Structural characterization of the human respiratory syncytial virus fusion protein core

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Human respiratory syncytial virus (HRSV), a member of the pneumovirus subfamily of the Paramyxoviridae family (1), is a leading cause of severe respiratory infections in infants and young children worldwide (2). A safe and effective HRSV vaccine is not yet available, so it is important to pursue potential therapeutic measures against HRSV infection.

HRSV carries three surface glycoproteins: F, G, and SH (1). The HRSV F protein is an attractive target for drug and vaccine development as it is essential for viral entry, is highly conserved, and is the major virus neutralization antigen (1). In contrast, the G protein is highly variable among different strains of HRSV (3), and the SH protein has been found to be dispensable for virus growth (4).

The F protein is synthesized as a 67-kDa precursor (denoted F0) that is processed by proteolytic cleavage to yield two disulfide-linked subunits: F1 and F2 (Fig. 1). The fusion peptide region, a hydrophobic/glycine-rich segment that inserts into the target cellular membrane during the fusion process, is located at the N terminus of the F1 subunit (1). The transmembrane segment is close to the C terminus of the F1 subunit. Adjacent to both the fusion peptide and transmembrane segments are two regions containing 4,3-hydrophobic heptad repeats (HR), a sequence motif suggestive of coiled-coil structures (5–7). These regions are denoted HR-N and HR-C, respectively, and are separated by an intervening domain of ≈270 residues. In general, the arrangement of these structural elements in the F1 protein is highly conserved among the Paramyxoviridae family (8).

Previous studies with viral fusion proteins (ref. 7 and references therein), including hemagglutinin of influenza (9, 10), TM subunit of Moloney murine leukemia virus (11), gp41 of HIV-1 and simian immunodeficiency virus (12–17), GP2 of Ebola (18, 19), gp21 of human T cell leukemia virus type 1 (20), and F1 of simian parainfluenza virus 5 (SV5) (21), suggest that HR-N and HR-C form trimeric hairpin-like structures, with the HR-C regions packing in an antiparallel manner against the inner coiled coiled formed by the HR-N regions. The likely role of the hairpin structure is to facilitate apposition of the viral and cellular membranes by bringing the fusion peptide, which inserts into the cellular membrane, close to the transmembrane segment, which is anchored in the viral membrane. Hence, the trimer-of-hairpins structure presumably corresponds to the fusion-active state, distinct from the native (nonfusogenic) state. But whether hairpin formation precedes the actual membrane-fusion event or occurs simultaneously with fusion is unknown.

There is extensive biochemical evidence to suggest that viral fusion proteins undergo conformational changes when activated (22, 23). Earlier work on the HIV-1 fusion protein gp41 led to a model for viral membrane fusion (Fig. 2) in which gp41 exists in at least three different conformations: (i) the native (nonfusogenic) form, where the fusion peptide and HR-N region are inaccessible, (ii) the prehairpin intermediate, where the HR-N region is exposed and the fusion peptide is projected into the target cellular membrane, and (iii) the fusogenic hairpin form, where the HR-N and HR-C regions associate and bring the viral and the cellular membranes together to facilitate fusion (24).

Agents that prevent these conformational changes by stabilizing the native or intermediate states are expected to prevent fusion activation and thus block viral entry. In the case of HIV-1 gp41, peptides corresponding to the HR-C region of gp41, referred to as C peptides, can effectively inhibit infection (24). One such C peptide is in clinical trials and shows antiviral activity in humans (25). C peptides function in a dominant-negative manner by binding to the transiently exposed coiled coil in the prehairpin intermediate and consequently blocking the formation of the fusion-active hairpin structures (24).

A similar approach may be effective in identifying inhibitors of HRSV infection. HR-C regions from HRSV have been shown to inhibit viral infection and thus provide evidence that the F protein also may display the prehairpin intermediate conformation (26). Analogous to the HIV-1 C peptides, these HRSV
HR-C peptides likely act in a dominant-negative manner to prevent the formation of final fusion-active structures, thereby inhibiting viral entry.

