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The structures of human rhinovirus and Mengo virus: relevance to function and drug design

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The structures of two serotypes (14 and 1A) of human rhinovirus and that of Mengo virus are reviewed, with particular attention paid to functionally important aspects. Discussion includes the relevance of structural studies to the immune reaction against viruses, receptor binding, uncoating, capsid assembly and development of antiviral agents.

Key words: rhinoviruses / Mengo virus / structure and function / receptors / antiviral agents

THIS REVIEW will discuss the structure-function relationships for two genera of the picornaviridae family, the rhinoviruses and the cardioviruses. Rhinoviruses are a major cause of the common cold.[1,2] Inhibition of replication in the host by vaccination has been successful for other picornaviruses, but not for rhinovirus because there are over 100 serologically distinct rhinoviruses.[1] However, with the help of structural studies, antiviral agents have been developed that bind to the capsid. Mengo virus is a member of the cardiovirus genus. Unlike the acid-labile rhinoviruses, the cardioviruses (like the enteroviruses), are stable at pH values as low as 3.[2] Murine Mengo virus is serologically indistinct from encephalomyocarditis virus (EMC), mouse-Elberfeld virus (ME), Columbia SK virus and Theiler's virus.[3] Despite the serological similarities between these viruses, they cause different diseases in mice including encephalitis, myocarditis and diabetes mellitus.[3] The M strain of Mengo virus, for which the structure has been solved, causes a fatal encephalitis in mice.[4]

The first picornavirus structure to be solved was that of human rhinovirus 14,[5] followed shortly thereafter by poliovirus serotype 1,[6] Mengo virus (a cardiovirus)[7] and foot-and-mouth disease virus (FMDV) (an aphthovirus).[8] A second rhinovirus serotype, 1A, has also been solved.[9] The structures of rhino- and Mengo viruses have been determined also when complexed with antiviral agents, at different pH values or ionic strengths, or when mutated to give drug resistance or acid stability.

Human rhinoviruses (HRV) can be subdivided into two groups based on host cell receptor specificity.[10] The major group viruses, containing at least 78 serotypes, utilize the host cell intercellular adhesion molecule-1 (ICAM-1) as a receptor.[11,12] The minor group viruses, which comprise at least 12 serotypes, bind to a second distinct receptor which has been characterized.[13,14]

This review will relate virus structure to function in order to come to a better understanding of the molecular mechanisms of the viral life cycles. Discussion will include coat protein assembly and processing, location and structure of the neutralizing immunogen (NIm) sites, viral adsorption, structural changes related to acidification (believed to be important in uncoating), inhibition of uncoating and adsorption via a class of antiviral compounds, and mutations which lend viral resistance to this class of antiviral compounds.

The protein shells of the rhino- and cardioviruses, like other picornaviruses, are comprised of four polypeptides. The larger proteins, VP1, VP2 and VP3, each of approximately 30,000 MW, make up the shell of the virus (Figure 1). The much smaller VP4 (MW about 8000) is on the interior of the virus shell, in contact with the viral RNA. Residues in the virus structures will be referred to by a four-digit number. The first digit indicates the viral polypeptide number (1-4) while the remaining three digits denote the sequence number within that polypeptide. Thus, residue H1220 refers to the histidine in VP1 with sequence number 220. The topologies of the three larger proteins, VP1, VP2 and VP3, are similar to each other and also similar to that found in other RNA viruses.[15-17] Each consists of a β-barrel with strands identified as βA, βB, . . ., βI (Figure 2). The surface corner between strands βB and βC (the βC loop) is the highly antigenic region in polio- and rhinoviruses used for inserting different epitopes.[18-20] The 'FMDV loop' in the rhinoviruses refers to the GH loop of VP1. The analogous loop in FMDV is

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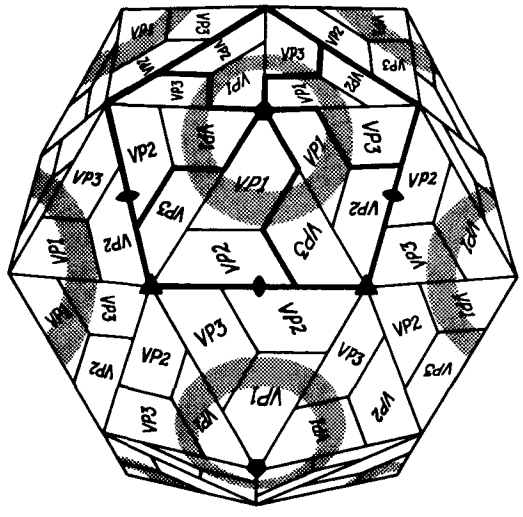

Figure 1. Organization of the viral polypeptides in the picornavirus. The biological assembly units, protomers comprised of VP1, VP2 and VP3, are outlined by heavy lines. 60 such protomers make up the viral icosahedron. The canyon is shown shaded here. (Reprinted from ref 48. Copyright by Wiley-Liss.)

strongly antigenic and may be the site of virus attachment to cells.[8]

Antibody binding

Sherry *et al*[21] isolated 62 neutralization-resistant mutants of HRV14 using 35 monoclonal antibodies. These mutants could be classified into four groups in which a mutant, selected according to resistance to neutralization by one antibody, would be resistant to most of the antibodies used to select other members of the group. This indicated that for a given group, the epitopes were overlapped. Partial sequencing showed that most of the mutations occurred at a small number of residues. Even though some of the epitopes were comprised of residues from disparate parts of the sequence on different proteins, all of the residues proved to have solvent-exposed side chains and were within one of four distinct regions protruding from the capsid surface as shown in Figure 3. These regions correspond to hypervariable sequences when comparing homologous polio- and rhinoviruses.[22]

To date, 21 escape mutants have been isolated from Mengo virus using four monoclonal antibodies,[23] and these map predominantly close to positions NIm-II and NIm-III in HRV14. However, Mengo virus differs in that cross-neutralization experiments indicated that the regions corresponding to NIm-II and NIm-III presented one contiguous antigenic area of roughly 30 Å by 12 Å.

The locations of neutralizing epitopes in poliovirus and FMDV have been determined and were compared with those of rhinovirus (ref 24 and references therein). Combining the information available for poliovirus, rhinovirus and FMDV, there are five or six distinct antigenic regions with most, but not all, applicable to each of the viruses. For each region there are a set of residues of which overlapping subsets are important for each virus.

