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## Viral cell recognition and entry

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### Abstract

Rhinovirus infection is initiated by the recognition of a specific cell-surface receptor. The major group of rhinovirus serotypes attach to intercellular adhesion molecule-1 (ICAM-1). The attachment process initiates a series of conformational changes resulting in the loss of genomic RNA from the virion. X-ray crystallography and sequence comparisons suggested that a deep crevice or canyon is the site on the virus recognized by the cellular receptor molecule. This has now been verified by electron microscopy of human rhinovirus 14 (HRV14) and HRV16 complexed with a soluble component of ICAM-1.

A hydrophobic pocket underneath the canyon is the site of binding of various hydrophobic drug compounds that can inhibit attachment and uncoating. This pocket is also associated with an unidentified, possibly cellular in origin, "pocket factor." The pocket factor binding site overlaps the binding site of the receptor. It is suggested that competition between the pocket factor and receptor regulates the conformational changes required for the initiation of the entry of the genomic RNA into the cell.

**Keywords:** antiviral compounds; ICAM-1 as receptor; regulation of entry; rhinovirus; structure; virus attachment; virus uncoating

### Viral receptors

Unlike plant viruses, most animal, insect, and bacterial viruses attach to specific cellular receptors that, in part, determine host range and tissue tropism. Viruses have adapted themselves to utilize a wide variety of cell-surface molecules as their receptors, including proteins, carbohydrates, and glycolipids (Table 1). Some viruses recognize very specific molecules (e.g., a large group of rhinoviruses recognize intercellular adhesion molecule-1 [ICAM-1]), whereas other viruses recognize widely distributed chemical groups (e.g., influenza viruses recognize sialic acid moieties). The tissue distribution of the receptor will in part determine the tropism of the virus and, hence, the symptoms of the infection. Similarly, species differences between receptor molecules can limit host range. For instance, only humans and apes have been shown to be susceptible to rhinovirus infections, a property correlated to the inability of human rhinoviruses to bind to the receptor ICAM-1 molecule in other species.

Although there are extensive similarities of sequence, structure, and physical properties among picornaviruses that show these viruses have evolved from a common ancestor (Rossmann et al., 1985; Palmenberg, 1989; Rueckert, 1990), they neverthe-

less recognize a variety of receptors (Table 2). Possibly the primordial virus had the ability to bind weakly to a large number of different molecules. With time, different viruses evolved that became progressively more efficient and specialized toward recognizing one particular molecule as a way of infecting specific cells. Indeed, the grouping of viruses might suggest such a scenario. Thus, all polioviruses appear to recognize the same receptor and most coxsackie A viruses recognize their own receptor, whereas coxsackie B viruses recognize yet another receptor. Therefore, it is surprising that rhinovirus serotypes can be divided into 3 groups that recognize different receptors (Abraham & Colonno, 1984; Uncapher et al., 1991). Furthermore, the receptor for the major group of rhinoviruses, ICAM-1, belongs to the immunoglobulin superfamily (Greve et al., 1989; Staunton et al., 1989), whereas the receptor for the minor group has been reported to be the low density lipoprotein (LDL) receptor (Hofer et al., 1994).

Receptor binding is only the first, albeit essential, step in the infection process. The virus, or the virus genome alone, then has to enter the cell, a process that requires translocation of the viral genome or a subviral particle across the membrane into the cytoplasm, and, in some cases, into the nucleus. Because delivery of the viral genome into the cell involves major rearrangements of the capsid structure, entry must be a tightly regulated process, which is triggered by the cell. The mechanism of entry can be, in the case of enveloped viruses, by fusion of the viral

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**Table 1.** Some known receptors for animal viruses

1. Sialic acid	Reoviruses Influenza virus Polyoma virus	Paul et al., 1989; Choi et al., 1990 Weis et al., 1988 Fried et al., 1981
2. Immunoglobulin family:		
(i) CD4	Human immunodeficiency viruses	Dalgleish et al., 1984
(ii) Poliovirus receptor	Polioviruses	Mendelsohn et al., 1989
(iii) ICAM-1	Major serotype group of HRV	Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989
3. Integrins	Foot-and-mouth disease virus	Acharya et al., 1989; Mason et al., 1993
4. Complement receptor type 2 (a B lymphocyte surface glycoprotein)	Epstein-Barr viruses	Moore et al., 1987; Tanner et al., 1987
5. Amino acid permeases	Ecotropic murine leukemia virus	J.W. Kim et al., 1991; H. Wang et al., 1991
6. Carcinoembryonic antigen	Mouse hepatitis virus (a coronavirus)	Williams et al., 1991
7. Erythrocyte P antigen	Human B19 parvovirus	Brown et al., 1993
8. LDL receptor	Minor serotype group of HRV (?)	Hofer et al., 1994

envelope with the limiting cellular membrane (Fig. 1). This process has been well characterized in several viruses (Semliki Forest virus [SFV], influenza virus, Sendai virus) where fusion is induced by specific viral envelope proteins, activated by conformational changes induced by the low pH environment of endosomes. The mechanism by which protein-encapsidated viruses, such as picornaviruses (Rueckert, 1990), enter the cytoplasm has not been well elucidated but must differ significantly in detail from the membrane-fusion strategy demonstrated by enveloped viruses in that RNA must be translocated through the membrane.

#### Rhinovirus structure and the canyon hypothesis

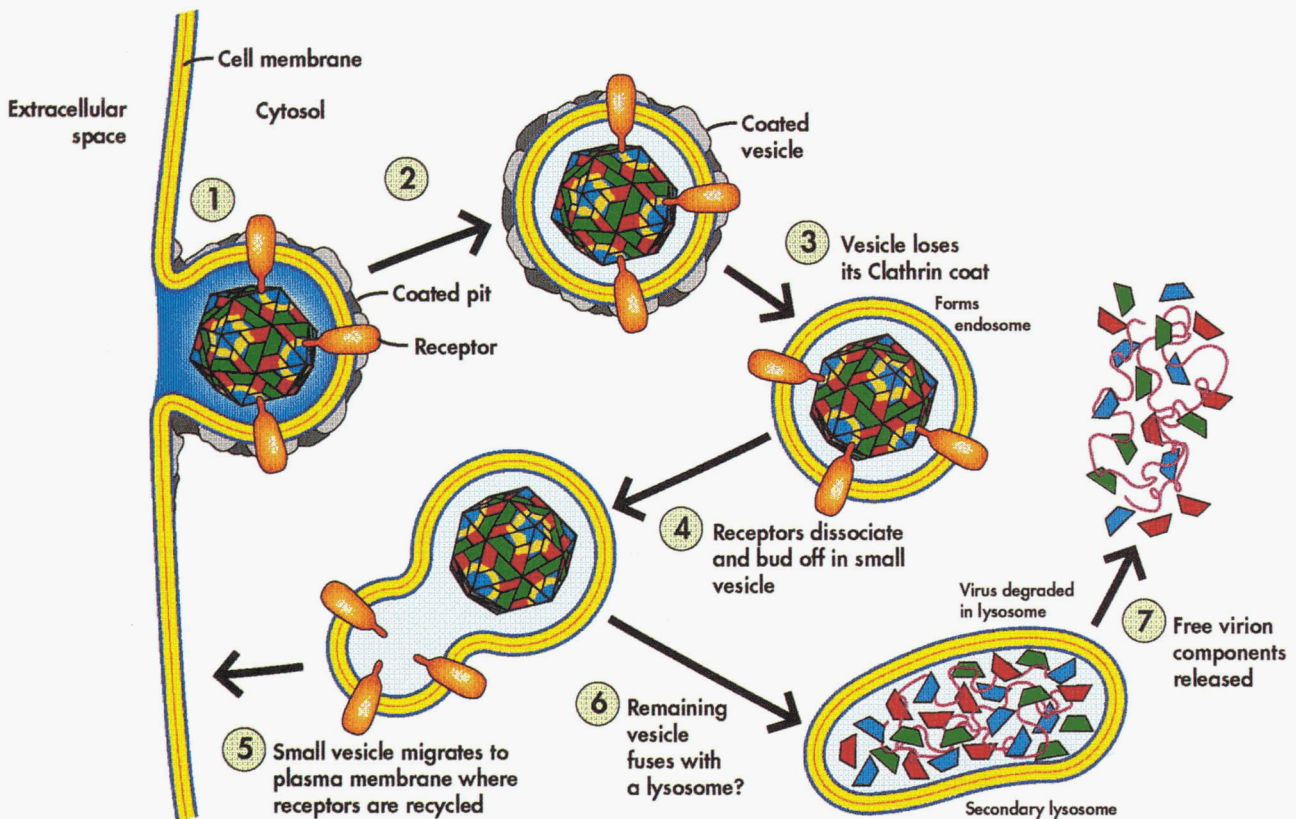
The genus *Rhinovirus* is composed of a group of over 100 serologically distinct viruses that are a major cause of the common cold in humans (Rueckert, 1990). These viruses belong to the picornavirus family, which also contains the genera *Enterovirus*, *Aphthovirus*, *Cardiovirus*, and hepatitis A virus. The picorna-