To verify that the HRSV F1 protein core indeed forms a trimer-of-hairpins structure and to provide a structural basis for the development of fusion inhibitors, we characterized the interaction of the two HR regions of HRSV F1 protein. We identified two peptides, HRSV-N57 and HRSV-C45, which correspond to the HR-N and HR-C regions of HRSV F1 protein, respectively, that form a stable trimer of heterodimers. This result is consistent with recent reports that indicate the HR-N and HR-C regions from HRSV associate to form an α-helical trimer of heterodimers (27, 28). The HRSV-N57/C45 complex characterized here was crystallized, and its x-ray structure was determined to 2.3-Å resolution. The structure confirms the similarity between the HRSV F1 protein core and several other viral fusion proteins, including HIV-1 gp41, suggesting that methods for discovery of potential therapeutics and prophylactics developed against HIV-1 gp41 also might apply to the HRSV F1 protein.

Materials and Methods

Gene Construction and Purification of recRSV-1. Using optimal codons for Escherichia coli expression (29), a synthetic gene sequence denoted recRSV-1 was constructed that encodes residues 153–209 and 476–524 of HRSV (strain RSS-2; Swiss-Prot accession no. P11209), connected by a glycine-rich linker (Fig. 1). A factor Xa cleavage site was incorporated upstream of the HRSV coding sequence. The constructed gene was inserted into the BamHI–HindIII restriction site of the hexahistidine expression vector pQE9 (Qiagen, Chatsworth, CA). The resulting plasmid, denoted pRSV-1, was transformed into E. coli XL1-Blue competent cells for protein expression. Cells were grown in Luria–Bertani medium to an optical density of 0.6 at 600 nm. Protein expression then was induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and cells were harvested after 3 h.

Cells were lysed in 6 M guanidine-HCl, and the lysate was clarified by centrifugation. The recombinant protein was purified by nickel-nitrioltriacidic metal-affinity chromatography, followed by reverse-phase HPLC (Waters) using a Vydac C18 preparative column (Vydac, Hesperia, CA) with a water/aetonitrile gradient of 0.1%/min in the presence of 0.1% trifluoroacetic acid. The mass of the purified protein was verified by mass spectrometry on a Voyager Elite matrix-assisted laser desorption ionization-time of flight mass spectrometer (PerSep tive Biosystems, Framingham, MA). The protein was lyophilized and resuspended in ultrapure water and dialyzed against factor Xa cleavage buffer (50 mM Tris–HCl, pH 8.0/100 mM NaCl/2 mM CaCl). To remove the His tag, factor Xa was added at a 1:500 wt/wt ratio of protease to tagged protein, and the reaction was incubated for 2 days at room temperature. The cleavage mixture then was purified by reverse-phase HPLC on a Vydac C18 preparative column. Peak fractions containing recRSV-1 were verified by mass spectrometry and lyophilized.

Proteolysis. All proteolysis reactions were performed with 1 mg/ml protein and 0.1 mg/ml protease in PBS, pH 7.4, at room temperature and quenched with 2 mM PMSF (final concentration). Proteolysis samples were analyzed by reverse-phase HPLC connected to an LCO electrospray mass spectrometer (Finnigan-MAT, San Jose, CA). Fragments were assigned by matching observed masses with a list of possible fragment masses predicted by the computer program FRAGMENT MASS (E. Wolf and P. S. Kim, http://www.wi.mit.edu/kim/computing.html). Assigned fragments were within 1 Da of their predicted values.

CD Spectroscopy. CD spectra were measured at 10 μM protein concentration in PBS buffer with an AVIV 62 DS spectrometer (Aviv Associates, Lakewood, NJ) as described (30). Protein concentrations were determined by absorbance at 280 nm in 20 mM phosphate-buffered 6 M guanidine-HCl (pH 6.5) (31).

Sedimentation Equilibrium Analysis. Sedimentation equilibrium analysis was performed on a Beckman XLA-90 analytical ultracentrifuge (Beckman Instruments) at 15,000 rpm and 20,000 rpm, and data were collected after spinning for 18 h at 20°C.

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Three protein samples at concentrations of 10, 50, and 100 μM were spun, following dialysis against PBS buffer overnight. Data analyses were performed as described (32).