While most of the viral surface is antigenic, only those antibodies that bind to the relatively few, highly exposed, neutralizing immunogen sites are capable of neutralizing the virus. One possible mechanism is through the cross-linking of virions, causing an immune precipitate. However, strong neutralizing antibodies[25,26] bind bivalently to a single virion and may inhibit attachment or subsequent stages in the viral life cycle. For instance, the presence of a few antibodies[27,28] on the virus surface may inhibit endocytosis by sterically hindering a sufficient number of receptors from binding to the virus during formation of an endocytolic vesicle. Alternatively, an antibody might alter the virus conformation. In an attempt to study these possibilities, the structures of a series of monoclonal antibodies, with known binding sites on the virus, are under investigation.[29] The antigen binding site of the antibody is complementary in charge and shape to that of the NIm-IA site on HRV14 to which it binds. The separation of two sites related by a twofold axis is about 120 Å, corresponding to the distance between the antigen binding sites of an antibody.

Receptor binding and adsorption

One of the most striking structural features of the rhinoviruses is a 25 Å deep depression or 'canyon' which encircles the icosahedral fivefold axis. A homologous depression exists in Mengo virus. Unlike rhinoviruses, in Mengo virus the surface depressions are not continuous about the fivefold axis but are broken into five deep pits. These pits are located in the region homologous to the deepest portions of the rhinovirus canyons.[30,31] In Mengo virus, the remainder of the canyon is filled by two insertions (loops I and II) between βC and βD of VP1 (Figure 2). A similar but smaller depression also exists at the receptor attachment site on the hemagglutinin spike of influenza virus.[32,33]

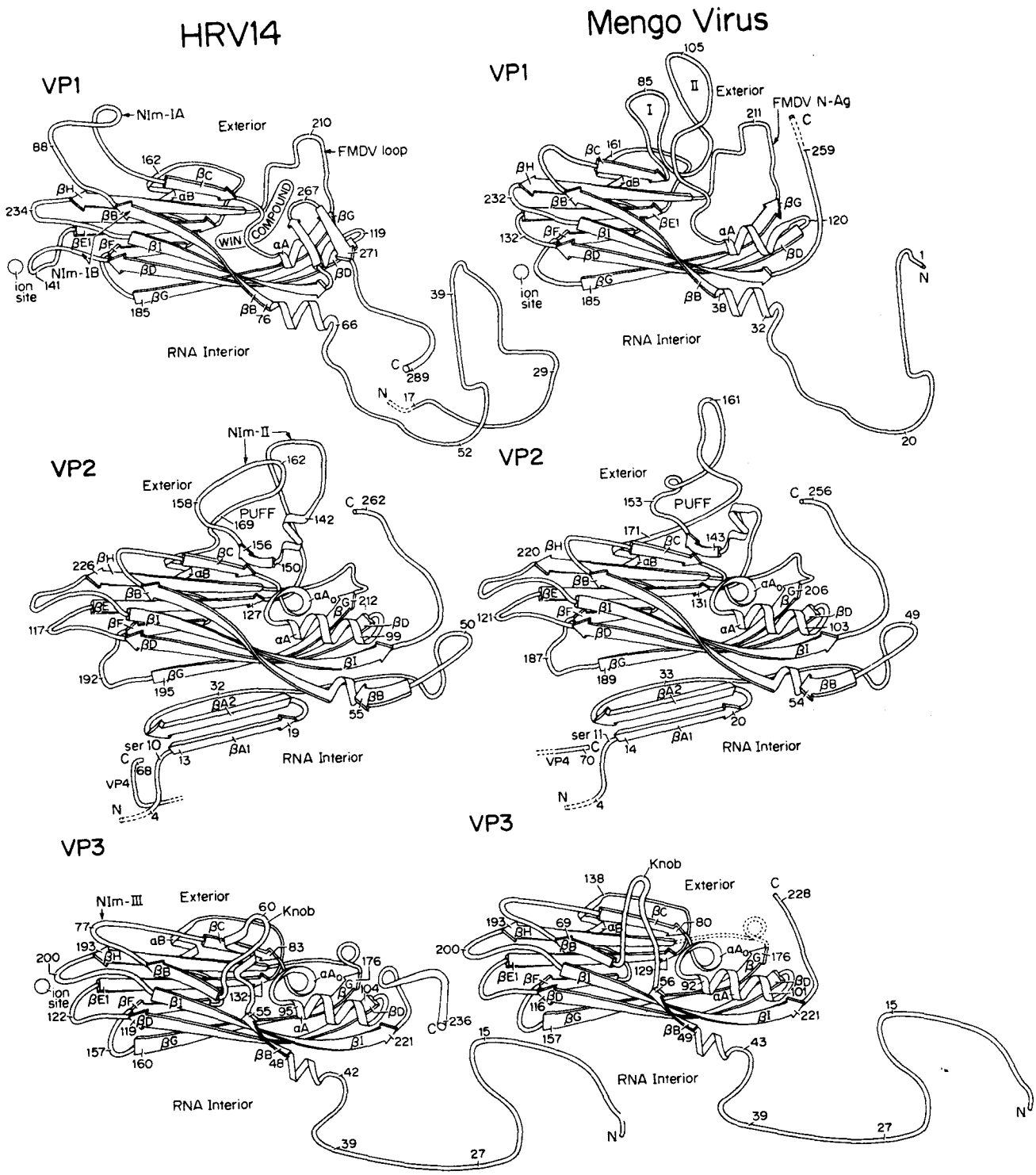

Figure 2. Ribbon drawings of the three surface polypeptides of both rhino- and Mengo virus. All polypeptides are eight-stranded β-barrels, which differ primarily at the ends of β-strands. The neutralizing immunogen sites, the WIN compound binding pocket, ion sites and secondary structure nomenclatures are shown. (Reproduced with permission from ref 7. Copyright by the American Association for the Advancement of Science.)

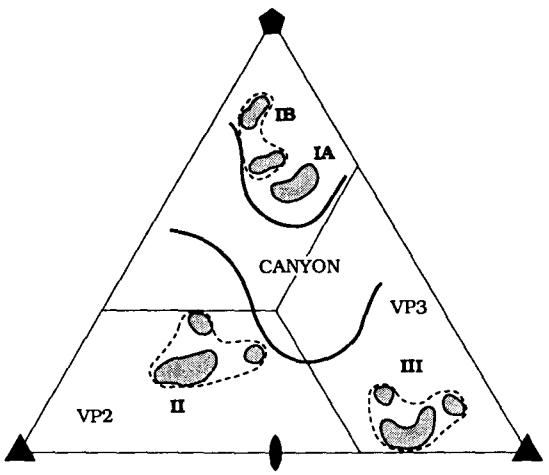

Figure 3. Location of the antibody binding sites for HRV14. Neutralizing immunogen (NIm) sites are shaded. The region shown is one of the triangles shown in Figure 5. The NIm sites have been identified by selecting escape mutations to monoclonal antibodies at the following positions: NIm-IA-1191 and 1195; NIm-IB-1083, 1085 and 1138-1139; NIm-II-1210, 2136, 2158-2159 and 2161-2162; NIm-III-1287, 3072, 3075, 3078 and 3203. (Adapted from ref 21.)