viruses are small, icosahedral, nonenveloped, single-stranded RNA viruses. X-ray crystal structures have been determined for at least 1 member in each picornavirus genus except for hepatitis A viruses (Rossmann et al., 1985; Hogle et al., 1985; Luo et al., 1987; Acharya et al., 1989; Filman et al., 1989; S. Kim et al., 1989). Polioviruses (genus *Enterovirus*) are structurally the most similar to rhinoviruses. Unlike the enteroviruses, rhinoviruses are unstable below pH 6. The infectious virion has a molecular weight of about  $8.5 \times 10^6$  Da and an external diameter of around 300 Å.

Each of the 60 icosahedral protomers in picornaviruses contains 4 viral polypeptides, VP1-VP4. VP1, VP2, and VP3 reside on the exterior of the virus and make up its protein shell (Fig. 2). These 3 peptides, each having a molecular weight of roughly 35 kDa, contain a common 8-stranded, antiparallel,  $\beta$ -barrel motif (Rossmann et al., 1985) (Fig. 3; Table 3). Their amino termini intertwine to form a network on the interior of the protein shell. Five VP3 amino termini form a 5-stranded

**Table 2.** Receptor families for picornaviruses based on virus competition for cell receptors

Virus	Receptor molecule	Receptor family	Reference
Human rhinovirus major group: 78 serotypes, including 3, 5, 9, 12, 14, 15, 22, 32, 36, 39, 41, 51, 58, 59, 60, 66, 67, 89	ICAM-1	Ig (5 Ig domains)	Abraham & Colonno, 1984; Greve et al., 1989; Staunton et al., 1989
Human rhinovirus minor group: 11 serotypes, including 1A, 2, 44, 49	Low density lipoprotein (LDL) receptor	LDLR	Abraham & Colonno, 1984; Hofer et al., 1994
Polioviruses	Poliovirus receptor (PVR)	Ig (3 Ig domains)	Mendelsohn et al., 1989
Coxsackievirus A13, 18, 21	ICAM-1	Ig (5 Ig domains)	Colonno et al., 1986; Roivainen et al., 1991
Coxsackievirus A2, 5, 13, 15, 18	?	?	Colonno et al., 1986; Roivainen et al., 1991; Schultz & Crowell, 1983
Coxsackievirus B3 and adenovirus 2	?	?	Lonberg-Holm et al., 1976
Echovirus 1	VLA-2	Integrin	Bergelson et al., 1992
Echovirus 6	?	?	Crowell, 1966
Foot-and-mouth disease viruses, types A <sub>12</sub> 119, O <sub>1B</sub> , C <sub>3Res</sub> ; SAT <sub>1,3</sub>	RGD integrin	Integrin	Sekiguchi et al., 1982; Mason et al., 1993
Mengo virus	?	Glycophorin (?)	Burness, 1981; Burness & Pardoe, 1983



**Fig. 1.** One possible endocytotic process (adapted from Rawn [1989]). Note, however, that in most cases it is not known when and where the receptor and virus part company, whether it is necessary for the virus to be bound to the receptor during uncoating, and what is the mechanism by which RNA translocates the membrane.

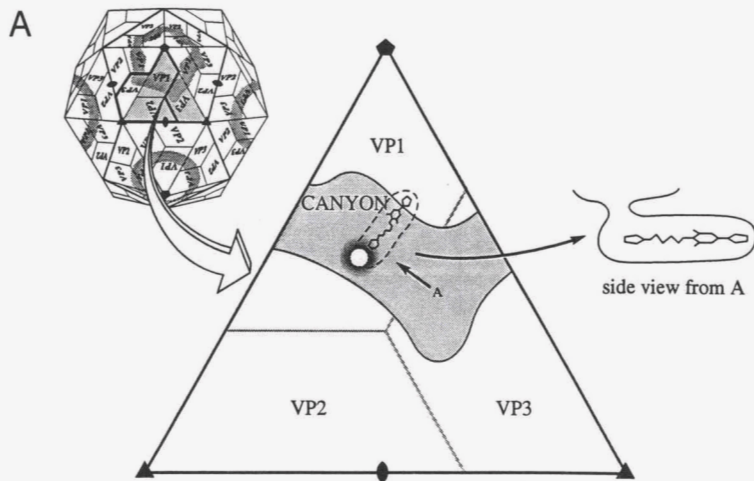
helical  $\beta$ -cylinder on the virion's interior about each icosahedral 5-fold axis. This  $\beta$ -cylinder stabilizes the pentamer and is thought to be important for its assembly (Hogle et al., 1985; Arnold et al., 1987).

VP4 is smaller than the other viral polypeptides and resides inside the virion's protein shell. VP4 is lost from the capsid as a result of virus uncoating, although the specific role of VP4 in uncoating or entry has not been elucidated. A mutant of human rhinovirus serotype 14 (HRV14) defective in VP4-VP2 cleavage (Lee et al., 1993) is able to bind to receptor and undergo cell-induced conformational transitions but is unable to initiate a new round of replication, suggesting that cleavage of VP0 into VP2 and VP4 (cf. Arnold et al., 1987; Luo et al., 1987) is an essential prerequisite for successful cell infection. The amino terminus of VP4 is myristylated, which may promote its association with lipid membranes during viral assembly or uncoating (Chow et al., 1987). In poliovirus, the myristylate moiety lies inside the virion coat close to the  $\beta$ -cylinder. The first 25-28 amino-terminal residues of VP4 are mostly disordered in rhinovirus structures, but a density consistent with myristylate is seen internally near the center of the pentamer in rhinoviruses 14, 1A, and 16 (Arnold & Rossmann, 1990; S. Kim et al., 1989; Oliveira et al., 1993).

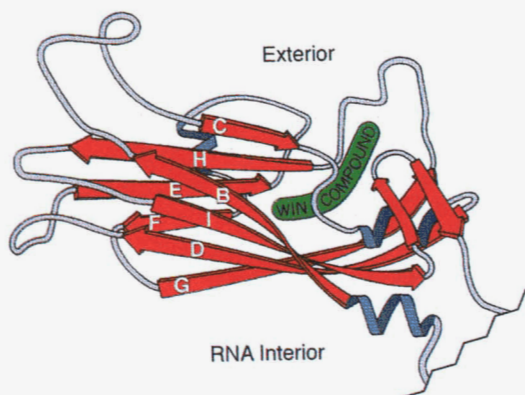
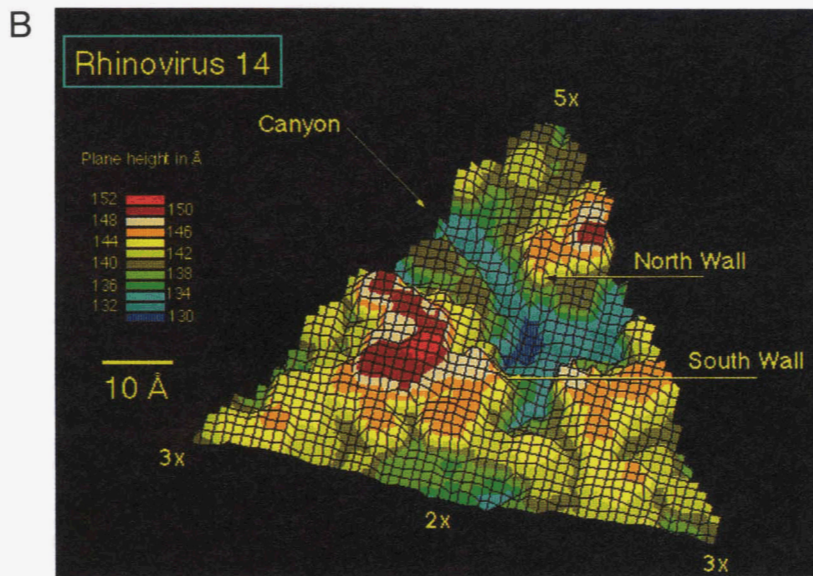
Each of the 3 larger capsid proteins has various insertions between the  $\beta$ -strands of the basic folding motif. Many of these insertions decorate the viral exterior and form "puffs" and loops that are hypervariable and have been shown to be the binding

