A dual-functional paramyxovirus F protein regulatory switch segment: activation and membrane fusion

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Introduction

Membrane fusion is a process central to biology that requires both the precise triggering of macromolecular assemblies and the metamorphosis of individual fusion protein machines. Membrane coalescence promoted by protein hairpin formation is a fusion mechanism shared by the type I viral fusion–mediating glycoproteins (vFGps) of retroviruses, lentiviruses, filoviruses, coronaviruses, orthomyxoviruses, and paramyxoviruses (Skehel and Wiley, 2000; Eckert and Kim, 2001). Type I vFGps are homotrimeric proteins that have to be cleaved by a protease to yield a metastable complex of membrane-distal and membrane-anchored subunits (for paramyxoviruses, the F0 precursor is cleaved to F1/H11001 F2; Fig. 1). The membrane-anchored subunits contain two hydrophobic domains, the fusion peptide and the transmembrane (TM) domain, each one adjacent to one of two conserved heptad repeat (HR) regions, designated HRA and HRB, respectively. Upon triggering by low pH or by receptor/coreceptor binding, the hydrophobic fusion peptide inserts into the target membrane (Hernandez et al., 1996). The native metastable state may also be triggered artificially by destabilizing agents such as heat or urea and by mutation (Ruigrok et al., 1986; Paterson et al., 2000; Wharton et al., 2000). A transient prehairpin intermediate formed after insertion of the fusion peptide into the target membrane is susceptible to binding by peptides derived from the HRB regions of some vFGps. The HRB-derived peptides are thought to inhibit membrane fusion by preventing HRA and HRB from refolding into the fusogenic six-helix bundle (6HB) structure, which couples protein refolding directly to membrane fusion (Melikyan et al., 2000; Russell et al., 2001). In this structure, a homotrimeric coiled coil formed by HRA is buttressed by three antiparallel COOH-terminal chains (in most cases consisting of residues from an HRB region) in an antiparallel fashion (Skehel and Wiley, 2000; Eckert and Kim, 2001).

A tightly regulated mechanism of fusion activation requires stabilization of the native metastable form to sequester the fusion peptide and keep the HR regions apart until the target membrane is within range. Influenza virus HA is the only type I vFGp for which the metastable structure is known at atomic resolution. The metastable structure of HA is stabilized by multiple interactions between regions of the protein that undergo a protein-refolding event associated with membrane fusion, such as the fusion peptide and HRA (Daniels et al., 1987; Bullough et al., 1994). Although a high resolution

Abbreviations used in this paper: 6HB, six-helix bundle; CF, carboxy-fluorescein; HR, heptad repeat; TM, transmembrane; vFGp, viral fusion–mediating glycoprotein; wt, wild type.
structure for the native metastable form of HIV gp41 has not yet been obtained, mutational experiments have revealed that some HR residues that stabilize the gp41 6HB also affect the stability of the metastable gp120–gp41 complex (Follis et al., 2002; Sanders et al., 2002).

Activation of most paramyxovirus F proteins at neutral pH is thought to be triggered by a three-step process including binding of the viral receptor-binding protein (RBP) HN or H to its receptor, HN interacting with F, and the HN/F interaction, leading to changes in F that mediate membrane fusion (Crennell et al., 2000; for review see Lamb and Kolakofsky, 2001; Russell et al., 2001; Takimoto et al., 2002; Colman and Lawrence, 2003). Most paramyxovirus F proteins require coexpression of their homotypic RBP for efficient fusion activity, and mutant RBPs have been discovered that retain receptor-binding activity, but have reduced or eliminated fusion-promotion activity (for review see Lamb and Kolakofsky, 2001). However, the SV5 W3A strain F protein mediates fusion when expressed alone, albeit to a lesser extent than when coexpressed with HN (Paterson et al., 1985). The anomalous HN-independent fusion activity of SV5 W3A strain F has been mapped to P22 near the NH₂ terminus of F₂ (Ito et al., 2000; Paterson et al., 2000; Tsurudome et al., 2001), and HN-independent fusion by NDV F results from an L289A mutation (Sergel et al., 2000) that is located on the NDV F atomic structure between the fusogenic and β-barrel domains (Chen et al., 2001; Fig. 1). Moreover, the SV5 W3A strain F mutation S443P (in a region adjacent to HRB) has a hyperactive fusion phenotype (Paterson et al., 2000). The mechanisms by which these mutations lower the energy required to activate metastable F and their effects on F protein intermediates and fusogenic F are unclear.

