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Role of the Simian Virus 5 Fusion Protein N-Terminal Coiled-Coil Domain in Folding and Promotion of Membrane Fusion

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Formation of a six-helix bundle comprised of three C-terminal heptad repeat regions in antiparallel orientation in the grooves of an N-terminal coiled-coil is critical for promotion of membrane fusion by paramyxovirus fusion (F) proteins. We have examined the effect of mutations in four residues of the N-terminal heptad repeat in the simian virus 5 (SV5) F protein on protein folding, transport, and fusogenic activity. The residues chosen have previously been shown from study of isolated peptides to have differing effects on stability of the N-terminal coiled-coil and six-helix bundle (R. E. Dutch, G. P. Leser, and R. A. Lamb, Virology 254:147–159, 1999). The mutant V154M showed reduced proteolytic cleavage and surface expression, indicating a defect in intracellular transport, though this mutation had no effect when studied in isolated peptides. The mutation I137M, previously shown to lower thermostability of the six-helix bundle, resulted in an F protein which was properly processed and transported to the cell surface but which had reduced fusogenic activity. Finally, mutations at L140M and L161M, previously shown to disrupt α-helix formation of isolated N-1 peptides but not to affect six-helix bundle formation, resulted in F proteins that were properly processed. Interestingly, the L161M mutant showed increased syncytium formation and promoted fusion at lower temperatures than the wild-type F protein. These results indicate that interactions separate from formation of an N-terminal coiled-coil or six-helix bundle are important in the initial folding and transport of the SV5 F protein and that mutations that destabilize the N-terminal coiled-coil can result in stimulation of membrane fusion.

Paramyxoviruses contain two major glycoproteins, both of which are essential for attachment and entry into target cells. The attachment protein (termed HN, H, or G) mediates primary attachment, while the fusion (F) protein promotes membrane fusion between the viral membrane and the membrane of the target cell (24). Paramyxovirus F proteins are type I membrane proteins which form homotrimers during initial protein folding (48). F proteins also undergo both N-linked glycosylation and proteolytic cleavage of the precursor protein, F_0, to the fusogenically active form, which consists of disulfide-linked F_1 and F_2 subunits (16, 49). The membrane-anchored F_1 subunit contains several regions important for promotion of membrane fusion. The fusion peptide, present at the F_1 subunit N terminus, has been demonstrated to insert into the target membrane upon initiation of membrane fusion (1). Also present in the F_1 subunit are two heptad repeat regions, one immediately C-terminal to the fusion peptide (HRA) and one adjacent to the transmembrane domain (HRB) (reviewed in reference 10).

Study of peptides corresponding to the F protein heptad repeats has provided important information concerning potential structures formed by these regions. Peptides corresponding to the HRA region from paramyxovirus F proteins, such as the simian virus 5 (SV5) F protein, the respiratory syncytial virus F protein, and the Newcastle disease (NDV) F protein, have been demonstrated to form trimeric α-helical structures termed coiled-coils (20, 27, 58). For each of these paramyxovirus F proteins, peptides corresponding to the HRB region did not have significant secondary structure in isolation. However, coincubation of HRA and HRB peptides corresponding to regions from paramyxovirus F proteins resulted in formation of a stable complex. Biochemical and crystallographic analyses demonstrated that this complex is a thermostable six-helix bundle consisting of a coiled-coil of three HRA peptides flanked in antiparallel orientation by three HRB peptides (3, 20, 27, 59). Similar six-helix bundle structures have been reported for a number of viral fusion proteins, including the human immunodeficiency virus (HIV) Env protein and the low-pH form of the influenza virus hemagglutinin protein (reviewed in reference 10). Interestingly, two mutations in SV5 F protein HRA peptides (L140M and L161M) have been shown to disrupt α-helical structure of the isolated HRA peptides without interfering with formation of a thermostable six-helix bundle, suggesting that the HRA/HRB complex can form in the absence of a stable HRA coiled-coil (13).

Research on a number of viral fusion proteins points to a critical role for six-helix bundle formation in promotion of membrane fusion. Mutations in the heptad repeat regions frequently lead to fusion defects (5, 7, 8, 25, 28, 44, 50, 51, 55, 56). Addition of peptides corresponding to the heptad repeat sequences has also been shown to inhibit fusion for many viral fusion proteins, including the paramyxovirus F and the HIV Env proteins (reviewed in reference 10). Considerable experimental evidence supports the model that, at least in the case of paramyxovirus F proteins and the HIV Env protein, formation of the six-helix bundle occurs during or after membrane merger and provides an energetic driving force for the membrane fusion event (3, 29).

The role of the heptad repeat regions in prefusogenic con-

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formations of the F protein is less clear. It has been suggested that formation of an N-terminal coiled-coil by the HRA region could be critical for trimerization of type I viral fusion proteins during initial protein folding in the endoplasmic reticulum (ER) (42). However, for the HIV Env protein, single point mutations in the HRA region generally give proteins which fold properly but are frequently fusion defective (7, 8, 25, 41, 55, 56). Mutations in the HRA region of the NDV F protein have been found to have effects both on initial protein folding and on promotion of membrane fusion (50, 51). However, to date no studies of paramyxovirus F proteins have compared the effects of mutations in the HRA region in peptide systems, where coiled-coil formation and six-helix bundle stability can be measured, to the effect of these same mutations on the folding, processing, and membrane fusion promotion of the entire F protein.

To investigate the role(s) of the N-terminal coiled-coil and six-helix bundle in protein folding, processing, and promotion of membrane fusion by paramyxovirus F proteins, we constructed four mutant F proteins containing substitutions in the N-terminal heptad repeat whose effects had been previously characterized in a peptide system (13). Protein folding and processing were only affected by a mutation which does not affect the α-helicity of isolated N-peptides or the stability of the six-helix bundle, indicating that interactions separate from formation of an N-terminal coiled-coil or six-helix bundle are important for initial protein folding. In addition, our results demonstrate that a mutation known to effect thermostability of the six-helix bundle leads to decreased fusogenic activity, confirming the important role this element plays in promotion of membrane fusion. Surprisingly, our results show that two mutations previously shown to affect α-helicity of isolated N-peptides without compromising thermostability of the six-helix bundle give properly folded proteins that are fully functional in fusion promotion. Indeed, the mutant SV5 F L161M gave increased syncytium formation and promoted fusion at lower temperatures than the wild-type (wt) SV5 F protein, indicating that some mutations which destabilize the N-terminal coiled-coil can stimulate membrane fusion promotion.

MATERIALS AND METHODS

Cells and viruses. BHK 21F, Vero, HeLa T4, and CV-1 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. The recombinant vaccinia virus vTF7-3, expressing the T7 polymerase, was grown in CV-1 cells as described previously (14).

Plasmids. The plasmids pGEM2X-SV5 F (35, 36), pCAGGS-SV5 F (33, 39), pGEM3X-SV5 HN, pCAGGS-SV5 HN, and pCAGGS-T7 (39) were kindly provided by Robert Lamb (Howard Hughes Medical Institute, Northwestern University, Evanston, Ill.). Site-directed mutagenesis was performed on the pGEM2X-SV5F plasmid using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, Calif.), and the F gene of all mutants was sequenced in its entirety to ensure that there were no secondary mutations. F genes containing the mutations were subcloned into pCAGGS as previously described (39). The plasmid pIntT7βgal was kindly provided by Edward Berger and Bernard Moss (National Institutes of Health, Bethesda, Md.).

