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Human rhinovirus capsid dynamics is controlled by canyon flexibility

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Abstract

Quantitative enzyme accessibility experiments using nano liquid chromatography electrospray mass spectrometry combined with limited proteolysis and isotope-labeling was used to examine the dynamic nature of the human rhinovirus (HRV) capsid in the presence of three antiviral compounds, a neutralizing Fab, and drug binding cavity mutations. Using these methods, it was found that the antivirals WIN 52084 and picovir (pleconaril) stabilized the capsid, while dansylaziridine caused destabilization. Site-directed mutations in the drug-binding cavity were found to stabilize the HRV14 capsid against proteolytic digestion in a manner similar to WIN 52084 and pleconaril. Antibodies that bind to the NIm-IA antigenic site and penetrate the canyon were also observed to protect the virion against proteolytic cleavage. These results demonstrate that quantifying the effects of antiviral ligands on protein "breathing" can be used to compare their mode of action and efficacy. In this case, it is apparent that hydrophobic antiviral agents, antibodies, or mutations in the canyon region block viral breathing. Therefore, these studies demonstrate that mobility in the canyon region is a major determinant in capsid breathing.

Keywords: Rhinovirus; Virus maturation; Mass spectrometry

Introduction

Picornaviruses are among the largest of animal virus families and include polio, hepatitis A, foot-and-mouth disease, and rhinoviruses. Rhinoviruses, of which there are more than 100 serotypes, are major causative agents of the common cold in humans (Rueckert, 1996). Human rhinovirus infections begin with binding of the virion to their receptor on the outside of the cell, translocation of the virus particles into the cell, and the release of its genomic material into the cytoplasm. The virus particle must be flexible enough to allow cellular binding and disassembly of the virions yet stable enough to survive in the extracellular milieu. In fact, several reports have shown that the viral capsid is a dynamic structure that transiently exposes deeply buried N-termini (Fricks and Hogle, 1990; Lewis et al., 1998; Li et al., 1994).

The human rhinovirus is nonenveloped and has an ~300-Å-diameter protein shell that encapsidates a single-stranded, plus-sense, RNA genome of about 7200 bases. The human rhinovirus 14 (HRV14) capsid exhibits a pseudo T = 3 (P = 3) icosahedral symmetry and consists of 60 copies each of four viral proteins, VP1, VP2, VP3, and VP4. Proteins VP1-3 have eight-stranded, antiparallel β-barrel motifs and comprise most of the capsid structure. VP4 is smaller, has an extended structure, and lies at the RNA/capsid interface, making it the most interior capsid protein (Rossmann et al., 1985). An ~20-Å-deep canyon lies roughly at the junction of VP1 (forming the "north" rim) with VP2 and VP3 (forming the "south" rim) and surrounds each of the 12 icosahedral fivefold vertices (Fig. 1). Four major neutralizing immunogenic (NIm) sites, NIm-IA, NIm-IB, NIm-II, and NIm-III, were identified by studies of neutralization-escape mutants with monoclonal antibodies (Sherry et al., 1986; Sherry and Rueckert, 1985) and mapped to four protruding regions on the viral surface

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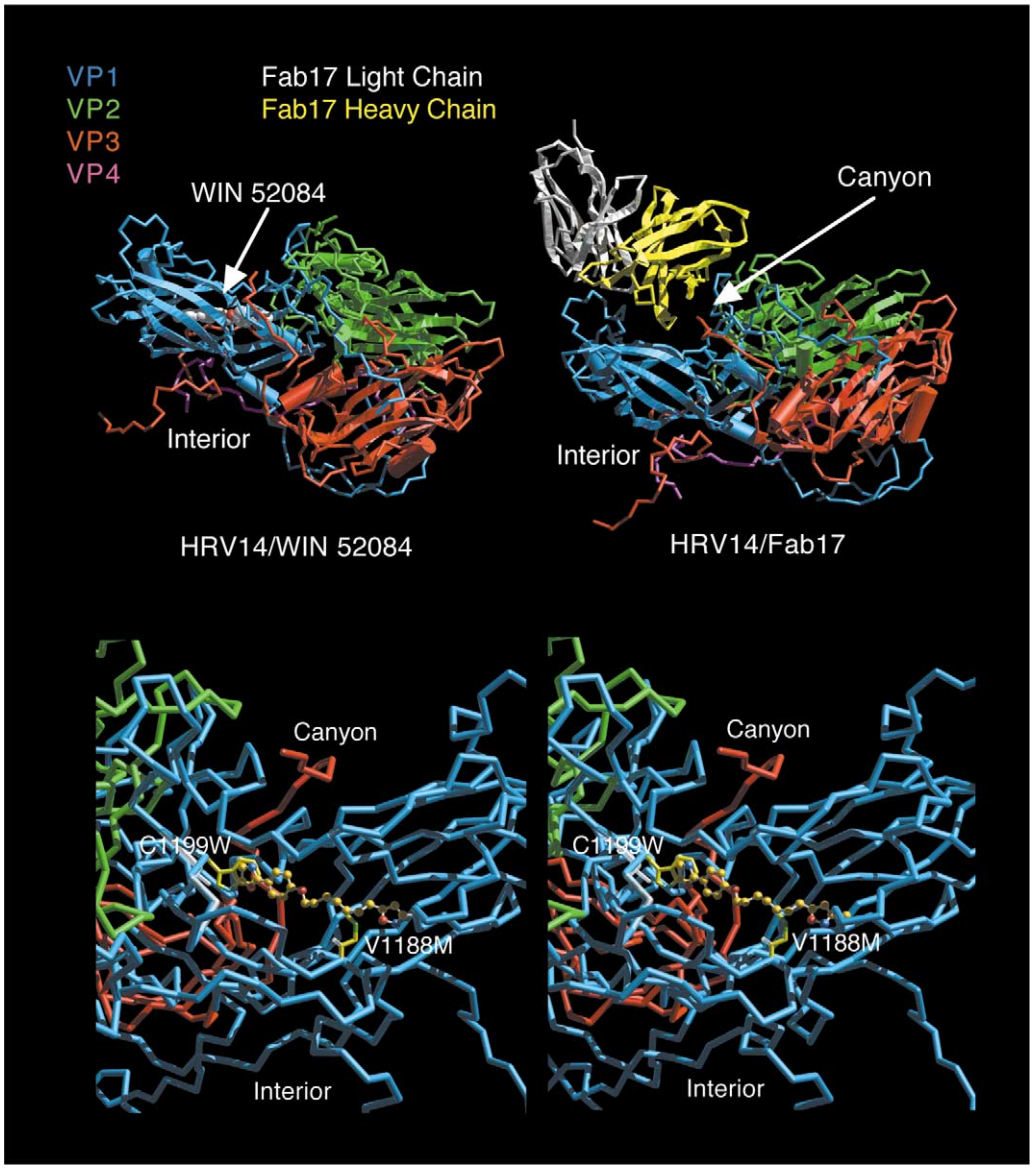

