The Paramyxovirus Fusion Protein C-Terminal Region: Mutagenesis Indicates an Indivisible Protein Unit

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Paramyxoviruses enter host cells by fusing the viral envelope with a host cell membrane. Fusion is mediated by the viral fusion (F) protein, and it undergoes large irreversible conformational changes to cause membrane merger. The C terminus of PIV5 F contains a membrane-proximal 7-residue external region (MPER), followed by the transmembrane (TM) domain and a 20-residue cytoplasmic tail. To study the sequence requirements of the F protein C terminus for fusion, we constructed chimeras containing the ectodomain of parainfluenza virus 5 F (PIV5 F) and either the MPER, the TM domain, or the cytoplasmic tail of the F proteins of the paramyxoviruses measles virus, mumps virus, Newcastle disease virus, human parainfluenza virus 3, and Nipah virus. The chimeras were expressed, and their ability to cause cell fusion was analyzed. The chimeric proteins were variably expressed at the cell surface. We found that chimeras containing the ectodomain of PIV5 F with the C terminus of other paramyxoviruses were unable to cause cell fusion. Fusion could be restored by decreasing the activation energy of refolding through introduction of a destabilizing mutation (S443P). Replacing individual regions, singly or doubly, in the chimeras with native PIV5 F sequences restored fusion to various degrees, but it did not have an additive effect in restoring activity. Thus, the F protein C terminus may be a specific structure that only functions with its cognate ectodomain. Alanine scanning mutagenesis of MPER indicates that it has a regulatory role in fusion since both hyperfusogenic and hypofusogenic mutations were found.

The *Paramyxoviridae* are enveloped, negative-strand RNA viruses that infect both humans and animals (23). The family includes many important pathogens, including parainfluenza viruses 1 to 5 (PIV1 to -5), mumps virus (MuV), measles virus (MeV), Newcastle disease virus (NDV), Sendai virus, Hendra virus, and Nipah virus (NiV). To enter cells, paramyxoviruses, like all enveloped viruses, have to fuse the viral envelope with a membrane of a host cell. For paramyxoviruses, this process involves two viral spike glycoproteins, the receptor-binding protein, variably called HN, H, or G, and the fusion protein (F) (22, 36).

The paramyxovirus F protein is a class I viral fusion protein that initially folds into a metastable prefusion form and, on triggering, it undergoes major irreversible refolding events to form the postfusion conformation, and this couples the energy released with membrane fusion (19). The F protein is synthesized as a precursor that has to be cleaved either by a host protease (furin) in the *trans*-Golgi apparatus or by an extracellular trypsin-like enzyme to form the biologically active molecule F1,F2. Cleavage creates a new N terminus that contains a conserved hydrophobic region known as the fusion peptide (9, 21).

For PIV5, the receptor-binding protein hemagglutinin-neuraminidase (HN) binds to sialic acid moieties on the cell surface, and HN is required for the activation of F that occurs at the plasma membrane and at neutral pH. It is thought that F interacts with the stalk domain of HN (5, 6, 11, 12, 24, 42, 46, 47, 54). On fusion activation, the fusion peptide inserts into the target cell membrane, and subsequent refolding events result in a trimer-of-hairpins structure, which is coupled with membrane merger (15, 34). The final most stable form of F, postfusion F, contains a six-helix bundle that is a highly stable structure formed by the coming together of two heptad repeats, HRA and HRB, to form an antiparallel helical bundle (3, 34, 53).

The atomic structures of soluble forms of several paramyxovirus F proteins have been determined by X-ray crystallography. These include PIV5 F in its prefusion conformation (53) and hu-

man parainfluenza virus 3 (hPIV3), NDV, and RSV F in their postfusion conformations (7, 25, 40, 41, 52). All of these proteins lack their natural C terminus, which was deleted to generate the soluble and secreted forms. Thus, we do not know the structure of the 7- to 8-residue membrane-proximal external region (MPER) (that exists between the last residue of heptad repeat B [HRB] and the transmembrane [TM] domain), the hydrophobic TM domain itself, or the cytoplasmic tail (see Fig. 1A).

Previous studies have shown that deletion of four residues of the MPER, or deletion of eight residues, including the entire MPER and the last residue of HRB, retain wild-type levels of surface expression and fusion activity, whereas deletion of two or six residues reduces fusion activity (3, 55). However, it has not been found possible to make insertions of residues into MPER and to retain biological activity (55).

The TM domain is defined as those hydrophobic residues sufficient to span a membrane and is usually bounded by charged residues. For paramyxovirus F proteins, the N-terminal boundary of the presumptive TM domain usually lacks a charged residue. Expression of an F construct that is truncated before the MPER and thus lacks the TM domain is secreted in the postfusion form (10, 52, 53). It has been suggested that the lack of a TM domain either causes F to fold directly to the postfusion form, or it drastically lowers the energy barrier to conversion from the prefusion to the postfusion conformation (10, 53).