**Purification, Crystallization, and Structure Determination of the HRSV-N57/C45 Trimer.** The HRSV-N57 and HRSV-C45 peptides were generated by trypsin digestion of recRSV-1 protein and purified to homogeneity by reverse-phase HPLC on a Vydac C18 preparative column. The purified HRSV peptides were lyophilized and dissolved in water and 10 mM Tris-HCl (pH 8.5), respectively. Equimolar amounts of HRSV-N57 and HRSV-C45 were mixed, and the complex was separated from the free peptide and aggregated species by gel filtration on a Sephacryl S-100 HR column (Amersham Pharmacia) in buffer (10 mM Tris-HCl, pH 8.5/50 mM NaCl). The purified HRSV-N57/C45 complex was concentrated to 10 mg/ml.

Crystals were obtained by using the hanging drop method by equilibrating 2-μl drops (protein solution mixed 1:1 with reservoir solution) against a reservoir solution containing 24–26% polyethylene glycol 4000/200 mM Tris-HCl, pH 8.5/300 mM Li₂SO₄. The crystals belong to the space group P1 (a = 67.9 Å, b = 71.5 Å, c = 76.5 Å, α = 81.3°, β = 73.8°, γ = 60.7°). There are four trimers per asymmetric unit that give rise to a solvent content of ~46%. For data collection, crystals were directly mounted in nylon loops (Hampton Research, Riverside, CA) from the drop and flash-frozen in liquid nitrogen. Diffraction data were collected at 100 K at the Howard Hughes Medical Institute Beamline X4A at Brookhaven National Laboratory, Brookhaven, NY, using a Quantum-4 CCD detector. Diffraction intensities were integrated by using denzo and scalepack software (33) and reduced to structure factors with the program TRUNCATE from the CCP4 program suite (34). The structure of the HRSV-N57/C45 complex was solved by molecular replacement with the program AMORE (47). A polyserine model derived from the SV5 N1/C1 trimer (21) was used as a search model. Initially, only three molecules per asymmetric unit were found. After applying solvent flattening, histogram matching, and 9-fold noncrystallographic averaging with the program DM (35), the electron density map was interpretable in most regions. Three HRSV-N57/C45 trimer molecules were built into the density using the program O (36). After a few cycles of refinement, the R_{free} value remained high, and the existence of unassigned continuous density was suggestive of a fourth molecule. Molecular replacement with a partially refined HRSV-unassigned continuous density was suggestive of a fourth molecule. Molecular replacement with a partially refined HRSV-N57/C45 trimer molecule readily located the fourth trimer in the unit cell. Crystallographic refinement of the structure (Table 1) was done with the CNS programs (37) (reflections beyond 2.3 Å were not used due to poor quality). Noncrystallographic symmetry restraints were applied during the first few cycles of refinement but then were removed and not used in the final refinement. The quality of coordinates was examined by PROCHECK (38). No residues were in disallowed regions, and 94.5% were in the most favored regions of the Ramachandran plot.

**Results**

**HRSV-N57 and HRSV-C45 Peptides Form a Stable Trimeric α-Helical Complex.** LEARNCOIL-VMF, a program for identifying coiled-coil-like regions in viral fusion proteins, predicts such regions in the HRSV F1 protein (7). The first region (residues 156–201) is near the putative fusion peptide, and the second region (residues 488–516) is adjacent to the transmembrane segment (Fig. 1).

To study the regions predicted by LEARNCOIL-VMF, we constructed a recombinant protein, recRSV-1, corresponding to residues 153–209 and residues 476–524 of HRSV F protein connected by a glycine-rich linker (see Materials and Methods) (Fig. 1). CD spectroscopy indicates that recRSV-1 is highly helical (~70% helix content) and extremely thermostable (the midpoint of thermal denaturation T_m = 90°C in the presence of 4 M guanidine-HCl, pH 7.4); recRSV-1 forms a trimer in PBS buffer as judged by sedimentation equilibrium analysis (X.Z. and P.S.K., unpublished results).

Proteolytic digestion of the recRSV-1 protein reveals a protease-resistant core containing the entire HR-N and HR-C regions predicted by LEARNCOIL-VMF. Digestion of recRSV-1 by trypsin generates two major fragments readily separated by reverse-phase HPLC. Mass spectrometry analysis unambiguously assigns these as an N-terminal fragment corresponding to residues 153–209, denoted HRSV-N57 [observed molecular weight (MW) = expected MW = 6,130], and a C-terminal fragment corresponding to residues 476–520, denoted HRSV-C45 (observed MW = expected MW = 5,111) (Fig. 1).

