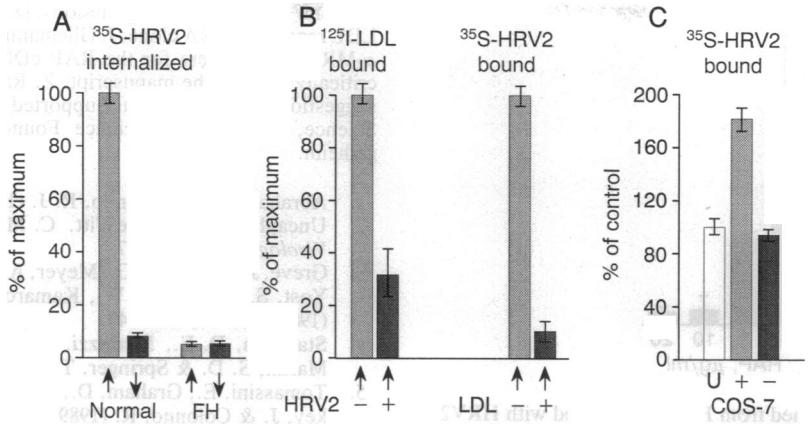

FIG. 2. Internalization of ^{35}S-HRV2 into human fibroblasts, competition of HRV2 and LDL for cell surface binding sites, and binding of ^{35}S-HRV2 to COS-7 cells transfected with pSVL-LDLr$^+$ and pSVL-LDLr$^-$. HRV2 was labeled with [^{35}S]methionine and purified (6). Purity of the labeled virus was confirmed by electrophoresis in 12.5% polyacrylamide gels. (*A*) Normal human fibroblasts or FH cells (NIH repository no. GM00486A) were grown in six-well plates (Nunc) in MEM containing 10% (vol/vol) delipidated fetal calf serum (PAA, Austria) either in absence (↑) or in presence (↓) of 12 μg of cholesterol and 2 μg of 25-hydroxycholesterol per ml for 24 hr. Cells were washed twice with PBS prior to addition of 10,000 cpm of ^{35}S-HRV2 in 0.5 ml of PBS containing 2% bovine serum albumin and 30 mM $MgCl_2$; the mixture was incubated for 60 min at 34°C. After removal of surface-bound HRV2 with PBS containing trypsin at 10 μg/ml and 25 mM EDTA, the cells were washed again and assayed for ^{35}S. Radiolabeled virus internalized per confluent monolayer (mean ± SD for triplicate samples in four independent experiments) is shown. Radioactivity from the normal fibroblasts (typically 1900 cpm per monolayer) grown in the absence of the sterols minus background is shown as 100%. Background was either determined with HRV2 that had been heated to 56°C for 30 min (6) or by competition with a 1000-fold excess of unlabeled HRV2 and was between 40 and 50 cpm per monolayer for both methods. (*B*) Normal fibroblasts were grown as in *A* in the absence of sterols; cells were incubated with 1.4×10^6 cpm of ^{125}I-labeled LDL (^{125}I-LDL) (18) (250 cpm/ng) in the presence (+) or in absence (−) of about 100 plaque-forming units [pfu; between 2400 and 24,000 particles (1)] per cell of purified unlabeled HRV2 or with 10,000 cpm of ^{35}S-HRV2 in the presence (+) or in absence (−) of 80 μg of unlabeled LDL for 60 min at 37°C. The cell-associated radioactivity was determined with a γ or a β counter, respectively. High-affinity binding of ^{125}I-LDL was calculated by subtracting the radioactivity bound in the presence of a 20-fold excess of unlabeled LDL (typically ≈40,000 cpm/mg of total-cell protein) from total LDL binding (≈150,000 cpm/mg). Binding of HRV2 was typically ≈1900 cpm in the absence of the competitor. Maximum binding of each ligand in the absence of the respective competitor was set to 100%. Results are expressed as the mean (±SD) for triplicate monolayers in two experiments. (*C*) COS-7 cells were transfected with pSVL-LDLr$^+$ (+) or pSVL-LDLr$^-$ (−), respectively. Cells were grown in 10% normal fetal calf serum in the presence of the sterols to suppress expression of endogenous LDLR. Binding of ^{35}S-HRV2 was determined 36 hr after transfection as in *B*. Radioactivity (≈250 cpm per monolayer) bound to untransfected cells (u) was set to 100%. Results are expressed as means ± SD for triplicate monolayers.

addition to the LDLR in extracts from normal fibroblasts; as expected, the band corresponding to the LDLR was absent in FH cells (Fig. 4).

Binding of a variety of ligands to α_2MR/LRP is inhibited by RAP (22, 23). When FH cells were infected in the presence of various concentrations of recombinant RAP, the yield of HRV2 was decreased to about 10% at the highest concentration tested (Fig. 5); no such effect was seen for HRV14 (not shown). This confirms that α_2MR/LRP is involved in the entry of HRV2 at least in this particular cell line. RAP, which specifically blocks α_2MR/LRP, did not reduce the virus yield in normal fibroblasts (not shown). This is consistent with the recent finding of Choi and Cooper (24), who have shown that on cells expressing the LDLR and α_2MR/LRP, the contribution of the latter is difficult to demonstrate for ligands shared by both proteins.

