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The Major Human Rhinovirus Receptor Is ICAM-1

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Summary

The major human rhinovirus receptor has been identified with monoclonal antibodies that inhibit rhinovirus infection. These monoclonal antibodies recognize a 95 kd cell surface glycoprotein on human cells and on mouse transfectants expressing a rhinovirus binding phenotype. Purified 95 kd protein binds to rhinovirus in vitro. Protein sequence from the 95 kd protein showed an identity with that of intercellular adhesion molecule-1 (ICAM-1); a cDNA clone obtained from mouse transfectants expressing the rhinovirus receptor had essentially the same sequence as ICAM-1. Thus, the major human rhinovirus receptor is ICAM-1. The gene for this receptor maps to human chromosome 19, which also contains the genes for a number of other picornavirus receptors.

Introduction

A major determinant of cell tropism and host range restriction in picornaviruses is cell surface receptor specificity (Holland, 1961; Lonberg-Holm et al., 1976). Rhinoviruses, a large family of acid-sensitive picornaviruses that are the major causative agent of the common cold (Hamparian et al., 1987), have been shown to bind to human cells by two classes of receptors (Abraham and Colonno, 1984; Colonno et al., 1986). These workers have shown that approximately 80% of the >100 serotypes of rhinoviruses and several serotypes of coxsackie A virus bind to a single common receptor, the "major" rhinovirus receptor; the remaining 20% bind to one or more other receptors. Tomassini and Colonno (1988) have provided evidence that a protein with an apparent molecular mass of 90,000 daltons is the major rhinovirus receptor.

The receptor binding site on the viral capsid should be a highly conserved structure, since there is very strong selective pressure to maintain it in a functional form. The primary structure of the capsid proteins of three serotypes of rhinovirus, HRV2 (Skern et al., 1985), HRV14 (Stanway et al., 1984; Callahan et al., 1985), and HRV89 (Duechler et al., 1987), have been deduced from the nucleotide sequences of their RNA genomes. The rhinovirus capsid protein sequences all show quite significant homology to one another in certain regions. In addition, the three-dimensional structure of HRV14, a serotype that binds to the major rhinovirus receptor, has been determined to atomic resolution (Rossmann et al., 1985). This work has revealed that HRV14 has an icosahedral protein capsid composed of 60 protomeric units, each composed of the four capsid proteins VP1, VP2, VP3, and VP4. Analysis of this structure revealed two important features of the viral capsid. First, a series of highly exposed peptide loops, which show the greatest variability in amino acid sequence between serotypes, contain residues that have been shown to be the most antigenic to the host immune system (Sherry et al., 1986). Second, a recessed "canyon" present in each protomeric unit contains amino acid residues that are more conserved than other solvent-exposed residues (Rossmann and Palmenberg, 1988), as would be expected of a receptor binding site. These observations, as well as the fact that this canyon has dimensions that would make it inaccessible to an antibody molecule (and therefore resistant to selective pressure from the host immune system), has led to the hypothesis that the canyon is a conserved receptor binding site on the viral capsid (Rossmann et al., 1985). The structure of the complex of influenza virus hemagglutinin protein and its receptor, sialic acid, has provided evidence in favor of this hypothesis in another virus–receptor system (Weis et al., 1988).

A full understanding of virus–receptor interaction will require identification of the viral receptor, characterization of the virus binding portion of the receptor, and, ultimately, elucidation of the molecular contacts between the viral capsid and receptor. In this report we describe the identification, transfection, purification, and reconstitution of the major human rhinovirus receptor (HRR). We also show that this receptor is identical to a previously described cell surface protein, intercellular adhesion molecule-1 (ICAM-1).

Results

Monoclonal Antibodies to HeLa Cells That Protect against Rhinovirus Infection

Monoclonal antibodies (MAbs) which recognize the HRR were raised using a strategy similar to that used by others to identify viral receptors (Nobis et al., 1985; Minor et al., 1984; Colonno et al., 1986; Crowell et al., 1986; Hsu et al., 1988). Three rhinovirus serotypes were used in this procedure: HRV2, which binds to the minor rhinovirus receptor; and HRV3 and HRV14, which bind to the major rhinovirus receptor (Abraham and Colonno, 1984). Hybridomas were prepared from mice immunized with HeLa cells, and hybridoma culture supernatants were screened for the ability to protect cell monolayers against infection by HRV14. Five hybridomas were obtained and cloned; the MAbs from these hybridomas prevented infection of HeLa cells by HRV14 and HRV3 and had no effect on HRV2 infection. These results demonstrate that these MAbs have the specificity expected of MAbs that recognize the major rhinovirus receptor. One of these antibodies, c78.4A, is discussed further in this report.