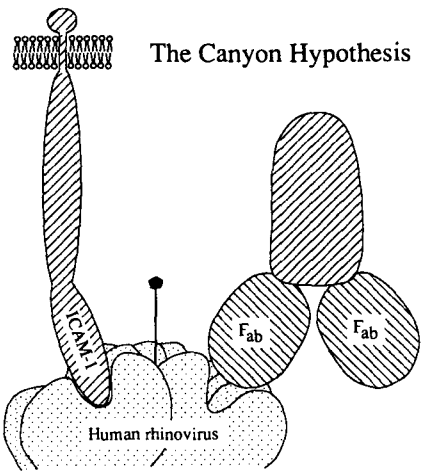

Figure 4. The canyon hypothesis suggests that the large variable end of an immunoglobulin cannot enter the canyon, while a narrower receptor molecule (ICAM-1) would be able to enter the canyon and interact with residues on the canyon floor. (Reprinted from ref 48. Copyright by Wiley-Liss.)

In rhinoviruses, the canyon is lined with residues from VP1 and VP3. Four β-strands (βB, βC, βD and βI), helix αB, and the FMDV loop (GH loop of VP1) participate from VP1. VP3 contributes the GH loop, helix αA and its carboxy terminus to the lining of the canyon. The FMDV loop of VP1 and the GH loop of VP3 are adjacent to each other and line the deepest portion of the canyon. The pit in Mengo virus is formed by homologous regions of VP3. The VP1 residues lining the Mengo pit are limited to the FMDV loop and a small portion of βE.

It had been surprising that the receptor binding site, shared by about 80 rhinovirus serotypes of the major receptor group and, therefore, probably highly conserved, could not elicit a cross-neutralizing response. With determination of the structure, Rossmann *et al*[5] hypothesized that the 25 Å deep 'canyon' on the viral surface is too narrow for an antibody to enter, that the bottom of the canyon might be the receptor binding site, and that the receptor binding sites of all picornaviruses might be similarly protected in a depression (Figure 4). Different lines of evidence have arisen to support the 'canyon hypothesis':

(1) Four sites in the canyon (K1103, P1155, H1220 and S1223) have been mutated to produce virus with altered binding properties.[34]

(2) The binding of antiviral compounds sometimes distorts the floor of the canyon.[35,36] In those cases where this distortion has been observed, the virus binds cells less well in the presence of compound than in its absence.[37]

(3) The canyon lining residues are conserved to a greater extent than non-canyon surface residues.[38]

(4) Changes in pH inhibit binding of Mengo virus to cell membranes and also cause conformational changes localized to the floor of the pit.[39]

The HRV major group receptor, ICAM-1, is a member of the immunoglobulin gene superfamily, a large family of gene products which share homology with immunoglobulins.[40] Other viral receptors[41,42] of the immunoglobulin superfamily include the poliovirus receptor,[43] CD-4 (used by human immunodeficiency virus, HIV, as a receptor), the H-2 MHC molecule (used by Semliki Forest virus as a receptor) and β-2 microglobulin (cytomegalovirus). ICAM-1 has also been reported to be a receptor for the malarial parasite *Plasmodium falciparum*.[44] The sequence of ICAM-1 suggests there are five (D1 to D5) extracellular immunoglobulin-like domains (seven-stranded β-sandwiches), a short transmembrane sequence and a cytosolic domain.[40] Electron microscopy of the five extracellular domains suggests that the immunoglobulin-like domains are arranged head-to-tail like beads on a string.[45] ICAM-1 is a glycoprotein that

is expressed widely in mammalian tissues.[46] In spite of the wide distribution of ICAM-1, HRV infections are limited to the pharyngeal region.[2] Thus, receptor expression cannot be the sole determinate of viral tissue tropism.

A soluble form of ICAM-1, which is known to inhibit infection by rhinoviruses,[47] exists as a monomer in solution, with dimensions which should allow entry into the canyon.[45] The ability of the N-terminal domain of ICAM-1 to reach into the canyon is also supported by modeling studies.[48] Site-directed mutational studies, when coupled with modeling, suggest that a large region of the ICAM-1 domain D1 surface is involved in HRV binding.[45] This result is different from that obtained in similar studies of the HIV-CD4[49,50] and the ICAM-1:LFA-1[45] interactions where less extensive regions of the receptors appear to be important for binding. The extensive interactions apparently made between ICAM-1 and HRV (Figure 5) could be accounted for by placing the ICAM-1 domain D1 into the canyon, thereby effectively surrounding D1 with rhinovirus.