High resolution structures have been determined for 6HBs formed by peptides derived from the HRA and HRB regions of the paramyxovirus F proteins from SV5 and HRSV F (Baker et al., 1999; Zhao et al., 2000). For SV5, the 6HB was formed by peptide N-1 containing all of HRA and part of the fusion peptide, and by peptide C-1 containing all of HRB and an extended chain region (Fig. 1). The
trimer-of-heterodimer structures of these complexes share a remarkable similarity to those of other type 1 vFGps (Skehel and Wiley, 2000; Eckert and Kim, 2001), including prominent cavities in the HRA cores to which residues adjacent to HRB such as SV5 F L447 and I449 pack tightly (Fig. 1). As predicted by the continuation of the HRA coiled coil into the fusion peptide and the proximity of the HRB helices to the TM domain (Baker et al., 1999), experiments on the arrest and capture of SV5 F intermediates are consistent with the formation of the 6HB being coupled directly to membrane merger (Russell et al., 2001). The atomic structure of NDV F has been determined from a crystal consisting of a mixture of uncleaved F, and cleaved F (cleaved at residue 139 in the fusion peptide region instead of the biological cleavage site at 117; Chen et al., 2001). Unfortunately, there is a lack of interpretable electron density for axial crystal contacts and for the fusion peptide, NH$_2$-terminal half of HRA, and HRB regions. Therefore, the roles of the HR regions in the structural transitions of F during the fusion process remain unclear (Colman and Lawrence, 2003).

To understand the role of the cavity-binding residues in 6HB formation and F-mediated fusion, we mutated SV5 F residues L447 and I449 adjacent to HRB in SV5 F. Mutation of the HRA cavity-binding residues destabilizes the 6HB structure and decreases the inhibitory potency of SV5 C-peptides in a correlative manner. These results identify in the F protein the physical target of the potent C-1 inhibitory peptide. Unexpectedly, the effects of the L447 and I449 mutations on F protein activation were even more drastic than their effects on 6HB formation. Residues 447 and 449 are shown here to be critical determinants of the energetic threshold required for activation of metastable F. In fact, mutant F proteins containing aromatic mutations at L447 and I449 cause hyperactive fusion despite having reduced 6HB stabilities, thus displaying a novel phenotype in which 6HB stability does not correlate with fusogenicity. Combined with previous data from this paper showing that residue 443 is also critical in the activation of F, the present data show that the residues comprising a linear segment adjacent to HRB (including but not limited to) residues 443, 447, and 449 regulate the stabilization and controlled activation of metastable F. Overall, the data are consistent with this linear segment having dual interaction sites in F, one in metastable F and one in the 6HB, thereby providing a key regulation of the precise switch between the metastable and fusion-active forms of the protein. We propose that other type I vFGps like HIV gp41 may have evolved similar dual-functional residues to control the precise switch between their native and fusogenic structures.

## Results

### Cavity-binding residues in C-1 stabilize the 6HB

In the 6HB structure, C-1 residues L477 and I449 pack into a prominent cavity formed by conserved N-1 residues (Fig. 1). To study the role of these residues in stabilizing the 6HB, we performed an experiment analogous to a study on HIV-1 gp41-derived peptides (Chan et al., 1998). C-1 peptides containing mutations at L447 and I449 of varying hydrophobic bulk (Gly, Ala, Val, Ile/Leu, Phe, and Trp) were incubated with N-1. Similar to the wild-type (wt) 6HB complex, all of the mutant peptide complexes formed trimers of heterodimers with high α-helical content, as determined by analytical ultracentrifugation and circular dichroism (Table I). Analysis of the thermal melts of the 6HB complexes showed that the mutations resulted in 6HB stabilities with the following rank order: Gly < Ala = Trp = Phe < Val < Ile/Leu < wt (Table I; Fig. 2, A and B). For the mutants containing aliphatic residues, 6HB stability correlated with hydrophobic bulk (Gly < Ala < Val < wt) and conservation of packing interactions (Ile/Leu < wt). The decrease in 6HB stability due to an increase in aromatic

### Table I. C-1 mutant peptides: 6HB stability and fusion inhibition

| M$_{av}$/M$_{ave}$ | [θ]$_{222}$ | T$_{m}$ | T$_{m}$ in 2 M GuHCl | IC$_{50}$ inhibition |
|-------------------|-------------|--------|---------------------|---------------------|
|                   | ($^o$C)     |        | ($^o$C)              | ($^o$C)              |
| W3A wt            | 2.9         | 30 167 | ≥98                  | 88                  | 38 ± 4          |
| L447G             | 2.7         | 28 922 | 86                   | 72                  | 150 ± 19        |
| L447A             | 2.8         | 29 328 | 88                   | 78                  | 92 ± 10         |
| L447V             | 2.8         | 29 495 | 91                   | 81                  | 83 ± 11         |
| L447I             | 2.8         | 29 820 | 94                   | 83                  | 50 ± 7          |
| L447F             | 2.9         | 29 407 | 90                   | 79                  | 100 ± 12        |
| L447W             | 3.1         | 29 381 | 89                   | 79                  | 106 ± 8         |
| I449G             | 2.9         | 25 390 | 79                   | 67                  | 271 ± 24        |
| I449A             | 2.8         | 27 384 | 87                   | 74                  | 115 ± 7         |
| I449V             | 3.0         | 28 847 | 96                   | 85                  | 58 ± 6          |
| I449L             | 2.8         | 29 291 | 97                   | 87                  | 54 ± 5          |
| I449F             | 2.9         | 27 408 | 87                   | 74                  | 151 ± 12        |
| I449W             | 2.9         | 27 362 | 85                   | 73                  | 146 ± 13        |

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* Sedimentation equilibrium data for N-1/C-1 complexes are reported as the ratio of the experimentally determined mol wt to the calculated mol wt for an N-1/C-1 heterodimer.