Pulse-chase analysis of wt F protein and F protein mutants. Duplicate samples of subconfluent HeLa T4 cells were transfected with pCAGGS-SV5 F wt or mutant constructs through use of Lipofectamine Plus reagents according to the manufacturer's specifications (Invitrogen Life Technologies, Carlsbad, Calif.). Following overnight incubation, cells were starved in cysteine- and methionine-deficient DMEM for 30 min and subsequently metabolically labeled with Tran^{35}S-label (100 μCi/ml; MP Biomedicals, Inc., Irvine, Calif.) for 30 min. Cells were then washed twice in phosphate-buffered saline (PBS) and either lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors (38) and 20 mM iodoacetamide or incubated in fresh DMEM plus 10% fetal bovine serum (FBS) for 3 h at 37°C prior to lysis. Immunoprecipitations were performed as described previously (23) using the antibody SV5 F 82–96 (9), which recognizes the F_2 domain of the SV5 F protein. Samples were separated on a 15% polyacrylamide gel to allow visualization of both the F_1 and F_2 subunits and analyzed by storage phosphor autoradiography using a STORM imaging system (Amersham, Piscataway, N.J.).

Flow cytometry. SV5 F wt or mutant proteins were expressed using the pCAGGS system in HeLa T4 cells as described above. After overnight (ON) incubation at 37°C, cells were prepared for flow cytometry as described previously (17) with the primary monoclonal antibody F1a (43) and the secondary antibody fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, Pa.). Fluorescence intensity of 10,000 cells was measured by flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, Calif.).

Chemical cross-linking. SV5 F wt and mutant proteins were expressed in HeLa T4 cells from pCAGGS constructs by use of Lipofectamine Plus reagents. Following ON incubation, cells were starved and labeled for 30 min with Tran^{35}S-label (100 μCi/ml), and samples were then chased in fresh DMEM plus 10% FBS for 30 min at 37°C. Chemical cross-linking using the reagent 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP; Pierce Biotechnology Inc., Rockford, Ill.) was performed in the presence of 1% NP-40 as described previously (48). Samples were immunoprecipitated as described above and resolved under nonreducing conditions on a 3.5% acrylamide gel.

Syncytium assay for fusion. The F protein, F protein mutants, and the SV5 hemagglutinin-neuraminidase (HN) protein were expressed using the pCAGGS system in Vero or BHK 21F cells as described above. Syncytia were examined 48 h (Vero) or 24 h (BHK) posttransfection using a Nikon TS100 inverted phase-contrast microscope (Nikon Inc., Garden City, N.Y.) and pictures taken using a Nikon Coolpix995 digital camera.

Reporter gene assay for content mixing. The assay for cytoplasmic content mixing, as measured by activation of the reporter gene β-galactosidase, was a modification of previously published protocols (2, 11, 15, 39). Vero cells coexpressing the SV5 F wt protein or mutant F proteins and the SV5 HN protein via the pCAGGS system and containing the plasmid pGINT7βgal were incubated ON at 37°C in fresh DMEM plus 10% FBS. Cells were then overlaid with Vero cells containing pCAGGS-T7, and the two populations of cells were incubated ON at 37°C. Cells were then washed in PBS and lysed by addition of 10% NP-40 in 0.25 M Tris (pH 8.0). Lysates were then mixed with equal volumes of chlorophenol red-β-D-galactopyranoside (Roche Diagnostics, Indianapolis, Ind.) substrate and incubated at 37°C until color developed in the reaction mixture. Cell fusion was measured by reading light absorbance at 590 nm for each sample.

Analysis of lipid mixing by fluorescence microscopy. For analysis of lipid mixing by fluorescence microscopy, the SV5 F protein or F protein mutants were coexpressed with the SV5 HN protein using the recombinant vaccinia virus-T7 RNA polymerase transient expression system (14). Monolayer cultures of CV-1 cells in 35-mm dishes were infected with recombinant vTF7-3 at 10 PFU per cell and incubated for 1 h at 37°C. The cells were then transfected with 2.5 μg of pGEM-SV5 F and 1.25 μg of pGEM-SV5 HN by using cationic liposomes prepared as described previously (45). At 5 h posttransfection, cells were washed and incubated overnight at 33°C in DMEM supplemented with 10% FBS. Cells were subsequently washed twice with PBS and incubated for 1 h at 37°C with 50 mU of neuraminidase (*Clostridium perfringens* type V; Sigma Chemical Co., St. Louis, Mo.) per ml. Freshly drawn human erythrocytes (RBCs) were labeled with the lipid probe octadecyl rhodamine B (R18; Molecular Probes, Eugene, Oreg.) as described previously (2, 30). Following two washes with PBS, cells were incubated with 1 ml of R18-labeled RBCs for 1 h at 4°C, and unbound RBCs were removed by multiple washes with ice-cold PBS. Fusion was initiated by addition of PBS prewarmed to the desired temperature, and cells were incubated for 10 min in a CO_2 incubator set to the appropriate temperature. Fusion was terminated by addition of ice-cold PBS followed by transfer of cells to ice. Dye transfer was examined using a Zeiss Axiovert 200 microscope (Zeiss Instruments Inc., Thornwood, N.Y.).

RESULTS

Expression and folding of F protein mutants. Upon formation of a coiled-coil or six-helix bundle, the first (a) and fourth (d) residue of each set of seven in the HRA region face into the interior of the HRA coiled-coil (3) (Fig. 1B). Four methionine substitutions in a or d residues of the SV5 F protein HRA

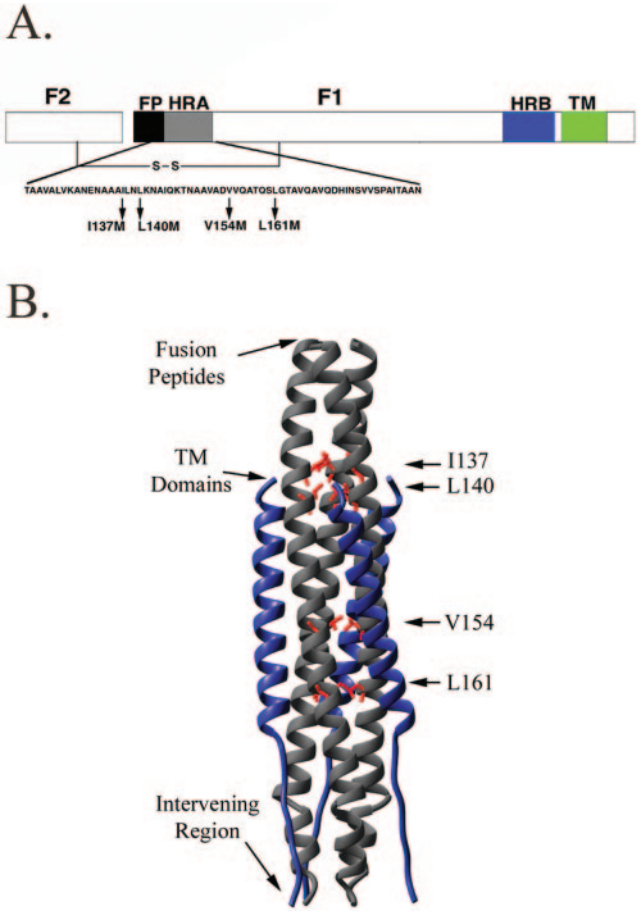

FIG. 1. (A) Schematic representation of the SV5 F protein showing the mutations analyzed in this study. The fusion peptide (FP [black]), HRA (grey), HRB (blue), and putative transmembrane domain (TM [green]) are shown. The sequence of HRA and the mutations made are indicated. (B) Positions of the four mutations in the six-helix bundle of SV5 (3). The side chains for the four residues mutated in this study are shown in red, the three helices from the HRA region are colored grey, and the three helices from the HRB region are colored blue.