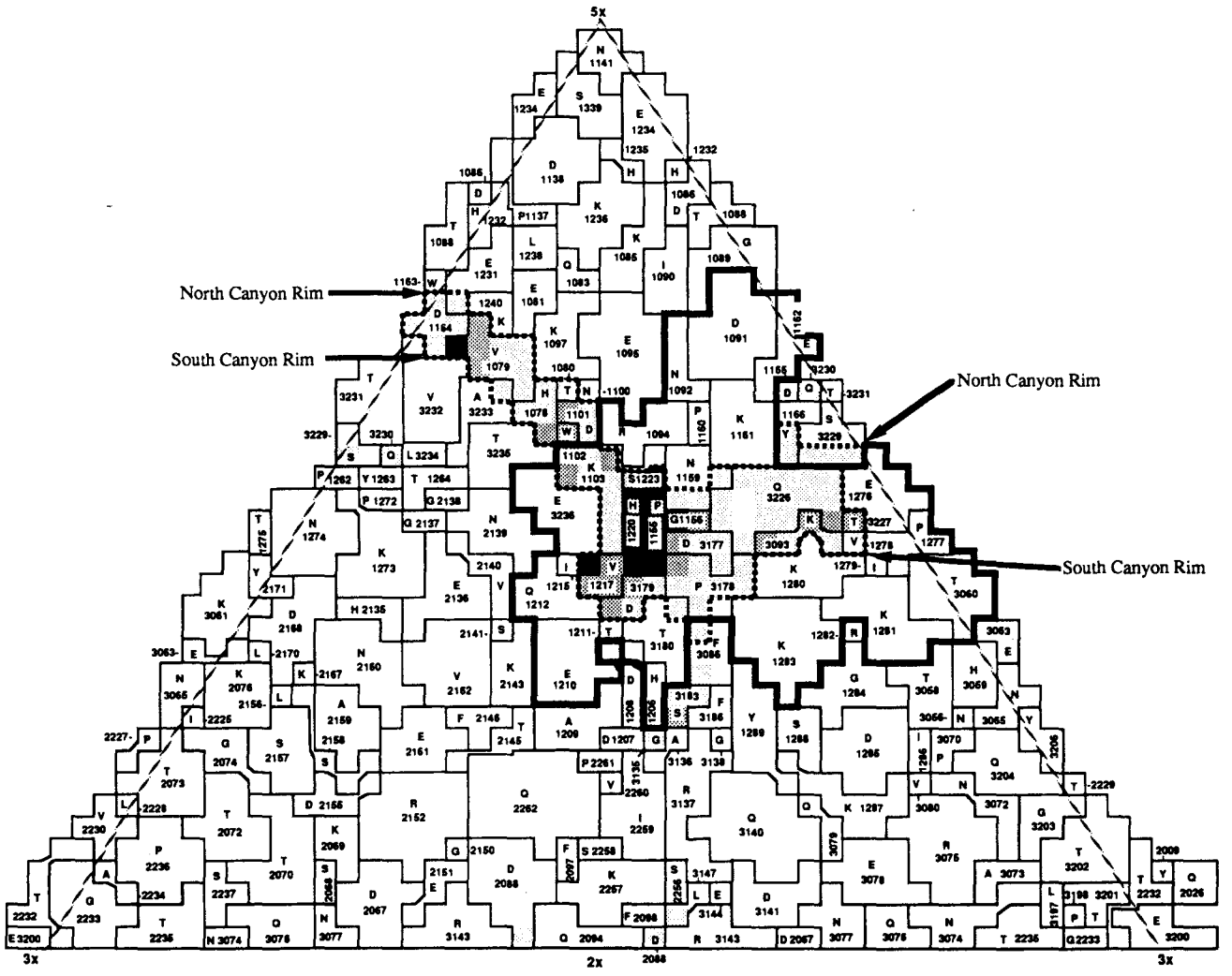

Figure 5. A 'road map' of the solvent accessible surface of HRV14 showing the canyon (heavy dotted line) and the predicted footprint of ICAM-1 (heavy solid line) on the virus. The triangle represents an icosahedral asymmetric unit, bounded by a fivefold vertex at the top and threefold vertices on either side of the base (compare with Figure 1). The surface residues of HRV14 are shown using the one-letter amino acid code. Canyon residues are shaded progressively darker with respect to their depth in the canyon. The ICAM footprint includes all surface residues within 5 Å of the predicted ICAM-1 D1 structure.

Comparisons of sequences between major and minor group rhinoviruses illuminate some differences between the canyon regions of these viruses.[9] Five surface residues which differ between the major and minor group viruses, yet are conserved within each group, have been identified. These residues are located on the canyon rim. Also, electrostatic potential calculations of the major and minor group viruses show important differences in the region of the canyon rim, while potentials at the floor of the canyons appear similar. These data argue that if charge distribution is responsible for receptor specificity, the specificity determining region is likely to be the canyon rim.

The Mengo viruses have some interesting physiological properties which have been exploited to obtain structural information pertaining to viral adsorption. In the presence of a high phosphate concentration, binding of Mengo viruses to cells is decreased approximately fivefold and infectivity decreases approximately twenty-fold.[39] The phosphate-induced changes can be ameliorated by decreasing the pH. The crystal structures of Mengo virus in high phosphate concentration at low or high pH have been solved.[39] Those crystals at low pH should be in a conformation which displays a greater binding affinity than those crystals at high pH. The differences between the two structures are localized in the pit region (Figure 6). Specifically, four changes were noted on acidification of the Mengo virus: the FMDV loop (residues 1204-1214) moves; the GH loop (residues 3176-3182) becomes more ordered; a bound phosphate near the FMDV loop is displaced; and the carboxy terminus of VP2 moves. The striking localization of these acid-induced changes in the Mengo virus pit, together with the physiological consequences of acidification in the presence of high phosphate concentration, provide evidence that the pit region of the Mengo virus is important in viral-to-cell adsorption.

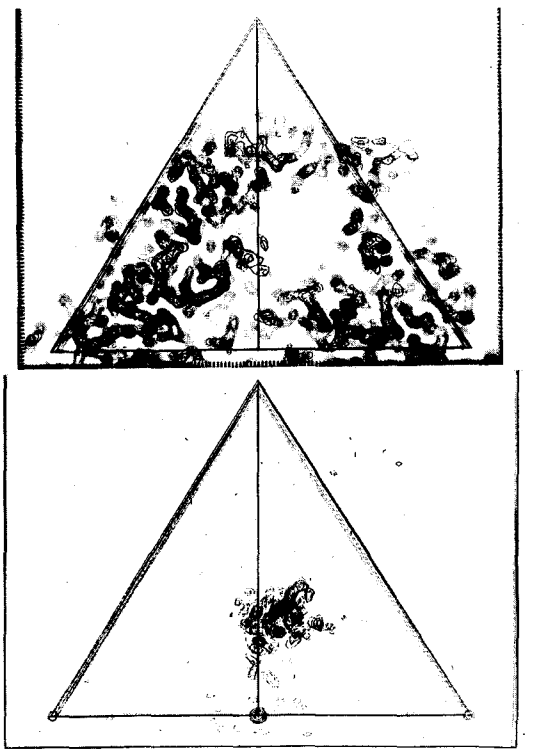

Figure 6. Electron density showing the difference in structure between Mengo virus at pH 7.4 and 4.6. (top) Original Mengo virus structure, at pH 7.4, in which the putative receptor attachment site (the pit) is clearly visible. (bottom) Difference electron density map between Mengo virus at pH 4.6 and pH 7.4. The large conformational changes are confined to the pit region. (Reproduced with permission from ref 39. Copyright by Academic Press.)

Antiviral agents

The development of antiviral drugs has lagged far behind antibacterials. There are only about a dozen drugs with documented beneficial effect in man, covering only a small fraction of viral diseases (for a review see ref 51). The shortage of antiviral drugs arises because viruses metabolize little extracellularly, but reproduce within the cell using enzymes of the host cell in many different ways. It is difficult to find antiviral agents that can pass through the plasma membrane and be selectively toxic to viral metabolism. The requirement is, therefore, that such agents bind specifically to viral products. This section will discuss structural aspects of the development of antiviral agents that hold promise because they can bind to the capsid proteins (unique to viruses) and can exert their effect prior to the metabolism of viral reproduction. This has the advantages of simplifying drug delivery, avoiding high cytotoxicity, and perhaps having a broad spectrum of activity.