Fig. 1. Structures of WIN drugs and Fab17-IA bound to HRV14. Shown in the top left figure is a ribbon diagram of one protomer of HRV14 complexed with WIN 52084 (Smith et al., 1986). VP1–VP4 are colored blue, green, red, and mauve, respectively. In this view, the RNA interior is toward the bottom of the figure and the nearest five-fold axis runs approximately vertical on the left side. WIN 52084 is represented by a space-filling model beneath the canyon region. In the upper right diagram is a ribbon diagram of the Fab17-IA/HRV14 complex (Smith et al., 1996) in approximately the same orientation. Here the variable regions of the heavy and light chains of Fab17-IA are shown in yellow and white, respectively. The bottom panel is a stereo diagram of the WIN 52084/HRV14 complex with the V1188M and C1199W mutations modeled to show how they fill the drug binding cavity and sterically block WIN binding. For clarity, the view is rotated 180° about the vertical axis compared to the top panels. For this figure, the drug and associated conformational changes were taken from the WIN 52084 structure (Smith et al., 1986). The C1199W mutation was modeled by replacing residues 1196–1201 of the WIN/HRV14 complex with those of the structure of the C1199Y mutant (Badger et al., 1989) and then replacing the TYR with a TRP using the graphics program "O" (Kleywegt and Jones, 1994). The V1188M mutation was modeled using "O" to replace the VAL with a MET and orienting the side chain in the most favored rotomer position. The original structures about these residues are shown in white in the figure.

(Rossmann et al., 1985). The canyon regions of HRV14 and HRV16, both major receptor group rhinoviruses, contain the binding site of the cellular receptor, intercellular adhesion molecule 1 (ICAM-1) (Colonno et al., 1988; Kolatkar et al., 1999; Olson et al., 1993).

X-ray crystallographic studies have shown that antiviral compounds (WIN drugs) that inhibit uncoating bind to a hydrophobic pocket immediately beneath the floor of the canyon (Smith et al., 1986) (Fig. 1). While these structural studies have yielded a wealth of information as to drug–virus interactions, they do not elucidate the conformational changes associated with the uncoating process. Modern mass spectrometry techniques such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), combined with limited proteolysis, have been used to monitor the dynamic nature of virus particles (Bothner et

al., 1999). More specifically, viral capsid mass mapping experiments have been used to obtain information regarding the dynamic nature of the viral capsid in the presence of antiviral compounds (Lewis et al., 1998). In these experiments, peptide fragments from limited proteolysis can be identified by MALDI-MS, thereby elucidating the relative accessibility of protein regions. Such experiments have shown that the HRV14 capsid transiently extrudes internal VP1 and VP4 N-termini in a "breathing" process (Lewis et al., 1998). Since this dynamic process is affected by a number of antiviral compounds, this technique is proving especially useful in the evaluation of such drugs in a rapid, mass-spectrometry-based manner without the complications of cell toxicity.

While the original WIN compounds tended to have relatively narrow serotype specificity, a derivative of these compounds, picovir (pleconaril), has demonstrated even greater efficacy for treatment of a range of rhinovirus serotypes and is currently in clinical trials (Kaiser et al., 2000). Pleconaril and other similar isoxazole analogs bind to the VP1 pockets primarily through hydrophobic interactions with residues beneath the canyon floor. This results in stabilization of the virus particle that prevents the capsid from uncoating and the RNA from entering cells (Romero, 2001). In contrast to the non-covalent binding of pleconaril, aziridine compounds have recently been shown to block virus infectivity by covalently modifying the bases on the viral RNA or DNA of various types of viruses (Broo et al., 2001). In a manner similar to the replacement of wild-type flock house virus RNA with cellular RNA (Bothner et al., 1999), the aziridine-alkylated nucleotide bases destabilize the RNA–protein contacts and therefore the entire viral capsid structure. Due to their high reactivity and lack of selectivity, these alkylating agents are currently under investigation for their potential in the development of antiviral vaccine preparations (Burrage et al., 2000a,b). While the targets (i.e., RNA versus protein) are different for these two types of antiviral agents, both rely upon the flexibility of the protein capsid to neutralize infectivity.

Here we further examine and quantify the breathing phenomena in HRV14. Limited proteolysis experiments using serine proteases were employed to monitor the effects of WIN 52084, pleconaril, and dansylaziridine on the viral protein capsid with and without O^{18} labeling. By analyzing the virus digestions with several mass spectrometry (MS) techniques including capillary nano liquid chromatography) LC-MS and MALDI mass spectrometry, we demonstrate that WIN 52084 and pleconaril decrease HRV14 dynamics, while an aziridine derivative causes an increase. It was previously suggested that the binding of hydrophobic compounds to the drug-binding pocket causes stabilization by a change in entropy (Phelps and Post, 1995; Smith, 1989; Tsang et al., 2000). This is supported here by the demonstration that filling the drug-binding pocket with hydrophobic side chains decreases capsid breathing in a manner similar to the WIN compounds. It has also been suggested that motion in the canyon might be the dynamic process being affected by these drugs (Lewis et al., 1998). This contention is supported by results presented here showing that Fab17, that penetrates the canyon region, does not induce conformational changes in the virion and yet also inhibits virus breathing. Together, these results imply that movement of the canyon region is a prerequisite for breathing. Further, since viral breathing is correlated with infectivity, it also suggests that canyon motion is essential for the normal uncoating process.