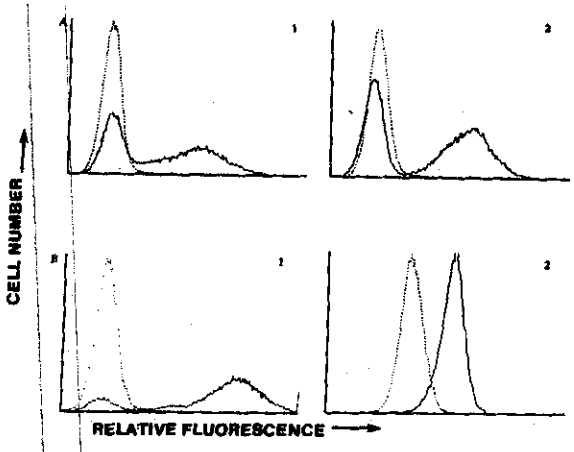


Figure 1. Expression of a Human Rhinovirus Receptor on Mouse L Cell Transfectants

(A) Analysis of primary transfectants sorted using HRV14-specific staining. Dotted lines: no virus or normal serum control. Solid lines: virus or antibody staining. (1) Virus-stained cells after three sorts. (2) Antibody c78.4A–stained cells after three sorts.

(B) Analysis of secondary transfectants. (1) Secondary transfectants after four sorts. Dotted line: control staining. Solid line: antibody c78.4A staining. (2) Comparison of c78.4A staining on cloned primary transfectant HRR1 (dotted line) and HE1 secondary transfectant (solid line).

Isolation of a Mouse Transfectant Expressing Elevated Levels of HRR

As a parallel approach to identifying the HRR, mouse L cell transfectants were isolated that exhibit a virus binding phenotype. These transfectants were obtained from a pool of mouse transfectants (transfected with high molecular weight genomic DNA from HeLa cells) by fluorescence-activated cell sorting (FACS) and a rhinovirus cell binding assay (see Experimental Procedures). To provide further evidence that the MAbs described above were directed against the HRR, one of the MAbs, c78.4A, was tested for the ability to bind to the virus receptor–positive transfectant pool. As shown in Figure 1A, this MAb bound to HeLa and the transfectant pool but not to Ltk$^-$ cells. These data suggest that the molecule(s) defined by the MAbs that inhibit infection and from the transfection of a rhinovirus binding phenotype are the same.

Cloned cell lines derived from the primary transfectants were used to prepare secondary transfectants. A fraction of the secondary transfectant population exhibited reproducibly higher relative fluorescence than either the primary transfectants or HeLa cells. These cells were selected by FACS with MAb c78.4A, and a cloned cell line designated HE1 was established that stably exhibited 5- to 10-fold greater relative fluorescence than HeLa cells (Figure 1B). Based on quantitative binding studies using ^{125}I-c78.4A IgG, HeLa cells were shown to express 6×10^4 sites per cell and HE1 cells were shown to express 3×10^5 sites per cell, consistent with the FACS analysis. This overexpressing cell line was used as a source of material for many of the experiments described below.

Chromosomal Mapping of the HRR Gene

A human–mouse somatic cell hybrid panel was used to map the HRR to a human chromosome. Hybrid cells were labeled by indirect immunofluorescence with MAb c78.4A and analyzed by fluorescent microscopy and by FACS. The pattern of reactivity with cell lines of the hybrid panel is indicated in Figure 2. Correlation of the expression of the antigen recognized by c78.4A with the partial human genomes in the hybrid cells allows the unambiguous assignment of the HRR gene to human chromosome 19.

	Human Chromosome																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	c4A
I MA9- 4.11	+	+	−	+	+	+	−	−	−	−	−	−	−	−	+	−	+	+	−	+	+	−	+	−
III MA9- 3	−	−	+	+	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−
III MA9- 4	−	+	−	−	−	−	−	+	−	−	−	+	+	+	+	−	+	+	+	−	+	−	+	+
III MA9-13	−	+	−	+	−	+	−	−	+	−	+	+	+	+	+	+	−	+	+	−	+	−	+	+
III MA9-14	+	−	−	+	−	+	+	+	−	+	+	+	+	+	+	−	+	+	−	+	+	−	+	−
VI MA9- 3	+	−	+	+	−	−	−	+	−	−	−	+	+	+	−	+	+	+	−	−	−	−	+	−
VI MA9-14B	+	−	+	+	+	+	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	+	+	+
CONCORDANT	2	5	3	2	4	3	4	4	5	4	5	4	4	5	4	4	3	4	7	3	5	5	3	
DISCORDANT	5	2	4	5	3	4	3	3	2	3	2	3	3	2	3	3	4	3	0	4	2	2	4	

Figure 2. Assignment of the HRR Gene to Human Chromosome 19

Shown is the distribution of human chromosomes among the hybrid cell lines as determined by karyotype and isozyme analysis (Kamarck et al., 1984). Results of indirect immunofluorescence assays with monoclonal antibody c78.4A are summarized in the column headed "c4A." Perfect concor-

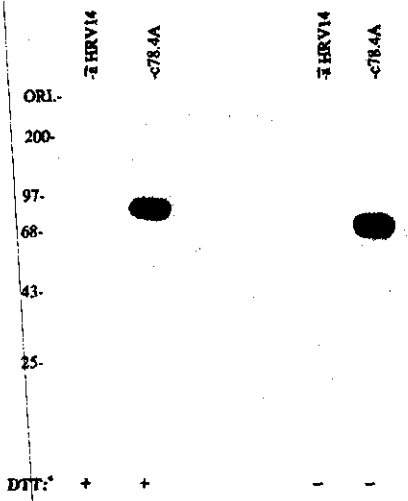


Figure 3. Immunoprecipitation of HRR from ^{125}I-Labeled HeLa Cells

Autoradiogram of an 8% SDS–polyacrylamide gel of immunoprecipitates analyzed under reducing (+DTT) and nonreducing (−DTT) conditions, as indicated. Cell extracts were immunoadsorbed with either a control IgG–resin (α-HRV14) or anti-HRR IgG–resin (c78.4A). Positions of protein molecular weight standards (× 10^{-3}) are indicated on the left.