The isolated HRSV-N57 peptide folds into an α-helical structure in PBS (>40% helix content) as determined by CD but does not form a monodisperse species according to sedimentation equilibrium analysis (X.Z. and P.S.K., unpublished results). The apparent T_m of HRSV-N57 varies (~40–50°C at 10 μM in PBS), presumably due to aggregation. In contrast, the isolated HRSV-C45 peptide is unfolded, and the CD signal does not show a thermal unfolding transition (X.Z. and P.S.K., unpublished results). The CD spectrum of an equimolar mixture of the HRSV-N57 and HRSV-C45 peptides shows a substantially higher α-helical signal than the weighted average of the spectra of the individual peptides, indicating a major conformational change upon association of the two peptides (Fig. 3 A). The HRSV-N57/C45 complex has an apparent T_m of 88°C at 10 μM in PBS (X.Z. and P.S.K., unpublished results). Sedimentation equilibrium centrifugation clearly indicates that in the HRSV-N57/C45 complex the peptides are present in a 3:3 ratio (Fig. 3 B). These results are in agreement with those of Lambert and colleagues (28).

**HRSV-N57/C45 can be further trimmed by protease K to generate smaller fragments denoted HRSV-N51 (residues 157–207) and HRSV-C39 (residues 482–520) (Fig. 1). Protease K is not sequence specific; therefore, its proteolytic fragments are expected to better represent the boundaries of a well-folded domain. The HRSV-N51/C39 complex has an apparent T_m of 88°C at 10 μM in PBS (X.Z. and P.S.K., unpublished results), the same as that of HRSV-N57/C45. Thus, the extra-terminal

**Table 1. X-ray data collection and refinement statistics**

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Resolution range, Å</th>
<th>20.0–2.20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed reflections</td>
<td>119,905</td>
<td></td>
</tr>
<tr>
<td>Unique reflections</td>
<td>56,973</td>
<td></td>
</tr>
<tr>
<td>Completeness, %</td>
<td>91.2 (64.7)</td>
<td></td>
</tr>
<tr>
<td>R_{free}</td>
<td>0.041 (0.269)</td>
<td></td>
</tr>
</tbody>
</table>

**Refinement**

| Resolution range, Å | 10.0–2.30 |
| Protein nonhydrogen atoms | 8073 |
| Water molecules | 888 |
| R_{cryst} | 0.233 |
| R_{free} | 0.286 |
| rms difference from ideal geometry |
| Bond lengths, Å | 0.008 |
| Bond angles, ° | 1.2 |
| Average B-factor, Å² | 45.4 |

*Values in parentheses correspond to highest resolution shell 2.28 to 2.20 Å. |
residues in HRSV-N57 and HRSV-C45 do not seem to contribute to the stability of the core complex.

Crystal Structure of HRSV-N57/C45. Crystals of HRSV-N57/C45 contain four trimers of HRSV-N57/C45 heterodimers per unit cell, giving rise to 12 independently refined heterodimers. Both the final 2Fo–Fc map and simulated annealing omit map are readily interpretable (Fig. 4A). However, some terminal residues, including 153–159, 208–209, 476–479, and 517–520, are disordered to a different degree among different chains. Although it is possible that this variation is due to the differences in local lattice contact environments, these terminal residues are more likely to be intrinsically flexible, especially in solution, as they are removed in the proteinase K-digested HRSV-N57/C45.

We focused our structural analysis on the well-defined regions, including residues 160–207 in HRSV-N57 and residues 480–516 in HRSV-C45. For these regions, the structural differences between the 12 copies of the HRSV-N57/C45 heterodimer are small (e.g., the average rms difference in Cα positions between any pair of heterodimers is 0.34 Å).

HRSV-N57/C45 forms a rod-shaped, six-helix bundle of approximately 68 Å in length and 27 Å in diameter (Fig. 4B and C). The HRSV-N57 peptides form a three-stranded coiled coil. Three HRSV-C45 peptides pack in an antiparallel manner against long, hydrophobic grooves formed on the surface of the HRSV-N57 core. This packing arrangement would put the fusion peptide segment, located immediately before HRSV-N57, and transmembrane segment, located immediately after HRSV-C45, in close proximity.