There is little knowledge of the secondary structure of the TM domain of viral fusion proteins. Our previous study of the TM

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domain of PIV5 F provided data indicating that it formed a three-helix bundle, with the nature of the side chain at residues L486 and L488, located in the membrane close to the extracellular side, being important for fusion (4). For other viral fusion protein TM domains, e.g., MeV F, influenza virus HA, vesicular stomatitis virus (VSV) G, human immunodeficiency virus (HIV) gp41, and murine leukemia virus Env, point mutations have been identified that reduce or completely block fusion (8, 20, 26–28, 30, 39, 42, 43, 50).

The TM domain of some viral fusion proteins do have specific sequence requirements to retain fusion activity; for example, substitution of the TM domain of HIV-1 gp41 with leucine residues results in a surface-expressed protein that is not fusogenic (39). PIV5 F containing an all-leucine TM domain is not expressed on the cell surface, presumably because of defective protein folding (4). The TM domain of NDV F, when replaced with that of Sendai virus F, MeV F, or VSV G, results in lowered surface expression levels and reduced fusion activity of the chimeric proteins (18). On the other hand, the TM domain of influenza virus HA can be replaced with that of Sendai virus F, Rous sarcoma virus Env, or even the polyimmunoglobulin receptor, an unrelated protein, without losing fusion activity (26, 37). Similarly, the TM domain of VSV G and HIV gp41 can be replaced with sequences from other membrane proteins (8, 29, 51). For influenza virus HA, the minimum required length of the TM domain has been shown to be 17 residues to retain surface and fusion activity (2). Also, a basic residue bordering the C terminus of the TM domain has been shown to be important for fusion activity (2, 43).

The cytoplasmic tails of viral fusion proteins have also been implicated in fusion activity in several studies, and many mutations have been identified that reduce fusion activity (1, 16, 38, 44). For PIV5 F, deletion of the cytoplasmic tail impairs fusion pore enlargement (16), and increasing the length of the cytoplasmic tail such that it has the propensity to form a three-helix bundle abolishes fusion (48).

To study the sequence requirements of the C terminus of PIV5 F for fusion, we made chimeras containing the ectodomain of PIV5 F and either the MPER, the TM domain, or the cytoplasmic tail of the F proteins of the paramyxoviruses MeV, MuV, NDV, hPIV3, and NiV (Fig. 1B). We analyzed the proteins for surface expression using conformation-specific antibodies and assayed cell-cell fusion. We also examined the sequence of the MPER in a greater detail by making alanine mutants that span the region, and we found key residues that affect the stability of the protein.

Our results indicate that the MPER, TM domain, and cytoplasmic tail act in a concerted fashion to cause fusion, with each region having an effect on fusion activity. We also established the MPER as a regulatory region, containing both stabilizing and destabilizing residues.

MATERIALS AND METHODS

Cells, viruses, and plasmids. BHK-21F, Vero, 293T, BSR-T7, and HeLa-CD4-LTR- β gal cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. The medium for BHK-21F cells was supplemented with 10% tryptose phosphate broth, and the medium for HeLa-CD4-LTR- β gal cells was supplemented with 200 μ g of Geneticin/ml, 100 μ g of hygromycin B/ml, and 20 mM HEPES (pH 7.4).

The recombinant vaccinia virus (vTF7-3) expressing T7 polymerase was grown in CV-1 cells as described previously (17). pCAGGS and pGEM2X expression plasmids encoding PIV5 F (strain W3A), PIV5 HN (strain W3A), and PIV5 S443P F have been described previously (32). The

C-terminal chimeras and alanine substitution mutants were made by four-primer PCR with *Vent* DNA polymerase and cloned into pCAGGS or pGEM2X vector. The nucleotide sequences used for making chimeras were from the Edmonton strain of MeV, the Miyahara strain of MuV, the AV strain of NDV, and the 47885 strain of hPIV3. Mutations were confirmed by DNA sequencing using an Applied Biosystems 3100-Avant DNA sequencer (Carlsbad, CA).

Syncytium assay. Monolayers of BHK-21F cells in six-well plates were transiently transfected with 1 μ g each of pCAGGS-PIV5 F and pCAGGS-PIV5 HN DNA using the Lipofectamine Plus expression system (Invitrogen, Carlsbad, CA), and incubated at 37°C for 20 h. Cells were fixed and stained using Hema-3 stain (Thermo Fisher Scientific, Pittsburgh, PA) according to the manufacturer's instructions, and photomicrographs were taken with a digital camera (DCS 760; Kodak, Rochester, NY) using an inverted phase-contrast microscope (Diaphot; Nikon, Melville, NY).