region were previously studied in peptide systems, with methionine utilized due to the potential use of these peptides in crystallographic analysis (13). Interestingly, these four mutations were found to have differing effects on the structure of isolated N-peptides containing these mutations, or on the thermostability of the six-helix bundle formed from incubation of the mutant N-terminal peptides with peptides corresponding to the C-terminal heptad repeat (13) (summarized in Table 1). Introduction of a methionine at position 137 resulted in N-terminal peptides which were still α-helical in isolation, but the six-helix bundle formed with this mutation showed a biphasic melt as judged by loss of a 222-nm circular dichroism signal, indicating that the bundle was destabilized by introduction of this mutation. Substitution of either residue 140 or residue 161 resulted in peptides which were no longer α-helical in isolation but which could still form a thermostable six-helix bundle when incubated with C-terminal heptad repeat peptides. Finally, mutation of residue 154 to methionine resulted in N-terminal peptides which, like peptides with wt sequence, were α-helical in isolation and formed a thermostable six-helix bundle. As the effect of these four point mutations on the key elements of N-terminal coiled-coil formation and six-helix bundle stability were drastically different, examination of these mutations within the context of the entire SV5 F protein was undertaken.

Cell surface expression analyzed by flow cytometry. To study the effect of these mutations in the context of the entire SV5 F protein, site-directed mutagenesis was employed to create the mutants SV5 F I137M, SV5 F L140M, SV5 F V154M, and SV5 F L161M (Fig. 1A). The positions of these mutated residues within the six-helix bundle are also shown (Fig. 1B). To examine the effect of these mutations on surface expression, the wt and mutant SV5 F proteins were expressed in HeLa T4 cells using the pCAGGS expression vector system (33) and surface expression was monitored by flow cytometry using the F1a monoclonal antibody (43). The mutants SV5 F I137M, L140M, and L161M showed both similar percentages of transfected cells and mean fluorescent intensities compared to the wt SV5 F protein, indicating that these mutations did not affect surface expression (Table 1). However, mutant SV5 F V154M gave a mean fluorescent intensity that was only one-third of that seen with the wt SV5 F protein, indicating that this mutation severely decreased surface expression. Similar results were seen when the wt and mutant proteins were expressed using the recombinant vaccinia virus-T7 RNA polymerase system in CV-1 cells, with ON incubation at 33°C. This indicates that the defect seen for mutant V154M is observed in several expression systems and cell types and that ON incubation at a lowered temperature does not rescue surface expression (data not shown).

Proteolytic cleavage determined by pulse-chase analysis. The SV5 F protein undergoes proteolytic cleavage by the host cell protease furin in the *trans*-Golgi network (21). To determine whether the introduced mutations affected either transport to the *trans*-Golgi network or proteolytic cleavage by furin, HeLa T4 cells expressing the wt SV5 F protein or the F protein mutants via the pCAGGS expression system were labeled with Tran^{35}S-label for 30 min, and then the cells were either lysed immediately or were incubated in chase medium for 3 h to permit transport through the secretory pathway and proteolytic cleavage. Proteins were immunoprecipitated and polypeptides were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized using the STORM imaging system (Fig. 2). The wt SV5 F protein and all four mutants

TABLE 1. Summary of effect of heptad repeat mutations on structure of peptides and on surface expression levels when incorporated in full-length SV5 F protein

Mutation	Position in heptad repeat	N-terminal peptides[a]	Melting point of 6-helix bundle[a] (°C)	% Total transfected[b]	MFI[c]
wt F		α-Helical	>90	53.8	212.4
I137M	a	α-Helical	Biphasic	51.4	201.2
L140M	d	Nonhelical	>90	53.7	220.6
V154M	d	α-Helical	>90	38.8	69.8
L161M	d	Nonhelical	>90	47.1	204.9

[a] Results published by Dutch et al.(13). α-Helicity was judged by circular dichroism at 208 and 222 nm; melting point was determinined by loss of 222-nm signal.

[b] Total percent cells positive for F proteins, determined by flow cytometry as described in Materials and Methods.

[c] MFI, mean fluorescent intensity, determined by flow cytometry.

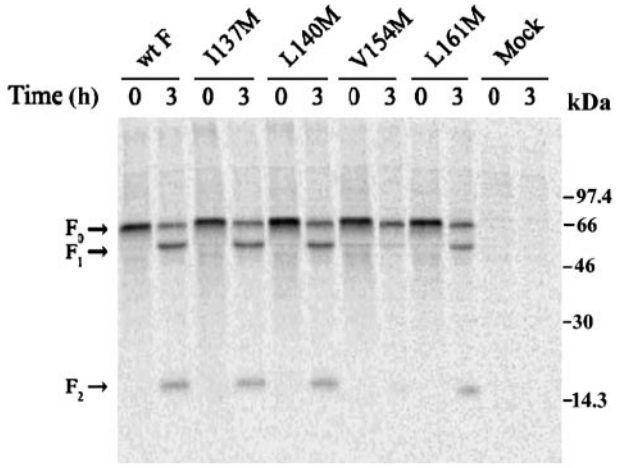

FIG. 2. Expression and proteolytic cleavage of the wt and mutant SV5 F proteins. HeLa cells were transfected with plasmids encoding the wt F or mutant F proteins as described in Materials and Methods. Following ON incubation, transfected cells were metabolically labeled with Tran^{35}S-label (100 μCi/ml) for 30 min and incubated in chase medium for 0 or 3 h. Polypeptides were immunoprecipitated, run on a 15% polyacrylamide gel, and analyzed on a STORM PhosphorImager system. The positions of the uncleaved F_0 and the cleaved subunits F_1 and F_2 are indicated.

showed equivalent amounts of the F_0 precursor protein at the zero-hour time point. After a 3-h chase, proteolytic cleavage to the F_1 and F_2 subunits was seen for the wt F protein and for the mutants I137M, L140M, and L161M. However, for the mutant SV5 F V154M, only very faint bands were seen at the positions corresponding to the F_1 and F_2 subunits, indicating that proteolytic processing was severely impaired by the mutation. Similar results were again seen when expression was performed at 33°C or when the recombinant vaccinia-T7 polymerase system was used (data not shown). These results, together with the low surface density, suggest that this mutation leads to a protein folding defect that markedly decreases transport through the secretory pathway and that this folding defect cannot be corrected by expression at lowered temperature.