Prior to the structure determination of HRV14, several classes of compounds had been found to be active against HRV replication (Figure 7). Although some of these compounds proved to be clinically ineffective[52] when delivered as nasal sprays, some are of interest because of the likelihood that they will be developed into commercially available drugs. R 61837 has been shown to be prophylactically active in human volunteers to the extent that it (at least) postponed the onset of a cold for the duration of 48 h

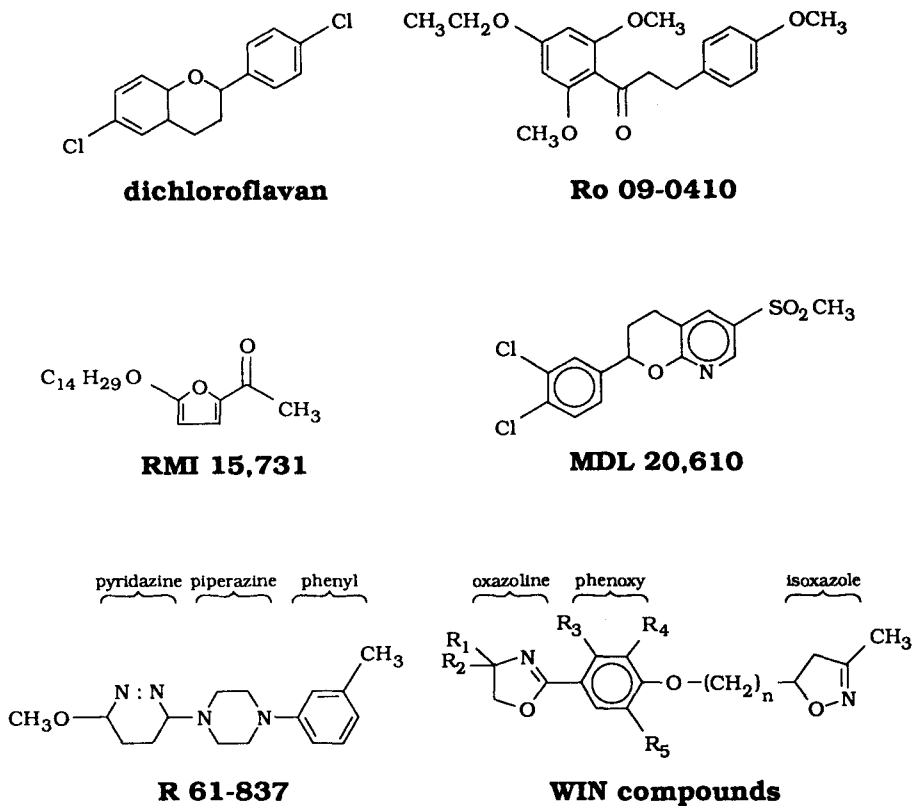

Figure 7. Examples of anti-rhinoviral agents: Sodium dodecyl sulfate (SDS) (not shown);[88] dichloroflavan;[89-91] Ro 09-0410;[92,93] RMI 15,731;[94] MDL 20,610 (representing 'MDL' compounds and pyridinecarbonitriles from Merrell Dow);[95,96] R 61837 (representing a series herein denoted R*number* from Janssen Research Foundation);[58,97] 'WIN' compounds (developed by Sterling Research Group).[56,57] WIN compounds discussed here have various length alkyl chains (n) and substituents for R_1 through R_5. For example, WIN 51711 has $n = 7$, (R_1-R_5 = H).

trials.[53] Oral doses of WIN compounds have been shown to be effective in mice infected with other picornaviruses,[54,55] and WIN compounds are currently being clinically tested. Most enteroviruses and rhinoviruses are susceptible to these compounds.[56-58] The structures of these antiviral agents when bound to rhinoviruses may suggest possible homologous targets in other viruses including HIV.[59]

The anti-rhinoviral agents are chemically diverse (Figure 7), but are all hydrophobic with the exception of SDS (amphiphilic). Their minimal inhibitory concentrations (MIC) are between 100 and 0.1 μM. Smith *et al*,[35] and Dutko *et al*[60] have shown that MIC and binding coefficients are roughly correlated, suggesting that a significant proportion of the biological activity is a consequence of the ability to bind well to the virus. (MIC is the maximum concentration required to halve a plaque count.) Scatchard plot analyses and crystallographic investigations[36] show that at concentrations equal to the MIC values, well above 50% of the 60 sites per virion are occupied. The structures of complexes of about 25 antiviral agents with HRV have been studied at Purdue University. Most were from the WIN series, but also two from Janssen Research Foundation. Most of the complexes involve HRV14, but some are with HRV1A. Many of the results discussed arise from a fruitful collaboration between Purdue, Sterling Research Group and the University of Wisconsin and have given insight into functional aspects of coat proteins.

Smith *et al*[35] showed by X-ray crystallography that both WIN 51711 and WIN 52084 (like WIN 51711, except that $R_1 = CH_3$) bind in the hydrophobic 'WIN' pocket in the interior of the β-barrel of VP1 of HRV14, in a position corresponding to some non-viral electron density in poliovirus.[6] It was suggested that this pocket might be the binding

site for an essential co-factor in the viral life cycle[35] and this may be the putative lipids found in Mahoney type 1 and type 3 Sabin polioviruses.[61] All this class of antiviral compounds bind to the same site in HRV14.[36,62,63] They bind similarly in other viruses such as HRV1A[9,62] and poliovirus (Hogle *et al*, personal communication). This is the binding site relevant to virucidal action as has been shown through the selection of spontaneous mutants of HRV14 that are resistant to high levels of WIN52084.[64] Sequencing of 56 selected mutants showed that the mutations mapped to 2 amino acids in the pocket (see section on mutational studies). Andries *et al*[65] selected mutants of HRV9 and HRV51 that were resistant to R 61837 and R 66703, respectively. At least one of these mutants showed cross-resistance to heat and acid destabilization in the presence of other types of antiviral agents (Figure 7), indicating that they all share the same binding site.