site of neutralizing antibodies (Rossmann et al., 1985; Sherry & Rueckert, 1985; Sherry et al., 1986). The surfaces of rhinoviruses (and polioviruses) contain a series of remarkably deep crevices or "canyons" (Fig. 2), unlike anything observed in plant virus structures. The canyon is formed roughly at the junction of VP1 (forming the "north" rim) with VP2 and VP3 (forming the "south" rim). The GH loop in VP1 (often referred to as the "FMDV loop" because of its immunodominance in the homologous foot-and-mouth disease virus [FMDV] structure) forms much of the floor of the canyon. Together with the carboxy termini of VP1 and VP3, the GH loop of VP1 also participates in the formation of the "south" rim of the canyon.

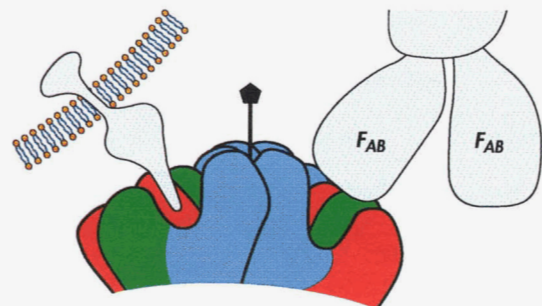
It was hypothesized (Rossmann et al., 1985) that the canyon (1 around each 5-fold vertex; Fig. 2) in HRV was the site of receptor attachment, largely inaccessible to the broad antigen-binding region seen on antibodies. Thus, residues in the lining of the canyon, which should be resistant to accepting mutations that might inhibit receptor attachment, would avoid presenting an unchanging target to neutralizing antibodies. Indeed, the neutralizing immunogenic sites that had been mapped by escape mutations were not in the canyon, but on the most exposed and variable parts of the virion in both HRV (Rossmann et al., 1985; Sherry & Rueckert, 1985; Sherry et al., 1986) and poliovirus (Hogle et al., 1985; Page et al., 1988). The "canyon hypothesis" suggests that a strategy for viruses to escape the host's immune surveillance is to protect the receptor attachment site in a surface depression (Fig. 4). Similar depressions related to host-cell



**Fig. 2. A:** Top left, diagrammatic view of picornavirus showing VP1, VP2, and VP3 and the deep cleft or “canyon” running around each 5-fold vertex. The 6S protomeric assembly unit (which differs from the geometric definition of the asymmetric unit) is shown in heavy outline on the icosahedron. Center, enlargement of one icosahedral asymmetric unit showing the outline of the canyon and the entrance to the WIN pocket. The terms “north” (top) and “south” rims of the canyon refer to this standard orientation. (Reprinted with permission from Oliveira et al. [1993]. Copyright by Current Biology Ltd.) **B:** Topological view of one icosahedral asymmetric unit of HRV14 showing the somewhat asymmetrically placed canyon. (Prepared by Jean-Yves Sgro, University of Wisconsin, Madison.)



**Fig. 3.** Schematic representation of the VP1 fold of HRV14. The folding topology of the 2 sheets “BIDG” and “CHEF” is the same in VP2 and VP3 as well as in most other viral capsid proteins. The binding site of antiviral WIN compounds within the hydrophobic interior of VP1 is also shown.



**Fig. 4.** The presence of depressions on the picornavirus surface suggests a strategy for the evasion of immune surveillance. The dimensions of the putative receptor binding site, the “canyon,” sterically hinder an antibody’s (top right) recognition of residues at the base of the site, while still allowing recognition and binding by a smaller cellular receptor (top left). This would allow conservation of receptor specificity while at the same time permitting evolution of new serotypes by mutating residues on the viral surface, outside the canyon.

**Table 3.** *The common  $\beta$ -barrel fold*

Virus <sup>a</sup>	Kingdom	Symmetry of capsid	Genome	Comments <sup>b</sup>	First reference
<b>Plant</b>					
TMV	Plant	Helical	RNA		
TBSV	Plant	$T = 3$	RNA	1	Harrison et al., 1978
SBMV	Plant	$T = 3$	RNA	1	Abad-Zapatero et al., 1980
STNV	Plant	$T = 1$	RNA	1	Liljas et al., 1982
CPMV	Plant	Pseudo $T = 3$	RNA	1	Stauffacher et al., 1987
BPMV	Plant	Pseudo $T = 3$	RNA	1, 2	Chen et al., 1989
STMV	Plant	$T = 1$	RNA	1, 2	Larson et al., 1993
<b>Insect</b>					
BBV	Insects	$T = 3$	RNA	1	Hosur et al., 1987
FHV	Insects	$T = 3$	RNA	1, 2	Fisher et al., 1993
<b>Bacterial</b>					
$\phi$ X174	<i>E. coli</i>	$T = 1$	DNA	3, 4	McKenna et al., 1992a
<b>Animal</b>					
Influenza	Human	Globular head Hemagglutinin spike	RNA	1	I.A. Wilson et al., 1981
Adeno	Human	Capsid hexon		3	Roberts et al., 1986
HRV14, 1A, 16	Human	Pseudo $T = 3$	RNA	1	Rossmann et al., 1985; S. Kim et al., 1989; Oliveira et al., 1993
Coxsackievirus B3	Human	Pseudo $T = 3$	RNA	1	J.K. Muckelbauer, M. Kremer, I. Minor, L. Tong, A. Zlotnick, J.E. Johnson, & M.C. Rossmann, submitted for publication
Polio 1, 2, 3	Human	Pseudo $T = 3$	RNA	1	Hogle et al., 1985
Cardio	Mice	Pseudo $T = 3$	RNA	1	Luo et al., 1987
FMDV	Cattle	Pseudo $T = 3$	RNA	1	Acharya et al., 1989
Parvo	Dogs and cats	$T = 1$	DNA	3, 4	Tsao et al., 1991

<sup>a</sup> BBV, black beetle virus; BPMV, beanpod mottle virus; CPMV, cowpea mosaic virus; FHV, flock house virus; SBMV, southern bean mosaic virus; STMV, satellite tobacco mosaic virus; STNV, satellite tobacco necrosis virus; TBSV, tomato bushy stunt virus; TMV, tobacco mosaic virus.

<sup>b</sup> 1, There are mostly small insertions between  $\beta$ -strands. 2, There is a significant amount of ordered RNA. 3, There are very large insertions between  $\beta$ -strands. 4, There is some ordered ssDNA.

attachment have also been found on the surface of the hemagglutinin spike of influenza virus (I.A. Wilson et al., 1981; Weis et al., 1988), and may be the case for human immunodeficiency virus (Matthews et al., 1987).