* Circular dichroism data for 10 µM each of N-1 and C-1 peptides in PBS, pH 7.0 (units = deg cm$^2$ dmol$^{-1}$).

* The midpoint of thermal denaturation (T$_{m}$) was estimated from the maximum of the first derivative of [θ]$_{222}$ plotted as a function of temperature.

* Thermal melts in the presence of 2.0 M GuHCl.

* C-1 mutant peptide IC$_{50}$ values for inhibition of cell–cell fusion using the T7 luciferase reporter gene assay.
sidechain bulk is also consistent with the conservation of packing interactions stabilizing the 6HB. Together, the data indicate that specific hydrophobic contacts between L447 and I449 and N-1 stabilize the SV5 F 6HB. However, the C-peptide with the most destabilizing mutation studied here, I449G, still contributed to the formation of a 6HB with a T_m of 79°C (only ~20°C lower than the wt 6HB).

C-peptide inhibitory potency is directly proportional to 6HB stability
To examine the relationship between the abilities of mutant C-peptides to stabilize the 6HB and their abilities to inhibit membrane fusion, we determined the IC_{50} values of the C-peptides and compared them to their corresponding 6HB T_m values. The L447 and I449 sidechains contribute to C-1 inhibitory potency with the rank order of IC_{50} value varying by guest residue as follows: Gly > Trp = Phe > Ala > Val > Ile/Leu > wt (Table I; Fig. 2, C and D). However, the C-peptide with the lowest inhibitory potency studied here, I449G, still had an IC_{50} value only approximately sevenfold higher than the wt C-1 peptide. The C-peptide IC_{50} values correlate with their corresponding 6HB thermal stabilities (Fig. 2 F). The apparent linear relationship between log IC_{50} and T_m indicates C-peptide inhibitory potency is directly related to C-peptide affinity for the N-1 core. The data are consistent with C-1 binding to a triple-stranded coiled-coil conformation of HRA and inhibiting membrane fusion by preventing the formation of the 6HB by the F protein.

Most of the L447 and I449 mutations are tolerated in the full-length protein
We constructed the L447 and I449 mutants in the background of the F protein and expressed these F protein mutants using both the pGEM-VacT7 and pCAGGS expression systems (see Materials and methods). The expression levels of individual F mutants did not vary between the two expression systems (Table II). The percentage of cells transfected did not differ significantly between wt and mutant F proteins (unpublished data); however, the mean fluorescence intensity values of transfected cells varied significantly (Table II). The L447F and I449 mutations caused a large reduction in F protein cell surface expression levels relative to wt, perhaps due to misfolding or a transport defect. The F protein aromatic mutants L447W, I449F, and I449W had cell surface expression levels between 50 and 75% of wt. The F protein aliphatic mutants L447G, I449A, and I449L had expression levels of 65–85% wt, and L447A, L447V, and L447I had expression levels similar to wt. Variable cell surface expression levels of the mutants requires quantitative membrane fusion assays to control for differential cell surface expression.
The F proteins containing aromatic mutations exhibit an altered conformation associated with hyperactive fusion activity

The F protein of SV5 WR strain requires HN coexpression to mediate fusion under physiological conditions (Ito et al., 2000). In contrast, the F protein of SV5 W3A strain mediates fusion in the absence of HN, albeit to a reduced extent when compared with HN coexpression (Paterson et al., 1985). Although the amino acid sequences of the two strains differ at residues 22, 443, and 516, previous work has shown that proline residues at positions 22 and 443 enhance membrane fusion and contribute to HN-independent fusion (Ito et al., 2000; Paterson et al., 2000). The WR and W3A strain F proteins have at position 22 the residues Leu and Pro, respectively, and have at position 443 the residues Pro and Ser, respectively. The conformation-specific mAb 21-1 is highly reactive to the fusion-hyperactive WR F mutant L22P, but shows little reactivity to the HN-dependent WR F unless WR F is heated to 47°C (Tsurudome et al., 2001). Although the epitope for mAb 21-1 has been mapped to the NH2-terminal half of F2 and the β-sheet domain in F1 (Tsurudome et al., 2001), the fusion-hyperactive SV5 W3A F mutant S443P (residue 443 is not in the mAb 21-1 epitope) has also increased mAb 21-1 reactivity (Table II). Thus, mAb 21-1 can be used as a sensitive probe for F protein conformational changes related to heightened fusion activation. Flow-cytometric analysis of mAb 21-1 reactivity to the W3A strain F mutants at L447 and I449 in the absence of HN coexpression showed that F proteins containing the aliphatic mutations maintain wt-like reactivity, whereas F proteins containing the aromatic mutations have increased reactivity (Table II). The results are consistent with the following W3A F mutations resulting in partial triggering of F in the absence of HN coexpression or target binding: S443P, L447F, L447W, I449F, and I449W.