The entrance to the WIN pocket is at the bottom of the canyon. Antiviral agents lie roughly parallel to the floor of the canyon. WIN 51711 binds to HRV14 with the oxazoline group innermost and the isoxazole group near the entrance (pore) (Figure 8).[35] Other compounds occupy almost exactly the same region,[36,62,63] but their (head-to-toe) orientation within the pocket appears to depend on the length of the compound.[36] The addition of even a methyl group can alter the MIC by an order of magnitude. Thus, the addition of an *S* methyl to the oxazoline ring of WIN 51711 not only alters the drug orientation but also greatly enhances its efficacy. When two compounds are compared, both binding with the isoxazole group innermost, one with $R_1 = CH_3$ and the other with $R_1 = H$, the former is shifted relative to the latter by 0.8 Å towards the pore.[36] Binding is primarily by hydrophobic interactions. The inner end of the pocket is lined by residues which are more hydrophobic than those at the pore end. Without an aliphatic chain

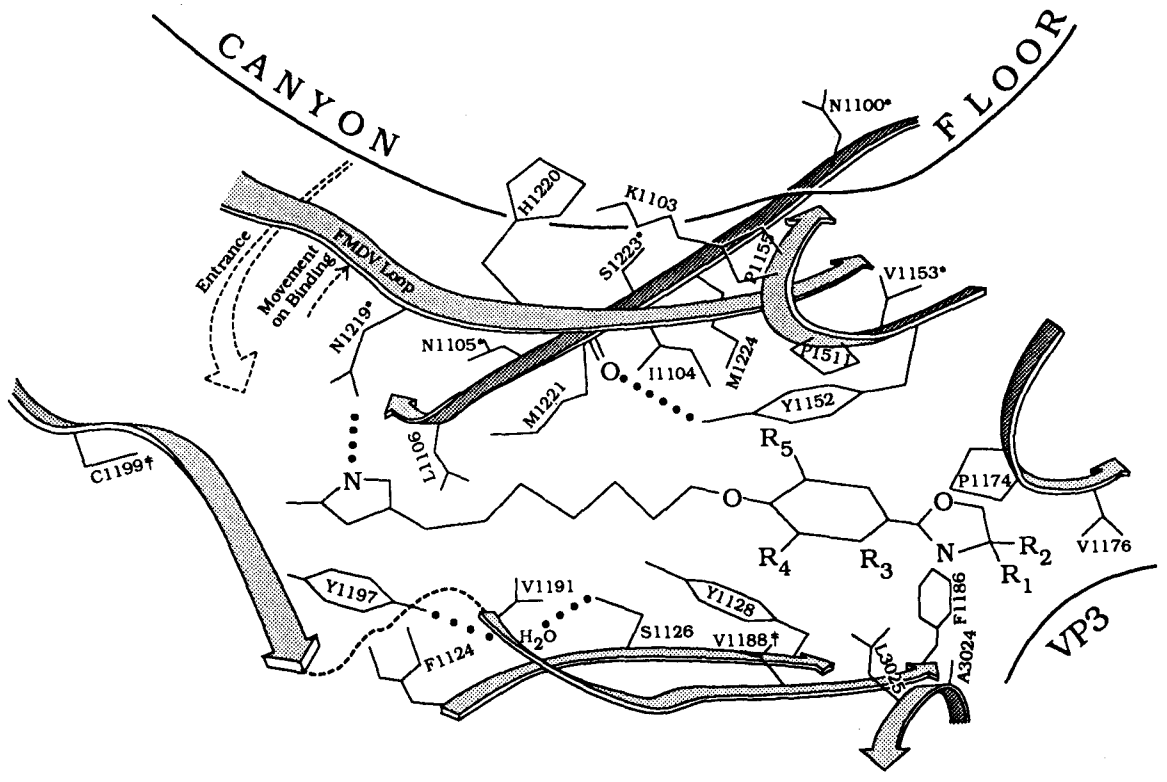

Figure 8. Schematic of the binding site in HRV14 in which antiviral agents bind. The binding direction illustrated is that for WIN 51711. The view is of a thin section through the surface of the virus running approximately from the fivefold axis (left) to a diad (right), and looking from VP3 of an adjacent protomer (cut away). Mutants resistant to high concentrations of WIN 52084 are labelled with ‡, and those to low concentrations with *.[64] Hydrogen bonds are shown as dotted lines.

or an isoxazole ring, 'short' WIN compounds bind in the innermost end of the pocket at a site corresponding to where they go in larger compounds (J. Bibler *et al*, unpublished results). However, R 61837, which is shorter than WIN 51711, does not penetrate as far into the end of the pocket.[63] In HRV14, this might be due to a lack of flexibility of R 61837 to bend around Y1128, but this cannot be the cause in HRV1A[62] where isoleucine replaces tyrosine. The extra bulk of the puckered piperazine ring (cf WIN compounds) may be sufficient to make it difficult for R 61837 to penetrate beyond a 7 Å constriction near the isoleucine. Relative to HRV14, antiviral compounds bind in a slightly different orientation and conformation in HRV1A (Figure 9). The differences can be simplified to a rigid body rotation about the pore end of the drug so that the innermost end moves about 4 Å towards residue I1125 of HRV1A (Y1128 in HRV14), and a rotation of the rings at each end relative to one another.[62] It is not yet clear whether the differences between binding in HRV14 and HRV1A reflect (a) differences between major and minor receptor group serotypes, (b) differences between the two drug groups proposed by Andries *et al*,[66] (c) a difference between HRV14 and all other serotypes on account of the unique Y1128 in HRV14 or (d) a wide variation among serotypes.

On binding to HRV14, all antiviral agents cause similar conformational changes.[36] M1221 must be displaced for the compounds to enter. This causes the FMDV loop to move its C_α backbone up to 4.5 Å away pushing it into the canyon. Part of the connecting β-strand is also moved, as are, by smaller amounts, adjacent strands and loops on either side of the β-strand (residues 1151-1159 and 1101-1110). Small differences in conformational change also occur near V1188, on the other side of the pocket. WIN 54954 ($n = 5$, $R_4 = R_5 = Cl$) is a compound with a particularly bulky phenoxy ring causing V1188 to move by as much as 1 Å (M. S. Chapman *et al*, unpublished results). Conformational changes in HRV1A on binding of antiviral compounds are less than those in HRV14 because the 'native' HRV1A structure contains a small 'co-factor' in the pore which might hold the pocket 'open'.[9] A detailed study of the smaller changes in HRV1A has had to await the collection of high resolution data,[62] inherently more difficult than for HRV14. In all of the WIN compounds studied, no matter which way round they bind, there is a putative strained hydrogen bond from N1219 (HRV14) or N1215 (HRV1A) to the isoxazole or oxazoline rings.[35] The hydrogen bond to the pyridizine is indirect, mediated through a water molecule, for R 61837 in HRV14.[63] However, in HRV1A, no such interaction is apparent.[62] Of further interest is that in progressing from a direct hydrogen bond to an indirect one, and then to none, the FMDV loop moves further from the compound towards the canyon. This suggests that once displaced from its native conformation, it is the compound that sometimes tethers the FMDV loop.