Identification and Purification of the HRR Protein

The protein recognized by MAb c78.4A was identified by immunoabsorption of ^{125}I-surface-labeled HeLa cell extracts with monoclonal antibody c78.4A IgG–Sepharose. Analysis by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single labeled polypeptide with an apparent molecular mass of 95,000 daltons; a slightly lower apparent molecular mass of 87,000 daltons under nonreducing conditions indicates the presence of intrachain disulfide bond(s) in the protein (Figure 3). All of the other monoclonal antibodies described above also recognized a 95,000 dalton cell surface protein on HeLa cells (data not shown). Receptor protein from mouse transfectant cell lines had approximately the same size, but greater heterogeneity in apparent molecular mass than HRR from HeLa cells (see Figure 5).

Using metabolic labeling in the presence of [^{35}S]cysteine, it was possible to partially characterize the HRR protein. When HRR was synthesized in the presence of tunicamycin, a poison that inhibits asparagine-linked glycosylation, a species with an apparent molecular mass of 54,000 daltons was obtained, indicating that the HRR is a glycoprotein (Figure 4). In the presence of swainsonine, an inhibitor of mannosidase II and the processing of core-glycosylated asparagine-linked oligosaccharides, a species of 80,000 daltons was obtained in HeLa cells. In HE1 cells a species of 78,000 daltons was obtained in the presence of swainsonine, indicating either an underutilization of all the carbohydrate acceptor sites or a difference in the form or stability of the core oligosaccharide unit in the two cell types. Partial digestion of core-glycosylated HE1 HRR with endoglycosidase H indicated the presence of 7–8 oligosaccharides on the HRR protein (data not shown).

For further biochemical analysis the receptor protein was purified from HeLa cells and HE1 cells by a combination of lectin and monoclonal antibody affinity chromatography. A typical preparation of HRR protein purified from HE1 cells is shown in Figure 5. The yield of receptor protein was approximately 5 fg per HeLa cell and about 25 fg per HE1 cell. Assuming a protein molecular mass of 54,000 daltons (based on the apparent molecular mass of unglycosylated protein), these values would translate into 6×10^4 molecules per HeLa cell and 3×10^5 molecules per HE1 cell, in agreement with the number of MAb binding sites per cell (assuming quantitative recovery of HRR protein).

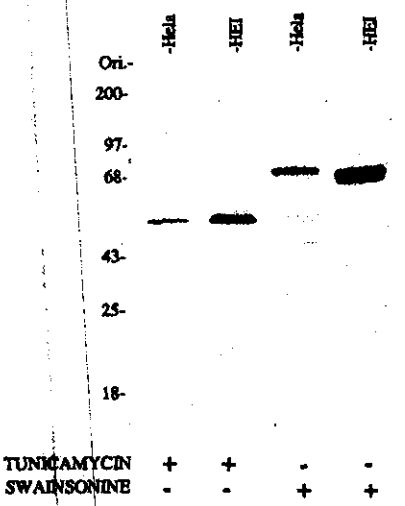


Figure 4. Analysis of Biosynthetic Precursors of HRR

[^{35}S]cysteine-labeled HRR was synthesized in the presence of tunicamycin or swainsonine in HeLa or HE1 cells, as indicated. Samples were analyzed on 8% SDS–polyacrylamide gels and subjected to fluorography.

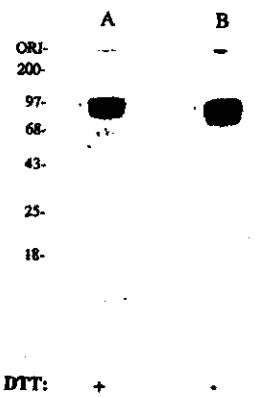


Figure 5. Purified HRR Protein

A silver-stained 7%–20% gradient SDS–polyacrylamide gel of 500 ng of HRR protein purified from HE1 cells. Samples were analyzed under reducing (+DTT) and nonreducing (−DTT) conditions, as indicated. Positions of protein molecular weight standards (× 10^{-3}) are indicated on left.

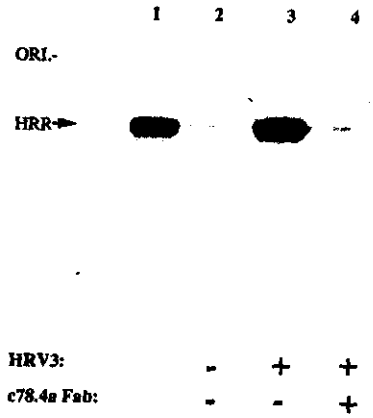


Figure 6. Demonstration of Virus–Receptor Binding with Purified Components

^{35}S-labeled native HRR from HE1 cells (final concentration 0.5 μg/ml, 10^{-8} M) was incubated with or without HRV3 (final concentration 10 μg/ml, 1.7×10^{-9} M) or c78.4A Fab (final concentration 200 μg/ml, 1.3×10^{-6} M), as indicated, and subjected to centrifugation as described in Experimental Procedures. Irrelevant monoclonal antibodies had no effect on binding (data not shown). Pellets were resuspended in SDS gel loading buffer, run on a 7%–20% SDS–polyacrylamide gel, and subjected to fluorography. Lane 1: one-tenth of the HRR added to reactions. Lanes 2–4: material pelleted from indicated reaction mixtures.