Analysis of oligomeric structure by chemical cross-linking. Protein folding of the SV5 F protein within the ER is accompanied by trimerization (48). To ascertain whether the introduced mutations, especially the 154M mutation, resulted in oligomerization defects, the wt F protein and F protein mutants were expressed in HeLa T4 cells by using the pCAGGS expression system. Following a 30-min label with Tran^{35}S-label and a 30-min incubation in chase medium, cells were subjected to chemical cross-linking with the reagent DTSSP in the presence of 1% NP-40. Samples were immunoprecipitated and subsequently analyzed on a 3.5% acrylamide gel (Fig. 3). A shift of the majority of expressed protein to the trimeric species upon addition of cross-linker was seen for the wt SV5 F protein and the mutants I137M, L140M, and L161M, with the presence of dimeric species also seen due to incomplete cross-linking. A slight increase in the trimeric form was also seen for the mutant V154M when cross-linker was added, suggesting that a small population of this mutant protein undergoes folding and trimerization. However, the percentage of trimer was low compared to the other tested proteins, indicating that much of the population of the mutant SV5 F V154M fails to undergo proper folding and trimerization. These results indicate that mutations which affect stability of either the N-terminal coiled-coil (L140M and L161M) or the six-helix bundle (I137M) do not interfere with initial protein folding and transport through the secretory pathway, suggesting that these elements are not critical for these processes. However, the folding defect seen with SV5 F V154M, which incorporates a mutation that has no effect on stability of the N-terminal coiled-coil or the six-helix bundle, strongly indicates that interactions separate from formation of these two structures are critical during initial protein folding.

Analysis of membrane fusion activity. Formation of a six-helix bundle has clearly been demonstrated to be critical for promotion of fusion by the SV5 F protein (20, 46, 47). However, little is known about the role of the N-terminal coiled-coil prior to bundle formation. Therefore, three different types of membrane fusion assays were conducted to analyze the effect of the four heptad repeat mutations on the fusogenic activity of the SV5 F protein.

Syncytium formation. First, the extent of syncytium formation was determined, as this has previously been demonstrated to occur upon expression of the SV5 F protein from cDNA (18, 37). Vero cells were transfected with pCAGGS expressing the SV5 HN protein and either the wt SV5 F protein or an F protein mutant. Syncytium formation was analyzed 48 h post-transfection (Fig. 4A). Multinucleated giant cells were observed when wt SV5 F was present. The mutant SV5 F I137M, containing a mutation that destabilized the six-helix bundle,

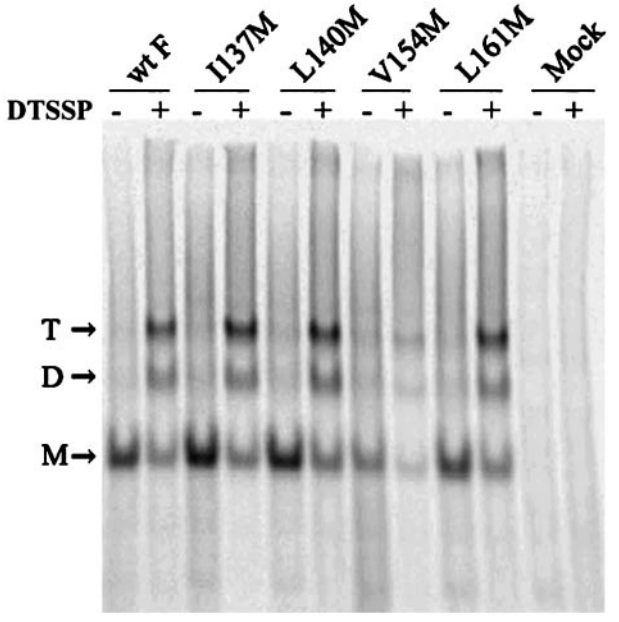

FIG. 3. Oligomerization of the wt and mutant F proteins. HeLa cells expressing the wt and HRA mutant F proteins were metabolically labeled with Tran^{35}S-label (100 μCi/ml) for 30 min and incubated in chase medium for 30 min. Cross-linking reactions were performed on cell suspensions in the presence of 1% NP-40 and the cross-linking reagent DTSSP as described in Materials and Methods. Following immunoprecipitation, F protein species were analyzed on a 3.5% gel in the absence of reducing agent. The positions of monomer (M), dimer (D), and trimer (T) are indicated.

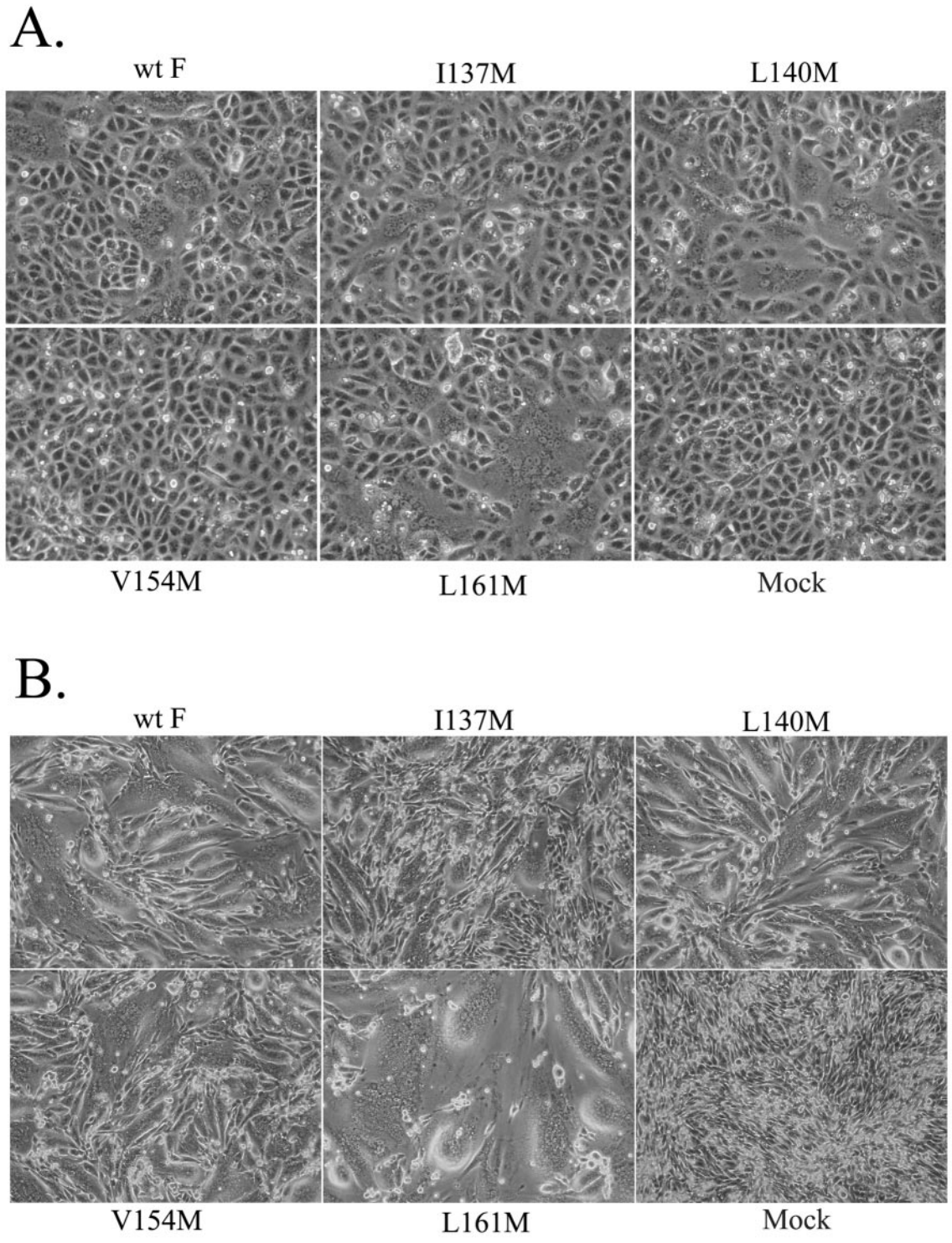

FIG. 4. Syncytium assay. Monolayers of Vero (A) and BHK 21F (B) cells were transfected with plasmids encoding the SV5 HN protein and either the wt F protein or the HRA mutants. At 48 h (for Vero) or 18 h (for BHK) posttransfection, monolayers were examined for the presence of syncytia. Representative photomicrographs are shown.