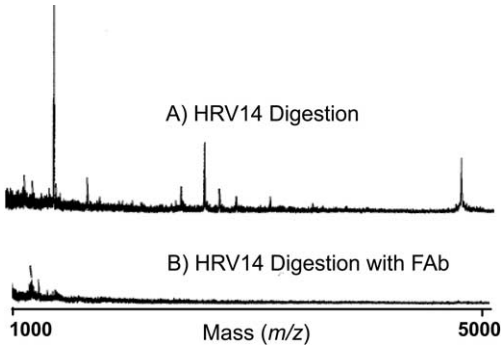

Fig. 2. Limited proteolysis of HRV14 in the presence or absence of FAb. MALDI-TOF spectra from a 15-min limited proteolysis of HRV14 in the presence (B) and absence (A) of Fab17. Note that Fab17 greatly inhibits proteolysis even at this low pH (10 mM ammonium acetate, pH 4.3). Spectra were set to the same relative scale.

Results

Fab17 effects on HRV14 breathing

Antibodies are a major component of the immune response to rhinovirus infection. There are four immunogenic regions (NIm sites) on the HRV14 surface: NIm-IA, NIm-IB, NIm-II, and NIm-III. The major mechanism of antibody-mediated neutralization for antibodies to all of these antigenic sites appears to be abrogation of cell attachment (Che et al., 1998; Smith et al., 1993). However, antibodies to the NIm-IA site appear to also stabilize the virion (Che et al., 1998). This was proposed to be due to these antibodies binding in the canyon region (Fig. 1) that is involved in the conformational changes associated with the uncoating process. HRV14 complexed with the neutralizing Fab fragment. Fab17-IA, resisted enzymatic degradation on the protein capsid as evidenced by MALDI-TOF mass analysis of digests on intact viruses. Even at a pH of 4.3, below the pH required to inactivate the virus, Glu-C enzymatic digestion of the virus particles was inhibited by the presence of HRV NIm-1A antibodies (Fig. 2). These effects cannot be due to Fab17 sterically interfering with the protease accessing the cleavage sites since, as we have previously shown, the initial and major protease cleavage sites are buried deep within the capsid (Lewis et al., 1998) and are therefore not

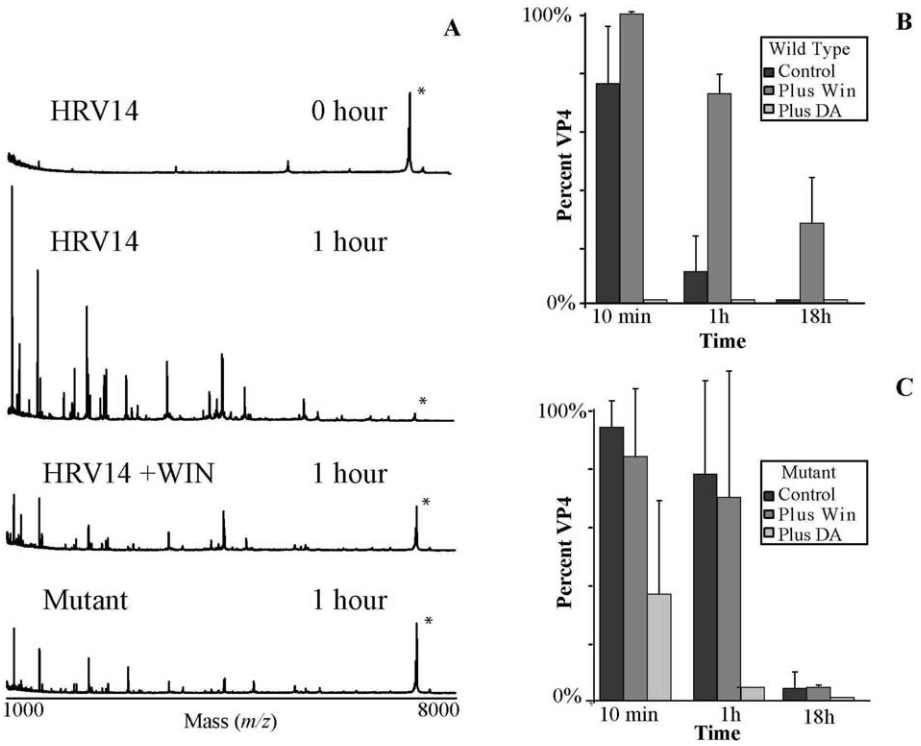

Fig. 3. Limited proteolysis of HRV14 under various conditions. (A) MALDI-TOF spectra of HRV14 wild-type and C1199W/V1188M mutant in the presence and absence of WIN compound. Spectra were set to the same relative scale. While wild-type virus is readily digested when exposed to trypsin for 1 h (spectrum second from the top) compared to undigested HRV14 (top spectrum), both mutant and wild-type virus + WIN are resistant to proteolysis (bottom spectrum). Asterisk (*) denotes the intact VP4 protein. (B) Time course of VP4 digestion of wild-type virus in the presence and absence of WIN and DA compounds. Note that, compared to the wild-type control, WIN compounds protect VP4 while DA facilitates proteolysis. Graphs B and C were generated from individual MALDI spectra, such as in Fig. 2A, using the peak height of VP4. For each treatment (Control, WIN, or DA), the VP4 peak at zero time was set to 100% and the percentage of VP4 from the remaining time points were calculated based on this value. (C) Time course of the C1199W/V1188M mutant form of HRV14 in the presence and absence of WIN and DA. WIN does not appear to significantly change the sensitivity of VP4 to cleavage compared to the mutant control. In addition, while the mutant is more resistant to digestion than wild-type virus, it is less sensitive than wild-type virus + WIN. Similar to wild-type virus, DA increases sensitivity of VP4.

in contact with the bound Fab17. Therefore, the fact that the internal protease sites on the VP1 and VP4 termini are protected by Fab17 is consistent with the global stabilizing effects of this class of antibodies. Further, this suggests that the antibody-mediated stabilization coincides with a decrease in capsid mobility.