The Interface Between the HRSV-N57 Core and HRSV-C45. Approximately 3,000 Å² of solvent-accessible surface area are buried at the interface between the HRSV-N57 core and each HRSV-C45 peptide. Twenty amino acid residues from each HRSV-C45 peptide and 26 residues from two adjacent HRSV-N57 peptides contribute to the interface interaction. These residues are highly conserved among different HRSV strains, including 20 HRSV isolates and seven bovine respiratory syncytial virus isolates (analysis based on sequences available in GenBank and Swiss-Prot as of September 2000) (Fig. 5A). Most of the amino acid differences between strains are limited to conservative mutations.

Various features of the deep groove on the surface of the HRSV-N57 trimer (Fig. 5B) suggest that this groove is a poten-
temporarily attractive drug target. The carboxyl-terminal end of the groove features a hydrophobic cavity. Within the cavity region, the majority of contacts are between six cavity-lining residues from the HRs in the HRSV-N57 coiled coil. This supports the idea that the 3-4-4-4-3 pattern will be conserved among paramyxovirus F proteins because it can maintain hydrophobic residues within the coiled-coil core (21). However, because the stutter in HRSV-N57 occurs at the end of the peptide helix, structural irregularities resulting from helix end effects cannot be ruled out.

It has been suggested that the C peptides of HRSV and other paramyxoviruses will have a similar main-chain conformation as observed in the SV5 crystal structure (21). However, our results show that the HRSV C peptide has one additional helical turn not observed in the SV5 C peptide. As a consequence, the position of the C peptide residues that pack into the hydrophobic cavity is shifted in HRSV compared with SV5 (Fig. 6B), and such a packing arrangement is not readily predicted from primary sequence alignment of the two proteins.

Discussion

The structure of HRSV-N57/C45 adds to the repertoire of viral fusion proteins that have been shown to form a trimer-of-hairpins motif. The remarkable similarity between the HRSV F1 and HIV-1 gp41 core structures, as well as similar C peptide inhibition phenomena, suggest a conserved mechanism of fusion. Similar to HIV-1 gp41, HRSV F1 likely undergoes a series of conformational changes to become fusion active. The distinct conformational states proposed for HIV-1 gp41 (24), including the native state, the prehairpin intermediate, and fusion-active hairpin state, also may apply to HRSV F1. Electron microscopy studies of the full-length F protein reveal two distinct conformations, one cone-shaped and the other lollipop-shaped, which may represent the native and fusion-active states, respectively (42). The HRSV-N57/C45 structure presented here likely corresponds to the fusion-active hairpin conformation, as the hairpin formation is structurally coupled to the apposition of viral and cellular membranes.

The existence of the prehairpin intermediate conformation for the HRSV F protein is strongly supported by the observation that peptides corresponding to the HRSV HR-C regions can efficiently inhibit viral fusion (26). Presumably, these HR-C peptides function by binding to the exposed HR-N regions, thereby blocking the conformational transition to the fusion-active form.

If small, oral, bioavailable molecules that disrupt hairpin formation are identified, they may be effective drugs against viral infection. In the case of HIV-1, a strategy to block hairpin formation has been developed to find D peptide inhibitors of viral entry that bind to a hydrophobic pocket on the surface of the central coiled coil consisting of HIV-1 gp41 N peptides (39). The cavity and well-defined groove on the surface of the central coiled coil of the HRSV-N57/C45 complex identified here may serve as useful drug targets.

Finally, the HRSV-N57/C45 structure may provide a new direction in vaccine development against HRSV infection. Antibodies from vaccine trials using purified, native, full-
length F protein have very low neutralizing activities compared with those generated by live HRSV (1). One suggested strategy for eliciting neutralizing HIV-1 antibodies is to target transient intermediates or fusion-competent conformations (39, 43). The N-terminal coiled coil observed in the current structure might be formed in a prehairpin intermediate analogous to that found in HIV-1 and thus may be a viable target for antifusion antibodies. A neutralizing antibody against HRSV has been mapped to the N-terminal coiled-coil region (44); it will be interesting to see whether it targets a prehairpin intermediate.

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