showed greatly reduced syncytium formation, suggesting a fusion defect. No syncytium formation was observed in Vero cells for the mutant SV5 F V154M, consistent with our finding that the majority of this protein is not properly folded and transported (Table 1; Fig. 2 and 3). Finally, the two mutants incorporating changes known to affect α-helicity of N-terminal heptad repeat peptides (L140M and L161M) were both capable of promoting syncytium formation, though at different levels. SV5 F L140M gave similar-sized syncytia as wt F, while SV5 F L161M showed an increase in syncytium formation. Syncytium

formation was also studied in BHK 21F cells, which have been previously shown to be highly fusogenic in this assay (12, 39). Twenty-four hours posttransfection, formation of large multinucleated giant cells was observed in samples with the wt F protein and the mutant L140M, with decreased levels of syncytia observed for the mutant I137 (Fig. 4B). The mutation L161M again gave considerably higher levels of syncytium formation compared to the wt F protein. Finally, though only a small percentage of the mutant SV5 F V154M folded properly and was present on the cell surface (Table 1; Fig. 2 and 3), this surface population was able to promote syncytium formation in BHK 21F cells, though at lower levels than seen with the wt F protein. This indicates that the small population of this mutant that folds and reaches the cell surface is competent to promote membrane fusion in this highly fusogenic cell line.

Reporter gene assay for membrane fusion. To more accurately quantitate promotion of membrane fusion, the SV5 F protein and heptad repeat mutants were analyzed in a reporter gene assay. Vero cells expressing the SV5 F protein or F protein mutants along with the SV5 HN protein and containing a plasmid with the β-galactosidase gene under the control of a T7 polymerase promoter were overlaid with Vero cells expressing the T7 polymerase. Twenty-four hours after overlay, cells were lysed and the lysates were analyzed for β-galactosidase activity (Fig. 5). Mutations I137M and V154M both resulted in severely debilitated membrane fusion promotion, consistent with the reductions in syncytium formation observed earlier (Fig. 4). Mutant SV5 F L140M gave fusion that was approximately 50% of that seen with the wt F protein, while the mutant SV5 F L161M gave fusion levels equivalent to those seen with the wt SV5 F protein. Reporter gene assays utilizing BHK 21F cells were not performed due to the high background fusion levels in this cell type.

Fluorescence microscopy assay for lipid mixing. To examine the early steps of membrane fusion and the effect of temperature on the reaction, CV-1 cells were transfected with plasmids expressing the SV5 F protein or F protein mutants along with the SV5 HN protein via the vaccinia virus-T7 RNA polymerase system. Following ON incubation at 33°C, transfected cells were incubated at 4°C with human RBCs (previously labeled with the lipid probe R18) to allow binding of the RBCs to the HN protein. The temperature was subsequently raised to allow promotion of membrane fusion, and the transfer of the lipid probe to the CV-1 cells following membrane fusion was visualized by fluorescence microscopy. After 5 or 10 min of incubation at 37°C, the wt F protein and mutant L161M showed equivalent numbers of labeled cells per field, with L140M giving demonstrable but reduced levels of fusion (data not shown). The mutants I137M and V154M gave only a small number of fusion events, consistent with the reduced levels of fusion seen in other assays (data not shown). As the mutant SV5 F L161M promoted increased syncytium formation, a phenotype that suggests increased fusion activity in certain cell types or conditions, the temperature dependence of fusion promotion for this mutant relative to that of the wt F protein was determined. Following binding of labeled RBCs at 4°C, the cold PBS was removed, PBS warmed to room temperature, 30, 33, or 37°C was added, and the plates were placed for 10 min in incubators set to the appropriate temperatures. Fusion for

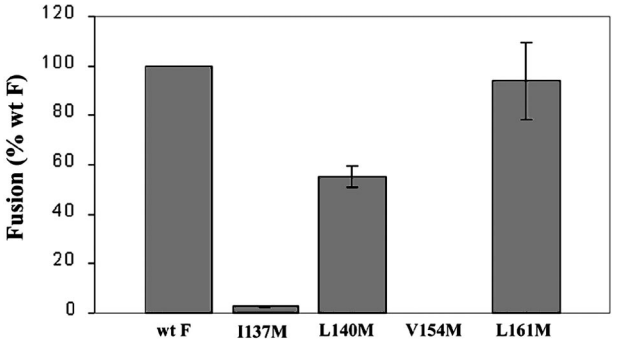

FIG. 5. Reporter gene assays of the wt F protein and the HRA mutants. Vero cells were cotransfected with pCAGGS plasmids encoding the SV5 HN protein and either the wt F protein or the HRA mutants under the control of the chicken β-actin promoter. In addition, these cells were transfected with the plasmid pINT7βgal, containing the β-galactosidase gene under the control of the T7 polymerase promoter. A second set of Vero cells was transfected with the plasmid encoding the T7 polymerase under the control of the β-actin promoter. After ON incubation, the T7 polymerase-expressing cells were overlaid on the cells expressing the F and HN proteins. Subsequent membrane fusion between the cell populations allowed the T7 polymerase to transcribe the β-galactosidase gene. β-Galactosidase activity was assayed as described in Materials and Methods. Samples were done in triplicate. The experiment presented is representative of five separate experiments.

the wt F protein was significantly decreased at 33°C compared to 37°C, and little fusion was seen at 30°C or at room temperature (Fig. 6). In contrast, SV5 F L161M efficiently promoted fusion at 37, 33, and 30°C, and one to two fusion events per field were seen when incubation was performed at room temperature (Fig. 6). These results demonstrate that mutation of L161 to methionine results in an F protein mutant that is capable of promoting fusion at lower temperatures than is the wt SV5 F protein.