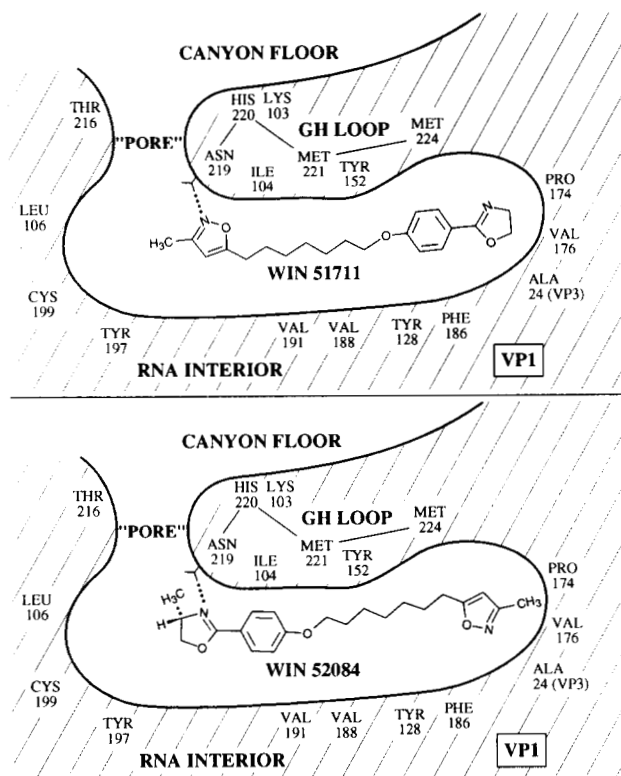
A number of lines of evidence emerged to support the canyon hypothesis. First, a comparison of the variability of surface-exposed residues between a number of picornaviruses indicated that amino acid residues lining the canyon are significantly more conserved than other surface-exposed residues (Rossmann & Palmenberg, 1988; Chapman & Rossmann, 1993). Second, the hypothesis rationalized the contrast between many vertebrate virus structures and plant viruses (e.g., tomato bushy stunt virus [Harrison et al., 1978], southern bean mosaic virus [Abad-Zapatero et al., 1980], satellite tobacco necrosis virus [Liljas et al., 1982], and cowpea mosaic virus [Stauffacher et al., 1987]) or insect viruses (e.g., black beetle virus [Hosur et al., 1987]). Namely, animal viruses tend to have surface depressions (a notable exception is FMDV [Acharya et al., 1989]), but viruses whose hosts did not have immune systems tend to have smooth surfaces or protrusions on their surfaces. Third, site-directed mutagenesis of HRV14 indicated that modification of several amino acid residues located in the base of the canyon has an impact upon virus-receptor affinity (Colonno et al., 1988). Specifically, mutants with substitutions at residues 1273,<sup>1</sup> 1223,

1103, and 1220 exhibited an alteration in virus-receptor affinity. Fourth, certain capsid-binding "WIN" antiviral compounds block the binding of some of the major receptor rhinoviruses, including HRV14 (Pevear et al., 1989). These compounds bind to many picornaviruses in a hydrophobic pocket located under the canyon floor (Fig. 2) and, in most cases, block virus from uncoating (Smith et al., 1986; Badger et al., 1988; K.H. Kim et al., 1993). Upon binding to HRV14, a conformational change occurs in the roof of the pocket, which is also the floor of the canyon (Fig. 5). Several amino acid residues are displaced by as much as 4 Å in their  $C_{\alpha}$  positions. These findings suggested that the conformational changes at the base of the canyon prevent viral attachment to cells. Although the observations for rhinovirus were consistent with the canyon being the receptor binding site, they did not provide conclusive proof nor did they identify a complete footprint of the receptor on the virus surface.

#### **Binding of ICAM-1, the major group rhinovirus receptor, to virus surface**

There are at least 78 serotypes (Tomassini et al., 1989) that bind to ICAM-1, the major group rhinovirus receptor (Greve et al., 1989; Staunton et al., 1989). The ICAM-1 molecule has 5 immunoglobulin-like domains (D1–D5, numbered sequentially from the amino end), a transmembrane portion, and a small cytoplasmic domain (Simmons et al., 1988; Staunton et al., 1988). Domains D2, D3, and D4 are glycosylated (Fig. 6). Unlike im-

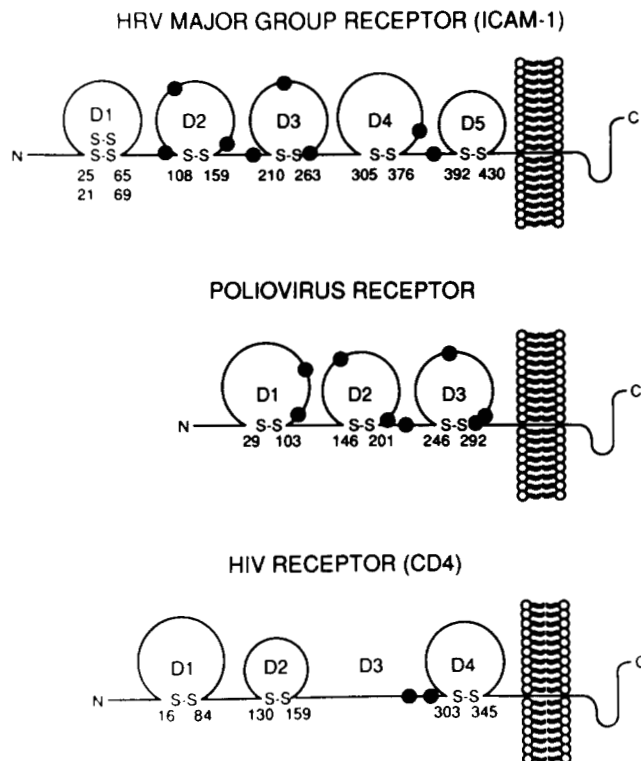
<sup>1</sup> Residues are numbered sequentially for each of VP1, VP2, VP3, and VP4, but start at 1001, 2001, 3001, and 4001, respectively.



**Fig. 5.** Schematic representation of the binding of the antiviral agents WIN 51711 and 52084 into a pocket underneath the canyon in HRV14. This causes enlargement of the pocket and conformational changes in the floor of the canyon, inhibiting attachment of the virus to HeLa cells in some cases and also increasing the stability of the virus in all cases. (Reprinted with permission from Dutko et al. [1989]. Copyright by Springer-Verlag, New York Inc.)

munoglobulins, ICAM-1 appears to be monomeric (Staunton et al., 1989). Mutational analysis of ICAM-1 has shown that domain D1 contains the primary binding site for rhinoviruses as well as the binding site for its natural ligand, lymphocyte function-associated antigen-1 (LFA-1) (Staunton et al., 1988, 1990; Lineberger et al., 1990; McClelland et al., 1991). Other surface antigens within the immunoglobulin superfamily that are used by viruses as receptors include CD4 for human immunodeficiency virus type 1 (Dalglish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986; Robey & Axel, 1990), the poliovirus receptor (Mendelsohn et al., 1989), and the mouse coronavirus receptor (Williams et al., 1991). In ICAM-1, in the poliovirus receptor (Freistadt & Racaniello, 1991; Koike et al., 1991), and in CD4 (Arthos et al., 1989), the primary receptor-virus binding site is domain D1. The structures of the 2 amino-terminal domains of CD4 have been determined to atomic resolution (Ryu et al., 1990; J. Wang et al., 1990; Brady et al., 1993). Truncated proteins corresponding to the 2 amino-terminal domains of ICAM-1 (D1D2, consisting of 185 amino acids) as well as the intact extracellular portion of ICAM-1 (D1-D5, consisting of 453 amino acids) have been expressed in CHO cells (Greve et al., 1991). The desialated form of D1D2 has been crystallized (Kolatk et al., 1992).