Luciferase reporter assay. Vero cell monolayers in six-well plates were transfected as described above with 1 μ g each of pCAGGS-PIV5 F, pCAGGS-PIV5 HN, and a plasmid containing the luciferase gene under the control of the T7 RNA polymerase promoter. At 16 h posttransfection, the monolayers were overlaid with BSR-T7 cells endogenously expressing T7 RNA polymerase and incubated for 8 h at 37°C (or 42°C in Fig. 2C). The cells were then lysed in reporter lysis buffer (Promega, Madison, WI), and the luciferase activity was quantified by using luciferase assay substrate (Promega) and a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Dye transfer assay. Human red blood cells (RBCs) were dually labeled with the lipid probe octadecyl rhodamine F chloride (R18; Invitrogen) and 6-carboxyfluorescein (6-CF; Invitrogen) as described previously (35). CV-1 cells were grown on glass coverslips, and pGEM F and pGEM HN were expressed using the Vac-T7 expression system. Labeled RBCs were bound to transfected cells at 4°C for 1 h and then allowed to fuse at 37°C for 15 min. Imaging was done by using a scanning confocal microscope (LSM, 5 Pascal; Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Flow cytometry. To quantify the F protein surface expression levels, monolayers of HeLa-CD4-LTR- β gal cells in six-well plates were transfected with 1 μ g of pCAGGS DNA that express the various F protein constructs as described above, and flow cytometry was performed using the conformation-specific antibodies, F1a (1:100 dilution) (33) or 6-7 (1:50 dilution) (45), or polyclonal antibody R9176 (1:100 dilution), followed by a fluorescein isothiocyanate-labeled secondary antibody (1:100 dilution) as described previously (48). The F intensity of 10,000 cells was measured by using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Radioimmunoprecipitation. HeLa-CD4-LTR- β gal cells in six-well plates were transfected with pCAGGS-F, and its mutants and cells at 18 h posttransfection were starved for 30 min in cysteine- and methionine-free DMEM, labeled with 50 μ Ci of [35 S]Promix, incubated in DMEM for various times, and then lysed in radioimmunoprecipitation assay buffer containing protease inhibitors as described previously (31). Lysates were clarified as described above, and the proteins were immunoprecipitated with a polyclonal anti-F antibody (1:100 dilution) overnight at 4°C, bound to protein A Sepharose beads, and washed as described above. The samples were then resuspended in protein loading buffer containing sodium dodecyl sulfate (SDS) and boiled for 5 min, and the polypeptides were analyzed by SDS-PAGE on 15% polyacrylamide gels. Autoradiography was performed to visualize and quantify proteins using an FLA-5100 phosphorimager (Fujifilm, Inc., Stamford, CT).

RESULTS

F protein C-terminal region and chimeric DNA constructs. An alignment of the amino acid sequences of the C termini of the paramyxovirus F proteins used in the present study is shown in Fig. 1A. For each F protein, HRB is followed by a mostly serine-threonine-rich MPER, after which follows a 23- to 28-residue hydrophobic sequence that is the presumptive TM domain. The F

FIG 1 Construction of C terminus chimeras in PIV5 F. (A) Alignment of C termini of the F proteins of PIV5, MuV, NDV, MeV, NiV, and hPIV3. Chimeras were constructed by replacing residues of PIV5 F with the corresponding residues of the heterotypic F proteins. (B) Schematic diagram of the various chimeras made and used in the present study. The light-colored regions indicate wt PIV5 F sequences and the dark-colored regions indicate heterotypic F sequences (from MeV, MuV, NDV, hPIV3, or NiV F).

cytoplasmic tails show no homology in sequence to each other (Fig. 1A). To investigate the roles of the MPER, TM domain, and cytoplasmic domain on the ability of the PIV5 F ectodomain to cause fusion, we constructed a series of chimeric proteins that contained the PIV5 F ectodomain from residues 1 to 477 that includes the last residue (I477) of HRB. We appended to the PIV5 F ectodomain, in various combinations, the F protein MPER, TM domain and cytoplasmic tail from MeV, MuV, NDV, hPIV3, and NiV. The nomenclature of the constructs follows the form P-X-Y-Z, where P is the PIV5 ectodomain, X is the MPER, Y is the TM domain, and Z is the cytoplasmic tail. The virus of origin of each region is indicated as follows: P, PIV5; Me, MeV; Mu, MuV; N, NDV; h, hPIV3; and Ni, NiV. A schematic diagram illustrating the chimeric F proteins used in the present study is shown in Fig. 1B, and the nomenclature is used consistently in each subsequent figure.

The C terminus chimeras are defective in causing cell-cell fusion. To study the role of the complete MPER, TM domain, and cytoplasmic tail in fusion, we replaced the PIV5 region with that from MeV, MuV, NDV, hPIV3, or NiV F (Fig. 2A). The chimeric proteins were expressed transiently in HeLa-CD4-LTR-βgal cells, and their levels of cell surface expression were examined by flow cytometry using a conformation-specific monoclonal antibody (MAb), F1a, that recognizes the cleaved form of prefusion PIV5 F

(10, 33). The NDV and hPIV3 chimeras were expressed on the cell surface at levels comparable to those of PIV5, whereas the MeV, MuV, and NiV chimeras showed reduced surface expression (Fig. 2B). Since MAb F1a is specific for the prefusion form of F, flow cytometry with a polyclonal antibody to F that recognizes pre- and postfusion F was also used to assay the total amount of chimeras expressed on the cell surface. The use of different antibodies did yield some differences in the values for surface expression in case of the MeV and NiV chimeras, e.g., P-Me-Me-Me and P-Ni-Ni-Ni, and some other chimeras used later in the study, such as P-Me-Me-P and P-Ni-Ni-P (Table 1). This indicates the possibility of these chimeras being folded in a conformation other than the prefusion F conformation. The kinetics and extent of cleavage of F₀ to F₁ and F₂ was also measured (Table 1). The rate and extent of cleavage broadly corresponded to the levels of surface expression, as detected by flow cytometry (see Table 1 for the extents of cleavage at 150 min postlabeling).

