Viral cell recognition and entry

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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 comparison 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 which 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.

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. Some viruses recognize very specific molecules (e.g. a large group of rhinoviruses recognize intercellular adhesion molecule-1 (ICAM-1)), while 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 which show that these viruses have evolved from a common ancestor[1-3], nevertheless they recognize a variety of receptors (Table 1). Possibly the primordial virus had the ability to weakly bind to a large number of different molecules. With time, different viruses evolved which became progressively more efficient and specialized towards 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, while coxsackie B viruses recognize yet another receptor. Therefore, it is surprising that rhinovirus serotypes can be divided into three groups which recognize different receptors[4,5]. Furthermore, the receptor for the major group of rhinoviruses, ICAM-1, belongs to the immunoglobulin superfamily[6,7], whereas the receptor for the minor group has been reported to be the low density lipoprotein (LDL) receptor[8].

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 which requires translocation of the viral genome or a sub-viral particle across the membrane into the cytoplasm, and, in some cases, into the nucleus. Since 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 envelope with the limiting cellular membrane (Figure 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 like picornaviruses[3] 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, which are a major cause of the common cold in humans[3]. These viruses belong to the picornavirus family, which also contains the genera *Enterovirus*, *Aphthovirus*, *Cardiovirus* and

Table 1. Receptors 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 and Colonno[4], Greve *et al.*[6], Staunton *et al.*[7]
Human rhinovirus minor group: 11 serotypes, including 1A, 2, 44, 49	Low-density lipoprotein (LDL) receptor	LDLR	Abraham and Colonno[4], Hofer *et al.*[8]
Polioviruses	Poliovirus receptor (PVR)	Ig (3 Ig domains)	Mendelsohn *et al.*[35]
Coxsackievirus A13, 18, 21	ICAM-1	Ig (5 Ig domains)	Colonno *et al.*[84], Roivainen *et al.*[85]
Coxsackievirus A2, 5, 13, 15, 18	?	?	Colonno *et al.*[84], Roivainen *et al.*[85], Schultz and Crowell[86]
Echovirus 1	VLA-2	Integrin	Bergelson *et al.*[87]
Echovirus 6	?	?	Crowell[88]
Foot-and-mouth disease viruses, types $A_{12}119$, O_{1B}, C_{3Res}:SAT_{1-3}	RGD integrin	Integrin	Sekiguchi *et al.*[89], Mason *et al.*[90]
Mengo virus	?	Glycophorin (?)	Burness[91], Burness and Pardoe[92]

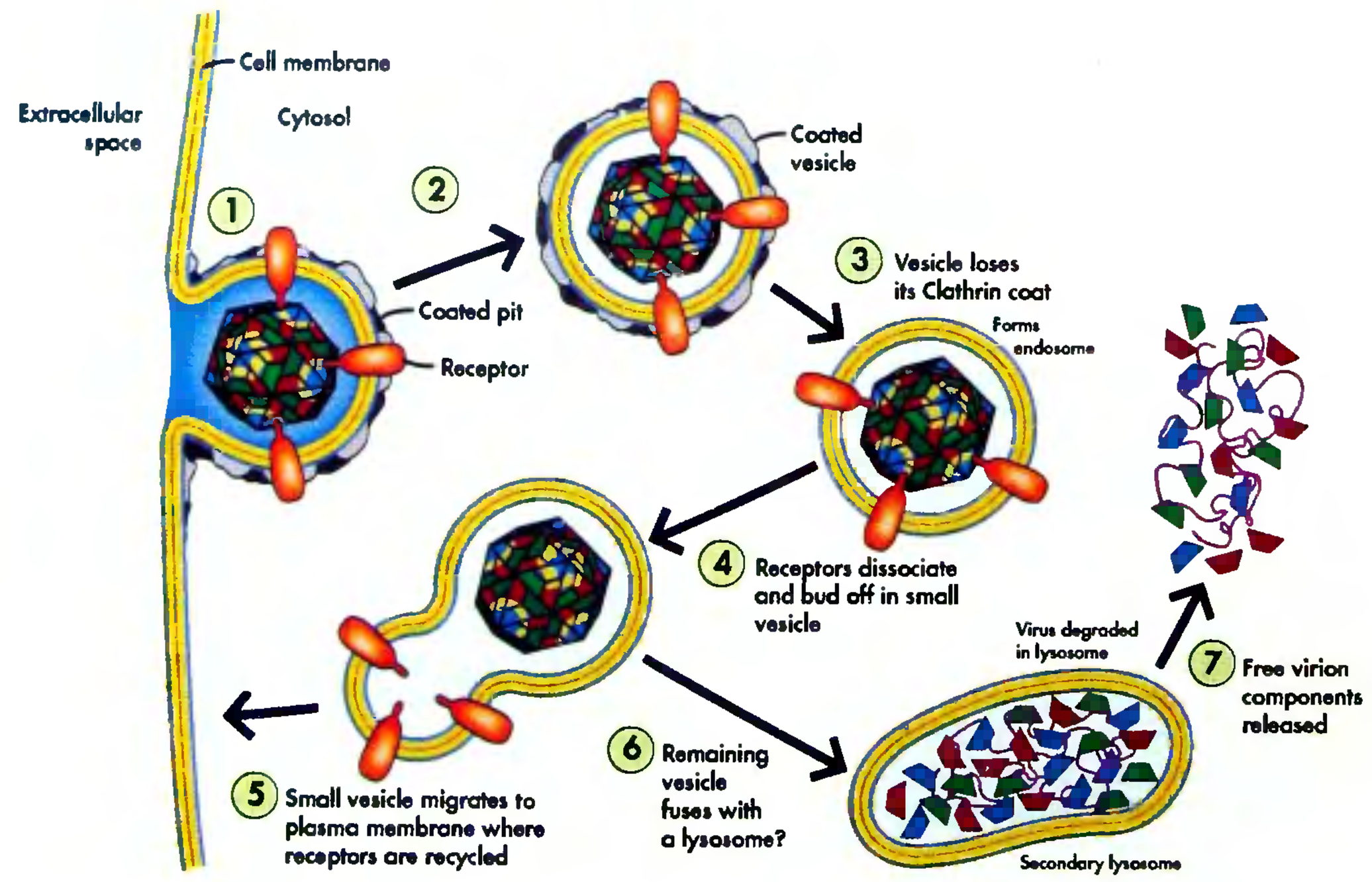

Figure 1. One possible endocytotic process (adapted from Rawn[103]). Note, however, that in most cases it is not known when and where the receptor and virus part company, whether it is necessary for the virus to be bound to the receptor during uncoating and what is the mechanism by which RNA translocates the membrane.

Hepatitis A virus. The picornaviruses are small, icosahedral, nonenveloped, single-stranded RNA viruses. X-ray crystal structures have been determined for at least one member in each picornavirus genus except for hepatitis A viruses[1,9-13]. 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 8.5×10^6 daltons and an external diameter of around 300 Å.