Effects of ligands on HRV14 breathing

The WIN class of antiviral drugs has been shown to have a general stabilizing effect on the viral capsid in addition to reducing cell attachment and inhibiting the uncoating process (Lewis et al., 1998). Our current results confirm these data; WIN 52084 decreased proteolysis of HRV14 as seen by the reduced amount of peptide fragments during a 1 h digestion with trypsin (Fig. 3A). Peptides were confirmed to have resulted from proteolysis of HRV14 as previously described (Lewis et al., 1998). There are no proteolytic fragments in wild-type prior to digestion. WIN 52084 has previously been shown to decrease the accessibility of trypsin to cleavage sites on the viral coat rather than inhibiting the enzymatic activity of trypsin itself (Lewis et al., 1998). It is apparent from these and other experiments that the WIN drug not only affects local conformation but also has a global stabilizing effect.

When HRV14 is grown in the presence of high concentrations of WIN drugs, naturally occurring mutants at two residues are commonly selected for V1188 and C1199 (Heinz et al., 1989). These mutations reside in the pocket region where the WIN drug normally binds and render the pocket inaccessible. This has been shown both crystallographically (Heinz et al., 1989) and through binding studies that these compounds do not bind to the V1188 mutations and that there is a direct correlation between drug efficacy and affinity (Fox et al., 1991). In the case of the C1199Y, filling the cavity with a more hydrophobic residue stabilizes the virion against thermal denaturation (Shepard et al., 1993). To ascertain whether thermal denaturation of HRV14 is related to the breathing phenomenon, and if filling the pocket with hydrophobic residues blocks this process, a double mutation of HRV14 was made, V1188M/C1199W, and proteolytic sensitivity was measured. Similar to the single mutant, this double mutant was found to be more stable than wild-type, but less stable than wild-type +

WIN (data not shown). Furthermore, the double mutant was not further stabilized by the addition of WIN compound and is consistent with the assumption that the drug did not bind to this double mutant. With regard to plaque size and viral yield, this particular mutant exhibits a wild-type phenotype. As shown in Fig. 3A, this double mutation dramatically reduced proteolysis when compared to wild-type HRV14. In the absence of WIN 52084, the mutant viral coat undergoes proteolysis in a manner similar to wild-type virus in the presence of WIN 52084. DMSO, the solvent used for WIN, alone had no apparent effect on proteolysis of HRV14 or the V1188M/C1199W mutant. These results strongly suggest that thermal denaturation of HRV14 is directly related to breathing and that the drugs stop this dynamic process through entropic effects.

By measuring the degradation of VP4 over increasing digestion times, we can also compare the long-term stabilization effects conferred by the WIN compounds and the mutations. Because the levels of VP4 consistently decreased as the proteolytic fragments increased, it proved useful to compare digestion of wild-type virus to the mutant virus using VP4 intensities. It should be noted that the graphs presented are only semiquantitative and are used to express the trend in proteolysis. As shown in Fig. 3B and C, VP4 from wild-type virus is almost completely digested within 1 h in the absence of WIN but is present after 18 h in the presence of WIN. By contrast, although the mutant is somewhat affected by WIN, there is a significant amount of VP4 present after 1 h (also see Fig. 2A, bottom spectrum). In addition, after 18 h VP4 is almost entirely digested in the mutant, regardless whether or not WIN is present. This is in contrast to what is observed in wild-type virus, where WIN causes a much more dramatic effect. Digestions performed for 24 h resulted in no apparent differences in VP4 levels when compared to 18 h (data not shown). From this analysis, it is clear that the V1188M/C1199W mutant is more stable than wild-type virus but not as stable as wild-type virus plus WIN compounds. As expected, the stability of the mutant virus is less affected by the presence of WIN compounds than wild-type. These results are consistent with infectivity studies (U. Katpally and T. J. Smith, unpublished data). Since these mutations all fill the drug cavity with hydrophobic side chains, these results support the contention that WIN compounds act via entropic stabilization (Phelps and Post, 1995; Smith, 1989; Tsang et al., 2000).

In contrast to pleconaril, dansylaziridine (DA) acts by using the dynamic nature of the capsid to penetrate the virion and modify the RNA genome, resulting in a destabilization of the capsid (Broo et al., 2001). This phenomena is also observed in the current experiments (Figs. 3B and C). In the presence of DA, VP4 of wild-type virus is cleaved in less than 10 min. Similarly, DA sensitizes VP4 cleavage in the mutant, but to a slightly lesser extent than that observed with wild-type virus. In the presence of the solvent for DA, acetonitrile, alone, digestion of the HRV14, and the V1188M/C1199W mutant are comparable to controls.