DISCUSSION

From the time of synthesis in the ER to the completion of promotion of membrane fusion, the SV5 F protein undergoes multiple changes in conformation. Initial protein folding in the ER of the precursor protein, F_0, is accompanied by trimerization (48), though the domains involved in this oligomerization process remain unclear. During subsequent transport through the secretory pathway, F_0 is proteolytically processed to the disulfide-linked heterodimer, $F_1 + F_2$, by the cellular enzyme furin (21, 40). This proteolytic cleavage event has been shown to lead to major conformational changes in the SV5 F protein and other paramyxovirus F proteins (9, 19, 22, 53). Once the F protein is present on the cellular surface or on the surface of a virion, promotion of membrane fusion can be initiated. While the triggering event for paramyxovirus F protein-mediated fusion remains unclear, considerable evidence points to a role for the SV5 attachment protein HN (reviewed in reference 24). The promotion of membrane fusion is hypothesized to occur with a series of conformational changes in the F protein, beginning with rearrangement of the protein to allow insertion of the fusion peptide into the target membrane (34), followed by refolding of the protein and finally formation of the six-helix

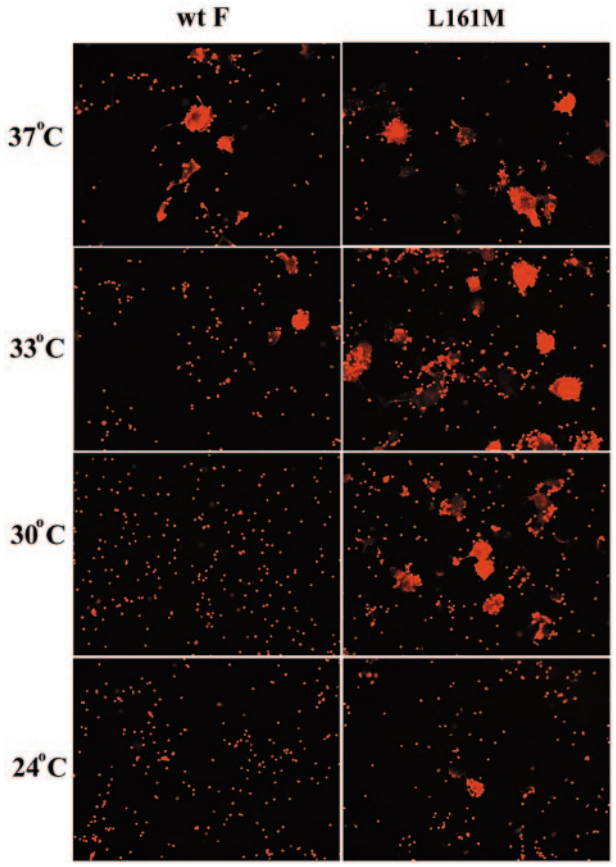

FIG. 6. Transfer of lipid dye by the wt F protein and the L161 M mutant. Human RBCs labeled with the lipid probe R18 were bound at 4°C to CV-1 cells coexpressing the SV5 HN protein and either the wt F protein or the L161 M mutant. Cells were incubated for 10 min at the indicated temperatures to allow membrane fusion and were examined by fluorescence microscopy.

bundle by the heptad repeat regions to drive fusion of the lipid bilayers (3, 20, 46, 47).

Coiled-coil formation by N-terminal heptad repeat regions has been hypothesized to play a critical role in initial protein folding and oligomerization of a number of type I viral fusion proteins (41). However, mutational analyses of a number of other viral fusion proteins, including the HIV gp41 protein (4, 7, 8, 25, 41, 55, 56), the Ebola virus Gp (54), and the Mason-Pfizer monkey virus transmembrane protein (52), found that fusion defects were common outcomes of mutations within this region, while folding defects were rare. For paramyxovirus fusion proteins, mutational analysis of the HRA region of the NDV F protein has found that both folding and fusion defects can result from single point mutations within this domain (50, 51), and those authors suggested that residues in the a positions of the heptad repeat might be more critical for folding, while those in the d positions of the heptad repeat were generally more critical for fusion promotion (31, 50). Of the four heptad repeat mutations in our study, V154M (d residue) led to a folding defective phenotype, while I137M (a residue) and L140M and L161M (d residues) were competent for oligomerization (Fig. 3), proteolytic processing (Fig. 2), and transport to the cell surface (Table 1). As the V154M mutation had no effect on coiled-coil formation of HRA peptides (13) while both the L140M and L161M mutations disrupted formation of an α-helical structure, our results strongly indicate that formation of a coiled-coil by the HRA region is not critical for initial protein folding and oligomerization of the SV5 F protein. However, both our data and the studies of the NDV F protein (50, 51) indicate that residues within the HRA region may play a role in initial folding, separate from their role in coiled-coil formation.

The formation of a six-helix bundle is clearly critical for promotion of membrane fusion by type I viral fusion proteins. Considerable evidence indicates that the six-helix bundle forms either during or immediately after fusion pore formation (3, 26, 29), providing a driving force for the membrane fusion event. Peptide studies have previously demonstrated that mutation I137M within the SV5 HRA region leads to formation of a less stable six-helix bundle with a biphasic temperature melt as judged by circular dichroism (13). Consistent with the importance of six-helix bundle formation in promotion of membrane fusion, we found that an SV5 F protein incorporating the I137M mutation showed drastically reduced membrane fusion activity, as judged by both syncytium assays (Fig. 4) and a reporter gene assay (Fig. 5). Russell and coworkers have examined a series of mutations in the HRB region that lower the temperature of thermal denaturation of the six-helix bundle and have not found a consistent correlation with inhibition of fusion activity (47). However, the effect of the I137M mutation on thermal denaturation of the six-helix bundle is more drastic than those seen for the HRB mutations, with much of the bundle undergoing denaturation with a midpoint at 55°C (13). In addition, while the mutations studied in the HRB region lie at the end of the six-helix bundle farthest from the transmembrane and fusion peptide domains (47), I137 is positioned at the opposite end of the six-helix bundle, near the fusion peptide (3) (Fig. 1B). Thus, mutations which negatively influence six-helix bundle stability can lead to inhibition of fusion promotion, but the relative effect of an individual mutation may be determined by the position of the residue within the six-helix bundle and the relative destabilization caused by the mutation. Further comparative analysis of the effects of mutations in HRA on both peptide structures and folding and function of the entire F protein will be necessary to confirm this hypothesis.

Our results and those of others indicate that the paramyxovirus F protein HRA coiled-coil is not critical for initial protein folding in the ER, but the coiled-coil is a component of the six-helix bundle structure formed during or immediately after fusion pore formation. It is unknown whether the HRA coiled-coil forms during transit of the protein to the cell surface, in the period prior to or during initiation of membrane fusion, or whether the elements of the six-helix bundle fold as a unit. A portion of the coiled-coil is present in the NDV F crystal structure (6), but it is unclear which conformational stage of the F protein is represented in the structure (32). For the NDV F protein, inhibition by HRA peptides was only noted when added prior to proteolytic activation of the protein (57), a result which could occur if a coiled-coil formed upon proteolytic activation and was blocked by HRA peptide binding. For the SV5 F protein, HRA and HRB peptides have clearly been shown to block at different stages along the fusion pathway

(46). Our mutational analyses indicate that mutants (L140M and L161M) that abolish α-helical structure, and thus coiled-coil formation, in peptide systems are still capable of promoting fusion. Indeed, one of these mutants (L161M) promoted syncytium formation to a greater extent than the wt SV5 F protein (Fig. 4) and promoted fusion at a lower temperature than the wt F protein (Fig. 6), demonstrating that this mutation gives a hyperfusogenic phenotype. A hyperfusogenic phenotype has been reported for several mutations in or near the HRB region of the SV5 F protein. Mutation of S443 to proline (39) and mutation of residues L447 and I449 to aromatic residues (47) all resulted in increased levels of fusion and fusion promotion at lower temperatures. I449 and L447 are present in the HRB region of the SV5 F protein six-helix bundle immediately after the structure moves from α-helix to extended chain (3) (Fig. 1B) and, thus, lie in close proximity to L161. Russell and coworkers have suggested that the region immediately N-terminal to HRB may play a role in activation of fusion, explaining the hyperfusogenic phenotypes of mutants at 447 and 449 (47). Our results suggest that residue 161 may be involved in a similar activation switch. One intriguing possibility is that residues from both HRA and HRB in this region distal from the membrane domains may direct folding into the bundle and that mutations which change interactions in this seed domain may drive the F protein towards six-helix bundle formation and promotion of membrane fusion.