Each of the 60 icosahedral protomers in picornaviruses contains four viral polypeptides, VP1–VP4. VP1, VP2 and VP3 reside on the exterior of the virus and make up its protein shell (Figure 2). These three peptides, each having a molecular weight of roughly 35 kDa, contain a common eight-stranded, antiparallel, β-barrel motif[1] (Figure 3, Table 2). Their amino termini intertwine to form a network on the interior of the protein shell. Five VP3 amino termini form a five-stranded helical β-cylinder on the virion's interior about each icosahedral five-fold axis. This β-cylinder stabilizes the pentamer and is thought to be important for its assembly[9,14].

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[15] 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. refs 10, 14) 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[16]. In poliovirus, the myristylate moiety lies inside the virion coat close to the β-cylinder. The first 25 to 28 amino-terminal residues of VP4 are mostly disordered in rhinovirus structures, but density consistent with myristylate is seen internally near the center of the pentamer in rhinoviruses 14, 1A and 16 (refs 13, 17, 18).

Each of the three larger capsid proteins has various insertions between the β-strands of the basic folding motif. Many of these insertions decorate the viral exterior and form 'puffs' and loops which are hypervariable and have been shown to be the binding site of neutralizing antibodies[1,19,20]. The surfaces of rhinoviruses (and polioviruses) contain a series of remarkably deep crevices or 'canyons' (Figure 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[1] that the canyon (one around each five-fold vertex; Figure 2) in HRV was the site of receptor attachment, largely inaccessible to the broad antigen-binding region seen on antibodies. Thus, residues

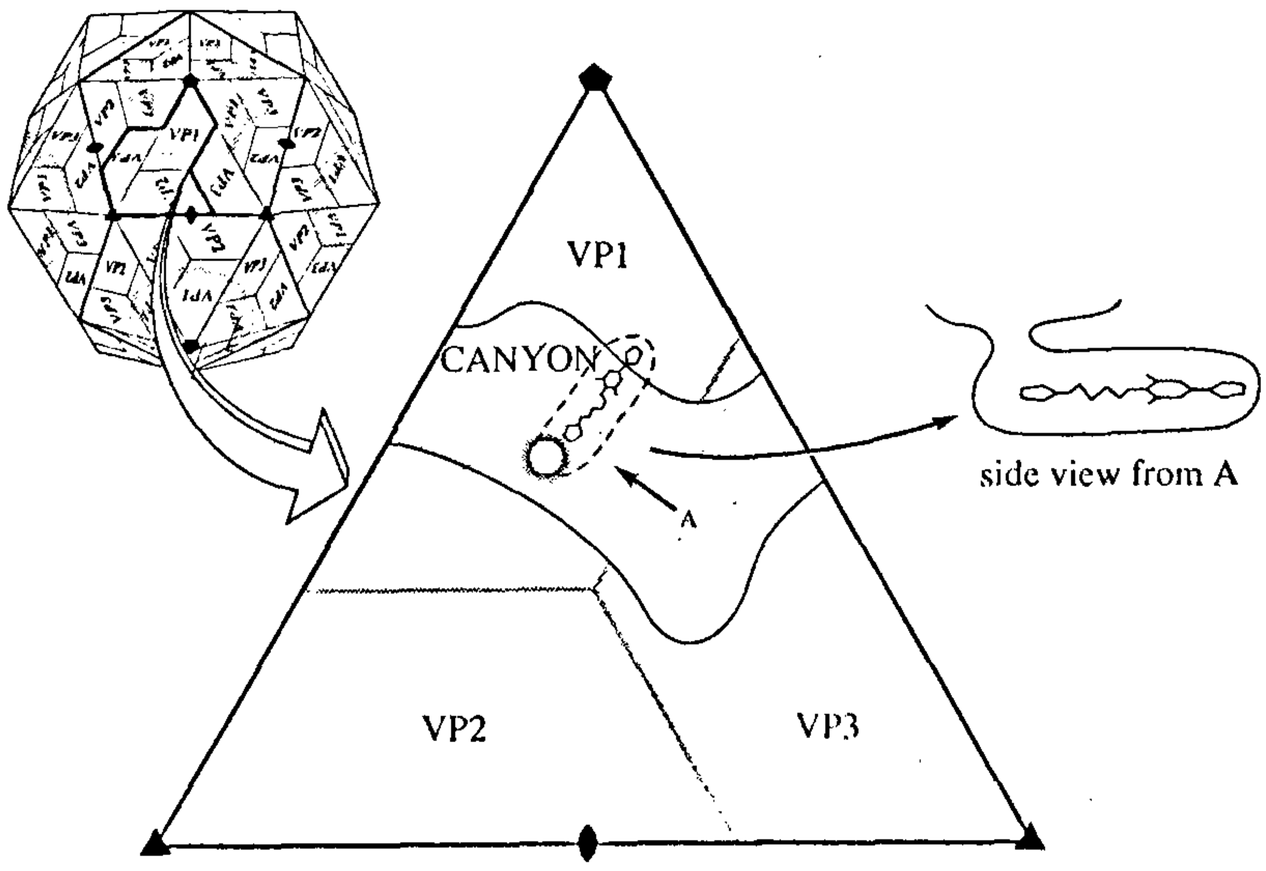

Figure 2. *Top left*: Diagrammatic view of picornavirus showing VP1, VP2 and VP3 and the deep cleft or 'canyon' running around each five-fold vertex. The 6S protomeric assembly unit (which differs from the geometric definition of the asymmetric unit) is shown in heavy outline on the icosahedron. *Center*: 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.*[18]. Copyright by *Current Biology*.]

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 both in HRV[1,19,20] and in poliovirus[9,21]. The 'canyon hypothesis' suggests that one strategy for viruses to escape the host's immune surveillance is to protect the receptor attachment site in a surface depression (Figure 4). Similar depressions related to host cell attachment have also been found on the surface of the haemagglutinin spike of influenza virus[22,23], tick-borne encephalitis virus (Harrison *et al.*, private communication) and may be the case for human immunodeficiency virus[24].

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

There are at least 78 serotypes[25] that bind to ICAM-1, the major group rhinovirus receptor[6,7]. The ICAM-1 molecule has five immunoglobulin-like domains (D1–D5, numbered sequentially from the amino end), a transmembrane portion and a small cytoplasmic domain[26,27]. Domains D2, D3 and D4 are glycosylated (Figure 5). Unlike immunoglobulins, ICAM-1 appears to be monomeric[7]. 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)[27–30]. Other surface antigens within the immunoglobulin superfamily that are used by viruses as receptors include CD4 for human immunodeficiency virus type 1 (refs 31–34), the poliovirus receptor[35] and the mouse coronavirus receptor[36]. In ICAM-1 in the poliovirus receptor[37,38] and in CD4[39], the primary receptor virus binding site is domain D1. The structures of the two amino-terminal domains of CD4 have been determined to atomic resolution[40–42]. Truncated proteins corresponding to the two amino-terminal domains of ICAM-1 (D1D2 consisting of 185 amino acid) as well as the intact extracellular portion of ICAM-1 (D1–D5 consisting of 453 amino acids) have been expressed in CHO cells[43]. The desialated form of D1D2 has been crystallized[44].