Even though the chimeric proteins were expressed largely in the prefusion conformation, they did not cause cell-cell fusion on coexpression with HN using either a luciferase assay (Fig. 2C), a syncytium assay (data not shown), or a dye transfer assay (data not shown). To investigate the possibility that the chimeras were folded in the postfusion conformation, the constructs were expressed in HeLa-CD4-LTR-βgal cells, and flow cytometry was

FIG 2 The C terminus chimeras are defective in causing fusion, but this defect can be partially overcome by introduction of the PIV5 (W3A) S443P destabilizing mutation. (A) Schematic of the C terminus chimeras. (B) Surface expression of the C terminus chimeras in wt F and (D) surface expression of C terminus chimeras in the F S443P background (P*). Surface expression levels were determined by flow cytometry of transfected HeLa-CD4-LTR- β gal cells using prefusion conformation-specific MAb F1a. The mean fluorescence intensity (MFI) is indicated as a percentage of the wt F levels. (C and E) A luciferase assay was used to quantify fusion activity of C-terminus chimeras (C) and C-terminus chimeras in an S443P (P*) background (E). Vero cells transfected with PIV5 F and HN and luciferase under the control of T7 promoter at 20 h posttransfection were overlaid with BSR cells that stably express T7 polymerase. After 8 h, the cells were lysed, and the luciferase activity was measured. Relative luciferase units (RLU) for mutants are expressed as the percentage of wild type (%WT) F RLU. (F) Dye transfer assay to visualize fusion of C terminus chimeras with S443P mutation (P*). CV-1 cells were transfected with PIV5 F and HN and expressed using the Vac-T7 expression system. Human red blood cells (RBCs) labeled with R18 and Calcein-AM were bound to the transfected cells at 4°C for 1 h, followed by warming to 37°C for 15 min, and the cells were visualized by confocal microscopy. Chimeras are named as described in the text. MCS, empty vector with a multiple cloning site. $n = 3$. The statistical significance between wt and mutant is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

TABLE 1 Expression of the chimeras was tested by flow cytometry with MAb F1a and an F-specific pAb^a

Construct	% of F2 at 150 min of chase (%WT)	Amt (mean $\pm$ SD) of F on the cell surface (%WT)	
		F1a	pAb
PIV5 F	100.0	100.0	100.0
P-Me-Me-Me	68.5	37.9 $\pm$ 4.2**	69.7 $\pm$ 3.3*
P-Mu-Mu-Mu	22.0	23.8 $\pm$ 5.1**	28.6 $\pm$ 6.5*
P-N-N-N	73.6	91.3 $\pm$ 8.5*	79.2 $\pm$ 4.5*
P-h-h-h	107.5	88.2 $\pm$ 6.6*	81.3 $\pm$ 2.9*
P-Ni-Ni-Ni	76.9	22.9 $\pm$ 3.8*	41.2 $\pm$ 3.9*
P-Me-Me-P	117.8	37.7 $\pm$ 3.4*	98.9 $\pm$ 7.5*
P-Mu-Mu-P	30.7	23.9 $\pm$ 8.2**	32.7 $\pm$ 4.5*
P-N-N-P	58.8	40.1 $\pm$ 5.3***	57.1 $\pm$ 3.0*
P-h-h-P	62.9	50.9 $\pm$ 7.8**	61.8 $\pm$ 5.8*
P-Ni-Ni-P	19.4	14.9 $\pm$ 6.3**	17.7 $\pm$ 5.2*
P-P-Me-Me	32.6	31.1 $\pm$ 4.8**	96.7 $\pm$ 7.9*
P-P-Mu-Mu	20.4	36.4 $\pm$ 9.2*	42.9 $\pm$ 2.8*
P-P-N-N	93.0	78.0 $\pm$ 3.9*	90.4 $\pm$ 0.6*
P-P-h-h	92.9	96.7 $\pm$ 4.9*	99.3 $\pm$ 2.2*
P-P-Ni-Ni	46.9	24.6 $\pm$ 0.1***	49.4 $\pm$ 4.8*
P-P-P-Me	107.6	56.4 $\pm$ 7.3*	55.6 $\pm$ 4.1*
P-P-P-Mu	65.8	37.9 $\pm$ 2.8**	41.4 $\pm$ 6.5*
P-P-P-N	173.1	102.0 $\pm$ 7.3*	115.1 $\pm$ 3.5*
P-P-P-h	103.6	99.0 $\pm$ 4.5*	99.4 $\pm$ 1.4*
P-P-P-Ni	35.9	25.8 $\pm$ 0.5***	40.8 $\pm$ 0.9*
P-P-Me-P	28.0	25.6 $\pm$ 6.3**	60.4 $\pm$ 4.7*
P-P-Mu-P	23.9	38.9 $\pm$ 12.1*	42.3 $\pm$ 0.9*
P-P-N-P	65.7	62.3 $\pm$ 5.3*	64.0 $\pm$ 8.7*
P-P-h-P	90.7	93.4 $\pm$ 4.0*	100.9 $\pm$ 0.3*
P-P-Ni-P	74.9	76.8 $\pm$ 9.9*	94.4 $\pm$ 3.0*
P-Me-P-P	27.5	42.3 $\pm$ 3.9**	64.4 $\pm$ 4.6*
P-Mu-P-P	75.1	95.7 $\pm$ 11.2*	91.1 $\pm$ 1.1*
P-N-P-P	81.4	105.1 $\pm$ 11.2*	80.6 $\pm$ 4.9*
P-h-P-P	78.2	61.8 $\pm$ 5.6*	57.0 $\pm$ 9.6*
P-Ni-P-P	30.0	43.2 $\pm$ 3.8**	52.5 $\pm$ 8.9*