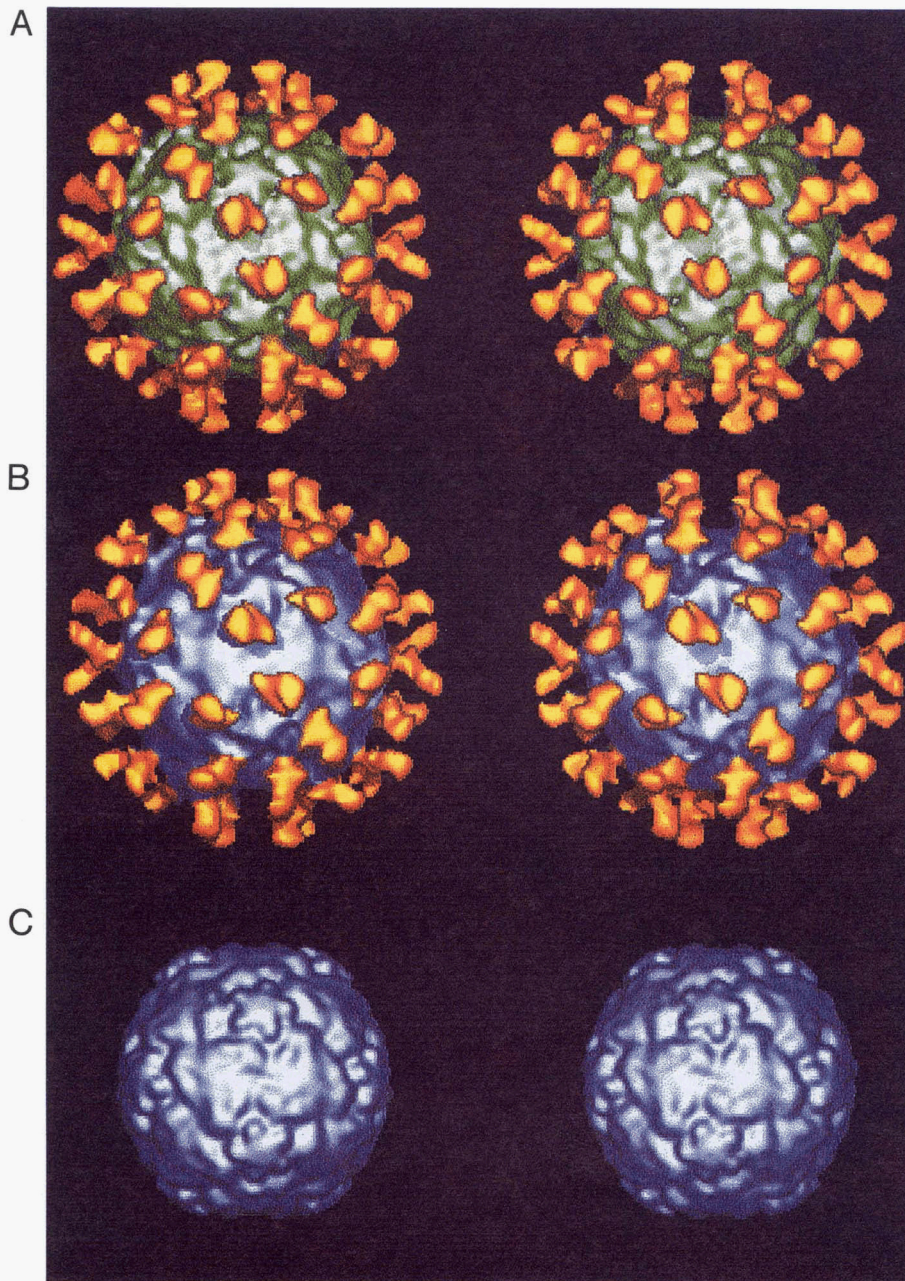
The structure of the complex of D1D2 with HRV16 (Olson et al., 1993) and with HRV14 (P.R. Kolatk, N.H. Olson, C. Music, J.M. Greve, T.S. Baker, & M.G. Rossmann, unpubl.



**Fig. 6.** Schematic diagram of viral receptors. The relative size and distribution of immunoglobulin-like domains are shown. The black circles show the position of potential glycosylation sites. Numbers indicate the amino acid positions of Cys residues involved in predicted disulfide (S-S) bridges. (Reprinted with permission from Colonna [1992]. Copyright by Academic Press Limited.)

results), and of D1D5 with HRV16 (Kolatk et al., unpubl. results), has been determined using cryoelectron microscopy and image reconstruction procedures (Fig. 7). The position of the ICAM-1 molecule relative to the icosahedral symmetry axes of the virus is unambiguous (Kolatk et al., unpubl. results) and shows the receptor binding into the canyon (Fig. 8). Each D1D2 molecule has an approximate dumbbell shape, consistent with the presence of a 2-domain structure. A difference map between the EM density and the 20-Å resolution HRV16 or HRV14 densities confirmed that the D1D2 molecule binds to the central portion of the canyon roughly as predicted by Giranda et al. (1990). There are some small differences in orientation of D1D2 when complexed to HRV16 or HRV14 that may relate to the change in length of the VP1 BC loop forming the north rim of the canyon (Kolatk et al., unpubl. results). The D1D2 ICAM fragment is oriented roughly perpendicular to the viral surface and extends to a radius of about 205 Å. Its total length is about 75 Å.

Extensive structural similarity between D1D2 of ICAM-1 and CD4 was shown by means of a cross-rotation function between the known structure of D1D2 for CD4 (Ryu et al., 1990; J. Wang et al., 1990) and the crystal diffraction data for ICAM-1 D1D2 (P.R. Kolatk, J.M. Greve, & M.G. Rossmann, unpubl. results). Thus, it seemed reasonable to use the known structures of CD4 for fitting the reconstructed density map (Fig. 7), although there was slightly too little density for domain D1 and too much density for D2. A better assessment of the fit of domain D1 to the density was obtained by taking the predicted D1

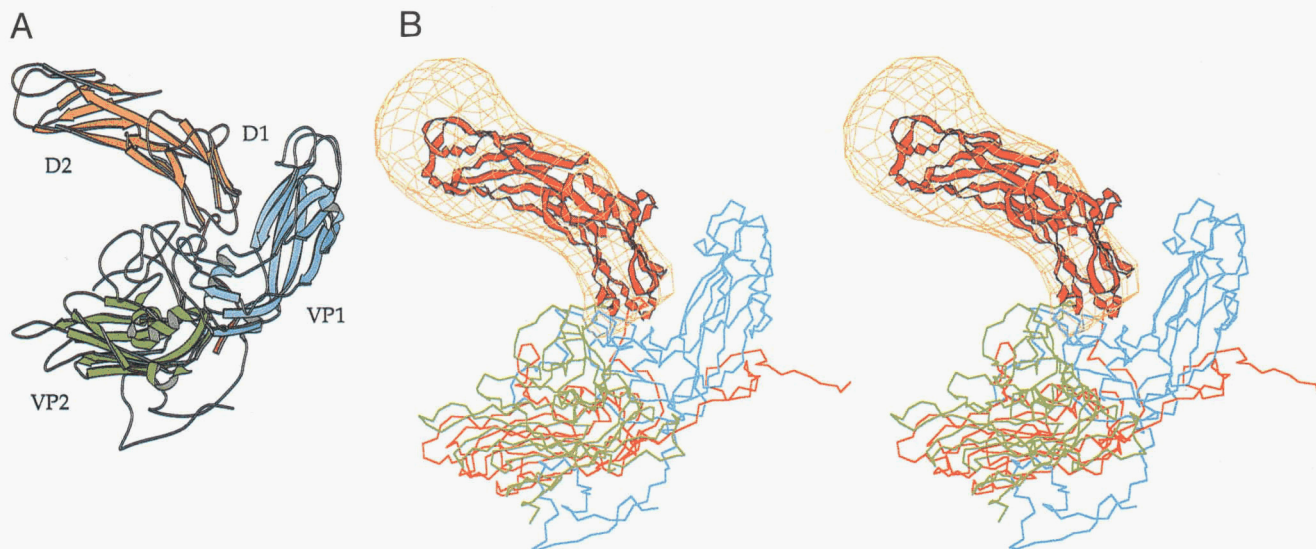


**Fig. 7.** Stereo views of cryo EM image reconstructions of (A) HRV16 (green)–D1D2 (orange) and (B) HRV14 (blue)–D1D2 (orange) complex, viewed along an icosahedral 2-fold axis in approximately the same orientation as in Figure 2. Both A and B show 60 D1D2 molecules bound to symmetry-equivalent positions in the canyons on the virion surface. C: Shaded-surface view of HRV14 (blue), computed from the known atomic structure (Rossmann et al., 1985), truncated to 20 Å resolution.

structure of ICAM-1, including all side chains, and superimposing it onto the fitted  $C_{\alpha}$  backbone of CD4. One major difference is that, although domain D1 of CD4 resembles a variable immunoglobulin-like domain with 2 extra  $\beta$ -strands, the ICAM-1 sequence is shorter and more like a constant C1 domain (Giranda et al., 1990); however, Berendt et al. (1992) suggest that the topology might be like a constant C2 domain in which strand C is not part of either sheet region. This gives domain D1 of ICAM-1 a sleeker appearance, consistent with the observed difference density. The extra density in D2 (in the region farthest from the virus) compared with domain D2 of CD4 is probably due to the 4 associated carbohydrate groups located in this region.