The structure of the complex of D1D2 with HRV16 (ref. 45) and with HRV14 (P. R. Kolatkar, N. H. Olson,

Table 2. The common β-barrel fold

Virus[a]	Kingdom	Symmetry of capsid	Genome	Comments[b]	First reference
Plant					
TMV	Plant	Helical	RNA		
TBSV	Plant	$T=3$	RNA	1	Harrison *et al.*[93]
SBMV	Plant	$T=3$	ss + RNA	1	Abad-Zapatero *et al.*[94]
STNV	Plant	$T=1$	ss + RNA	1	Liljas *et al.*[95]
CPMV	Plant	Pseudo $T=3$	ss + RNA	1	Stauffacher *et al.*[96]
BPMV	Plant	Pseudo $T=3$	ss + RNA	1, 2	Chen *et al.*[97]
STMV	Plant	$T=1$	ss + RNA	1, 2	Larson *et al.*[98]
Insect					
BBV	Insects	$T=3$	ss + RNA	1	Hosur *et al.*[99]
FHV	Insects	$T=3$	ss + RNA	1, 2	Fisher *et al.*[100]
Bacterial					
ϕX174	*E. coli*	$T=1$	ss + DNA	3, 4	McKenna *et al.*[101]
Animal					
Influenza	Human	Globular head haemagglutinin spike	ss + RNA	1	Wilson *et al.*[22]
Adeno	Human	Capsid hexon		3	Roberts *et al.*[102]
HRV 14, 1A, 16	Human	Pseudo $T=3$	RNA	1	Rossmann *et al.*[1], Kim *et al.*[13], Oliveira *et al.*[18]
Coxsackievirus B3	Human	Pseudo $T=3$	RNA	1	Muckelbauer *et al.*, in preparation
Polio 1, 2, 3	Human	Pseudo $T=3$	RNA	1	Hogle *et al.*[9]
Cardio	Mice	Pseudo $T=3$	RNA	1	Luo *et al.*[10]
FMDV	Cattle	Pseudo $T=3$	RNA	1	Acharya *et al.*[11]
Parvo	Dogs and cats	$T=1$	ss + DNA	3, 4	Tsao *et al.*[65]

[a] 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.

[b] 1 – There are mostly small insertions between β-strands.
2 – There is a significant amount of ordered RNA.
3 – There are very large insertions between β-strands.
4 – There is some ordered ss + DNA.

C. Music, J. M. Greve, T. S. Baker and M. G. Rossmann, unpublished results) and of D1D5 with HRV16 (Kolatkar *et al.*, unpublished results) has been determined using cryoelectron microscopy and image reconstruction procedures (Figure 6). The position of the ICAM-1 molecule relative to the icosahedral symmetry axes of the virus is unambiguous (Kolatkar *et al.*, unpublished results) and shows the receptor binding into the canyon (Figure 7). Each D1D2 molecule has an approximate dumbbell shape, consistent with the presence of two-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.*[46]. There are some small differences in orientation of D1D2 when complexed to HRV16 or HRV14 which may relate to the change in length of the VP1 BC loop forming the north rim of the canyon (Kolatkar *et al.*, unpublished 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.

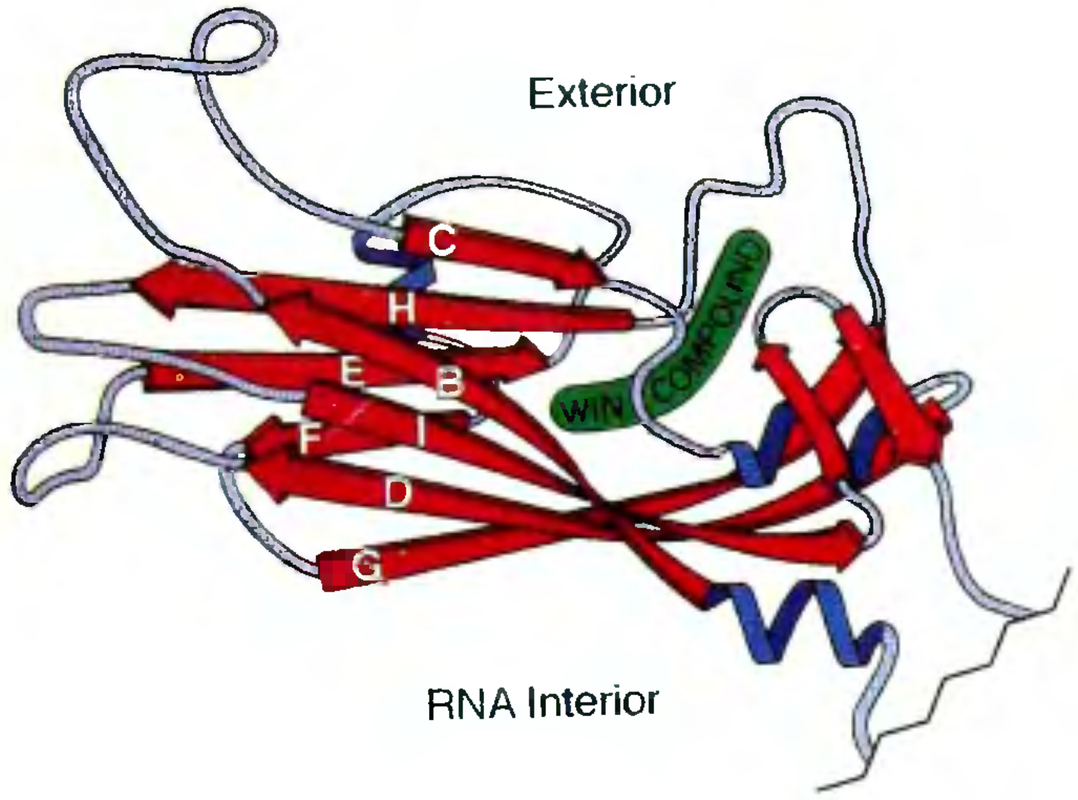

Figure 3. Schematic representation of the VP1 fold of HRV14. The folding topology of the two sheets 'BIDG' and 'CHEF' is the same in VP2 and VP3 as well as in most other viral capsid proteins. The binding site of antiviral WIN compounds within the hydrophobic interior of VP1 is also shown.

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 (refs 40, 41), and the crystal diffraction data for ICAM-1 D1D2 (P. R. Kolatkar and M. G. Rossmann, unpublished results). Thus, it seemed reasonable to use the known structures of CD4 for fitting the reconstructed density map (Figure 6), 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 structure of ICAM-1, including all side chains, and superimposing it onto the fitted C_α backbone of CD4. One major difference is that although domain D1 of CD4 resembles a variable, immunoglobulin-like domain with two extra β-strands, the ICAM-1 sequence is shorter and more like a constant C1 domain[46], although Berendt *et al.*[47] suggest that the topology might be like a constant C2 domain in which strand C is not part of either sheet