^a As determined by flow cytometry ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The extent of cleavage was determined by ³⁵S radiolabel, pulse-chase immunoprecipitation using an F-specific polyclonal antibody (pAb) and SDS-PAGE. The amount of F2 at 150 min after chase is indicated as the percentage of the wild type (%WT).

performed using a PIV5 postfusion specific MAb, 6-7 (10, 45). None of the chimeras showed reactivity to MAb 6-7, indicating that the chimeras were not folded in the postfusion conformation (data not shown). To test whether the chimeras had a higher energy barrier for fusion or whether they were in an “off-pathway” conformation, the luciferase assay was performed at 42°C, to examine whether the higher temperature enabled the chimeras to cause fusion. We observed small increase in amounts of fusion for the MeV, MuV, NDV, and hPIV3 chimeras (Fig. 2C). Similar results were obtained in a dye-transfer assay performed at 55°C (data not shown).

A destabilizing mutation rescues fusion of the C terminus chimeric proteins. The destabilizing S443P mutation in PIV5 F has been characterized as rendering the mutant hyperfusogenic, with a reduced energy of activation, faster kinetics of fusion, and HN-independent fusion (32). The mutation destabilizes an intricate hydrogen-bonding network at the base of the head in PIV5 prefusion F (53).

Since the C terminus chimeras showed reactivity to MAb F1a and increased fusion at 42°C, we further investigated whether the

chimeras were trapped in the course of the conformational changes from pre- to postfusion form due to increased activation energy rather than being in an off-pathway conformation. The C terminus chimeras were constructed in the background of the S443P mutation (P*).

These chimeric F proteins were expressed on the cell surface to various levels, as measured by flow cytometry using MAb F1a (Fig. 2D). It was observed that introduction of the destabilizing mutation S443P into the chimeric F proteins rescued fusion to various extents, as measured by the luciferase assay (Fig. 2E), dye transfer assay (Fig. 2F), and syncytium formation (data not shown). The low surface expression level of the MeV and NiV chimeric F proteins (P*-Me-Me-Me and P*-Ni-Ni-Ni) was also reflected in the comparatively low levels of fusion activity of these mutants. In contrast, the hPIV3 chimera (P*-h-h-h) showed fusion activity that was greater than that of wild-type (wt) F. These data suggest that the F chimeras are expressed at the cell surface and have a higher energy barrier for F activation than does the wt F protein and that introduction of the destabilizing S443P mutation lowers the energy barrier of fusion activation for these chimeric F proteins.

Substituting the wt PIV5 cytoplasmic tail on the C-terminal chimeras partially restores fusion by the PIV5 ectodomain. The MPER, TM domain, and cytoplasmic tail are usually considered as distinct regions of the F protein C terminus, and they may have independent functions in regulating fusion activity. To investigate the contribution of individual heterotypic regions toward the fusion-negative phenotype of the C terminus chimeras, we made a series of chimeras, replacing individual regions of native PIV5 F protein back into the C terminus chimeras. First, the PIV5 F cytoplasmic tail was put back into the C terminus chimeras (Fig. 3A). The F chimeras were tested for surface expression by flow cytometry of surface proteins as described above (Fig. 3B, Table 1) and also tested for the extent of F cleavage (Table 1). The amount of MAb F1a-reactive species on the surface was 50% or less than that of the wt F, although the total amount of cell surface expression was higher when analyzed by the polyclonal antibody. The level of fusion activity was assayed by luciferase assay and syncytium assay as described above, and it was found that the MuV and NDV chimeras (P-Mu-Mu-P and P-N-N-N-P) showed ~60% fusion and that the hPIV3 chimera (P-h-h-P) showed ~40% fusion compared to wt levels (Fig. 3C; data not shown for the syncytium assay). These data indicate that in the right sequence context the native cytoplasmic tail sequence contributes to fusion. However, with the wrong MPER and TM domain the PIV5 cytoplasmic tail is not able to drive the PIV5 ectodomain to cause fusion.