The F mutants show variable dependence on HN for fusion activation

To examine the abilities of the mutant F proteins to cause cell–cell fusion, syncytium formation was examined. Representative photomicrographs of syncytium formation in BHK cells transfected with the F mutants are shown in Fig. 3 A. All of the mutants except I449G (which has a cell surface expression level <10% of wt) promoted syncytium formation when coexpressed with SV5 HN (Table III). In the presence of HN coexpression, I449A and I449V promoted syncytium formation less efficiently than wt F, whereas the remainder of the F proteins containing aliphatic mutations promoted syncytium formation similar to wt F. When coexpressed with HN, the F proteins containing aromatic mutations promoted larger syncytium formation than wt F (Fig. 3 B). Therefore, the effects of the L447 and I449 mutations on syncytium formation do not correlate with their effects on 6HB stability.

In the absence of HN coexpression, L447I and I449L were the only F proteins with aliphatic mutations that promoted syncytium formation, although to a reduced extent than wt F. In contrast, the F proteins with aromatic mutations promoted larger syncytium formation than wt in both the presence and absence of HN coexpression. The F proteins containing aromatic mutations appear to have a less stringent requirement for HN coexpression for F-mediated syncytium formation, whereas the F proteins containing aliphatic mutations are more dependent on HN coexpression. Again, the effects of the mutations on syncytium formation in the absence of HN coexpression do not correlate with their effects on 6HB stability.

To quantify the fusion phenotypes of the mutants, we used a luciferase gene-reporter assay to measure content mixing. As the extent of fusion mediated by SV5 F is dependent on cell surface expression levels and the expression levels of the mutants varied, we first measured the luciferase activities...
resulting from various amounts of wt F cell surface expression, as indicated by the lines connecting the open squares in Fig. 3 (B and C). The fusion activity of wt F as a function of expression in the presence and absence of HN coexpression is reported in Fig. 3 (B and C, respectively). In the presence of HN coexpression, the F proteins containing aliphatic mutations had fusion activities similar to wt when compared with equivalent expression levels (Fig. 3 B). However, F proteins containing aliphatic mutations were much less efficient than wt in causing fusion in the absence of HN coexpression (Fig. 3 C). In contrast, the F proteins containing aromatic mutations had fusion-hyperactive phenotypes both in the presence and absence of HN coexpression (Fig. 3, B and C; points above the lines). These results are consistent with the syncytia and mAb 21–1–binding analyses (Table II).

The F mutants are not blocked in a prehairpin conformation or in a form that only causes hemifusion. The L447 and I449 mutations in C-1 reduce 6HB stability (Table I; Fig. 2), and the available data indicate that 6HB formation is coupled directly to membrane merger (Russell et al., 2001). In principle, the reduction in fusion activity by the F proteins containing aliphatic mutations could be due to a decrease in energy released per 6HB given a fixed number of activated trimers. If this were the case, fusion might be arrested at either a hemifusion stage (characterized by the ability to promote lipid but not content mixing; Melikyan et al., 1999; Armstrong et al., 2000) or after formation of a target membrane–inserted prehairpin intermediate (characterized by the ability to retain bound RBCs independent of HN; Russell et al., 2001). To test the effects of the mutations on late steps in fusion, we performed a dye transfer assay for fusion using dual-labeled (lipidic R18 and aqueous carboxyfluorescein [CF]) RBCs. There were no significant differences between the extents of transfer of R18 lipidic dye and CF aqueous dye for any individual mutant, whether coexpressed with SV5 HN or when coexpressed with uncleaved influenza virus HA (fusion inactive) as a binding protein (Table III). Therefore, the mutants do not exhibit a hemifusion phenotype. Consistent with the results obtained from syncytia analysis and from the luciferase assay, the F proteins containing aliphatic mutations had a great reduction in dye transfer in the absence of HN coexpression.

Unexpectedly, we found the F proteins containing aromatic mutations L447F and L447W did not promote dye transfer, and that I449F and I449W promoted dye transfer inefficiently. To address the apparent anomaly of these mutants having a fusion-hyperactive phenotype in syncytium formation and the luciferase assay, but a fusion-deficient phenotype in the dye transfer assay, we performed the dye-transfer assay using CF-labeled RBC target cells and SYTO-17–labeled CV-1 effector cells. With the modified assay, both syncytium formation by CV-1 effector cells and dye transfer from RBC target cells were visualized simultaneously. Moreover, the differential labeling of CV-1 cells and RBCs also allows for analysis of F-dependent retention of target RBCs resulting from a prehairpin conformation of the F protein that is likely anchored to both cell membranes through the TM and fusion peptide regions (Russell et al., 2001). Incubation of the effector–target cell complexes at