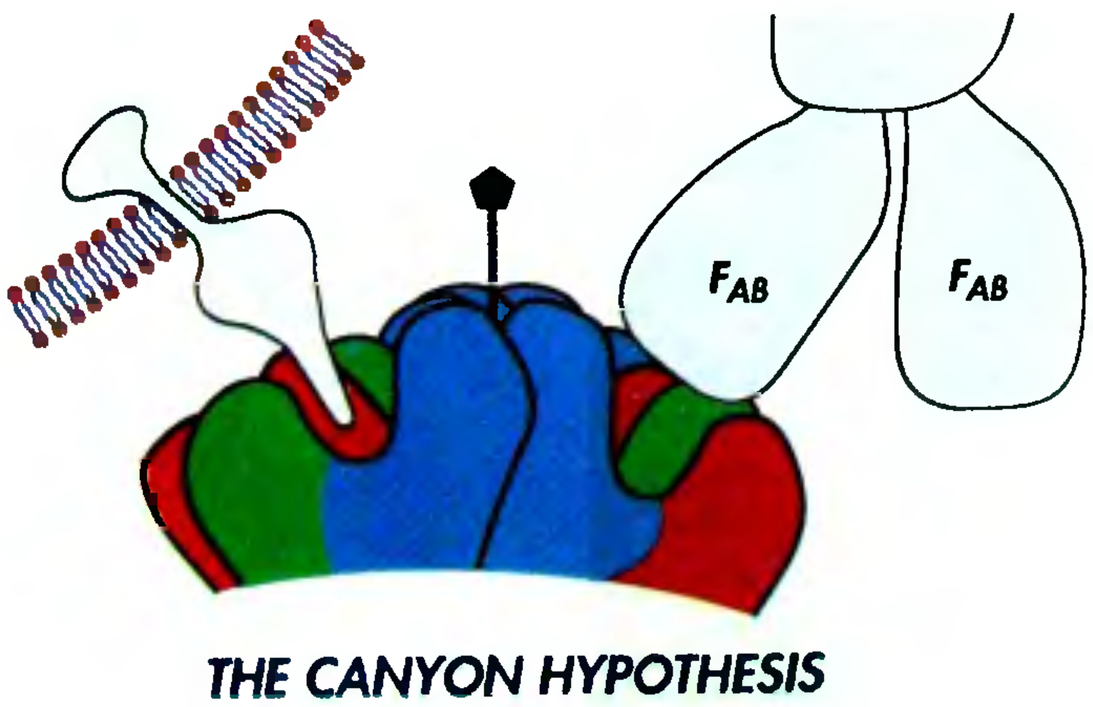

Figure 4. The presence of depressions on the picornavirus surface suggests a strategy for the evasion of immune surveillance. The dimensions of the putative receptor binding sites, the 'canyon', sterically hinder an antibody's (*top right*) recognition of residues at the base of the site, while still 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.

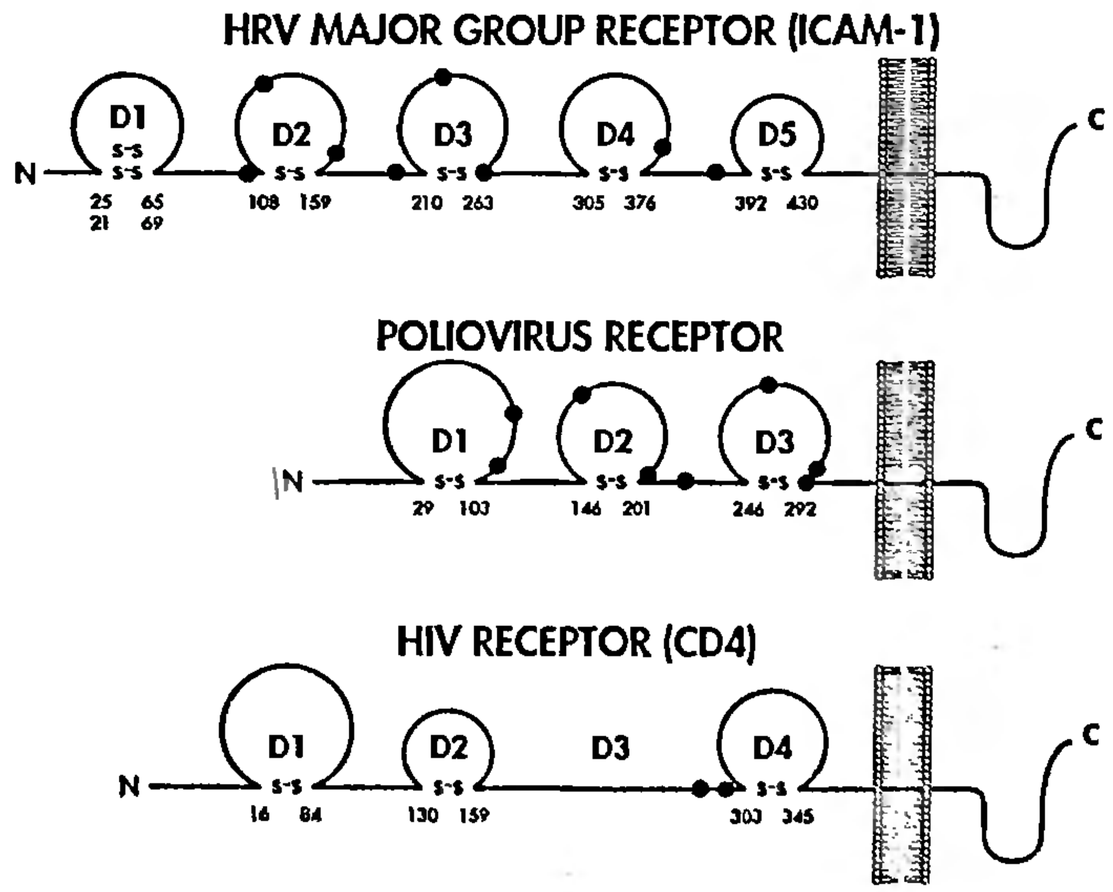

Figure 5. Schematic diagram of viral receptors. The relative size and distribution of immunoglobulin-like domains are shown. The black circles show the position of potential glycosylation sites. Numbers indicate the amino acid positions of Cys residues involved in predicted disulfide (S–S) bridges. [Reprinted with permission from Colonno[104]. Copyright by Academic Press.]

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 away from the virus) compared with domain D2 of CD4 is probably due to the four associated carbohydrate groups located in this region.

The footprint of ICAM-1 onto the HRV14 structure (Figure 8) correlates very well with Colonno's mutational studies of residues in the canyon which alter affinity of the virus to HeLa cell membranes[48]. 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[49–51].