Chimeras with a heterotypic TM domain and cytoplasmic tail show reduced fusion. To extend the analysis further mutants were made containing the PIV5 ectodomain and the PIV5 MPER and the TM domain and cytoplasmic tails from heterotypic F proteins (Fig. 4A). The chimeras were tested for surface expression by flow cytometry using the F-specific polyclonal antibody and the prefusion conformation-specific antibody F1a (Table 1, Fig. 4B). The hPIV3 chimera was expressed at wt levels, and the NDV chimera was expressed at 65% of wt levels. The MeV, MuV, and NiV chimeras had much lower surface expression levels. The mutants were also analyzed for the extent of cleavage, which was found to correspond to the surface expression levels (Table 1). These chimeras showed reduced fusion (<40% of wt levels) in luciferase (Fig. 4B) and syncytium assays (data not shown), except the

FIG 3 PIV5 wt F cytoplasmic tail substituted into the C-terminus chimeras partially restores fusion. (A) Schematic of the chimeras with the PIV5 F ectodomain and cytoplasmic tail and the heterotypic MPER and TM domain. (B) Surface expression of the chimeric F proteins was determined as described in the legend to Fig. 2. The mean fluorescence intensity (MFI) is indicated as the percentage of wt F levels (%WT). (C) Fusion activity was measured using the luciferase assay as described in the legend to Fig. 2. Relative luciferase units (RLU) for mutants are expressed as the percentage of wt F RLU. MCS, empty vector with a multiple cloning site. $n = 3$. The statistical significance between wt and mutant is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

hPIV3 chimera (P-P-h-h), which fused at 65% of wt levels. These data suggest that the TM domain and cytoplasmic tail can influence fusion activity.

Heterotypic cytoplasmic tails impair fusion. To investigate the activity of cytoplasmic molecules containing the replacement of single regions, we began by constructing chimeras in which the PIV5 cytoplasmic tail was substituted for a heterotypic cytoplasmic tail (Fig. 5A). The chimeras were expressed in HeLa-CD4-LTR- β gal cells and tested for surface expression and cleavage as described above (Fig. 5B, Table 1). The chimeras containing cytoplasmic tails derived from NDV and hPIV3 reacted with MAb F1a at levels similar to that of wt F, whereas the MeV and MuV chimeras only expressed at 40 to 55% the level of wt F, and the construct containing the NiV cytoplasmic tail (P-P-P-Ni) was expressed poorly on the cell surface. As anticipated from the surface expression levels when fusion activity was measured by the luciferase assay (Fig. 5B) or syncytium assay (data not shown), the chimeras showed low levels of fusion. The most fusogenic among these cytoplasmic tail chimeras was the one containing the NDV cytoplasmic tail (P-P-P-N) that showed 47% fusion activity compared to wt F despite higher levels of cleavage compared to wt (Table 1).

FIG 4 Chimeras with non-native TM domain and cytoplasmic tail are impaired in fusion. (A) Schematic of the chimeras with PIV5 F ectodomain and MPER and the heterotypic TM domain and cytoplasmic tail. (B) Surface expression of the chimeras was determined as described in the legend to Fig. 2. The mean fluorescence intensity (MFI) is indicated as the percentage of wt F levels (%WT). (C) Fusion activity was measured by the luciferase assay as described in the legend to Fig. 2. Relative luciferase units (RLU) for mutants are expressed as the percentage of wt F RLU. MCS indicates the empty vector with a multiple cloning site. $n = 3$. The statistical significance between wt and mutant is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

The data indicate that the correct cytoplasmic tail has a great influence on surface expression levels and fusion activity.

TM domain chimeras cause minimal fusion. Chimeras were made in which the TM domain of PIV5 was substituted with the TM domain of heterotypic F proteins. The extent of cleavage of F mirrored the surface expression levels (Fig. 6B and Table 1). The hPIV3 and NiV TM domain chimeras (P-P-h-P and P-P-Ni-P) were expressed at wt levels on the surface, and NDV chimera (P-P-N-P) was expressed at 65% the level of wt F. In contrast the MuV and MeV chimeras (P-P-Mu-P and P-P-Me-P) were expressed at <40% of wt F. In fusion assays, all of the TM domain chimeras showed <40% fusion, emphasizing the importance of the TM domain in fusion (Fig. 6C).

MPER chimeras show varied phenotypes in fusion. The last residue of HRB is a highly conserved hydrophobic residue (either I, V, or M) in paramyxovirus F proteins (Fig. 1A). MPER in PIV5 F has been designated as the region between the end of HRB at I477 and the postulated beginning of the TM domain at V485 (4). In PIV5 F, six of the seven residues in this region are serine or threonine. However, there is no conservation in the residues of the MPER among the F proteins (Fig. 1A). The MPER chimeras (Fig. 7A) were made by replacing residues