The footprint of ICAM-1 onto the HRV14 structure (Fig. 9) correlates very well with Colonna's mutational studies of residues in the canyon that alter affinity of the virus to HeLa cell membranes (Colonna et al., 1988). All the residues are part of the canyon floor and lie centrally within the footprint of the D1D2 molecule binding site. Similarly, there is excellent agreement between the ICAM-1 footprint and residues on the virus surface whose conformation is changed by antiviral agents (Smith et al., 1986; Heinz et al., 1989; Pevear et al., 1989).

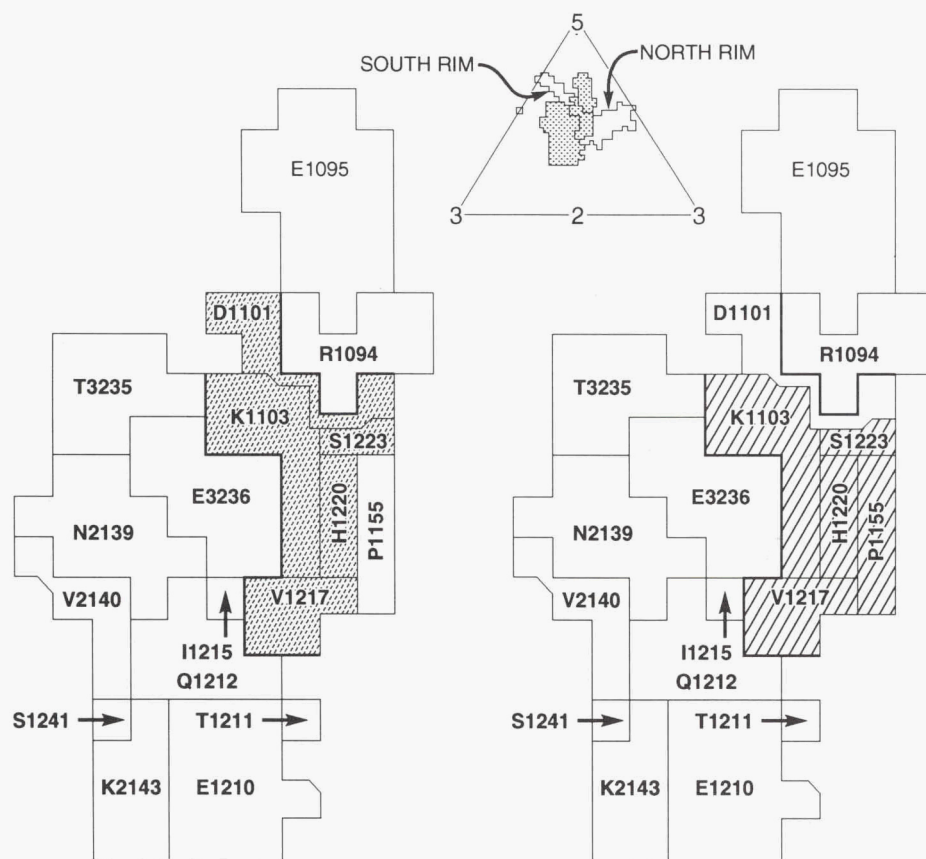
Immunoglobulin-like domains consist of 7  $\beta$ -strands ( $\beta A$ – $\beta G$ ) arranged into 2  $\beta$ -sheets that form a  $\beta$ -sandwich (Fig. 8). The sequence of the first domain of ICAM-1 (D1) has 2 unusual features for an immunoglobulin-like domain: it is relatively short,



**Fig. 8. A:** Structure of HRV16 VP1 (blue), VP2 (green), and part of VP3 (red) complexed with D1D2 of ICAM-1 (orange) modeled from the known structure of CD4. **B:** The difference map between those shown in Figure 7A and B.

being 88 residues instead of the more typical size of approximately 100 residues; and, instead of the typical 2 cysteine residues, located in the  $\beta$ B strand and the  $\beta$ F strand, there are 4 cysteines (Fig. 6). The  $\beta$ B and the  $\beta$ F cysteines usually participate in an

intrachain disulfide bond across the  $\beta$ -sandwich in most members of the immunoglobulin supergene family. However, the additional 2 cysteine residues in ICAM-1 D1 have an  $i + 4$  spacing relative to Cys 21 and Cys 65, which in a  $\beta$ -strand would place



**Fig. 9. Top:** View of the icosahedral asymmetric unit bounded by adjacent 5- and 3-fold axes, outlining residues on the HRV14 surface. The limits of the canyon are shown, arbitrarily demarcated by a 138-Å radial distance from the viral center (Rossmann & Palmenberg, 1988). Residues under the ICAM-1 footprint are stippled. Improved resolution of the electron density could only marginally alter the HRV residues at the virus-receptor interface. **Left and right:** Enlarged view of the residues in the ICAM-1 footprint showing the residues (hatched areas) that, when mutated, affect viral attachment (right) (Colonno et al., 1988), and the residues (stippled areas) altered in structure by the binding of antiviral compounds that inhibit attachment and uncoating (left) (Smith et al., 1986). (Reprinted with permission from Olson et al. [1993]. Copyright by the National Academy of Sciences.)



them in proper register for forming a second disulfide bond between the  $\beta$ B and  $\beta$ F strands.

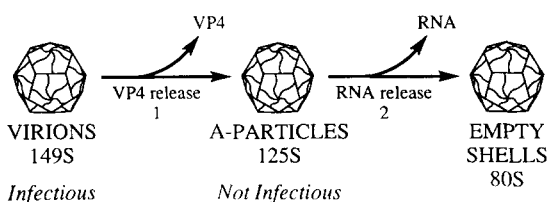
The parts of the predicted ICAM-1 structure (based on Giranda et al. [1990]) that contact HRV14 or HRV16 are the amino-terminal 4 residues and loops BC (residues 24–26), DE (residues 45–49), and FG (residues 71–72). This is roughly in correspondence with the “malarial” binding side of ICAM-1, rather than the LFA-1 binding region (Berendt et al., 1992). This part of ICAM-1 has been associated with adherence to erythrocytes infected with the malarial parasite *Plasmodium falciparum*. Staunton et al. (1990), McClelland et al. (1991), and Register et al. (1991) have examined the effects of a number of site-directed mutations and mouse-human substitutions in domain D1 of ICAM-1 on rhinovirus binding (mouse ICAM-1 does not bind to rhinoviruses). There is correspondence to the 4 regions of ICAM-1 seen to be in contact with rhinovirus, and 4 of the 7 regions implicated in virus binding by site-directed mutagenesis, but there are also inconsistencies between the mutational and structural data. These should be resolved when the crystal structure of ICAM-1 (Kolatkhar et al., 1992), or better still of the complex, has been determined.

### Virus entry and uncoating

Productive viral uncoating requires that the RNA move from inside the viral protein shell, through a cellular membrane, into the cytosol. Such displacement probably requires large conformational changes in the rhinovirus coat. For poliovirus or rhinovirus, acidification of endosomes may be required for an infection to proceed normally as measured by either progeny virus production or cytopathic effects (Madshus et al., 1984a, 1984b; Zeichhardt et al., 1985; Neubauer et al., 1987; Gromeier & Wetz, 1990), although Pérez and Carrasco (1993) conclude that acidification is not essential.

Rhinovirus and poliovirus 149S infectious virions undergo several progressive transformations (Lonberg-Holm & Korant, 1972; Everaert et al., 1989) when bound to cells (Fig. 10) that can be followed by sedimentation through sucrose gradients. The 149S virions are initially converted to 135–125S particles, which have lost VP4 but retain RNA (altered or “A”-particles). Subsequently, the RNA is released with the formation of 80S empty capsids, as well as small capsid fragments.