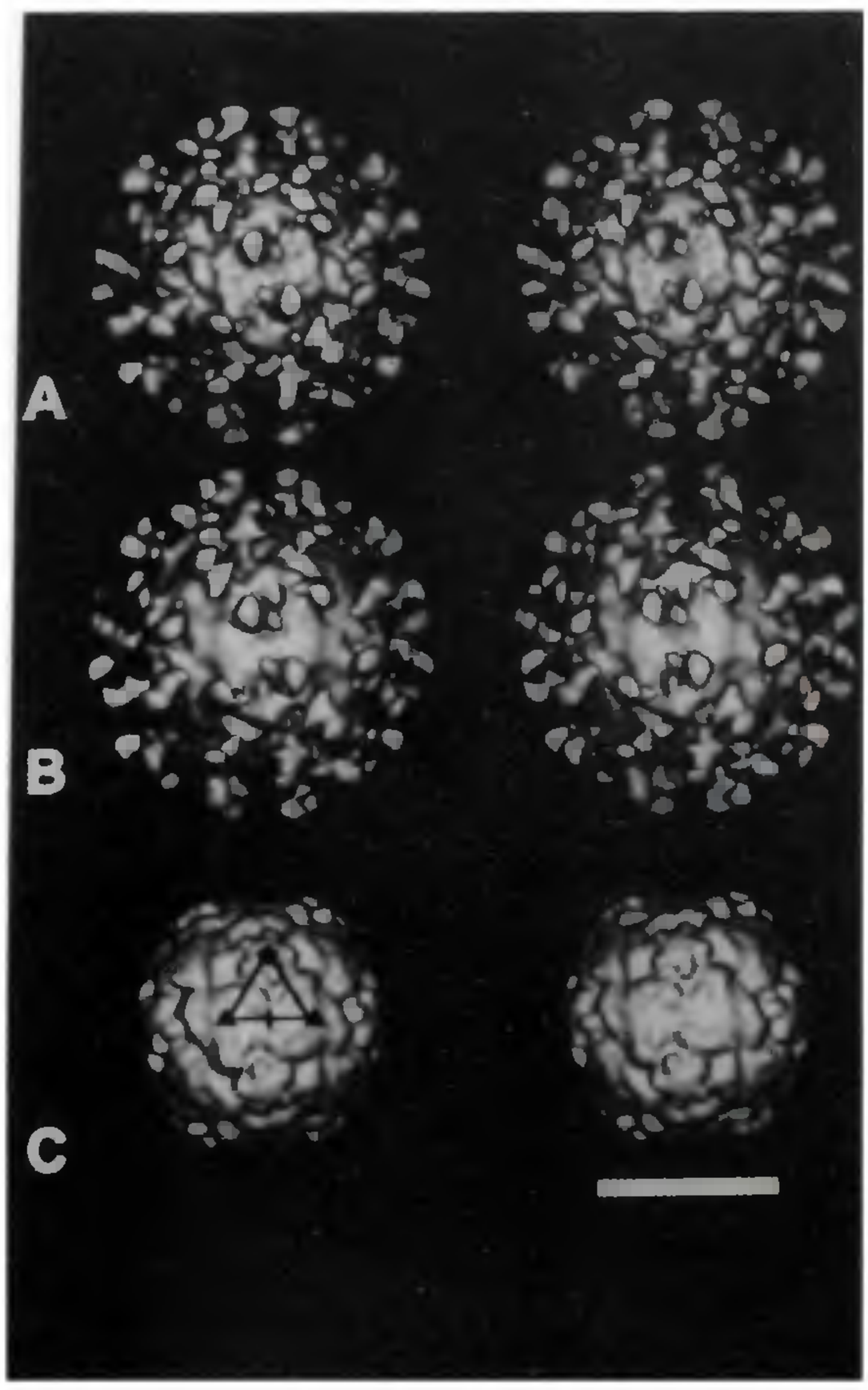

Figure 6. Stereo views of cryo EM image reconstructions of (A) HRV16 (green)–D1D2 (orange) and (B) HRV14 (blue)–D1D2 (orange) complex, viewed along an icosahedral two-fold axis in approximately the same orientation as in Figure 2. Both (A) and (B) show sixty D1D2 molecules bound to symmetry-equivalent positions in the canyons on the virion surface. (C) Shaded-surface view of HRV14 (blue), computed from the known atomic structure[1], truncated to 20 Å resolution.

Virus entry and uncoating

Productive viral uncoating requires that the RNA moves 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[52–56].

Rhinovirus and poliovirus 149S infectious virions undergo several progressive transformations[57,58] when bound to cells, which can be followed by sedimentation through sucrose gradients. The 149S virions are initially converted to 135 to 125S particles which have lost VP4 but retain RNA ('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 which suggest a role in virus entry. They have been shown to be hydrophobic and able to bind to liposomes[59,60]. It has also been shown that the formation of poliovirus 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[61].

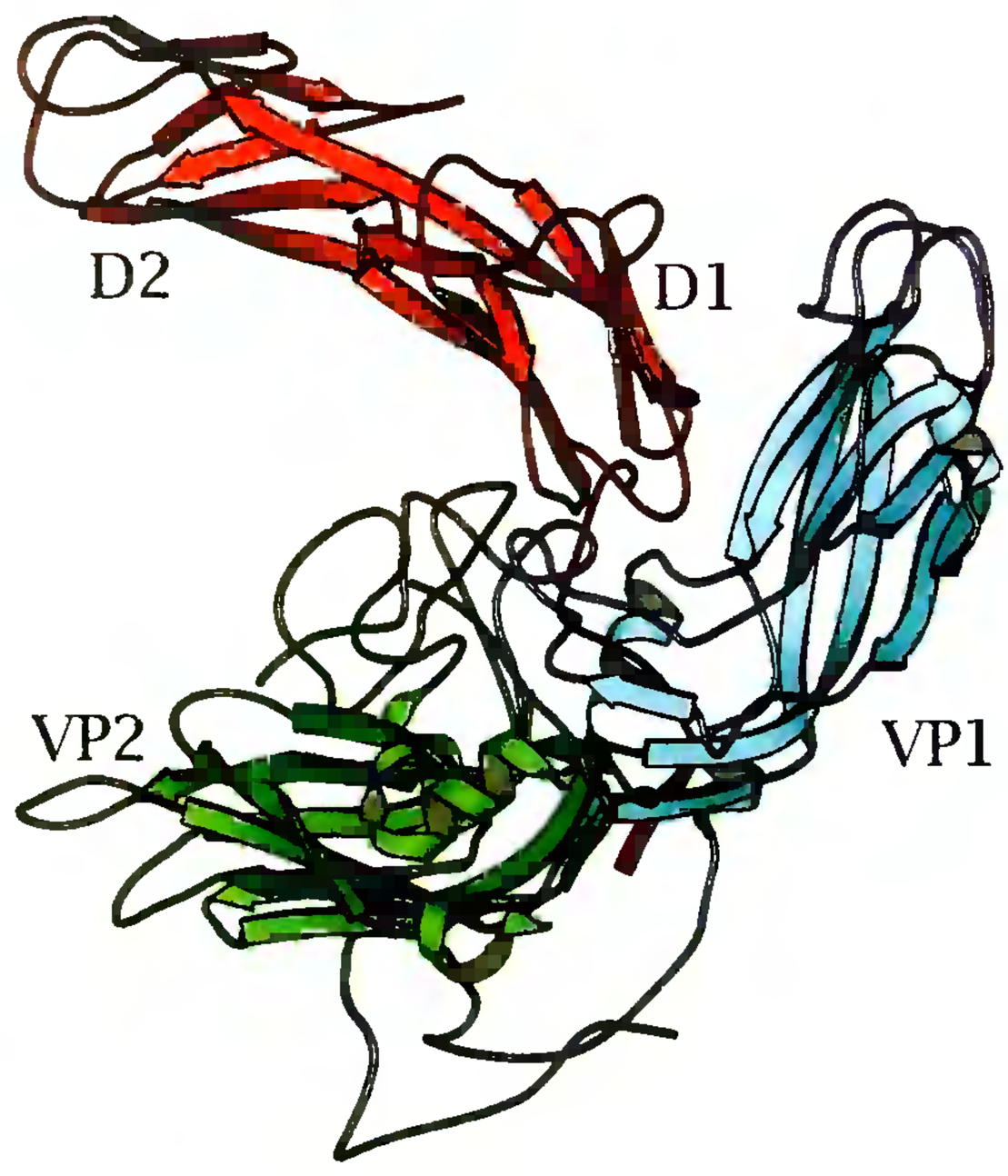

Figure 7. Structure of HRV16 VP1 (blue), VP2 (green) and part of VP3 (red) complexed with D1D2 of ICAM-1 (orange) modeled from the known structure of CD4.