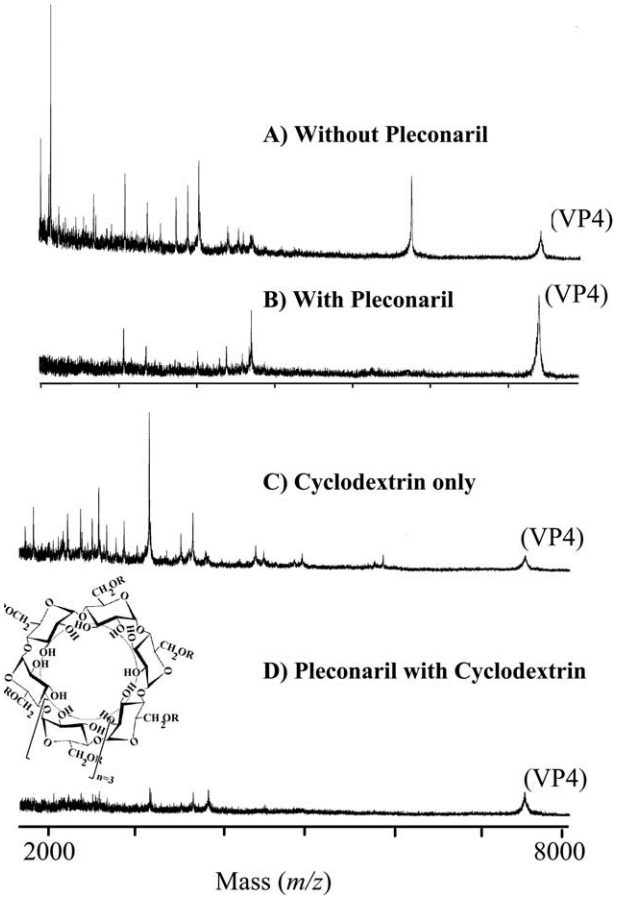

Fig. 4. MALDI-TOF spectra from a 30-min limited proteolysis of HRV-14 (A) alone, (B) after incubation with pleconaril, (C) in the presence of γ-cyclodextrin, and (D) in the presence of γ-cyclodextrin and pleconaril. All spectra are displayed on the same intensity scale. All incubations and subsequent proteolytic digestions were performed on HRV at 1.0 mg/mL and at 37°C. The concentration of pleconaril (at 112 mM) was six times the concentration of cyclodextrin.

Effects of pleconaril

Similar to WIN compounds and Fab17-IA, incubation of HRV with pleconaril under physiologically relevant conditions (i.e., 37°C, pH 7.5) yielded significantly fewer peptide products than HRV solutions absent of pleconaril (Fig. 4). Figs. 4A and B show the MALDI spectra from 15-min tryptic digestions following a 1 h incubation with pleconaril (Fig. 4B) and an incubation absent of the drug (Fig. 4A). The presence of pleconaril inhibited digestion as ascertained by the reduced amount of peptide fragments. Additional analysis of aliquots from longer digestion times continued to demonstrate this trend (data not shown).

The hydrophobic character of pleconaril and its analogs contribute to its selective and high-affinity binding in the VP1 pocket, but also limits its solubility in aqueous solutions. Acetonitrile at a concentration of more than 75% (v/v) was required to dissolve 2 mg pleconaril per milliliter of solution. Serial dilutions minimized the organic solvent content (less than 1% v/v) and the amount of drug was less

than 50 μM. However, delivering more drug to the virus solution without additional organic solvent was investigated as a possible means of further inhibiting the viral capsid dynamics. Cyclodextrin has previously been used to enhance drug solubility by acting as a "cage" molecule (Rajewski and Stella, 1996), and more specifically as a vehicle for pirodavir, an antiviral with in vitro activity against rhinoviruses (Hayden et al., 1992). As a simple test, γ-cyclodextrin was used to increase the amount of drug added to the virus solution with only trace amounts (less than 0.2% v/v) of organic solvent. With the addition of cyclodextrin at a concentration of approximately six times less than the concentration of pleconaril, the HRV proteins were further protected from digestion (compare Fig. 4B with D). The presence of cyclodextrin alone had no effect on HRV protease sensitivity (compare Fig. 4A with C). While it is possible that cyclodextrin increases the solubility of some peptides, and hence changes the mass spec profile slightly, cyclodextrin itself does not decrease proteolysis. An even further decrease in the proteolytic susceptibility is seen when a larger amount of pleconaril was delivered to the HRV solution (Fig. 4D) than without cyclodextrin (Fig. 4B). These results suggest that cyclodextrin is an efficacious drug carrier system.

Drug mechanism of action

The qualitative results shown thus far demonstrate the general stabilizing effect of pleconaril. However, to determine the relative effectiveness of this drug requires a more quantitative approach. Nano-LC ion-trap mass spectrometry is a particularly sensitive method for characterizing one or more peptides in the midst of other constituents and is appropriate for the quantitative analysis of peptide products from proteolytic digestion of intact virions. Isotopically labeled tryptic peptides were monitored by nano-LC-MS to quantitatively show how pleconaril and dansylaziridine altered the stability of the HRV. Incorporation of oxygen^{18} (O^{18}) by enzyme-catalyzed hydrolysis has been an effective means of quantitatively monitoring numerous substrates including proteolytic peptides with several mass spectrometry techniques (Desiderio and Kai, 1983; Kosaka et al., 2000; Mirgorodskaya et al., 2000; Pickett and Murphy, 1981), and more recently for comparative proteomics (Yao et al., 2001). Peptide bonds hydrolyzed for short time periods in the presence of O^{18}–water incorporated an O^{18} atom into the carboxy-terminus of the resulting peptides. This is visualized as a peak that is apparent at two mass units above the monoisotopic ion.

The O^{18}-isotope labeling in this study was performed in a manner that would not bias the results with respect to the natural abundances of the MH^+ (monoisotopic) and [MH^+ + 2] isotopes. For biomolecules below 1000 D, the contribution from monoisotopic ions dominates other isotopes (Yergey et al., 1983); thus, an insignificant amount of [MH^+ + 2] isotopes is typically observed in mass spectra of small peptides. Controls for limited proteolysis experiments were performed with samples where the aqueous solvent was exchanged with O^{18}–water, and in the absence of any antiviral agents (Fig. 5A). Virus digestions containing antiviral agents were performed in O^{16}–water. Therefore, when equal amounts of these digests were mixed, the effect on the viral capsid would be noted in the relative abundance of the monoisotopic peak relative to the O^{18}-labeled peptide (Figs. 5B and C). Considering the normally dominant abundance of the monoisotopic peak for peptides in this mass range, the relative intensity of this isotope in the presence of these antiviral agents (in O^{16}–water) establishes how each antiviral agent affects the virus dynamics.