It is an open question why FH cells, which internalize less virus than do normal fibroblasts with down-regulated LDLR expression (Fig. 2*A*), yield a higher number of infectious

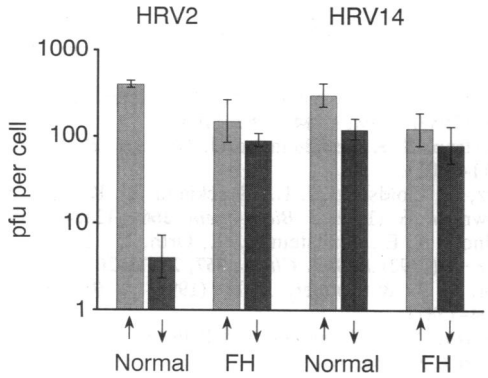

FIG. 3. Fibroblasts with upregulated LDLR yield a 100 times more infectious HRV2 than do fibroblasts with suppressed LDLR expression. Cells were grown as described in Fig. 2*A* and infected with a multiplicity of infection of ≈100 of either HRV2 or HRV14. The cells were carefully washed and incubated for 24 hr at 34°C. Cells were broken by three freeze/thaw cycles, debris was removed, and virus progeny was determined by standard plaque tests on HeLa cells and calculated as pfu per cell. Results are expressed as means ± SD for three duplicate independent determinations. For the symbols, see Fig. 2.

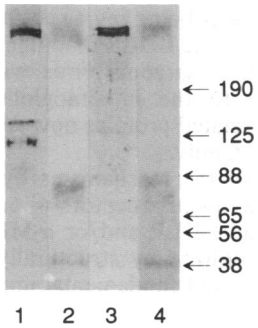

FIG. 4. HRV2 binds to a polypeptide comigrating with the α_2MR/LRP on a 4–12% polyacrylamide gradient gel. Membrane proteins from normal fibroblasts (lanes 1 and 2) and from FH cells (lanes 3 and 4) corresponding to about 2×10^5 cells each were electrophoresed under nonreducing conditions, transferred to nitrocellulose, and probed with ^{35}S-HRV2 (lanes 1 and 3) or with antiserum against α_2MR/LRP (19) (lanes 2 and 4). Note that the nonreduced LDLR (and its presumed degradation product; see also ref. 14) migrate somewhat differently in linear 7.5% gels (compare with Fig. 1). Molecular masses are shown in kDa.

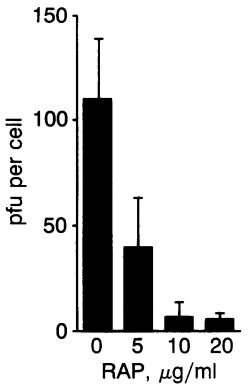

FIG. 5. Virus yields obtained from FH cells infected with HRV2 in the presence of various concentrations of recombinant RAP (21, 22). Cells were grown in 24-well plates and incubated with RAP at 4°C for 2 hr at the concentrations indicated. HRV2 was added at a multiplicity of infection of ≈100, and incubation was continued for an additional 2 hr. The plates were heated to room temperature for 10 min, washed three times with PBS, and incubated for 16 hr at 34°C. Infectious virus was determined by plaque assays as described in Fig. 3. Results are expressed as means ± SD for two duplicate independent determinations.

particles (Fig. 3). At the present time we hypothesize that the internalization and/or uncoating efficiency of the two receptors could be quite different. It is possible that the affinity of the virus for LDLR is higher than for α_2MR/LRP, and therefore most of the virus is internalized by LDLR in those cells that express both macromolecules. However, if this were the case, we have to assume that the uncoating via LDLR would be a rather inefficient process leading to a large percentage of abortively infecting virions. In FH cells, where the LDLR is absent, all virions have to enter via α_2MR/LRP, and a much higher percentage of the internalized virus gives rise to productive infection. Apparently, the low amount of residual LDLR present on down-regulated normal fibroblasts might still be able to overcome any effect of α_2MR/LRP (24). Also, since the cells employed in this study are not isogenic, their α_2MR/LRP might behave differently. To shed light onto these processes we are currently investigating the kinetic parameters of virus attachment and internalization via the two different receptors. Both receptors are ubiquitous in terms of their presence in almost all tissues of a large variety of species (25). Therefore, the question of what determines the host specificity as well as the tissue specificity of minor group HRVs remains to be answered.

While this paper was being reviewed, a report on a small protein also related to the LDLR that functions as a receptor for subgroup A Rous sarcoma virus was published (26). Therefore, similar to the immunoglobulin superfamily, LDLR and LDLR-related proteins now seem to emerge as a new virus receptor family.

The elucidation of the mechanisms by which two completely different classes of receptors—i.e., ICAM-1 for major-group HRVs and LDLR and/or α_2MR/LRP for minor-group HRVs—are utilized by structurally and functionally very similar viruses is of fundamental importance.

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