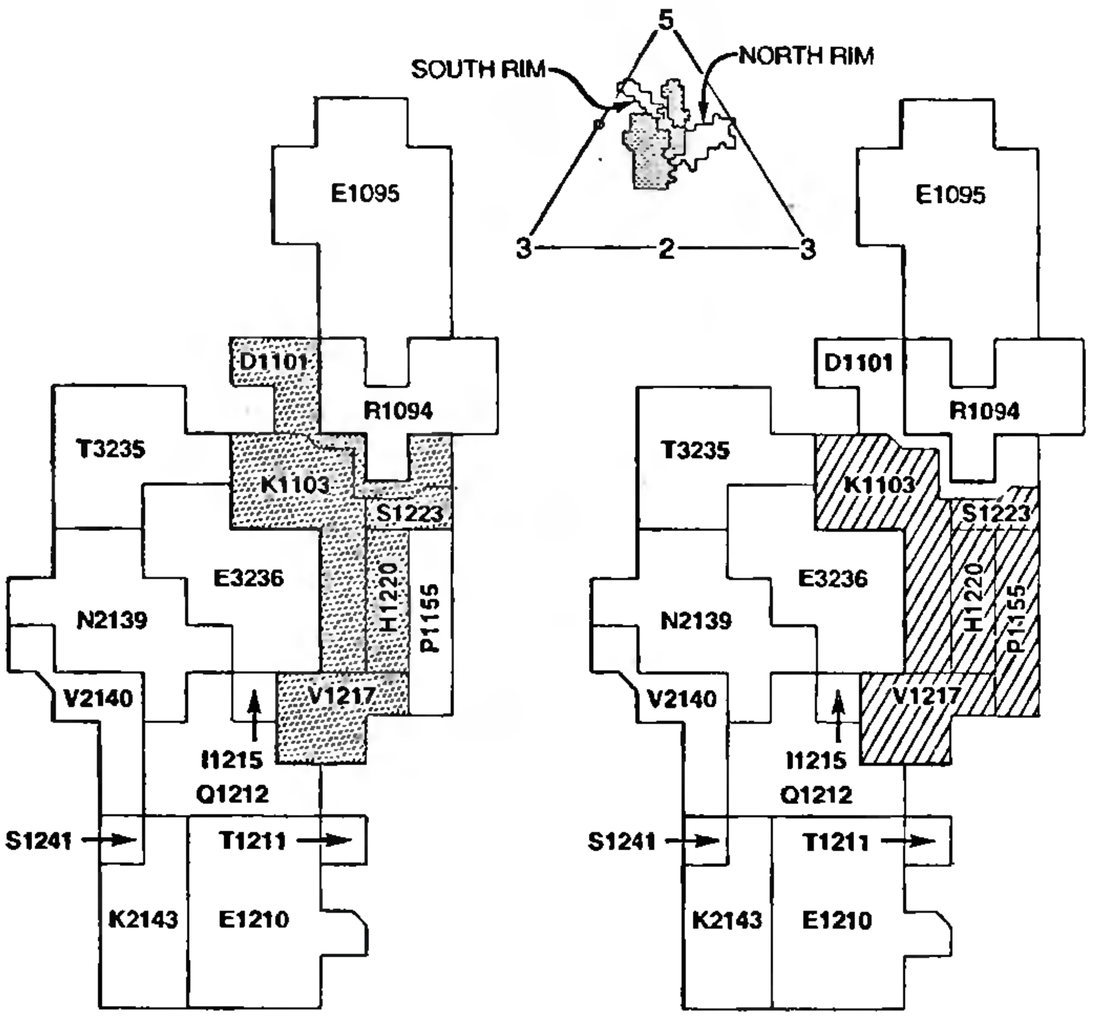

Figure 8. (*Top*) View of the icosahedral asymmetric unit bounded by adjacent five- and three-fold axes, outlining residues on the HRV14 surface. The limits of the canyon are shown, arbitrarily demarcated by a 138 Å radial distance from the viral center[105]. Residues under the ICAM-1 footprint are stippled. 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)[48], and the residues (stippled areas) altered in structure by the binding of antiviral compounds that inhibit attachment and uncoating (left)[49]. [Reprinted with permission from Olson *et al.*[45]. Copyright by the National Academy of Sciences.]

The sequence of the amino-terminal 23 residues of VP1 suggests that it could form an amphipathic α-helix and, thus, could promote interactions with lipid bilayers.

A-like particles can be generated under certain conditions *in vitro*[60,62,63]. 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[60].

Since the conformational changes required for uncoating which 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[64]. This permitted the rapid recording of the diffraction pattern before the crystals completely disintegrated. It was found that an ion-binding site (Figure 9) on the icosahedral five-fold axes, the interior of the virus shell near the five-fold axes including the amino end 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. The expansion of the β-cylinder and cation release, therefore, may be among the first events permitting eventual escape of VP4s, possibly along the five-fold axial channels. There are parallels to this process in the externalization of VP1 through