The A-particles have a number of properties that suggest a role in virus entry. They have been shown to be hydrophobic and able to bind to liposomes (Korant et al., 1975; Hoover-Litty & Greve, 1993). It has also been shown that the formation of po-



**Fig. 10.** Two steps in the uncoating of picornaviruses. The first step, release of VP4, is mediated by interaction with viral receptor, by decreasing pH or by heating to 52 °C. The second step, release of RNA, may be caused by acidification of the membrane-bound particle. (Reprinted with permission from Giranda et al. [1992]. Copyright by the National Academy of Sciences.)

liovirus A-particles is associated with externalization of the N-terminus of VP1 and that removal of approximately 30 residues from the N-terminus of VP1 by proteolysis abolishes the ability of poliovirus to bind to liposomes (Fricks & Hogle, 1990). The sequence of the amino-terminal 23 residues of VP1 suggests that it could form an amphipathic  $\alpha$ -helix and, thus, could promote interactions with lipid bilayers.

A-like particles can be generated under certain conditions in vitro (Koike et al., 1992; Hoover-Litty & Greve, 1993; Yafal et al., 1993). HRV14 incubated at pH 5–6, the pH likely to be found in endosomes, is converted to 135S A-particles. HRV14 incubated with soluble ICAM-1 is converted, through a virus-receptor complex intermediate, to 80S empty capsids, suggesting that receptor binding can destabilize the virion (Hoover-Litty & Greve, 1993).

Because the conformational changes required for uncoating that occur on acidification are probably similar to those that occur on viral interaction with receptor, a structural determination of these changes could be useful. It has been possible to study the initial changes that occur in wild-type HRV14 crystals upon lowering the pH by using a very high-intensity synchrotron X-ray source (Giranda et al., 1992), permitting the rapid recording of the diffraction pattern before the crystals completely disintegrated. It was found that an ion binding site on the icosahedral 5-fold axes, the interior of the virus shell near the 5-fold axes (including the amino-terminal residue of VP3), much of the ordered part of VP4, and the GH loop of VP1 all became disordered. Furthermore, the magnitude of the disorder increased as the time of acid exposure increased. An expansion of the  $\beta$ -cylinder (even beyond the first residue) and cation release, therefore, may be among the first events permitting eventual escape of VP4s, possibly along the 5-fold axial channels. There are parallels to this process in the externalization of VP1 through the 5-fold axial channels of canine parvovirus (Tsao et al., 1991) and the ejection of single-stranded DNA through the 5-fold ion channel of  $\phi$ X174 (McKenna et al., 1992b, 1994). An alternative proposal made by Fricks and Hogle (1990), based on mutational analyses and a comparison with properties of tomato bushy stunt virus (Robinson & Harrison, 1982), suggests that the first step in uncoating and the externalization of VP1 is a weakening of the contacts between protomeric units (Fig. 2).

### Inhibition of uncoating and the pocket factor

Capsid-binding antiviral agents such as the “WIN” compounds bind into a hydrophobic pocket in VP1 below the canyon floor. Not only do they inhibit attachment in HRV14 and other major group rhinoviruses, but they also stabilize major and minor group rhinoviruses in vitro to acidification (Gruenberger et al., 1991) and heat (Fox et al., 1986). HRV14 differs from other picornaviruses in that its pocket is empty in the native structure. For example, there is electron density in the homologous pockets of poliovirus Mahoney 1, poliovirus Sabin 3, and in a chimera of poliovirus 2 (Hogle et al., 1985; Filman et al., 1989; Yeates et al., 1991). This density has been interpreted as a sphingosine or palmitate-like molecule because of the hydrophobic nature of the pocket and the polar environment at one end of the pocket. Similarly, the somewhat smaller electron density in the pocket of HRV1A (S. Kim et al., 1989; K.H. Kim et al., 1993) and HRV16 (Oliveira et al., 1993) has been tentatively interpreted as a fatty acid, 8 or more carbon atoms long. A rather

longer "pocket factor" is found in this pocket for coxsackievirus B3 (CVB3) (J.K. Muckelbauer, L. Tong, M.J. Kremer, & M.G. Rossmann, submitted for publication). Although it is possible that the pocket factor might be a small impurity picked up in the extraction procedure with detergent or during crystallization with polyethylene glycol, these conditions differ greatly among the known structures. Smith et al. (1986) imply, whereas Filman et al. (1989) and Flore et al. (1990) propose, that the pocket factor might be cellular in origin and might regulate viral assembly and uncoating.

Binding of WIN compounds to HRV14 causes major conformational changes in the pocket and, hence, also in the canyon floor (the receptor attachment site). These changes were correlated to inhibition of attachment in the presence of the antiviral compounds (Heinz et al., 1989; Pevear et al., 1989). In contrast, in HRV1A (a minor receptor group virus) and polioviruses, where the WIN compounds merely displace the pocket factor without a correspondingly large conformational change, there is inhibition of uncoating but not of attachment. Preliminary results suggested that rhinoviruses of the minor receptor group exhibited no inhibition of attachment, whereas those of the major receptor group behaved like HRV14, for which attachment is inhibited. Thus, it was a surprise to find "pocket factor" electron density in HRV16, causing the shape of the pocket to resemble that of the "WIN-filled" form of HRV14 (S. Kim et al., 1989; K.H. Kim et al., 1993).

In HRV16 and CVB3, the height for the density of the pocket factor is comparable to that of amino acid side chains, indicating that most pockets are fully occupied. However, in HRV16, the height decreases beyond the sixth carbon atom, suggesting that the density might represent a mixture of fatty acids 6, 8, or 10 carbon atoms long.

In HRV1A and HRV16, the more active antiviral compounds tend to have an aliphatic chain less than or equal to 5 carbon atoms long (Mallamo et al., 1992), correlating with the available space within the binding pocket (Diana et al., 1990, 1992; K.H. Kim et al., 1993). In HRV14, the most active antiviral agents tend to be longer, with 7-carbon aliphatic chains. For example, WIN 56291 has an aliphatic chain of only 3 carbons (compare Fig. 5) and is equally active against HRV16 and HRV1A but less active against HRV14. Thus, for each serotype, there is an optimal drug size that displays the greatest activity and binding affinity (Diana et al., 1990, 1992) and best fills the volume of the pocket. It follows that the smaller pocket factors, which can be easily displaced by WIN compounds in HRV16 and HRV1A (K.H. Kim et al., 1993; Oliveira et al., 1993), bind with less affinity than the antiviral compounds. Nevertheless, the pocket factors seen in the electron densities remain in the pocket even after extensive dialysis of the virus sample. The WIN compounds have a binding constant comparable to their minimal inhibitory concentrations of  $\sim 10^{-8}$  M (Fox et al., 1986, 1991).

#### Role of the pocket factor

When the antiviral binding pocket in HRV14 is filled with WIN compounds or fragments of WIN compounds that do not inhibit infectivity, there is an increase in the thermal stability of the virus (Heinz et al., 1990; Bibler-Muckelbauer et al., 1994), presumably as a consequence of placing a hydrophobic molecule into an internal hydrophobic cavity (Eriksson et al., 1992a,

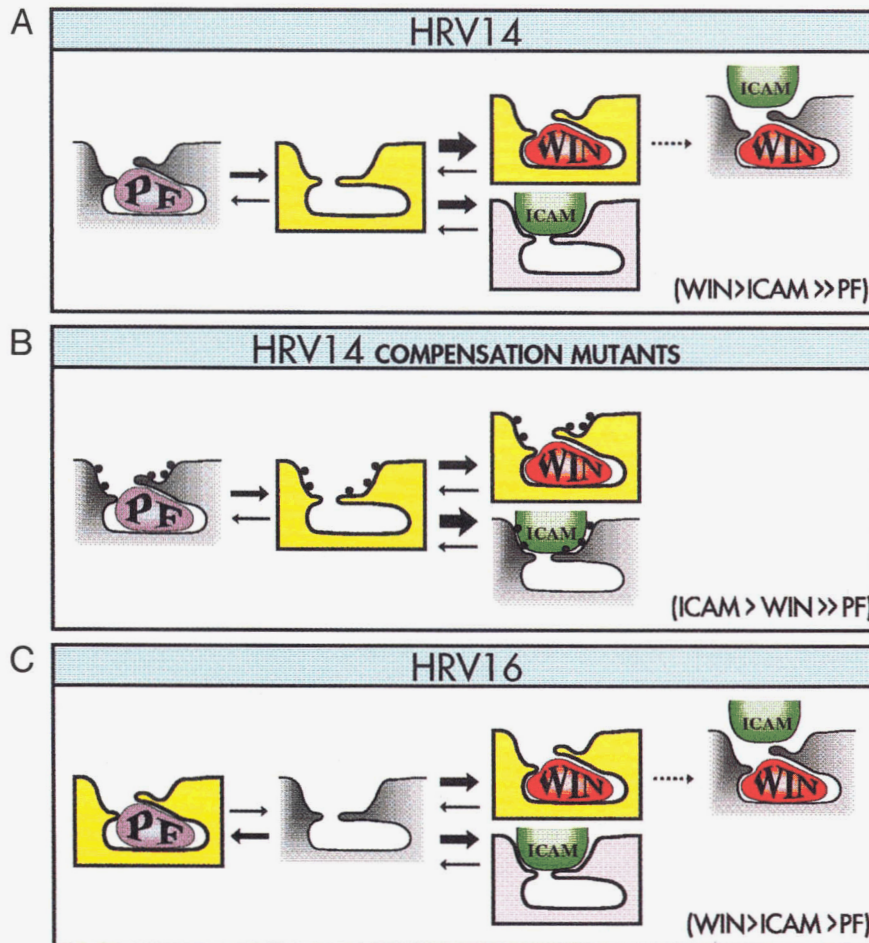
1992b). Similarly, drug-dependent mutants of poliovirus require WIN compounds to maintain their stability (Mosser & Rueckert, 1993). The pocket factor may, therefore, be required to stabilize the virus in transit from one cell to another. However, the delivery of the infectious RNA into the cytoplasm must require a destabilizing step that might be effected by expulsion of the pocket factor during receptor-mediated uncoating.