Figure 3. Cell–cell fusion assays. (A) Representative photomicrographs of syncytia formed between BHK cells expressing the F mutants in the presence and absence of HN coexpression (20 h after transfection). Arrows point to syncytia. Bar, 200 μm. (B and C) Cell–cell fusion monitored by the luciferase reporter gene assay in the presence (B) and absence (C) of HN coexpression. The data in both panels are normalized to 100% fusion corresponding to the luciferase activity resulting from coexpression of HN and F (2 μg F DNA). F protein expression levels were determined by flow cytometry using the mAb F1a. Open boxes correspond to titrating increasing amounts of wt F DNA. Closed circles correspond to transfection of 2 μg F mutant DNA. Error bars represent triplicate experiments.
The lack of F-dependent RBC retention by mutants with inefficient dye transfer is consistent with the mutations affecting an F protein intermediate preceding prehairpin formation (Russell et al., 2001), and not due to differential binding of RBCs because all of the mutant F proteins bound similar numbers of RBCs (unpublished data). Differential levels of syncytium formation occurred between CV-1 cells expressing HN and the F mutants before incubation of effector–target cell complexes (Fig. 4). The large syncytia formed between cells coexpressing HN and the F proteins containing aromatic mutations (Fig. 4, arrows) indicates these mutations cause the F pro-

Table III. Membrane fusion efficiencies of L447 and I449 mutant F proteins

|       | Syncytia<sup>a</sup> | Syncytia<sup>b</sup> | R18 dye<sup>c</sup> | CF dye<sup>c</sup> | R18 dye<sup>d</sup> | CF dye<sup>d</sup> |
|-------|----------------------|----------------------|---------------------|--------------------|---------------------|--------------------|
|       | +HN<sup>e</sup>       | −HN<sup>e</sup>      | +HN<sup>e</sup>     | −HN<sup>e</sup>    | +HN<sup>e</sup>     | −HN<sup>e</sup>     |
| SV5 F wt<sup>f</sup> | +++                  | +++                  | 105 ± 10            | 107 ± 13           | 34 ± 4              | 34 ± 5              |
| L447G | ++                   | −                    | 65 ± 5              | 66 ± 6             | 1 ± 1               | 1 ± 1               |
| L447A | ++                   | −                    | 82 ± 7              | 80 ± 6             | 0 ± 1               | 0 ± 1               |
| L447V | +++                  | −                    | 83 ± 13             | 83 ± 10            | 3 ± 1               | 3 ± 1               |
| L447F | +++                  | +                    | 97 ± 9              | 94 ± 9             | 16 ± 3              | 17 ± 3              |
| L447W | +++                  | +++                  | 0 ± 0               | 0 ± 0              | 0 ± 0               | 0 ± 0               |
| I449G | −                    | −                    | 0 ± 0               | 0 ± 0              | 0 ± 0               | 0 ± 0               |
| I449A | +                    | −                    | 83 ± 6              | 84 ± 7             | 1 ± 1               | 1 ± 1               |
| I449V | +                    | −                    | 89 ± 8              | 88 ± 7             | 2 ± 1               | 2 ± 1               |
| I449L | +++                  | +                    | 67 ± 6              | 65 ± 4             | 2 ± 1               | 1 ± 1               |
| I449F | +++                  | +++                  | 12 ± 3              | 11 ± 3             | 5 ± 2               | 5 ± 2               |
| I449W | +++                  | +++                  | 18 ± 2              | 18 ± 3             | 3 ± 1               | 3 ± 2               |
| S443P | +++                  | +++                  | 168 ± 7             | 166 ± 6            | 139 ± 8             | 138 ± 8             |

<sup>a</sup>Synctia assays performed 20 h after transfection on monolayers of BHK 21F cells. The extents of syncytia formation were ranked as follows: ++++, noticeably greater than wt; ++, similar to wt; +, noticeably less than wt; −, no syncytia formation.

<sup>b</sup>F protein coexpressed with SV5 HN.

<sup>c</sup>F protein coexpressed with SV5 HN.

<sup>d</sup>Average number of R18 lipid dye transfer events per microscopic field from target RBCs to effector CV-1 cells expressing F mutants.

<sup>e</sup>Average number of CF aqueous dye transfer events per microscopic field from target RBCs to effector CV-1 cells expressing F mutants.

<sup>f</sup>F protein coexpressed with uncleaved influenza virus HA as an RBC-binding protein.

Figure 4. Cell–cell fusion monitored by dye transfer. Target cell erythrocytes (RBCs) were labeled with the green fluorescent dye 6-CF, and effector CV-1 cells coexpressing SV5 F mutants and HN 37°C resulted either in dye transfer or the release of unfused RBCs (due to a decrease in affinity of HN for its receptor sialic acid upon heating at 37°C; Corey et al., 2003; Fig. 4).
teins to exhibit a hyperactive fusion-promotion ability after reaching the cell surface, provided target cells are proximal. However, the lack of efficient dye transfer after 18 h incubation at 33°C before target RBC coincubation for 1 h at 4°C and 15 min incubation at 37°C is completely consistent with these F proteins containing aromatic mutations becoming inactivated in the absence of fusion targets. It should be noted that for the luciferase assay, the transiently transfected Vero cells were incubated for 18 h at 37°C before target T7-BSR cell coincubation for 8 h at 37°C, so that the combination of a long coincubation time (8 h vs. 15 min) and continuous (further) expression of the F protein mutants did not reveal the “do-or-die” phenotype of the F proteins containing aromatic mutations that was observed in the RBC dye-transfer assay. Additionally, Vero cells were specifically chosen for the luciferase assay because Vero cells do not form syncytia after 18 h of transient transfection with the F protein mutants, whereas transiently transfected BHK cells and infected/transfected CV1 cells do form syncytia 18 h after transfection. The fusion-hyperactive S443P mutant did not become inactivated under the conditions of the RBC dye-transfer assay (Table III), suggesting that the hyperactive fusion phenotype of the aromatic mutations at L447 and I449 differs from that of S443P, despite both S443P and the aromatic mutations at L447 and I449 having increased reactivity to mAb 21-1, increased syncytium formation, and increased luciferase activity.