FIG 5 Chimeras with non-native cytoplasmic tail cause reduced fusion. (A) Schematic of the chimera with PIV5 F ectodomain, the MPER and TM domain, and a heterotypic cytoplasmic tail. (B) Surface expression of the chimera was determined as described in the legend to Fig. 2. (C) To measure fusion activity, a luciferase assay was used as described in the legend to Fig. 2. Relative luciferase units (RLU) for mutants are expressed as the percentage of wt RLU (%WT). MCS, empty vector with a multiple cloning site. $n = 3$. The statistical significance between wt and mutant is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

corresponding to this region from the alignment shown in Fig. 1A. The chimeras were expressed and tested for surface expression and cleavage as described above (Fig. 7B, Table 1). The MuV and NDV chimeras were expressed to wt F levels, the hPIV3 chimera was expressed at 63% of wt F, and the MeV and NiV chimeras were expressed at 43% of wt F levels, as seen with F1a reactivity. The extent of cleavage mirrored the surface expression level. In fusion assays, the chimeras showed variable phenotypes. The MuV chimera was 80% as fusogenic as the wt F protein, and the NiV chimera was moderately fusogenic (~60% of wt F levels). On the other hand, the MeV and hPIV3 chimeras showed close-to-background levels of fusion, both in luciferase assays (Fig. 7C) and in syncytium assays (data not shown). In contrast, the NDV chimera was hyperfusogenic, consistently causing fusion at ca. 150% of wt F levels (Fig. 7C), and fusion by the P-N-P-P chimera did not require coexpression of HN (data not shown). Thus, in this respect P-N-P-P behaves in a manner similar to the destabilized PIV5 (W3A) mutant S443P, which shows an HN-independent hyperfusogenic activity.

MPER is an important regulatory region in PIV5 F. Since the MPER sequences of many of the heterotypic F proteins are enriched in serine and threonine but do not show sequence identity

FIG 6 Chimeras with non-native TM domain are debilitated in fusion. (A) Schematic of the chimera with PIV5 F ectodomain, MPER and cytoplasmic tail, and heterotypic TM domain. (B) Surface expression of the chimera was determined as described in the legend to Fig. 2. (C) To measure fusion activity, a luciferase assay was used as described in the legend to Fig. 2. Relative luciferase units (RLU) for mutants are expressed as the percentage of wt RLU (%WT). MCS, empty vector with a multiple cloning site. $n = 3$. The statistical significance between wt and mutant is indicated as follows: *, $P < 0.05$; and **, $P < 0.01$.

and the chimeras result in a range of activity from nonfusogenic to hyperfusogenic proteins compared to wt F protein, the role of the MPER in PIV5 F was studied in greater detail. Alanine point mutations were made in each residue of the PIV5 MPER (except residue 480, which is a native A). A mutant was also constructed in which all of the residues of MPER were substituted with alanine (478-484A). The mutants were tested for surface expression by flow cytometry using MAb F1a as described above (Fig. 8B). The T478A, S479A, T481A, and T482A mutants were expressed at ~80% of wt F levels, the T483A mutant was expressed at ~80% of wt F levels, and the S484A mutant was expressed at 35% of wt F levels. Functionally, the mutations T481A, T482A, and S484A made the mutant F protein hyperfusogenic, as observed in syncytium assays (Fig. 8A) and recapitulated in luciferase assays, where it was found that these mutants cause ~180% of wt F fusion levels (Fig. 8C). It was observed that mutant S484A had a hyperfusogenic phenotype despite its reduced surface expression measured using MAb F1a. T478A mutant retained fusion at wt F levels, whereas mutants S479A and T483A showed reduced fusion (37 and 48% of wt F levels, respectively). The 478-484A mutant only caused fusion at slightly over the background levels, but its cell surface expression level measured using MAb F1a was also greatly reduced.

FIG 7 Chimeras with non-native MPER show varied phenotypes. (A) Schematic of the chimeras with PIV5 F ectodomain, TM domain and cytoplasmic tail, and heterotypic MPER. (B) Surface expression of the chimeras was determined as described in the legend to Fig. 2. (C) To measure fusion activity, a luciferase assay was used as described in the legend to Fig. 2. Relative luciferase units (RLU) for mutants are expressed as the percentage of wt RLU (%WT). MCS, empty vector with a multiple cloning site. $n = 3$. The statistical significance between wt and mutant is indicated as follows: *, $P < 0.05$; and **, $P < 0.01$.

DISCUSSION

Viral fusion proteins are macromolecular machines that enable viral entry into cells by fusing viral envelopes with cellular membranes. Although the atomic structures of many viral fusion proteins are now known (reviewed in reference 19), the C-terminal regions of these proteins remain among their least understood regions both structurally and functionally. Biochemical analyses of several viral fusion proteins have identified length requirements of individual regions of the C termini (2, 3, 16, 49, 55), as well as specific residues that affect activity (1, 4, 8, 18, 20, 26–28, 30, 38, 39, 42, 43, 50). Individual domain replacements have shown various phenotypes from none to significant effects on fusion activity (8, 18, 26, 29, 37, 39, 51). However, extensive studies of the individual contributions and the interrelationship of the various regions of the C termini, in the context of a functional fusogenic molecule, have not been described previously.

In the present study we found that the replacement of the native PIV5 F protein sequences with the equivalent region from other paramyxovirus F proteins can affect cell surface expression, from having no effect to a significant reduction in surface expression. Most likely the reduced surface expression is because of misfolding of the chimeric proteins (13) due to addition of the het-

erotypic C-terminal regions. The rate of cleavage of F0 to F1,F2, which is indicative of the rate of transport to the *trans*-Golgi network, was also delayed for many chimeric F proteins (Table 1).