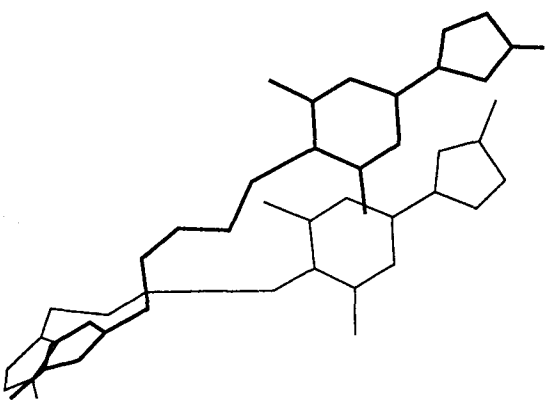

Figure 9. A WIN anti-rhinovirus compound bound to HRV14 (bold lines) and to HRV1A (thin lines). The two structures are superimposed such that the icosahedral symmetry axes of the two viruses coincide. The orientation of the axes is the same as in Figures 3 and 5.

The antiviral agents affect replication in two ways. Firstly, they inhibit uncoating. For the WIN compounds this has been shown[67] by infection with viruses encapsidating neutral red dye. In these the RNA could be disrupted (with a resultant drastic loss of infectivity) after a time normally sufficient for uncoating, indicating that the RNA was still encapsidated. Radiolabeling and sensitivity to neutralizing antisera were used to show that WIN 51711 had little or no effect on adsorption or penetration through the plasma membrane for poliovirus type 2 and HRV2 (a minor receptor group serotype).[67] This was confirmed by Zeichhardt *et al*,[68] who found that the RNA synthesis was reduced by the presence of WIN 51711 in poliovirus, in spite of apparently normal adsorption and receptor-mediated endocytosis as visualized by electron microscopy. This conclusion was supported by the *in vitro* protection of poliovirus and HRV against heat or acid inactivation[69,70] in the presence of antiviral compounds. It is likely that antiviral agents inhibit

uncoating through filling the hydrophobic pocket, making the capsid more stable and perhaps more rigid.[35,71]

After it was found that the conformation of the bottom of the canyon was altered with the binding of these compounds, it was suggested that receptor binding might be affected. A second mode of action of the antiviral compounds was indicated by an absence of a strong correlation between concentrations of R 61837 required to inhibit uncoating and concentrations required to inhibit replication.[65] Pevear *et al*[37] resolved this question when they showed that cell attachment of HRV14, a serotype from the major receptor group, unlike that of minor group viruses, was affected by the presence of antiviral agents. Similar results were also obtained by Heinz *et al*.[64] The lack of inhibition of attachment in HRV1A is presumably due to the absence of major conformational changes when WIN compounds attach. Perhaps the receptor for the minor group rhinoviruses (and perhaps poliovirus) recognizes a 'drug-bound' conformation, and perhaps the densities found in the WIN pockets of native HRV1A and poliovirus represent natural co-factors which maintain this conformation.

A thorough understanding of binding interactions might enable the design of more effective anti-rhinovirus drugs. To this end there have been a number of studies using the techniques of molecular dynamics. Lau and Pettitt[72] studied the contribution of the Asn 1219-oxazoline hydrogen bond and Lybrand and McCammon[73] have studied the Δ (ΔG) that should accompany the binding of WIN 51711 as compared to WIN 52084. However, it is simpler methods that have most facilitated development of the WIN compounds. For example, inspection of the structures shows that there are regions, especially near the alkyl chain, where WIN compound atoms are not in van der Waal's contact with the virus, suggesting that the addition of bulk might enhance the binding. This is, perhaps, why R 61837 binds effectively even though it does not reach to the hydrophobic innermost end of the pocket. Aberrantly low effectiveness of WIN 54954 in HRV14 can be rationalized in terms of a presumably unfavorably large movement required near Val 1188 due to the combined presence of the chlorines and Tyr 1128. Dutko *et al*[60] have extended such observations to a systematic study. They superimposed the van der Waal's volumes for active and inactive compounds in HRV14, concluding that larger volumes (especially at the innermost end) increased activity, while exceeding a critical bulk on the phenyl ring decreased it. Multivariate regression analysis of 12 phenyl-substituted compounds in HRV14 showed that activity was most correlated to lipophilicity and is reasonably well correlated to a combination of lipophilicity and molecular bulk.

Mutational studies

Heinz *et al*[64,70] have selected mutants that are resistant to the effects of antiviral agents and also to acid pH. The mutants were selected against a variety of WIN compounds and under different concentrations of the compounds. Two classes of mutations that are resistant to WIN compounds were selected, namely those when there was a high and those when there was a lower concentration of the compound. The 'high-resistant' mutations, when sequenced, occurred exclusively in the WIN pocket at two different sites. Presumably these mutations simply block the entry of the WIN compound into the pocket. In each case, the change is to a larger, hydrophobic residue. Analysis of the amino acids that line the pocket shows that the observed selected mutations are probably the only single base change mutations that could produce large hydrophobic amino acids.[64] Structural analysis of two of these mutants (C1199Y and V1188I) has been completed.[74] While V1188I has no effect on the surrounding virus structure, C1199Y has an extensive effect.

Both crystallographic and virological techniques have shown that these mutations inhibit entry of WIN compounds into the pocket. In the latter case[70] it was possible to show that the wild-type (WT) virus has enhanced stability at elevated temperatures when complexed with a WIN compound. Thus, the enhanced stability in the presence of WIN compounds at elevated temperatures can be used as an assay for determining the binding of WIN compounds to the virus. It is of interest that, on the basis of these experiments, C1199F blocks the entry of the longer WIN 52084 (length of the aliphatic chain is $n = 7$) into the pocket, but only to a lesser extent the entry of WIN 52035 (length of the aliphatic chain is $n = 5$). A structural investigation of C1199F with WIN 52035 is now in progress (M.A. Oliveira and M.G. Rossmann, unpublished results).

The low-resistant mutants map exclusively into the floor of the canyon (Figure 8) and the 'pore' or WIN pocket entrance.[64] These mutations do not directly block the entrance of WIN compounds into

the pocket. Indeed, both crystallographic and thermal stability results verify the ability of the compounds to bind to the mutated virions. Furthermore, they continue to impose a conformational change on the virion similar to that which occurs on binding the compounds to the WT virus. Growth curves (B.A. Heinz *et al*, unpublished results) suggest that there is a blockage of attachment but that, nevertheless, some virus attaches and continues to grow.

A series of acid-stable mutants have also been selected and are being studied structurally (B.A. Heinz *et al*, unpublished results). This work is not yet completed, but may help to differentiate between enteroviruses (which remain infective at low pH) and rhinoviruses (which require at least a neutral pH environment for propagation). Preliminary structural results suggest that the pH-stable mutants encourage the binding of a co-factor in the WIN pocket—as is also the case for poliovirus.