The L447 and I449 mutations change the level of the F protein activation barrier

Previous experiments on the temperature dependence of fusion by WR strain F, W3A strain F, and the W3A mutants S443P and P22L are consistent with the F protein being activated from a metastable state, as these experiments showed that F protein residues 22 and 443 modulate fusion activity by altering the energetic threshold for fusion activation (Paterson et al., 2000). To test whether the L447 and I449 W3A strain F mutants had altered fusion efficiencies due to altered activation thresholds, we studied the temperature dependence of their dye-transfer efficiencies (Fig. 5 A). Consistent with the F proteins containing aliphatic mutations having larger activation thresholds than wt F, these mutant F proteins caused increasing extents of fusion at increasing incubation temperatures. In contrast, the two F proteins containing aromatic mutations that caused a limited amount of dye transfer, I449F and I449W, reached their maximum (albeit low) fusion extents at or below 22°C (Fig. 5 A), promoted dye transfer at a slower initial rate than wt, and reached their final extents of fusion faster than wt (Fig. 5 B). Thus, dye transfer by F proteins I449F and I449W either occurs rapidly without a high thermal requirement or not at all.

In the RBC dye-transfer assays, although the F proteins containing aromatic mutations caused massive syncytium formation between CV1 cells (Fig. 4, arrows), they caused little or no dye transfer, but when observed, it occurred at low temperature (Fig. 5 B). Therefore, we hypothesized that the aromatic mutations were not only destabilizing the F protein upon surface expression (thereby enhancing syncytia formation), but were also inactivating the F protein relatively quickly (thereby diminishing dye transfer from RBC target cells coincubated with CV-1 effector cells hours after cell surface expression of the F mutants). To prevent the suspected inactivation of the F proteins containing aromatic mutations in the absence of CF-labeled RBC target cells, we introduced these mutations into the background of a mutant (FR3) that only contains three Arg residues in the F protein cleavage/activation site, and thus is not cleaved by furin, but can be cleaved by addition of exogenous trypsin (Ward et al., 1995). Cleavage of the double mutants (FR3 mutants containing aromatic substitutions at 447 and 449) at 4°C followed by coincubation with CF-labeled RBCs at 4°C and a warm-up to 22 or 37°C resulted in extremely ef-
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Discussion

Biochemical analyses are consistent with the paramyxovirus F protein adopting the following conformations during membrane fusion: (1) the native metastable structure; (2) an activated intermediate that is susceptible to N-peptide binding; (3) a prehairpin intermediate that is susceptible to C-peptide binding; and (4) a fusogenic or post-fusogenic form that has as its core a 6HB formed by HRA and HRB (Fig. 7 A; for review see Russell et al., 2001). The importance of the HR regions in the paramyxovirus fusion mechanism is suggested by their conservation (Fig. 1) and their requirement for fusion activity (Buckland et al., 1992; McGinnes et al., 2001; Sergel et al., 2001). Additionally, peptides derived from the HR regions of paramyxovirus F inhibit fusion (for review see Colman and Lawrence, 2003), presumably by preventing 6HB formation, which appears to be coupled directly to membrane fusion (Russell et al., 2001). Here, we define a relatively localized segment upstream of HRB (residues 443, 447, and 449) in the F polypeptide chain that provides a key regulatory switch between the native and fusogenic conformations of the F protein. First, residues 447 and 449 engage a hydrophobic pocket of the HRA coiled coil in the fusogenic 6HB conformation, with mutations of these residues affecting the overall stability of the final 6HB conformation. Second, the correlated patterns of 6HB stability and C-peptide inhibition of F-mediated fusion confirm that the HRA coiled coil is the physical target of the C-peptide in the intact F protein. Third, and most importantly, residues 443, 447, and 449 profoundly influence the activation barrier of the metastable, prefusion F conformation in a manner that is not anticipated from analysis of the 6HB structure (Fig. 7 B). Therefore, residues 443, 447, and 449 must play two distinct structural and functional roles in the prefusion and 6HB conformations.

Electron microscopic analyses show that HRSV F adopts cone- and lollipop-shaped morphologies that both have "head-out" orientations in rosettes (Calder et al., 2000). The cone-shaped form most likely represents a prefusogenic F, whereas the lollipop-shaped form most likely represents fusogenic F with its 100-Å long rodlike stalk corresponding to a 6HB formed by the HR regions. The crystal structure of NDV F is thought to correspond to common structures shared by both the native and fusogenic forms of F (Chen et al., 2001). However, the lack of interpretable electron density for the fusion peptide, HRB, and the NH2-terminal half of HRA suggests these regions adopt different structures in both forms. In the fusogenic or post-fusogenic structure, HRB and residues 447 and 449 bind in the grooves of the HRA coiled coil, forming a 6HB revealed in the structure of the SV5 core complex (Baker et al., 1999).