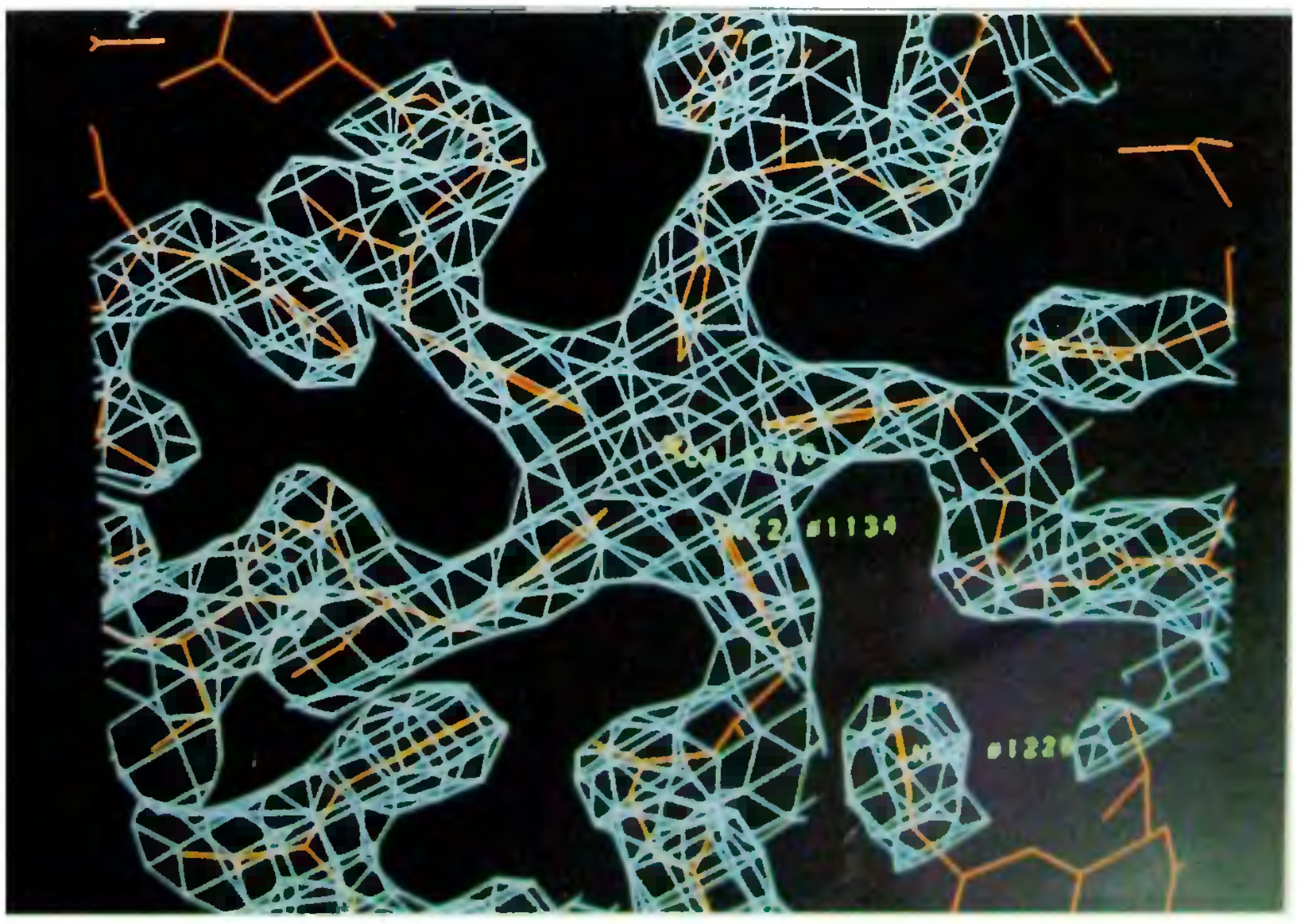

Figure 9. A putative Ca site in HRV16 with five His 1134 ligands (Hadfield *et al.*, unpublished results). Simila cation sites are found in CVB3 and HRV14. The ion comes off on acidification in HRV14.

the five-fold axial channels of canine parvovirus[65], and the ejection of single-stranded DNA through the five-fold ion channel of ϕX 174 (refs 66, 67). An alternative proposal made by Fricks and Hogle[61] based on mutational analyses and a comparison with properties of tomato bushy stunt virus[68] suggests that the first step in uncoating and the externalization of VP1 is a weakening of the contacts between protomeric units (Figure 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[69] and heat[70]. 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 (refs 9, 12, 71). 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 (refs 13, 72) and HRV16 (ref. 18) has been tentatively interpreted as a fatty acid, eight or more carbon atoms long. A rather longer 'pocket factor' is found in this pocket for coxsackievirus B3 (CVB3) (J. K. Muckelbauer,

Figure 10. Electron density in the hydrophobic interior of VP1 corresponding to the site of binding of certain antiviral compounds (Figure 2) of coxsackievirus B3 (Muckelbauer *et al.*, unpublished results).

L. Tong, M. G. Rossmann and M. J. Kremer, unpublished results) (Figure 10). While 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.*[49] imply, while Filman *et al.*[12] explicitly propose that the pocket factor could 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[50,51]. 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 (refs 13, 72).

In HRV1A and HRV16, the more active antiviral compounds tend to have an aliphatic chain less than or equal to five carbon atoms long[73], correlating with the available space within the binding pocket[72,74,75]. In HRV14, the most active antiviral agents tend to be longer with seven-carbon aliphatic chains. For example, WIN 56291 has an aliphatic chain of only three carbons (compare Figure 11) and is equally active against HRV16 and HRV1A, but less active against HRV14. Thus, for each serotype there is an optimal drug size which displays the greatest activity and binding affinity[74,75] and