The construction of an infectious clone of HRV14 (W. Lee and R.R. Rueckert, unpublished results) now makes it possible to make a large number of site-specific mutants. Thus, it will be possible to explore other mutations which might be drug-resistant or pH-resistant but have not been selected, to explore the extent of the footprint of monoclonal antibodies or of ICAM-1 or to investigate assembly or disassembly. The choice of mutations will depend on structural knowledge, the molecular biology and functional investigations.

Assembly and Disassembly

Assembly of picornaviruses proceeds through the formation of 6S protomers of VP1, VP3 and VP0, 14S pentamers of 6S protomers, to the full capsid, with maturation after the injection of RNA and cleavage of VP0 to VP2 and VP4. The biochemical and biophysical evidence for this consensus has been reviewed by Rueckert[2,75] and Putnak and Phillips.[76] The interpretation of which polypeptides constitute a protomer and pentamer is shown in Figure 1 and was based on the intertwining of neighboring polypeptide chains in HRV14.[5] This designation is supported through calculation of surface areas of contact between the peptide chains in HRV14[30] and Mengo virus,[31] as a first approximation to binding energies. Warwicker[77] has used the structure of HRV14 to perform theoretical calculations of changes in the electrostatic charges on lowering the pH to 5. These showed that the pentamer-pentamer interface and the canyon regions were likely to be the most affected by low pH.

Many of the features of rhinovirus uncoating are similar to features of poliovirus uncoating. Rhinoviruses, like polioviruses,[78] are known to uncoat in the endosome after uptake via receptor-mediated endocytosis.[79] The process is thought to be acid-dependent because agents which inhibit acidification of the endosome also inhibit uncoating.[78,79] Both rhinoviruses and polioviruses can exist in two isoelectric forms, one with neutral pH and one with acidic pH.[80,81] The isoelectric form with the acidic pH can be formed by exposure of the virus to acid solution. Accompanying this change in the pI of the virus is the loss of VP4 and a concomitant decrease in the viral sedimentation rate from 150S to 135S.[80] Isoelectric focusing of native rhinovirus, with VP4 present, also demonstrates a low pI form (140S). Neither of the low pI forms are able to bind to cells or initiate infection. An important difference between the rhino- and polioviruses is that in rhinovirus the conversion from the native isoelectric form (neutral) to the acid isoelectric form is irreversible, whereas in poliovirus it can be reversed.[81] It is unclear whether the low pH isoelectric form of rhinoviruses is an intermediate in the mechanism for uncoating, or a dead-end product.

VP4 plays an interesting role in the uncoating of rhinoviruses. It was noted that abortive infections could occur when VP4 was lost from the viruses but the RNA was still intact.[82] The resulting particles, devoid of VP4, are no longer infectious, implicating VP4 as a protein necessary for infection.[80] It has been suggested that VP4 may act to carry the RNA across the endosomal membrane. Credence was added to this theory upon the discovery that the VP4 of poliovirus,[83] and subsequently also of other picornaviruses,[84] are myristoylated. The hydrophobic myristoylate could act as a hydrophobic probe and insert into the membrane, initiating the transport of VP4 and the viral RNA across the endosomal membrane.[85] This myristoylate has been seen in the structure of poliovirus, clustered about the fivefold axes on the interior of the protein shell.[83] Less well-defined electron densities which are likely to correspond to myristoylate are seen in rhinovirus and Mengo virus.[30,31]

The mechanism of uncoating of Mengo virus appears to be quite different to that of rhinoviruses. Mengo virions dissociate into pentamers under physiological conditions when they interact with their

cellular receptors. This extracellular uncoating can be inhibited by raising the pH or lowering the temperature.[86] Some halide ions can induce uncoating in the pH range from 5.8 to 6.4.[87] The narrow pH range of the halide ion effects suggests that there are at least two titratable groups important in uncoating. It has been suggested that at least one of these groups is a histidine.

The structural changes (Figure 6) seen when the halide concentration is increased in crystals at pH 6.2 are the same as those seen in the crystals with lowered pH in the presence of phosphate.[39] This suggests that this set of structural changes is important in dissociation into pentamers as well as the aforementioned changes important in binding. From the structures of Mengo virus at different pH values and halide concentrations, two histidines which may be involved in disassembly of the virions have been proposed.[39] One of these histidines is in the FMDV loop at position 1205. This residue undergoes a significant conformational shift on acidification or on an addition of Cl^- to the Mengo virus crystals. His 1205, however, is not at a pentamer-pentamer interface and thus, if it is to mediate dissociation into pentamers, its effect must be propagated to that interface. A second histidine more proximal to the pentameric interface also shows movement upon acidification of the Mengo virus crystals. This is histidine 2250 which is located near the icosahedral twofold axis. Protonation of this histidine could disrupt the bridging between the six arginine and two glutamine residues at the interface (R2101, R2102, R2255, E2251 and the twofold-related residues).

Most of the structural changes in Mengo virus induced by pH changes or halide ions are confined to the pit area. The biggest changes are: (1) the GH loop of VP3, initially buried underneath the FMDV loop of VP1 of an adjacent protomer, becomes disordered and shows a second disordered conformation pulled out into the canyon; (2) the FMDV loop moves slightly away from the adjacent VP3. As part of this movement, H1205 changes its hydrogen bond partners from N1210 and N2138 to N1210 and the carbonyl of 1207.

Conclusion

The determination of the atomic structure of two rhinoviruses and Mengo virus has yielded results regarding the function and provided a framework about which other experiments have been designed. The ability of these viruses to mutate their surfaces rapidly to evade immune surveillance, but to conserve a receptor binding site, has been understood in terms of the canyon hypothesis. The mapping of the neutralizing antigenic surface has reinforced observations on the various mechanisms of neutralization by antibodies and possibly the nature of serotype specificity. It has been possible to predict which residues on the surfaces of the virus and receptor for HRV14 might interact. Site-directed and random mutagenesis (in the presence of antiviral agents) are being used to probe this interaction. Studies of the pH- and halide-dependent conformational changes in Mengo virus, and those induced by antiviral agents in HRV, together with mutagenesis studies are yielding information regarding both receptor binding and uncoating. The ubiquity of the β-barrel in the capsids of viruses suggests that there may be homologous pockets for antiviral agents of the WIN type in many viruses. The effect of these antiviral agents on other families of viruses is being examined. Crystallographic studies of HRV complexed with antiviral agents is contributing to the improvement of these agents and is providing a data base from which rules might be established for the design of quite different drugs that interact with proteins. Finally, differences between HRV1A and HRV14 have suggested the possible basis for receptor specificity.

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