In the native prefusion structure, we suggest that the residues adjacent to HRB (SV5 F residues 443, 447, and 449) make alternative contacts within F because they are key regulators of the stabilization and activation of native F, even in the absence of HN. The binding of these and potentially other residues in HRB within the native structure may contribute to its cone-shaped morphology (in comparison with the lollipop-shaped morphology; Calder et al., 2000). The data also suggest that the transition from the native to the fusogenic forms of F must include the formation of a prehairpin intermediate containing a triple-stranded HRA coiled coil, which is accessible for C-peptide binding, but not for HRB binding (Fig. 7 A). Conserved packing interactions between the cavity-binding residues 447 and 449 and the HRA hydrophobic cavity are critical in stabilizing
the 6HB structure. All of the mutations of cavity-binding residues studied here decrease 6HB stability. Moreover, the paramyxovirus F proteins generally compensate for non-identical residues in the HRA cavity with complementary changes in HRB cavity–binding residues (Zhao et al., 2000). The 6HB structures from filoviral, retroviral, and lentiviral vFGps such as Ebola virus GP2, HIV gp41, SIV gp41, MoMLV TM, HTVL-1 gp21, and Visna virus TM also reveal prominent hydrophobic HRA cavities that may be suitable drug targets (for review see Eckert and Kim, 2001). The importance of the cavity-binding residues in both the native and fusogenic structures of paramyxovirus F suggests that the evolution of drug-resistant strains against such inhibitors would occur infrequently.

Unexpectedly, the effects of the mutations on fusion activation appear to be greater than their effects on 6HB formation. The F proteins containing aromatic mutations form less stable 6HBs than wt F (Fig. 2), thereby coupling less energy to fusion per 6HB. The hyperactive phenotype of the F proteins containing aromatic mutations is consistent with these mutations destabilizing the F protein native metastable state so that more trimers become activated (Fig. 7 B). In contrast, it seems most likely that the F proteins containing aliphatic mutations have both higher energetic requirements for activation and lower amounts of energy per 6HB to couple to fusion (Fig. 7 B). For the F proteins containing the aliphatic mutations, the decrease in fusion activity is most likely due to the combination of decreases in the number of activated trimers and the amount of energy released per 6HB. The fusion deficiencies of the aliphatic mutants cannot be due to their reduced 6HB stabilities alone because the aliphatic mutants were restricted at fusion activation and not at prehairpin or hemifusion intermediate formation.

To avoid inactivation, the native metastable structures of the type I vFGps must prevent indiscriminate release of the fusion peptide and HR regions until the target membranes are within range. A general strategy for a tightly regulated fusion mechanism may include sequences within or near HR regions having dual structural roles; one in the native structure to regulate fusion activation and another in the final structure to determine hairpin stability. The linear segment from 443 to 449, adjacent to HRB, in the paramyxovirus F protein has been shown here to be critical in stabilizing both the native and fusogenic forms of the protein. Moreover, mutational analyses on HIV gp41–mediated fusion have identified individual HRA and HRB residues critical in the stabilization of both the native gp120–gp41 complex and the 6HB structure (Lu et al., 2001; Follis et al., 2002; Sanders et al., 2002). The strongest evidence for HR residues with dual, independent interaction sites comes from structural and genetic experiments on influenza virus HA. Numerous HRA residues in HA2 (in segments A and C) bind to other HRA residues in the native structure, but interact with the antiparallel buttressing strand (equivalent to a HRB region) in the

Figure 7. Model of the paramyxovirus fusion mechanism. (A) Previous reports have shown the paramyxovirus F protein adopts a series of conformations while mediating membrane fusion (for review see Russell et al., 2001); the native structure (which is in a metastable conformation), a temperature-arrested intermediate (which forms after HN binds its receptor at nonfusion-permissive temperatures and is susceptible to N-1 binding), a prehairpin intermediate (which adheres to target cells independent of HN and is susceptible to C-1 binding), and the fusogenic form of F (which couples 6HB formation to membrane merger). The present work is consistent with residues 447 and 449 having distinct interaction sites on the F protein, one in the native structure, and the other in the cavity formed by HRA trimers in the 6HB of the fusogenic form. The strong correlation between 6HB stability and C-peptide inhibitory potency in the present work and the coincidence of C-1 inhibition and F protein binding in a previous work (Russell et al., 2001) show that C-1 inhibits membrane fusion by binding to transiently exposed HRA triple-stranded coiled coils in the prehairpin intermediate. (B) Biophysical experiments on the N-1/C-peptide mutants show that the aliphatic (al) and aromatic (ar) mutations at L447 and I449 decrease the amount of energy released by mutant 6HB formation, whereas functional experiments on the F mutants are consistent with the aromatic (ar) mutations decreasing and the aliphatic (al) mutations increasing the activation energy of the native state. The hyperactive fusion phenotype of the aromatic mutants is consistent with the facile activation (and subsequent inactivation in the absence of a fusion target) of these F mutants overcompensating for the decrease in energy released by mutant 6HB formation.
fusogenic structure (Daniels et al., 1987; Bullough et al., 1994; Chen et al., 1999). Other residues identified as critical in the switch between the native and fusogenic structures of HA2 include those in and around the fusion peptide and those involved in interactions between HA2 and HA1 (Bullough et al., 1994), showing regions other than the HR regions may be critical in stabilizing the native states of other type I vFGps. Further structural and biochemical analyses on the native metastable forms of these proteins will be instrumental in better understanding the general principles governing type I fusion activation and its inhibition.