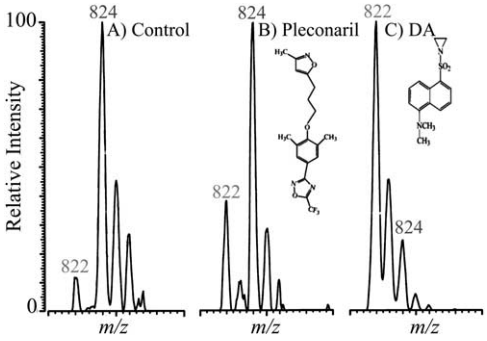

Fig. 5. Capillary LC-ESI mass spectrometric evaluation of isotopically labeled peptides from a 15-min limited proteolysis of (A) HRV alone in O^{18}–water, (B) an equal mixture of HRV in the presence of pleconaril in regular water and HRV alone in O^{18}–water, and (C) an equal mixture of HRV in the presence of dansylaziridine (DA) in regular water and HRV alone in O^{18}–water. Tryptic digests were quenched by the addition of acetic acid and either loaded onto the capillary or frozen immediately for further analyses.

Since VP1 98–103 represents an externally exposed portion of the capsid that was sensitive to proteolysis, it was chosen as a clear marker for evaluating these drugs. This relatively hydrophobic peptide is significant because it lies in the canyon that is directly affected by the presence of pleconaril. Although this peptide is only moderately accessible to trypsin, it is observed in adequate, measurable amounts in the presence or absence of antiviral compounds. Proteolytic peptides other than VP1 displayed similar trends for pleconaril and aziridine-treated HRV, but were observed as multiply charged peaks, or nonstructurally relevant regions of the virus (data not shown).

Control digests were conducted in O^{18}–water in the absence of antiviral drugs. The control experiment shown in Fig. 4A reveals that the digests performed in O^{18}–water with no drug present yields a dominant O^{18}-labeled peak at *m/z* 824 for the VP1 peptide. Since it is difficult to completely eliminate regular water from virus samples where maintaining the virion structure is critical, virus digests performed in O^{18}–water were expected to show a small contribution of monoisotopic peaks due to residual water that remained. The typical amount of the unlabeled monoisotopic species (*m/z* 822) observed was at 10–20% rel-

ative intensity to the labeled species (*m/z* 824) and was primarily due to residual water left in the virus solution (Fig. 5A).

For experiments where pleconaril or dansylaziridine was added, digests were performed in regular O^{16}–water. Subsequently, equal amounts of drug-positive digests (O^{16}–water) were mixed with control digests (O^{18}–water). In this situation, the effect on the viral capsid is apparent when the relative abundance of the monoisotopic peak is compared to the O^{18}-labeled peptide. For example, if these experiments were conducted using a drug that had no effect on the capsid proteins, the result would be an equal abundance of peaks at 822 and 824 *m/z* (data not shown). The control digest (O^{18}) contributes the peak at 824 *m/z* and the peak at 822 *m/z* comes from the experimental digest performed in regular O^{16}–water. If the drug had a suppressive effect, the peak at 822 *m/z* would be significantly lower than the peak at 824 *m/z*. As shown in Fig. 5A, the presence of pleconaril effectively suppressed proteolytic digestion as exemplified by the significantly lower abundance of the monoisotopic peak at *m/z* 822 (40 $\pm$ 11%) compared to the peak at 824 *m/z* (100%). In the middle panel, O^{18}–water from the control digest presumably contributes much of the peak at *m/z* 822, although there is likely to be limited digestion occurring in the presence of pleconaril. This data support the hypothesis that pleconaril acts by binding to the VP1 pockets and hence stabilizing the protein capsid, making it less susceptible to proteolysis.

In contrast to the effect of pleconaril, dansylaziridine drastically increased the abundance of the VP1-derived peptide at 822 *m/z* (Fig. 5C). As seen in the far right panel, the 822 *m/z* peak is significantly higher than the 824 *m/z* peak, indicating that the presence of dansylaziridine effectively increases tryptic digestion. The reactive dansylaziridine has previously been shown to destabilize the virus particle by disrupting the RNA/capsid protein interactions (Broo et al., 2001). However, the global virus particle structure is not compromised over the short term. Instead, the uncharged aziridine molecules pass through the dynamically mobile protein capsid to selectively alkylate the RNA rather than break up the virus particle to expose the RNA for reaction. Our current data support this hypothesis; the dynamic nature of the protein capsid in the presence of dansylaziridine allows for increased exposure to proteolytic digestion of the capsid.

The results from this study illustrate the subtle changes induced by the presence of antirhinovirus agents. While these protein–protein and protein–RNA disruptions do not compromise the structural integrity of the virus particles, they significantly decrease HRV infectivity. HRV incubated with the capsid-binding drug pleconaril and the alkylating agents at 37°C each showed a threefold decrease in infectivity, respectively (data not shown). No notable differences were observed between untreated HRV and HRV treated with dansylaziridine at 25°C. The increased capsid mobility at the elevated temperature of 37°C presumably allowed the dansylaziridine molecules to pass through the capsid to alkylate the viral RNA. The temperature effect was less pronounced for the capsid binding agent pleconaril, which has a viral target near the virus capsid surface.

Discussion

These studies demonstrate that the dynamics of the viral capsid is the "Achilles' heel" of picornaviruses that can be targeted by antivirals. Small quantities of pleconaril enhanced the stability of HRV, thereby decreasing the accessibility of the protein capsid to enzymatic degradation. In fact, pleconaril produced more than a 50% increase in stability compared to native virus as shown by proteolytic accessibility and quantitative mass spectrometry (Fig. 5B). WIN compounds have been shown to interfere with the receptor/virus interactions of the major group of rhinoviruses but not that of the minor group (Pevear et al., 1989). ICAM-I is the receptor for the major group of rhinoviruses and binds to the upper south wall of the canyon region (Olson et al., 1993). In contrast, the minor group of rhinoviruses uses the uppermost rim of the north canyon to interact with LDL receptors (Hewat et al., 2000). It has been suggested that the mechanism of action of the WIN compounds is abrogation of receptor interaction in the case of the major group of rhinoviruses (Pevear et al., 1989). However, it seems likely that these compounds have the primary effect of stabilizing the capsids of all rhinoviruses and, in the case of the major group, this stabilization of the canyon region affects the virus/receptor affinity. Since the receptor for the minor group is well outside the canyon region (Hewat et al., 2000), it follows that receptor–virus interactions are unaffected by the drugs. As has been previously suggested (Lewis et al., 1998), it seems likely that drug-mediated stabilization prevents capsid breathing that is in turn crucial for uncoating. In this way, all HRV's are inhibited by WIN compounds and their derivatives.