Demonstration of Virus–HRR Interaction with Purified Components

To demonstrate the virus binding activity of the purified 95,000 dalton protein, an assay was devised based on the ability of [^{35}S]cysteine-labeled native HRR to pellet with virus in an air-driven centrifuge. As can be seen in Figure 6, when HRV3 (a rhinovirus that belongs to the major receptor class) and ^{35}S-native HRR (prepared from HE1 cells) were incubated together for 30 min at 37°C, approximately 20% of the total HRR could be pelleted with the virus. The pelleting of HRR was completely inhibited by incubation in the presence of an Fab fragment of MAb c78.4A. These data clearly demonstrate that purified receptor binds specifically to rhinovirus in the absence of other cellular components.

Protein Sequence Analysis of HRR Protein

No sequence could be obtained from intact purified HRR protein, suggesting that it has a blocked amino terminus. HRR was then subjected to limited or complete proteolytic digestion to produce large protein fragments or small peptides, respectively. Discrete protein fragments from limited digestion were separated by SDS-PAGE and electroblotted onto aminopropyl-glass fiber filters or polyvinylidene difluoride membranes for sequencing. Peptides from complete digests were isolated by reverse-phase chromatography for sequencing. Eight unique peptide sequences derived from these experiments are shown in Table 1. One hundred five residues of HRR protein sequence were confidently assigned, and 36 additional residues were tentatively assigned. The majority of the sequence information was obtained from HRR purified from HE1 cells. In one case two peptides, one from protein isolated from human (HeLa) cells (RP/T-4; not shown) and one from HE1 mouse transfectants (RP/LE-19), yielded the same sequence (FPLPIGESVTVTR) over 13 residues, providing further evidence for the identity of receptor protein from human and transfectant sources.

These sequences were used to search the available protein and nucleic acid sequence data banks. A match of all of the peptide sequences was made with an entry in the MIPSX (Martinsrieder Institut für Proteinsequenzen) data base: ICAM-1 (Simmons et al., 1988). As can be seen in Table 1, 100% of the confidently assigned amino acid residues and 89% of the tentative assignments matched exactly, providing strong evidence that the HRR is the same as ICAM-1.

HRR Transfectants Contain and Express the Human ICAM-1 Gene

Having determined an identity between the rhinovirus receptor peptide sequences and ICAM-1, two unique 47 base antisense oligonucleotides from the published ICAM-1 cDNA sequence were synthesized and used to probe a Southern blot of human and transfectant cell lines (Figure 7A). A single hybridizing EcoRI fragment of 4.3 kb was present in HeLa and HE1 transfectants but was absent in untransfected Ltk$^-$ cells. This size is in agreement with the data of Simmons et al. (1988), but differs from the 8.0 kb EcoRI fragment reported by Staunton et al. (1988), presumably reflecting a polymorphism. To confirm the expression of the ICAM-1 gene in HRR transfectants, polymerase chain reaction (PCR) amplification was performed on HeLa, Ltk$^-$, and HE1 cDNA using primers derived from the amino-terminal and carboxy-terminal coding regions of the protein (Figure 7B). A specific band with the expected size of the ICAM-1 coding region (1612 bp) was amplified from HeLa and HE1 cells but was absent from Ltk$^-$ cells. A randomly primed cDNA library in λgt11 prepared from HE1 poly(A)$^+$ RNA was screened with two 47-mer oligonucleotide probes from the middle of the ICAM-1 coding sequence. A positive clone, designated λ19.1, was isolated that had an insert of 1.5 kb; an additional ICAM-1 cDNA clone, pHRR2, was obtained by subcloning the products of PCR amplification (Figure 7B, lane 3) into Bluescript. Both of these cDNAs were sequenced. The sequence of pHRR2 showed that it contained the entire ICAM-1 coding sequence extending from nucleotide 58 to 1652 (according to the numbering system of Staunton et al. [1988]) and was identical to the published sequence except for the substitution of an A for a G at position 1462, changing E-442 to K. The λ19.1 insert, which was not full length (corresponding to nucleotides 150 to 1639), was also found to have this substitution, indicating that the A-for-G substitution at position 1462 was unlikely to be due to an amplification error. These data indicate a 99.8% identity with ICAM-1 at the amino acid level.