Because ICAM-1 binds to HRV14 and to HRV16 (Fig. 13), the shape of the canyon for HRV16 should be similar to that in HRV14 when ICAM-1 binding occurs. As soluble ICAM-1 binds to purified HRV14, which does not contain any pocket factor, presumably the pocket is empty when ICAM-1 binds to HRV16. However, the structure of HRV16 shows the presence of a pocket factor in the purified virus (Oliveira et al., 1993). Hence, it must be assumed that the pocket factor is displaced before the receptor can seat itself into the canyon. In essence, there are 2 competing equilibria: the binding of ICAM-1 and the binding of the pocket factor to the virus. Although the sites of binding of ICAM-1 and of the pocket factor are not the same, they are in close proximity and interfere with each other. The floor of the canyon is also the roof of the pocket for the pocket factor or WIN compounds. When ICAM-1 binds, the floor is depressed downward, which is possible only when there is nothing in the pocket. Conversely, when there is a compound in the pocket, its roof is raised upward. The displacement of the pocket factor *per se* does not cause the virus to fall apart. For instance, when HRV14 is crystallized, it does not contain a pocket factor, and the complex of HRV16 with ICAM-1 is reasonably stable. Nevertheless, the absence of pocket factor increases the potential for disruption by lowered pH or by formation of the receptor-virus complex.

Presumably, the destabilization of the virus on cell attachment is made possible by the displacement of a sufficient number of pocket factors when the receptor competes for the overlapping binding site. Progressive recruitment of receptors is then sufficient to trigger release of the VP4s. The terminal myristate moieties of VP4 and the exposure of the amino terminus of VP1 will permit entry through the cell membrane, possibly by creating a channel along the 5-fold axes of the virus (Giranda et al., 1992).

A class of HRV14 drug-resistant (compensation) mutants can be selected by growing the virus in the presence of antiviral WIN compounds. Such mutants occur at a frequency of about 1 per  $10^4$  virions. They have been shown to be mostly single mutations (Heinz et al., 1990; Shepard et al., 1993), and 6 of the 7 characterized to date are situated near the walls and floor of the canyon. WIN compounds bind into the pocket of these mutant viruses and deform the canyon floor in a similar manner to their effect on wild-type viruses (M.A. Oliveira, I. Minor, R.R. Rueckert, & M.G. Rossmann, unpubl. data). In some of these mutants, the affinity of ICAM-1 for the virus is enhanced (R.R. Rueckert, pers. comm.; M.P. Fox, D.C. Pevear, & F.J. Dutko, unpubl. data). Thus, it is reasonable to conclude that ICAM-1 binds better to these mutant viruses than the WIN compounds (Fig. 11B).

In the case of poliovirus or HRV1A (a minor group rhinovirus), only uncoating is inhibited by WIN compounds, and not attachment. If the pocket factor needs to be absent for the virus to uncoat, binding of receptor to these viruses should lead to displacement of the pocket factor, just as is the case for the major group rhinoviruses. Similarly, the WIN compounds must



**Fig. 11.** Conditions for inhibition of viral attachment by WIN compounds. Crystallographically and electron microscopically determined structures are in yellow and pink, respectively, whereas hypothetical structures are in gray. **A:** In wild-type HRV14, the pocket factor binds weakly and is not observed in crystallographic studies. When WIN compounds bind into the pocket, they deform the roof of the pocket, which is also the floor of the canyon. This inhibits the attachment of the virus to the ICAM-1 receptor and, hence, presumably the binding affinity of WIN is greater than that of ICAM-1. When ICAM-1 recognizes the canyon floor, the putative pocket factor must be displaced by ICAM-1 and, hence, the binding affinity of ICAM-1 is greater than that of pocket factor. **B:** Drug-resistant compensation mutants of HRV14 cluster around the canyon walls and floor (crosses) and increase the affinity of ICAM-1 for the virus. Although WIN compounds can bind to the virus, they do not inhibit infectivity. Thus, the binding affinity of the mutant virus to ICAM-1 is greater than that of WIN. **C:** Wild-type HRV16 contains a pocket factor. This can be replaced by WIN compounds, which inhibit attachment. Hence, in this case, the affinity of HRV16 for WIN is greater than that of ICAM-1, which is greater than that of pocket factor.

also be displaced by the receptor because there is no inhibition of attachment, thus requiring the remaining WIN compounds to stabilize the virus sufficiently to inhibit uncoating.

## Conclusions

### Receptor attachment site

The canyon hypothesis, which suggested that the receptor binding site can be hidden from immune surveillance in a "canyon" on the surface of the capsid, has been verified for the major group of rhinoviruses. Mutational analyses indicate that the canyon is also the receptor attachment site for poliovirus (Racaniello, 1992). The receptor for the minor group of rhinovirus serotypes has been reported to be the LDL receptor (Hofer et al., 1994), a vastly different type of molecule than ICAM-1. Although no clear relationship has been established between sequence and receptor specificity, it may be relevant that the binding site of ICAM-1 on the minor group HRV1A is more basic in character (S. Kim et al., 1989) than the corresponding site is on major group rhinoviruses. The large positively charged residues that line the canyon of HRV1A correlate with the large positively charged patch on an LDL receptor binding protein (C. Wilson et al., 1991). This protein, as also minor group rhinoviruses, is able to bind to the LDL receptor.

It should not be concluded that a canyon is the only available strategy for hiding the receptor attachment site from the host's immune system. For instance, Acharya et al. (1990) suggest that a disordered RGD sequence, thought to be a part of the receptor binding site of FMDV, might be protected from immune recognition by surrounding it with hypervariable residues.

### Virus entry

A virus must be stable in the extracellular environment during transit between hosts but also must be destabilized once it has bound to or entered the host cell, shedding its protein coat to allow infection to proceed. In rhinoviruses and polioviruses, the need for reversible stabilization appears to be fulfilled by the binding of a small cellular aliphatic molecule, the "pocket factor," into a hydrophobic pocket in VP1. In the major group of rhinovirus serotypes, the binding site for ICAM-1, the virus receptor, overlaps with the binding site of the stabilizing pocket factor. Virus attachment is, therefore, a competition between 2 equilibria: (1) binding of the pocket factor into the pocket and (2) binding of the receptor into the canyon. Provided that the receptor competes successfully with the pocket factor, many pocket factors will be lost as receptor molecules are recruited, destabilizing the virus as a prelude for uncoating. Certain antiviral compounds also bind in the hydrophobic pocket, displac-

ing the pocket factor. If the affinity of an antiviral compound for the pocket is higher than that of ICAM-1, the antiviral compound will prevent receptor attachment and uncoating. Drug escape mutations in VP1 that improve binding affinity for ICAM-1 can shift this balance, overcoming the antiviral effect (Fig. 11).

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