The chimeric F proteins with the replacement of the entire C terminus are inactive in causing cell-cell fusion. Even the NDV and hPIV3 chimeras (P-N-N-N and P-h-h-h), which exhibit wt F surface expression levels, caused no fusion activity. The available data indicate that this sensitivity is a function of an increased stability of the chimeric protein because when the chimeras contain the destabilizing mutation S443P (32), some fusion activity was restored. The current model of F refolding from pre- to postfusion conformations requires as an early step the HRB trimeric stalk to separate, so that at a later stage the TM domains can rotate to allow formation of the six-helix bundle (53). It seems possible that the chimeras are unable to carry out this opening up of the C-terminal stalk due to an increased stability of the chimeric trimers. The data indicate that the chimeras are trapped at a prelipidic stage of fusion as in dye transfer assays the chimeras in a wt F background showed no transfer of the lipidic dye R-18 (data not shown). It has been proposed that the homotypic TM domain and the fusion peptide interact specifically (14) at a late stage in fusion. Thus, for the chimeras a failure of the TM domain and fusion peptide to associate does not seem to be a likely explanation for the lack of fusion.

To analyze the contribution of the individual regions of the PIV5 F protein C terminus, we constructed a panel of chimeric F proteins switching the corresponding sequences from the F proteins of MuV, MeV, NDV, NiV, and hPIV3. The data indicate that none of the native PIV5 F sequences can be replaced without an effect on fusion activity. We suggest the various regions of the C terminus are synergized (probably by forming a larger structural motif) such that the effects of individual replacements do not lead to a fully functional structural motif and hence the fusion level exhibited by the chimeras does not reach wt levels of fusion activity.

Mutations within a C-terminal segment can also affect fusion activity. We have shown previously that mutations within the TM domain can block fusion activity (L486A and I488A) (4). In addition, we have found that the PIV5 F MPER could not tolerate insertions of amino acids and retain biological activity but it could tolerate deletion of specific residues (3, 55). Here, we found that mutations within the MPER show both stabilizing and destabilizing activity (less fusion or increased fusion compared to wt F, respectively), suggesting the MPER is a highly sensitive region of the protein.

Structural studies have shown that the F protein region known as HRB forms α -helices in both the prefusion and the postfusion conformations of F (52, 53), and HRB is directly coupled to the serine/threonine-rich seven-residue MPER. In turn, MPER is coupled to the TM domain. Mutagenesis and modeling studies on the PIV5 TM domain indicate the domain is a three-helix bundle that contains a pi-bulge (4). Thus, it is tempting to model the MPER as an α -helical region continuous with HRB and the TM domain. The inability of two of the MPER substitutions to be biologically active may be due to disruption of specific protein structural motifs that are made from residues that span outside the MPER.

Our previous analysis has shown that most of the PIV5 F (W3A isolate) 20 residue cytoplasmic tail can be deleted and the biological activity retained (16, 49). However, different isolates of PIV5

FIG 8 MPER is an important regulatory region in PIV5 F. (A) BHK-21F cells cotransfected with PIV5 HN and PIV5 F WT or alanine point mutant or all-alanine mutant as indicated were imaged at 20 h posttransfection. (B) Surface expression of the chimeras was determined as described in the legend to Fig. 2. (C) To measure fusion activity, a luciferase assay was used as described in the legend to Fig. 2. Relative luciferase units (RLU) for mutants are expressed as the percentage of wt RLU (%WT). MCS, empty vector with a multiple cloning site. $n = 3$. The statistical significance between wt and mutant is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

have an F protein with either a short (20-residue) or long (42-residue) cytoplasmic tail and a long cytoplasmic tail suppresses fusion activity in a sequence-specific manner. Mutagenesis studies, together with the addition of a domain that has the propensity to form a 3-helix bundle suggested that a long 42-residue cytoplasmic tail forms a stable structure that suppresses fusion activation. It was inferred that F protein activation involves cytoplasmic signaling to the ectodomain (48). As discussed above, in transitioning from the prefusion conformation to the postfusion conformation, the F TM domains must rotate in the plane of the membrane to allow the 6-helix bundle to form. This provides a possible explanation for the effect of a structure in the longer cytoplasmic tail on suppressing fusion (48). Alternatively, a structure in the longer cytoplasmic tail might prevent clustering of trimeric pre-hairpin intermediates that are thought to form the fusion prepore complex (14). The inability of many of the PIV5 F TM domain chimeras and the cytoplasmic tail chimeras to cause fusion may be because specific protein structures in either each of the domains or both in combination are ablated.

In summary, the PIV5 F C-terminal region appears to be a specialized region that contributes to fusion in a sequence-specific manner. The individual parts of the C terminus act concertedly in the process, and the native protein in its entirety is required for obtaining wt levels of fusion. In addition, the MPER seems to provide another layer of regulation to the protein, containing both hyperfusogenic and hypofusogenic regions, giving rise to a switch to control fusion.

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