Discussion

The results presented in this communication allow two conclusions to be drawn. First, the "major" rhinovirus

Table 1. Comparison of Amino Acid Sequences of Peptides Isolated from HRR Protein with the Published Sequence of ICAM-1

Peptide	Sequence
RP/LE-19	k dgtFP LPIQE SVTVT RDLEG TYLCR Ar K DGTFP LPIGE SVTVT RDLEG TYLCR AR 406 433
RP/LE-10	k vYELS NVQED SQPMC YSNCP DGQSTA K VYELS NVQED SQPMC YSNCP DGQSTA 50 76
RP/LE-15a	k eLLLP GNNRK K ELLLP GNNRK 40 50
RP/LE-14	k kyRLQ QAQK K KYRLQ QAQK 483 492
RP/T-2	k gTPMK PNTQA K GTPMK PNTQA 492 502
EB/LE-34Kd	k -LKRE PAVGE PAEVT TTVLV RRDHH ga-f k ELKRE PAVGE PAEVT TTVLV RRDHH GANF 128 157
EB/T-50Kd	r tLEVD TQGTV V-SLD GLFPV sEA-V -l-l R VLEVD TQGTV VCSLD GLFPV SEAQV HLAL 198 227
RP/LE-15b	k viLPR K VILPR 8 13

The numbering scheme of Staunton et al. (1988) rather than that of Simmons et al. (1988) is used, beginning with Q as residue 1 of the mature protein (number 28 of translated coding sequence of mRNA) because it fits more closely the empirical signal peptidase rules (Von Heijne, 1983, 1984). RP or EB in peptide code names indicates that the peptide was isolated by reverse-phase chromatography or electroblotting from SDS–polyacrylamide gels, respectively. LE or T indicates cleavage with lysyl endopeptidase or trypsin, respectively. In many cases the given sequences were determined from more than one peptide; for the sake of clarity, only the longest is shown. For each peptide the sequence is given on the upper line and the corresponding match with ICAM-1 is given below, with the residue numbers indicated underneath. Uppercase letters are confident assignments, lowercase letters are tentative assignments or cycles at which two residues were obtained, and dashes indicate no assignment at that cycle. The space after the first residue indicates the cleavage point of the enzyme; in all cases the residue to the left of the space is inferred from the specificity of the enzyme. Asterisks above the peptide sequence indicate matches between a confidently assigned residue and ICAM-1.

receptor on HeLa cells is a glycoprotein with an apparent molecular mass of 95,000 daltons. Second, this 95,000 dalton glycoprotein is the same as ICAM-1 (Simmons et al., 1988; Staunton et al., 1988), an intercellular adhesion molecule.

The identification of the HRR as a 95,000 dalton protein is based on a number of results from several different experimental approaches, summarized below. First, several MAbs were isolated that specifically inhibit infectivity of rhinoviruses belonging to the major receptor group. All of these MAbs react with a cell surface protein on HeLa cells with an apparent molecular mass of 95,000 daltons.

Second, transfection of human genomic DNA into mouse L cells created a population of cells from which transfectants with a rhinovirus binding phenotype could be selected using virus labeling. These transfectants expressed a cell surface protein that reacted with MAb c78.4A and has an apparent molecular mass of 95,000 daltons. Finally, purified 95,000 dalton protein binds specifically to purified rhinovirus, demonstrating that this protein can serve as a receptor for rhinovirus in the absence of any other cellular components.

It is quite likely that this protein and the 90,000 dalton protein identified by Tomassini and Colonno (1986) are the same molecule. However, the number of virus binding sites per cell reported by these workers ($1–2 \times 10^3$ per HeLa cell) is at least 30-fold lower than the number of HRR molecules reported here (6×10^4 per HeLa cell), based on MAb binding, amount of HRR protein recovered from cells, and radioactive virus binding (Forte and Greve, unpublished results). The reason for this significant discrepancy is unclear.

The identification of the HRR as ICAM-1 is based primarily on the match of HRR amino acid sequence data and the predicted amino acid sequence of ICAM-1 deduced from the cDNA sequence. Out of 105 confidently assigned residues, 100% matched exactly (21% of the total ICAM-1 sequence), and out of 36 tentative assignments, 89% were correct. Other data also support the identity of HRR with ICAM-1. The apparent molecular

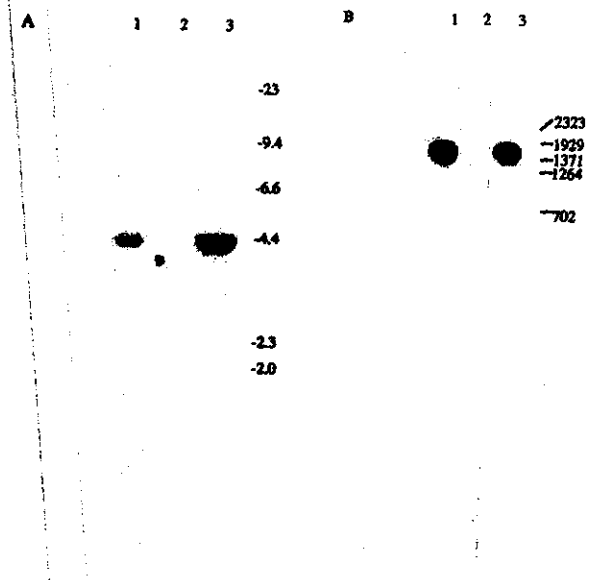


Figure 7. The ICAM-1 Gene and Its Expression in the HE1 Transfectant

(A) Southern blot: 20 μg samples of genomic DNA from HeLa cells (lane 1), Ltk⁻ cells (lane 2), and HE1 transfectant cells (lane 3) were restricted with EcoRI and probed with a 47 base oligonucleotide (ICAM1; see Experimental Procedures).