The dansylaziridine compounds differ from the WIN compounds in that they offer a "two-prong" attack on the viruses. In previous studies, it was shown that these compounds take advantage of capsid dynamics to penetrate the protein shell and covalently modify the RNA genome (Broo et al., 2001). The results presented here demonstrate that these alkylating agents have a second effect in that the modification of the RNA causes a marked destabilization of the capsid. Such destabilization would likely interfere with the efficacy of viral transmission and makes these compounds even more efficacious.

These studies also demonstrate how the combination of limited proteolysis and isotope labeling can be used to qualitatively and quantitatively ascertain the modes of inactivation. Traditionally, the efficacies of antiviral agents are often measured by plaque assays and lethal dosing (LD_{50}) experiments and often do not reveal the mode of inactivation. In this case, WIN and alkylating agents both

effectively neutralize infectivity but have completely opposite modes of action. The techniques described here offer an efficient means to discern these differences. Furthermore, these results show the key role capsid dynamics can play in virus inactivation and may be further exploited in antiviral drug development. The quantitative nature of these techniques will be useful to assess the relative effectiveness of antiviral agents. In addition, these methods can be used to determine the number of antiviral molecules required to neutralize the virion. Such studies on WIN compounds have been impossible using plaque assays since the concentration of drugs in solution is hard to ascertain because these hydrophobic compounds partition into cell membranes.

The results of the Fab17-mediated stabilization suggest that canyon motion is part of the capsid breathing process. Fab17 and other NIm-IA antibodies penetrate into the canyon region and stabilize the virus against pH and thermal denaturation (Che et al., 1998; Smith et al., 1996). What is shown here is that Fab17, by wedging into the canyon region but not inducing any conformational changes (Smith et al., 1996), blocks breathing in the same way as the antiviral compounds. The effects of the antiviral compounds could be relatively diffuse—affecting a number of conformational changes associated with uncoating. However, the antibodies are clearly filling the canyon without inducing conformational changes. This all implies that breathing requires conformational flexing of the canyon region and this process is being blocked by the extensive antibody/canyon interactions with the north wall, south wall, and base of the canyon. The WIN drugs accomplish the same effect by binding under the canyon floor. Interestingly, the receptor ICAM-I binding region overlaps with the antibody contact region on the south wall, causing the opposite effect compared to the antibody.

The effects of the drug-binding cavity mutations on capsid breathing are entirely consistent with these hypotheses. The results shown here demonstrate that filling this cavity with nonpolar elements mimics, to a degree, the effects of WIN drugs. The fact that these mutations do not affect viral infectivity (U. Katpally and T.J. Smith, unpublished data); however, makes it important to note that these breathing measurements are performed in the absence of receptor. With receptor bound, the differences between WIN and pocket mutations are expected to be more pronounced. Another possible conclusion from these results is that capsid breathing may be linked to conformational changes in the canyon and this motion facilitates improved interactions with the receptor. In this way, the WIN drugs can indirectly affect the binding of ICAM-1 to the major group of rhinoviruses by disrupting capsid breathing. The fact that filling the drug binding cavity with hydrophobic residues through mutagenesis causes stabilization similar to that of WIN compounds suggests that all of these effects are driven by changes in entropy as previously suggested for HRV14 (Phelps and Post, 1995; Smith, 1989) and experimentally demonstrated in the case of poliovirus (Tsang et al., 2000).

Materials and methods

HRV14 mutagenesis

HRV14 cDNA that produces infectious RNA (Wang et al., 1998) was used as a template for mutagenesis by the PCR overlap method. The two mutation sites, V1188 and C1199, are in the cDNA region, enclosed by the two unique restriction sites *Dra*III and *Avr*II. Two oligos were synthesized, one in 5′-3′ orientation, covering the *Dra*III site, and one in 3′-5′ orientation, covering the *Avr*II site. For each mutation, fragments of two portions of the cDNA were made by PCR using one primer for the end of the *Dra*III and *Avr*II region and the other primer containing the mutation. These fragments were then used as primers for subsequent PCR reactions to make the full-length *Dra*III/*Avr*II fragment. For the double mutation, the V1188M PCR product was used as a template for amplification with the C1199W primers. These fragments were inserted into the HRV14 cDNA using the *Dra*III and *Avr*II restriction sites. The mutated cDNA was sequenced to verify the mutations.

Transfection and amplification

The DEAE-dextran transfection procedure was described elsewhere (Wang et al., 1998). RNA transcripts from the full-length mutated HRV14 cDNA were made using in vitro transcription. The RNA transcripts were diluted in HEPES-buffered saline containing 200 μg DEAE-dextran/ml. The dilutions were added to HeLa cell monolayers and incubated at room temperature for 60 min. The cells were washed to remove DEAE-dextran and supplemented with 4 ml of AH medium in a liquid overlay method. The plates were incubated for 48 h before the cells were scraped and the virus particles were released with repeated freezing and thawing. Plaque assays were performed to check for particles. Virus titer amplification was accomplished using monolayers of HeLa cells on T75 flasks and later on roller bottles until the titer was $\sim 10^7$ PFU/ml. Virus was purified and RNA was extracted as described elsewhere (Erickson et al., 1983; Wang et al., 1998). cDNA corresponding to the structural proteins, VP1–VP4, was made by RT-PCR and sequenced. Even after several passages, RT-PCR and sequencing analysis of the entire capsid region showed that the mutations V1188M and C1199W were unaltered and no second site mutations were observed (data not shown).