Materials and methods

Peptides, cells, and plasmids

The N-1 (derived from HRA) and C-peptides (derived from HRB) were expressed and purified as described previously (Joshi et al., 1998). Monolayer cultures of Vero, BHK, T7-BSR, and CV-1 cells were grown as described previously (Paterson et al., 2000). pGEM and pCAGGS plasmids encoding SV5 W3A strain F, the SV5 F3 mutant, SV5 HN, and influenza virus A/Amur/72 HA have been described previously (Paterson et al., 2000). pGE4XAT C-1 has been described previously (Joshi et al., 1998). Mutant F proteins and C-peptides were constructed by subcloning four primer PCR products into pGEM2X SV5 F, pGEM2X SV5 F3, pCAGGS SV5 F, and pGE4XAT C-1.

Biophysical characterization of peptide 6HBs

Appropriate volumes of 6HBs were formed by mixing peptide stock solutions to yield a final concentration of 10 μM of each peptide in PBS (50 mM phosphate buffer, pH 7.0, and 150 mM NaCl) and dialyzing the mixtures twice against 1 PBS. Sedimentation equilibria were measured at 20°C after a 24-h centrifugation at 15,000, 20,000, and 25,000 rpm using an analytical ultracentrifuge (model XL-A; Beckman Coulter). The apparent mol wts of the complexes were determined by fitting to a single-ideal species model using Igor Pro (WaveMetrics). Circular dichroism experiments on 6HB solutions (10 μM of each peptide in PBS) were performed using a spectropolarimeter (model J-715; Jasco) as described previously (Joshi et al., 1998).

Expression of viral envelope glycoproteins

Viral envelope glycoproteins were expressed using two different expression systems: (a) Vero or BHK cells transfected with pCAGGS DNA and (b) CV-1 cells infected with the recombinant vaccinia virus vTF7-3 and transfected with pGEM DNA. Vero and BHK cells were transfected using the Li-cove ler (Promega), lysed by freeze-thawing, and clarified by centrifugation at 14,000 rpm in an Eppendorf microcentrifuge. From each clarified lystate, 40 μl solution was pipetted into a 96-well plate. The luciferase activity resulting from fusion of the two-cell populations was quantified using Luciferase Assay Substrate (Promega) and an Lmax luminescence microplate reader (Molecular Devices). For peptide inhibition experiments, BSR cells were aliquoted into Eppendorf tubes containing twofold serial dilutions of C-peptides before being overlaid onto the Vero cells. The IC50 values of the peptides were estimated by fitting the experimental data to the equation y = 100 − 100/(1 + [x/a]^k + [y/a]^k), where y is average percentage of luciferase activity (from triplicate samples), x is the bulk concentration of peptide, k is a scaling constant, and a is the IC50 value.

Lipidic and aqueous dye transfer

Human erythrocytes (RBCs) were single labeled with aqueous 6-CF (Molecular Probes, Inc.) or dual labeled with CF and the lipid probe octadecyl rhodamine B chloride (R18; Molecular Probes). CV-1 cells grown on glass coverslides were infected with vTF7-3 and transfected with pGEM DNA for 4 h at 37°C, followed by 18 h at 33°C. To allow binding of the dye-labeled RBCs but to prevent temperature-induced fusion, 1 ml ice-cold 0.1% hemocrit dye-labeled RBCs was added to each sample and the CV-1/RBC (effector-target) complexes were incubated on ice at 4°C for 1 h. The effector-target cell complexes were incubated at 37°C for 15 min to allow dye transfer to take place, before washing five times with ice-cold PBS+ to remove unbound RBCs. Dye transfer was assessed by scanning confocal microscopy (model LSM 410; Carl Zeiss MicroImaging, Inc.) and quantifying CF or R18 dye transfer from labeled RBCs to CV-1 cells. To study syncytium formation and HN-independent retention of RBCs, CV-1 effector cells were labeled with 1 μM SYTO-17 nucleic acid dye (Molecular Probes, Inc.) at 37°C for 30 min, and single-labeled CF-RBCs were used as fusion targets. For the temperature dependence of dye transfer, effector-target complexes were incubated for 5 min at the reported temperatures before washing with ice-cold PBS+. For the kinetics of dye transfer, effector-target complexes were incubated at 37°C for the reported times before washing with ice-cold PBS+.

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