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REFERENCES

1. **Asano, K., and A. Asano.** 1985. Why is a specific amino acid sequence of F glycoprotein required for the membrane fusion reaction between envelope of HVJ (Sendai virus) and target cell membranes? Biochem. Int. **10:**115–122.
2. **Bagai, S., and R. A. Lamb.** 1995. Quantitative measurement of paramyxovirus fusion: differences in requirements of glycoproteins between simian virus 5 and human parainfluenza virus 3 or Newcastle disease virus. J. Virol. **69:** 6712–6719.
3. **Baker, K. A., R. E. Dutch, R. A. Lamb, and T. S. Jardetzky.** 1999. Structural basis for paramyxovirus-mediated membrane fusion. Mol. Cell **3:**309–319.
4. **Bernstein, H. B., S. P. Tucker, S. R. Kar, S. A. McPherson, D. T. McPherson, J. W. Dubay, J. Lebowitz, R. W. Compans, and E. Hunter.** 1995. Oligomerization of the hydrophobic heptad repeat of gp41. J. Virol. **69:**2745–2750.
5. **Cao, J., L. Bergeron, E. Helseth, M. Thali, H. Repke, and J. Sodroski.** 1993. Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein. J. Virol. **67:** 2747–2755.
6. **Chen, L., J. J. Gorman, J. McKimm-Breschkin, L. J. Lawrence, P. A. Tulloch, B. J. Smith, P. M. Colman, and M. C. Lawrence.** 2001. The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. Structure (Cambridge) **9:** 255–266.
7. **Chen, S. S.-L., C.-N. Lee, W.-R. Lee, K. McIntosh, and T.-H. Lee.** 1993. Mutational analysis of the leucine zipper-like motif of the human immunodeficiency virus type 1 envelope transmembrane glycoprotein. J. Virol. **67:** 3615–3619.
8. **Dubay, J. W., S. J. Roberts, B. Brody, and E. Hunter.** 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. J. Virol. **66:**4748–4756.
9. **Dutch, R. E., R. N. Hagglund, M. A. Nagel, R. G. Paterson, and R. A. Lamb.** 2001. Paramyxovirus fusion (F) protein: a conformational change on cleavage activation. Virology **281:**138–150.
10. **Dutch, R. E., T. S. Jardetsky, and R. A. Lamb.** 2000. Virus membrane fusion proteins: biological machines that undergo a metamorphosis. Biosci. Rep. **20:**597–612.
11. **Dutch, R. E., S. B. Joshi, and R. A. Lamb.** 1998. Membrane fusion promoted by increasing surface densities of the paramyxovirus F and HN proteins: comparison of fusion reactions mediated by simian virus 5 F, human parainfluenza virus type 3 F, and influenza virus HA. J. Virol. **72:**7745–7753.
12. **Dutch, R. E., and R. A. Lamb.** 2001. Deletion of the cytoplasmic tail of the fusion (F) protein of the paramyxovirus simian virus 5 affects fusion pore enlargement. J. Virol. **75:**5363–5369.
13. **Dutch, R. E., G. P. Leser, and R. A. Lamb.** 1999. Paramyxovirus fusion protein: characterization of the core trimer, a rod-shaped complex with helices in anti-parallel orientation. Virology **254:**147–159.
14. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T_7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:** 8122–8126.
15. **He, B., G. P. Leser, R. G. Paterson, and R. A. Lamb.** 1998. The paramyxovirus SV5 small hydrophobic (SH) protein is not essential for virus growth in tissue culture cells. Virology **250:**30–40.
16. **Homma, M.** 1971. Trypsin action on the growth of Sendai virus in tissue culture cells. I. Restoration of the infectivity for L cells by direct action of trypsin on L cell-borne Sendai virus. J. Virol. **8:**619–629.
17. **Horvath, C. M., and R. A. Lamb.** 1992. Studies on the fusion peptide of a paramyxovirus fusion glycoprotein: roles of conserved residues in cell fusion. J. Virol. **66:**2443–2455.
18. **Horvath, C. M., R. G. Paterson, M. A. Shaughnessy, R. Wood, and R. A. Lamb.** 1992. Biological activity of paramyxovirus fusion proteins: factors influencing formation of syncytia. J. Virol. **66:**4564–4569.
19. **Hsu, M., A. Scheid, and P. W. Choppin.** 1981. Activation of the Sendai virus fusion protein (F) involves a conformational change with exposure of a new hydrophobic region. J. Biol. Chem. **256:**3557–3563.
20. **Joshi, S. B., R. E. Dutch, and R. A. Lamb.** 1998. A core trimer of the paramyxovirus fusion protein: parallels to influenza virus hemagglutinin and HIV-1 gp41. Virology **248:**20–34.
21. **Klenk, H.-D., and W. Garten.** 1994. Host cell proteases controlling virus pathogenicity. Trends Microbiol. **2:**39–43.
22. **Kohama, T., W. Garten, and H.-D. Klenk.** 1981. Changes in conformation and charge paralleling proteolytic activation of Newcastle disease virus glycoproteins. Virology **111:**364–376.
23. **Lamb, R. A., P. R. Etkind, and P. W. Choppin.** 1978. Evidence for a ninth influenza viral polypeptide. Virology **91:**60–78.
24. **Lamb, R. A., and D. Kolakofsky.** 2001. *Paramyxoviridae*: the viruses and their replication, p. 1305–1340. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott-Raven Press, New York, N.Y.
25. **Lu, M., M. O. Stoller, S. Wang, J. Liu, M. B. Fagan, and J. H. Nunberg.** 2001. Structural and functional analysis of interhelical interactions in the human immunodeficiency virus type 1 gp41 envelope glycoprotein by alanine-scanning mutagenesis. J. Virol. **75:**11146–11156.
26. **Markosyan, R. M., F. S. Cohen, and G. B. Melikyan.** 2003. HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation. Mol. Biol. Cell **14:**926–938.
27. **Matthews, J., T. F. Young, S. P. Tucky, and J. P. Mackay.** 2000. The core of the respiratory syncytial virus fusion protein is a trimeric coiled coil. J. Virol. **74:**5911–5920.
28. **McGinnes, L. W., T. Sergel, H. Chen, L. Hamo, S. Schwertz, D. Li, and T. G. Morrison.** 2001. Mutational analysis of the membrane proximal heptad repeat of the Newcastle disease virus fusion protein. Virology **289:**343–352.
29. **Melikyan, G. B., R. M. Markosyan, H. Hemmati, M. K. Delmedico, D. M. Lambert, and F. S. Cohen.** 2000. Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. J. Cell Biol. **151:**413–423.
30. **Morris, S. J., D. P. Sarkar, J. M. White, and R. Blumenthal.** 1989. Kinetics of pH-dependent fusion between 3T3 fibroblasts expressing influenza hemagglutinin and red blood cells. J. Biol. Chem. **264:**3972–3978.
31. **Morrison, T. G.** 2003. Structure and function of a paramyxovirus fusion protein. Biochim. Biophys. Acta **1614:**73–84.
32. **Morton, C. J., R. Cameron, L. J. Lawrence, B. Lin, M. Lowe, A. Luttick, A. Mason, J. McKimm-Breschkin, M. W. Parker, J. Ryan, M. Smout, J. Sullivan, S. P. Tucker, and P. R. Young.** 2003. Structural characterization of respiratory syncytial virus fusion inhibitor escape mutants: homology model of the F protein and a syncytium formation assay. Virology **311:**275–288.
33. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants by a novel eukaryotic vector. Gene **108:**193–200.
34. **Novick, S. L., and D. Hoekstra.** 1988. Membrane penetration of Sendai virus glycoproteins during the early stage of fusion with liposomes as determined by hydrophobic affinity labeling. Proc. Natl. Acad. Sci. USA **85:**7433–7437.
35. **Paterson, R. G., T. J. R. Harris, and R. A. Lamb.** 1984. Analysis and gene