(B) Southern analysis of PCR reactions: one-fifth of each PCR was run on a 1.4% agarose gel, transferred to GeneScreen, and probed with 47 base oligonucleotide ICAM1. Primers specific to amino-terminal and carboxy-terminal nucleotide sequences were used as described in Experimental Procedures. Lane 1: random-primed HeLa cDNA, 72 hr exposure. Lane 2: random-primed Ltk⁻ cDNA, 72 hr exposure. Lane 3: random-primed HE1 cDNA, 90 min exposure.

mass of the unglycosylated HRR and the estimated number of asparagine-linked glycosylation units are consistent with the data of Dustin et al. (1986) and the information predicted from the sequence (Simmons et al., 1988; Staunton et al., 1988). Southern blot analysis using ICAM-1–specific oligonucleotide probes confirm that HRR transfectants contain the human ICAM-1 gene. PCR amplification shows that the ICAM-1 mRNA is expressed in the HE1 cell line. Finally, a cDNA clone isolated from HE1 cells has a nucleotide sequence essentially identical to that of ICAM-1.

The identification of the HRR and the knowledge of its primary structure will now allow an examination of the role of the receptor in the pathogenesis of rhinovirus infection. First, the molecular basis of the high-affinity binding of virus to the receptor can be investigated by a combination of genetic and structural studies. A substantial amount has been learned about the receptor binding site on the viral capsid (reviewed by Rossmann and Palmenberg, 1988), but the absence of detailed molecular information about the receptor molecule until now has limited progress in this area. Second, conformational changes in picornavirus capsids occur as a result of interaction with receptor (Holland, 1962; Noble-Harvey and Lonberg-Holm, 1974) and may be related to membrane penetration (Chow et al., 1987; Abraham and Colonno, 1988); this phenomenon can now be studied at a biochemical and biophysical level with a purified and biologically active receptor. Third, the precise role of the receptor as a determinant of tissue tropism remains unclear; the widespread tissue distribution of ICAM-1 argues that cellular receptors are not the only determinant in vivo. In addition to considerations such as the route of infection and the acid sensitivity of rhinoviruses, it is possible that other factors may be important in determining tissue tropism in vivo, such as stimulation of ICAM-1 expression by immunoregulatory molecules (Pober et al., 1986, 1987; Dustin et al., 1986).

ICAM-1 is an integral membrane protein first identified based on its role in adhesion of leukocytes to T cells (Rothlein et al., 1986), which has been shown to be mediated by the heterotypic binding of ICAM-1 to LFA-1 (Marlin and Springer, 1987). Based on its widespread tissue distribution and regulation in response to inflammatory cytokines, it may be involved in cellular adhesion of many different cell types. The primary structure of ICAM-1 has revealed that it is homologous to the cellular adhesion molecules neural cell adhesion molecule (NCAM) and myelin-associated glycoprotein (MAG), and has led to the proposal that it is a member of the immunoglobulin supergene family (Simmons et al., 1988; Staunton et al., 1988). The relationship between the natural in vivo functions of this molecule and its function as a major rhinovirus receptor is at present unclear but merits intensive investigation.

We have mapped the HRR gene to human chromosome 19 by somatic cell genetic techniques. Other genes coding for picornavirus receptors have also been mapped to human chromosome 19: the poliovirus receptor (Medrano and Green, 1973; Siddique et al., 1988), the coxsackie B virus receptor (Couillin et al., 1987), and the echovirus receptor (Couillin et al., 1987). Since the capsid proteins of picornaviruses show a high degree of structural conservation, and since many picornavirus receptors map to the same human chromosome, it is reasonable to speculate that host cell receptors for different members of the picornavirus family might also be structurally related or belong to a gene family. Since the gene for the poliovirus receptor has been cloned (Mendelsohn and Racaniello, personal communication), it will now be possible to compare the structures of the two receptors and how two members of the picornavirus family recognize their respective receptors. In the same way that the comparative analysis of the three-dimensional structures of picornaviruses has advanced our understanding of their assembly and interaction with the host immune system (Rossmann et al., 1985; Hogle et al., 1985; Luo et al., 1987), a comparative study of picornavirus receptors may lead to a greater understanding of the mechanism of tissue tropism and host range restriction in this important family of viruses.

Experimental Procedures

Growth, Purification, and Assay of Rhinovirus

Rhinoviruses HRV2, HRV3, and HRV14 were obtained from the American Type Culture Collection, plaque purified, and isolated from lysates of infected HeLa-S3 cells. Purified rhinovirus was prepared by polyethylene glycol precipitation and sucrose gradient sedimentation, essentially by the method of Abraham and Colonno (1984). Viral purity was

assessed by SDS-PAGE analysis of capsid proteins and by electron microscopy. Infectivity was quantitated by a limiting dilution infectivity assay scoring for cytopathic effect, essentially as described by Minor (1985).