Virus purification

Human rhinovirus 14 was produced using previously described protocols (Erickson et al., 1983). In brief, HeLa cells were infected with HRV14 at a multiplicity of infection of 10. After incubating the infected cells at 34.5°C for 10.5 h, the virus was purified from lysed cells treated with *N*-lauryl-sarcosine to solubilize cellular debris. However, unlike the previously described protocol, the lysed cellular

material was not treated with trypsin since even this brief treatment resulted in cleavage of VP1 and VP4 (data not shown). Virus particles were pelleted by ultra centrifugation at a maximum centrifugal force of 278,434 *g* for 2 h and purified with a 7.5–45% sucrose gradient spun at a maximum centrifugal force of -2×10^5 *g* for 1.5 h. The virus bands were collected and dialyzed against 10 mM Tris buffer, pH 7.2. HRV14 concentration was determined spectrophotometrically using an extinction coefficient of 7.7 ml/mg · cm at 260 nm and stored at 4°C.

Fab 17-IA preparation

Monoclonal antibodies (Mabs) 17-IA were produced as previously described (Smith et al., 1993) using the Cellmax Quad 4 cell-culture system (Cellco Corp., Germantown, MD) and purified with a protein G affinity column. Fab17-IA fragments were made by papain cleavage using an enzyme-to-antibody ratio of 1:100 (w/w) and incubated at 37°C for 10 h in the presence of 25 mM β-mercaptoethanol. The digested fragments were dialyzed against 10 mM Tris–HCL, pH 7.5, and purified by ion exchange on a FPLC system (Pharmacia, Piscataway, NJ).

Mass spectrometry

Qualitative mass spectrometry experiments were conducted using a PerSeptive Biosystems (Framingham, MA) Voyager-DE.STR MALDI time-of-flight reflectron mass spectrometer equipped with a nitrogen laser. MALDI generated ions were accelerated into the time-of-flight mass analyzer by a 20-kV pulse after a 200-ns delayed extraction period. Detector voltages were turned on after ions greater than *m/z* 600 had passed the detector (with a "low mass gate") to improve detection sensitivity for the ions of interest. MALDI analyses utilized 3,5-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO) as the matrix dissolved in a 70% acetonitrile/30% water (0.1% trifluoroacetic acid) solution. Sample volumes of 0.5 μL were applied to the MALDI plate followed by 0.5 μL of the matrix solution and allowed to dry. All MALDI spectra were generated from an average of 128 laser pulses.

Quantitative virus-drug stability studies were performed using nano-LC electrospray on a LCQ ion-trap mass spectrometer (ThermoFinnigan Inc., San Jose, CA). The electrospray and the internal capillary line/skimmer potentials were set at 1800 and 57 V, respectively, with a capillary line temperature of 150°C. The injected ions were trapped and scanned between *m/z* 400 and 1800. For ion detection, the conversion dynode and electron multiplier were set at -15 and -1.05 kV, respectively. LC capillaries (100 μm i.d.) were drawn to approximately 5 mm i.d. tips using a laser-based micropipette/fiber puller (Sutter Instrument Co, Novato, CA) and packed with 5 μm C18 stationary phase particles using a high-pressure bomb built in-house. Samples volumes of 5–10 μl were loaded directly onto the capillary columns using the same high-pressure bomb. Chromatographic separations were performed by splitting the solvent flow from an Agilent 1100 binary HPLC pump to yield a final LC flow rate of 0.3–0.56 μl/min. Peptides were eluted with a gradient of 0 to 15% acetonitrile in 0.1% acetic acid for 1 min followed by a gradient of 15 to 100% with 80% acetonitrile in 0.1% acetic acid for 55 min.

Limited proteolytic digestions for isotopic labeling studies using pleconaril and dansylaziridine were performed at 37°C. Modified trypsin (Promega, Madison, WI) and Glu-C (Roche Diagnostics, Indianapolis, IN) digests were performed in 10 mM ammonium bicarbonate (pH 7.8) and 10 mM ammonium acetate (pH 4.3), respectively. Enzyme-to-virus ratios of 1:500 and 1:1000 (w/w) were used for these limited proteolysis experiments. Isotope-labeling was achieved by exchanging the aqueous buffer with O^{18}–water (Isotec Inc., Miamisburg, OH) buffer. Purified picovir (pleconaril) was supplied by Dan Pevear (Viropharma Inc., King of Prussia, PA), whereas dansylaziridine was synthesized and purified as previously described (Broo et al., 2001).

Wild-type HRV14 versus mutant HRV14

For studies comparing HRV14 and the mutant form of HRV14, virus samples were prepared to a final concentration of 1.0 mg/ml in 10 mM Tris buffer at pH 7.6. Modified trypsin (Promega) was dissolved in water and digests were performed at 25 or 37°C using a 1:100 enzyme-to-virus ratio. In samples where WIN 52084 was used, a final concentration of 10 μg/ml was added 10 min prior to the addition of trypsin. Where dansylaziridine was used, a 10 mM stock solution in 50% acetonitrile was added to samples for a final concentration of 1 mM. For control samples, a final concentration of 5% acetonitrile or 0.1% DMSO were added. Reaction volume was 10 μl and 0.75 μl was plated directly on the MALDI analysis plate. Subsequently, 0.75 μl of matrix [3,5-dimethoxy-4-hydroxy cinnamic acid] (Aldrich) in a saturated solution of acetonitrile/water (50:50)/0.25% trifluoracetic acid was added to the plate.

Mass spectrometry experiments for these studies were conducted using an Applied Biosystems (Framingham, MA) Voyager-DESTR MALDI time-of-flight reflectron mass spectrometer. MALDI-generated ions were accelerated into the time-of-flight mass analyzer by a 20-kV pulse after a 100-ns delayed extraction period. Detector voltages were turned on after ions greater than *m/z* 700 had passed the detector (with a low mass gate) to improve detection sensitivity for the ions of interest. Figs. 3B and C were generated from individual MALDI spectra, such as in Fig. 3A, by comparing the peak height of VP4 between time points of each treatment. For each treatment, the VP4 peak at zero time was set to 100% and the percentage of VP4 from the remaining time points was calculated based on this value. In cases where the value of VP4 exceeded the control value, this amount was set to 100% for simplicity. Experi-

ments were repeated a minimum of three times and an average of two to three experiments were used to obtain the resulting graphs and error bars.

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