assignment of mRNAs of a paramyxovirus, simian virus 5. Virology **138:** 310–323.
36. **Paterson, R. G., T. J. R. Harris, and R. A. Lamb.** 1984. Fusion protein of the paramyxovirus simian virus 5: nucleotide sequence of mRNA predicts a highly hydrophobic glycoprotein. Proc. Natl. Acad. Sci. USA **81:**6706–6710.
37. **Paterson, R. G., S. W. Hiebert, and R. A. Lamb.** 1985. Expression at the cell surface of biologically active fusion and hemagglutinin-neuraminidase proteins of the paramyxovirus simian virus 5 from cloned cDNA. Proc. Natl. Acad. Sci. USA **82:**7520–7524.
38. **Paterson, R. G., and R. A. Lamb.** 1987. Ability of the hydrophobic fusion-related external domain of a paramyxovirus F protein to act as a membrane anchor. Cell **48:**441–452.
39. **Paterson, R. G., C. J. Russell, and R. A. Lamb.** 2000. Fusion protein of the paramyxovirus SV5: destabilizing and stabilizing mutants of fusion activation. Virology **270:**17–30.
40. **Paterson, R. G., M. A. Shaughnessy, and R. A. Lamb.** 1989. Analysis of the relationship between cleavability of a paramyxovirus fusion protein and length of the connecting peptide. J. Virol. **63:**1293–1301.
41. **Poumbourios, P., W. el Ahmar, D. A. McPhee, and B. E. Kemp.** 1995. Determinants of human immunodeficiency virus type 1 envelope glycoprotein oligomeric structure. J. Virol. **69:**1209–1218.
42. **Poumbourios, P., K. A. Wilson, R. J. Center, W. El Ahmar, and B. E. Kemp.** 1997. Human immunodeficiency virus type 1 envelope glycoprotein oligomerization requires the gp41 amphipathic alpha-helical/leucine zipper-like sequence. J. Virol. **71:**2041–2049.
43. **Randall, R. E., D. F. Young, K. K. A. Goswami, and W. C. Russell.** 1987. Isolation and characterization of monoclonal antibodies to simian virus 5 and their use in revealing antigenic differences between human, canine and simian isolates. J. Gen. Virol. **68:**2769–2780.
44. **Reitter, J. N., T. Sergel, and T. G. Morrison.** 1995. Mutational analysis of the leucine zipper motif in the Newcastle disease virus fusion protein. J. Virol. **69:**5995–6004.
45. **Rose, J. K., B. Bonagurio, and M. A. Whitt.** 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechniques **10:**520–525.
46. **Russell, C. J., T. S. Jardetzky, and R. A. Lamb.** 2001. Membrane fusion machines of paramyxoviruses: capture of intermediates of fusion. EMBO J. **20:**4024–4034.
47. **Russell, C. J., K. L. Kantor, T. S. Jardetzky, and R. A. Lamb.** 2003. A dual-functional paramyxovirus F protein regulatory switch segment: activation and membrane fusion. J. Cell Biol. **163:**363–374.
48. **Russell, R., R. G. Paterson, and R. A. Lamb.** 1994. Studies with cross-linking reagents on the oligomeric form of the paramyxovirus fusion protein. Virology **199:**160–168.
49. **Scheid, A., and P. W. Choppin.** 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology **57:**475–490.
50. **Sergel, T. A., L. W. McGinnes, and T. G. Morrison.** 2001. Mutations in the fusion peptide and adjacent heptad repeat inhibit folding or activity of the Newcastle disease virus fusion protein. J. Virol. **75:**7934–7943.
51. **Sergel-German, T., C. McQuain, and T. Morrison.** 1994. Mutations in the fusion peptide and heptad repeat regions of the Newcastle disease virus fusion protein block fusion. J. Virol. **68:**7654–7658.
52. **Song, C., and E. Hunter.** 2003. Variable sensitivity to substitutions in the N-terminal heptad repeat of Mason-Pfizer monkey virus transmembrane protein. J. Virol. **77:**7779–7785.
53. **Umino, Y., T. Kohama, T. A. Sato, A. Sugiura, H.-D. Klenk, and R. Rott.** 1990. Monoclonal antibodies to three structural proteins of Newcastle disease virus: biological characterization with particular reference to the conformational change of envelope glycoproteins associated with proteolytic cleavage. Virology **176:**656–657.
54. **Watanabe, S., A. Takada, T. Watanabe, H. Ito, H. Kida, and Y. Kawaoka.** 2000. Functional importance of the coiled-coil of the Ebola virus glycoprotein. J. Virol. **74:**10194–10201.
55. **Weng, Y., and C. D. Weiss.** 1998. Mutational analysis of residues in the coiled-coil domain of human immunodeficiency virus type 1 transmembrane protein gp41. J. Virol. **72:**9676–9682.
56. **Weng, Y., Z. Yang, and C. D. Weiss.** 2000. Structure-function studies of the self-assembly domain of the human immunodeficiency virus type 1 transmembrane protein gp41. J. Virol. **74:**5368–5372.
57. **Young, J. K., D. Li, M. C. Abramowitz, and T. G. Morrison.** 1999. Interaction of peptides with sequences from the Newcastle disease virus fusion protein heptad repeat regions. J. Virol. **73:**5945–5956.
58. **Yu, M., E. Wang, Y. Liu, D. Cao, N. Jin, C. W. Zhang, M. Bartlam, Z. Rao, P. Tien, and G. F. Gao.** 2002. Six-helix bundle assembly and characterization of heptad repeat regions from the F protein of Newcastle disease virus. J. Gen. Virol. **83:**623–629.
59. **Zhao, X., M. Singh, V. N. Malashkevich, and P. S. Kim.** 2000. Structural characterization of the human respiratory syncytial virus fusion protein core. Proc. Natl. Acad. Sci. USA **97:**14172–14177.