Isolation of MAbs

BALB/cByJ female mice were immunized by intraperitoneal injection of 10^7 intact HeLa cells in 0.5 ml of phosphate-buffered saline (PBS) three times at 3 week intervals. Two weeks later the mice were bled and aliquots of serum were tested for protective effects against HRV14 infection of HeLa cells. Positive mice were boosted by a final injection of 10^7 HeLa cells, and 3 days later spleen cells were fused to P3X63-Ag8.653 myeloma cells (Galfre et al., 1977) to produce a total of approximately 700 hybridoma-containing wells. Each well was tested by incubating 3×10^4 HeLa cells in 96-well plates with 100 µl of supernatant for 1 hr at 37°C; the cells were then washed with PBS, and a sufficient amount of HRV14 was added to give complete cytopathic effect in 24–36 hr. Wells that were positive (protected from infection) were scored at 36 hr.

Transfection and Cell Sorting

Cell culture and calcium phosphate transfection of Ltk$^-$ cells were performed as described (McClelland et al., 1987). For labeling of transfected cells with virus, purified virus was rendered noninfectious by ultraviolet irradiation. Cells (5×10^6) were incubated for 30 min at 37°C with 10^{11} virus particles in 50 µl of DMEM, 2% serum. The cells were chilled to 4°C, washed with PBS, and incubated at 4°C with saturating amounts of anti-HRV14 MAb (kindly provided by R. Rueckert, University of Wisconsin). After a final incubation with fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories), the cells were subjected to analysis or preparative cell sorting on a FACS IV (Becton-Dickinson). Analysis and sorting of cells labeled with MAb were performed as described (McClelland et al., 1987). Somatic cell hybrids were produced by the fusion of primary human fibroblasts with HPRT$^-$ L cells and were selected and characterized by established methods (Kamarck et al., 1984).

MAb Binding

^{125}I-IgG was incubated with a single-cell suspension in DMEM, 2% fetal calf serum, 10 mM HEPES (pH 7.2) at 10^7 cells per ml at 4°C for 60 min. Bound ligand was separated from free by spinning the cells through 1 ml of 5% Ficoll in PBS at 12,000 × g for 30 sec in a microfuge and determining the radioactivity associated with the cell pellet. The concentration of ^{125}I-c78.4A IgG (12 µg/ml; 8.8×10^5 cpm/µg) needed to saturate the cell surface was attained by dilution with unlabeled IgG. IgG binding sites per cell were calculated from the number of cells recovered, the cpm bound, and the specific activity of the IgG.

Radioactive Labeling of HRR and Immunoabsorption

Surface labeling of cells with ^{125}I was done according to Hubbard and Cohn (1975). HeLa or HE1 cells were metabolically labeled with [^{35}S]cysteine (100 µCi/ml; 967 Ci/mmol) for 3 hr in cysteine-free medium; cells labeled in the presence of tunicamycin or swainsonine were preincubated for 2 hr with 5 µg/ml or 1 µg/ml of the poison, respectively, and then labeled in the presence of the drug. Labeled cells were extracted with lysis buffer (L buffer: 1% Triton X-100 in PBS) with protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM EDTA) for 1 hr at 4°C, and residual debris was removed by centrifugation. Immunoabsorptions were carried out by incubating 50–100 µl of cell lysate, supplemented with 1 mg/ml bovine serum albumin and 10 µg/ml human transferrin, with 5 µl of c78.4A IgG–Sepharose or anti-HRV14 IgG–Sepharose for 1 hr at 4°C with gentle agitation. The resin was then washed extensively with L buffer and then eluted in 50 µl of 1% SDS.

Purification of HRR Protein

HeLa or HE1 cells were extracted with L buffer with protease inhibitors for 1 hr at 4°C; the extract was then filtered through 0.22 µm Millipore filters. The extract was adsorbed to wheat germ agglutinin–Sepharose 6MB (Sigma) in the batch mode for 6–18 hr at 4°C with gentle shaking (1 ml of packed resin per 10^9 cell equivalents). The resin was washed with L buffer and eluted in 5 vols of 0.3 M N-acetylglucosamine at room temperature for 1 hr. The eluate was supplemented with 10 µg/ml human transferrin and then adsorbed in the batch mode to c78.4A IgG–Sepharose for 6–18 hr at 4°C (0.2 ml of packed resin per 10^9 cell equivalents). The resin was washed in L buffer and then eluted in 5 vols of 50 mM diethanolamine (pH 11.5), 0.1% Triton X-100 for 1 hr at room temperature. The eluate ("native" receptor) was neutralized by the addition of 0.2 vol of 1 M HEPES (pH 7.2) and dialyzed exhaustively against 10 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% Triton X-100 (N buffer). Some preparations were subjected to sedimentation through 5%–20% sucrose gradients in N buffer (17 hr at 49,000 rpm in a Beckman SW50.1 rotor) to remove minor high molecular weight contaminants. When necessary, receptor was concentrated in Centricon-30 concentrators (Amicon). Alternatively, the resin was eluted with 1% SDS for 1 hr at room temperature and lyophilized, and HRR was further purified by gel filtration on a GF-450 column (Du Pont) equilibrated in 0.1% SDS, 10 mM ammonium bicarbonate (pH 7.5). Receptor protein was quantitated by comparison with several dilutions of the standard proteins on silver-stained SDS–polyacrylamide gels and by amino acid analysis. [^{35}S]cysteine-labeled receptor was prepared in the same manner from HE1 cells labeled for 24 hr in the presence of 100 µCi/ml [^{35}S]cysteine (967 Ci/mmol, New England Nuclear) in cysteine-free medium. Specific activity of the purified receptor was 2×10^5 cpm/µg.