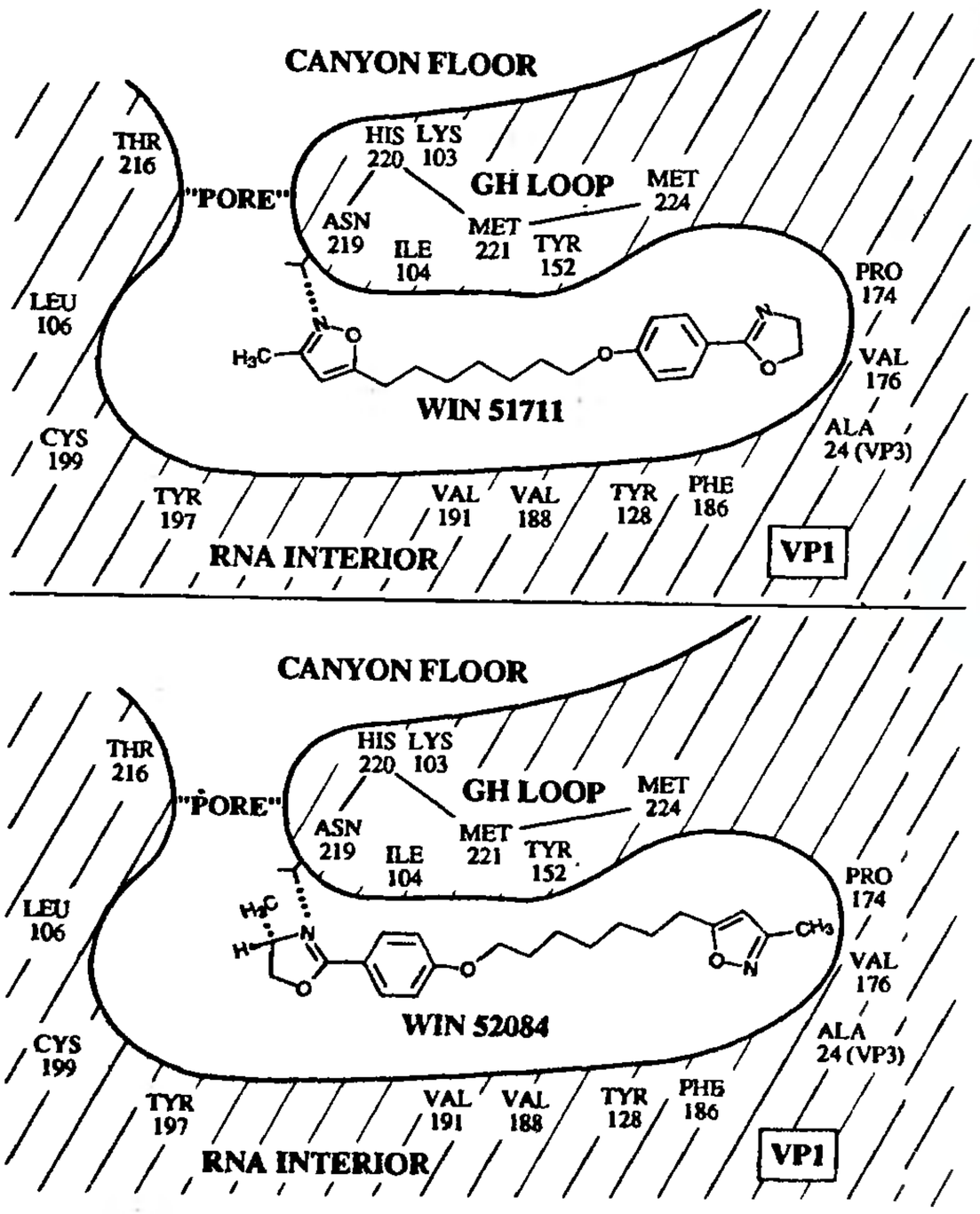

Figure 11. Schematic representation of the binding of the antiviral agents WIN 51711 and 52084 into a pocket underneath the canyon in HRV14. This causes enlargement of the pocket and conformational changes in the floor of the canyon, inhibiting attachment of the virus to HeLa cells in some cases, and also increasing the stability of the virus in all cases. [Reprinted with permission from Dutko *et al.*[106]. Copyright by Springer-Verlag, New York.]

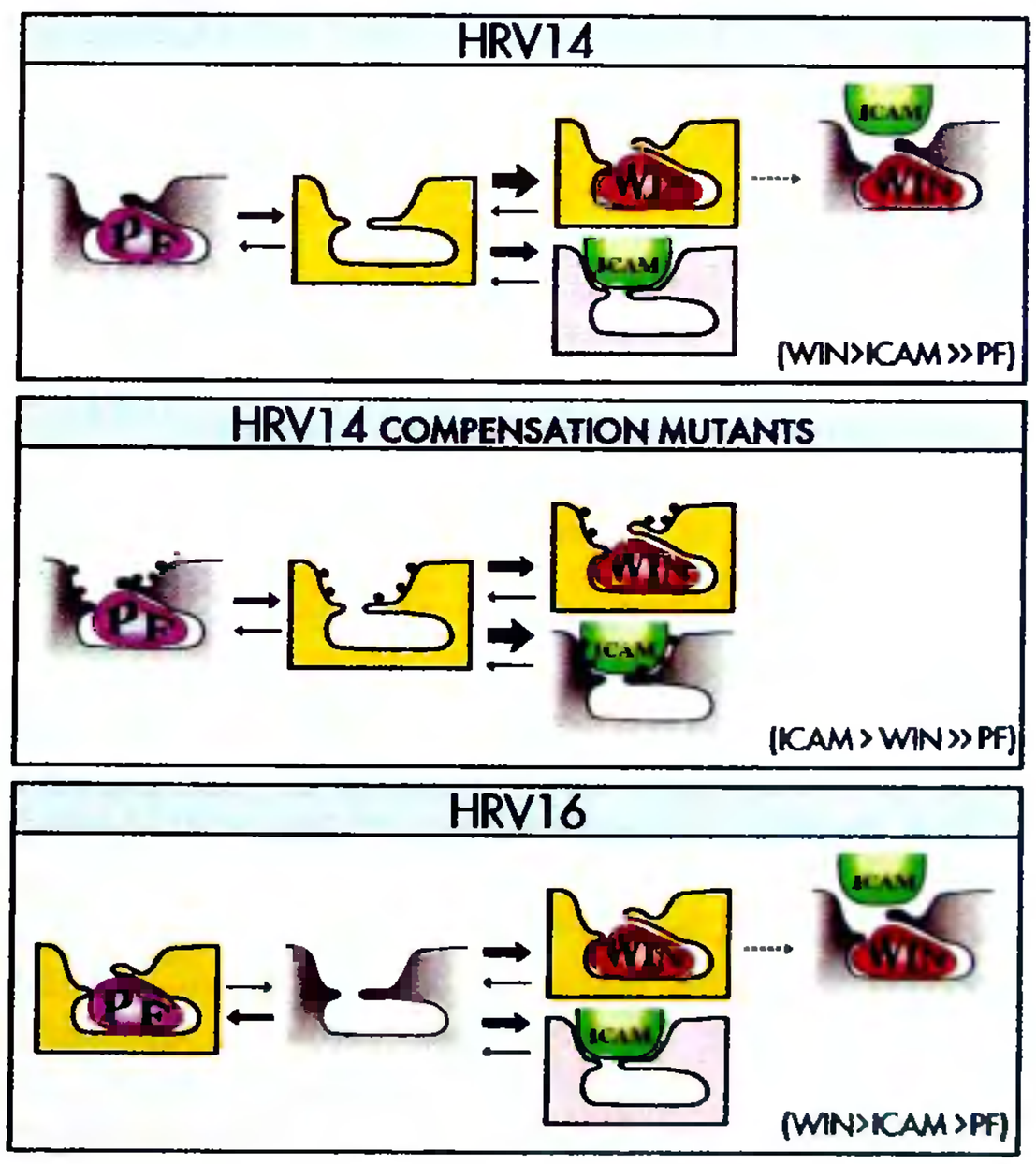

Figure 12. Conditions for inhibition of viral attachment by WIN compounds. Crystallographically and electron microscopically determined structures are in yellow and pink, respectively, while hypothetical structures are in gray. (*Top*) In wild-type HRV14, the pocket factor binds weakly and is not observed 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 displayed by ICAM-1 and, hence, the binding affinity of ICAM-1 is greater than that of pocket factor. (*Center*) Drug-resistant compensation mutants of HRV14 cluster around the canyon walls and floor (•) 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. (*Bottom*) Wildtype 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.

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 (refs 18, 72), 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 (refs 70, 76).

The 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[77,78], presumably as a consequence of placing a hydrophobic molecule into an internal hydrophobic cavity[79,80]. Similarly, drug-dependent mutants of poliovirus require WIN compounds to maintain their stability[81]. 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 which might be effected by expulsion of the pocket factor during the receptor-mediated uncoating.

Since ICAM-1 binds to HRV14 and to HRV16 (Figure 12), 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[18]. Hence, it must be assumed that the pocket factor is displaced before the receptor can seat itself into the canyon. In essence, there are two 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 downwards, which is possible only when there is nothing in the pocket. Conversely, when there is a compound in the pocket, its roof is raised upwards. 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 five-fold axes of the virus[64].

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 one per 10^4 virions. They have been shown to be mostly single mutations[77,82] and six of the seven 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 and M. G. Rossmann, unpublished data). In some of these mutants, the affinity of ICAM-1 for the virus is enhanced (R. R. Rueckert, private communication; M. P. Fox, D. C. Pevear and F. J. Dutko, unpublished data). Thus, it is reasonable to conclude that ICAM-1 binds better to these mutant viruses than the WIN compounds (Figure 12, center).

Conclusions

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 have indicated that the canyon is also the receptor attachment site for poliovirus[83].

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 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 two equilibria – (i) binding of the pocket factor into the pocket and (ii) binding of the receptor into the canyon. Provided that 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, displacing 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 (Figure 12).

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