In Vitro Virus Binding Assay

[^{35}S]cysteine-labeled native HRR was mixed with HRV3 in 100 µl of N buffer. The mixture was incubated for 30 min at 37°C, cooled on ice, layered on top of a cushion of 200 µl of 10% glycerol, 0.2 M triethanolamine (pH 7.5), and centrifuged in a Beckman air-driven centrifuge at 134,000 × g for 30 min at 4°C. The top 275 µl was removed, and the pellet was analyzed by SDS-PAGE and scintillation counting. Silver-staining of SDS gels of control experiments indicated that essentially all of the HRV3 is pelleted under these conditions and essentially all of the HRR remains in the supernatant.

Receptor Protein Fragmentation and Sequencing

Receptor protein was reduced and carboxymethylated by incubating the receptor in 1% SDS with 10 mM DTT for 3 hr at 37°C followed by addition of iodoacetic acid (approximately 2.5 molar excess over free sulfhydryl) and incubation for 30 min at room temperature. Receptor was dialyzed against 0.05% SDS, 100 mM ammonium bicarbonate (pH 8.6), 1 mM $CaCl_2$ (for trypsin), or 0.05% SDS, 100 mM Tris (pH 9.0) (for lysyl endopeptidase). Approximately 1 nmol (50 µg) of HRR was digested with 1% (wt/wt substrate) trypsin (Sigma, EC 3.4.21.4) for 6 hr at 37°C, or lysyl endopeptidase (Wako, EC 3.4.21.50) for 24 hr at 37°C by the addition of 1% (wt/wt substrate) at 0 hr and 12 hr. Digestion was terminated by the addition of trifluoroacetic acid to a final concentration of 1%, and the reaction mixture was separated on a Brownlee C-18 column (ODS 5 µm, 3 cm × 2.1 mm i.d.) using a Beckman HPLC system.

The peptides were eluted with a 0%–70% acetonitrile gradient in 0.1% trifluoroacetic acid at 0.2 ml/min; the elute was monitored at 215 nm. Selected peaks were subjected to amino acid analysis and sequencing. In some instances nonreduced receptor protein was subjected to limited digestion with trypsin and lysyl endopeptidase in the presence of 0.1% SDS, and discrete protein fragments were electroblotted onto aminopropyl-glass fiber filters (Aebersold et al., 1986) or polyvinylidene difluoride membranes (Matsudaira, 1987) and sequenced. Protein sequencing was performed on an Applied Biosystems model 470A protein sequencer (program 03CPTH) with on-line injection onto a model 120A PTH analyzer with a C-18 reverse-phase column (220 mm × 2.1 mm i.d., 5 µm). Data handling was performed with Nelson Analytical model 2600 chromatography software.

Oligonucleotide Hybridizations, PCRs, cDNA Cloning, and Nucleotide Sequencing

Southern blotting and oligonucleotide hybridizations were performed exactly as described by Devlin et al. (1988). Hybridizations were for 16 hr at 45°C, and final stringent washes were in 0.1× SSC at 65°C. Two 47 base antisense oligonucleotides were synthesized from the published ICAM-1 sequence (Staunton et al., 1988; Simmons et al., 1988), having the sequences GAGGTGTTCTCAAACAGCTCCAGCCCTTGG-GGCCGCAGGTCCAGTTC (ICAM1) and CGCTGGCAGGACAAAGGT-CTGGAGCTGGTAGGGGGCCGAGGTGTTCT (ICAM3). PCRs were per-

formed using 100 ng of randomly primed single-stranded cDNA as described by Saiki et al. (1988). Twenty-five extension cycles were performed for 4 min each at 72°C. Primers from the N- and C-terminal coding regions of the ICAM-1 sequence were PCR5.1 (ggaattcATGG-CTCCCAGCAGCCCCCGGCCC) and PCR3.1 (ggaattcTCAGGGAGG-CGTGGCTTGTGTGTT), respectively. (Uppercase letters denote ICAM-1 sequence, lowercase letters restriction site linkers.) A randomly primed cDNA library was constructed in λgt11 from HE1 poly(A)$^+$ RNA by Clontech Laboratories, Palo Alto, CA. Double-stranded DNA sequencing of Bluescript (Stratagene) subclones was performed as described by Toneguzzo et al. (1988).

Other Methods

SDS-PAGE was performed essentially as described by Laemmli (1970). Silver staining was performed according to Morrissey (1981), except that the formaldehyde fixation step was omitted. Fluorography was performed with Amplify (Amersham). Hybridoma IgG was isolated from ascites fluid of tumor-bearing mice by chromatography on an ABx column (Baker) according to the manufacturer's directions. MAb affinity columns were prepared by coupling IgG to CNBr–Sepharose 4B to approx. 5 mg of IgG per ml of packed resin, according to Parham (1983). Fab fragments of IgG were prepared as described by Greve and Gottlieb (1982). IgG was labeled with ^{125}I Bolton-Hunter reagent to a specific activity of 1.6 μCi/μg.

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