

Differences in the Role of the Cytoplasmic Domain of Human Parainfluenza Virus Fusion Proteins

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We have investigated the roles of the cytoplasmic domains of the human parainfluenza virus type 2 (PI2) and type 3 (PI3) fusion (F) proteins in protein transport and cell fusion activity. By using the vaccinia virus-T7 transient expression system, a series of F protein cytoplasmic tail truncation mutants was studied with respect to intracellular and surface expression and the ability to induce cell fusion when coexpressed with the corresponding hemagglutinin-neuraminidase (HN) proteins. All of the cytoplasmic tail truncation mutants of PI2F were expressed at high levels intracellularly or on cell surfaces as measured by immunoprecipitation and cell surface biotinylation assays. In addition, when coexpressed with PI2HN, these truncation mutants of PI2F were all found to be essentially unimpaired in the ability to induce cell fusion as measured by a quantitative cell fusion assay. In contrast, surface expression and cell fusion activity were found to be eliminated by a mutant of PI3F in which the entire cytoplasmic tail was deleted, and the mutant protein appeared to be unable to assemble into a high-molecular-weight oligomeric structure. To further investigate whether there is a specific sequence requirement in the cytoplasmic tail of PI3F, a chimeric protein consisting of the PI3F extracellular and transmembrane domains and the PI2F cytoplasmic tail was constructed. This chimeric protein was detected on the surface, and it was capable of inducing cell fusion when expressed together with PI3HN, although the fusogenic activity was reduced compared with that of wild-type PI3F. These results demonstrate that although PI2 and PI3 viruses belong to the same parainfluenza virus genus, these viruses show marked differences with respect to functional requirements for the cytoplasmic tail of the F glycoprotein.

The human parainfluenza viruses are members of the family *Paramyxoviridae* and encode two glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins (26). The HN protein provides an attachment function, allowing the virus to bind to sialic acid-containing receptors on the cell surface, and also plays an undefined role in subsequent membrane fusion (18, 20), whereas the F protein is directly involved in virus-cell or cell-cell membrane fusion (6, 7). The F protein is synthesized as a biologically inactive precursor (F₀) which is then cleaved into the disulfide-linked F₁-F₂ subunits, the biologically active form for fusion, by a cellular trypsin-like protease. The exposure of a hydrophobic domain at the amino terminus of the F₁ subunit appears to be essential in mediating membrane fusion (20, 38).

Previous studies in our laboratory showed that cell fusion caused by human parainfluenza virus type 2 (PI2) and type 3 (PI3) glycoproteins requires coexpression of F and HN proteins from the same virus type (18). Other reports showed similar type-specific interactions among paramyxovirus glycoproteins (3, 5, 13, 16, 22, 50). We have been interested in defining functionally important regions of the F and HN proteins that may be involved in the cell fusion process. A number of studies have reported on the functional regions of HN involved in cell fusion (23, 40, 41). Analysis of two deletion mutants of Newcastle disease virus (NDV) HN indicated that the attachment function could be separated from the fusion promoting activity (41). By using site-directed mutagenesis, evidence was obtained that mutations in the transmembrane domain of the HN protein of NDV affect the tetrameric structure, attachment activity, and fusion promotion activity (23). In contrast, fewer studies of the role of specific protein sequences in the F protein have been reported. It has been reported that

disruption of a leucine zipper motif of the measles virus F protein affects syncytium formation (4) and that mutations in the fusion peptide and heptad repeat regions of the NDV F protein also affect fusion (42, 49).

The cytoplasmic domains of integral membrane proteins are known to be involved in several biological processes. In several studies of cytoplasmic tail mutants of human immunodeficiency virus type 2 and simian immunodeficiency virus, it has been reported that truncation mutants significantly enhance cell fusion activity (27, 34) and alter the conformation of the external domain of the envelope protein (44). This altered conformation could play a role in enhancing the fusion activity of the truncated simian immunodeficiency virus glycoprotein. Members of the family *Paramyxoviridae* show high-level variability in the sequences of the cytoplasmic tail (17, 26), which might indicate functional differences for their cytoplasmic domains. In this report, we have constructed a series of cytoplasmic tail deletion mutants of the PI2F and PI3F glycoproteins and compared their expression, processing, and transport in HEp-2 and HeLa-T4 cells. Using a quantitative fusion assay, we compared the fusogenic activities of the different cytoplasmic tail deletion mutants of the PI2F and PI3F proteins. Finally, we constructed a PI3F-PI2F chimera to further examine the roles of specific sequences in the cytoplasmic tail in protein transport and cell fusion.

MATERIALS AND METHODS

Cells, viruses, and recombinant plasmids. HEp-2, CV-1, and HeLa-T4 cells were maintained in Dulbecco's minimal essential medium (DMEM) with 10% newborn calf serum (HyClone Laboratories, Inc., Logan, Utah). Vaccinia virus (VV; IHD-J strain) and the VV-T7 recombinant virus (WR strain) (10) were obtained from Bernard Moss (National Institutes of Health, Bethesda, Md.). VV-T7 was grown on HeLa-T4 cells, and titers of the virus were determined on CV-1 cells. The PI2F and PI2HN clones were described previously (17). They were subcloned in pGEM-3 and pGEM-3Zf(-) plasmids (Promega Biotech, Madison, Wis.). The PI3F and PI3HN cDNA clones were kindly provided by Mark Galinski (Cleveland Clinic Foundation, Cleveland, Ohio). They were then

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subcloned in pGEM-3 and pGEM-4. Plasmid pG1NT7 β -gal was kindly provided by Edward Berger (National Institutes of Health).

Construction of F protein cytoplasmic tail truncation mutants. Clones encoding PI2F cytoplasmic tail truncation mutants PI2F-d15, PI2F-d25, and PI2F-d34 (see Fig. 1A) were constructed by introducing premature stop codons by mutations at nucleotide positions 1781 (G to T), 1752 (C to A), and 1724 to 1725 (CT to TA), respectively, by PCR amplification. The left outside primer was 5'-GCCAATGACTGTGTCGTGACACCG, which contained an *AspI* site. The right outside primers were 5'-AAAGAAGGCATGCTTTTACCTGTGGAA for PI2F-d15, 5'-TGTTGTAGCATGCAGCTATCTGAATTG for PI2F-d25, and 5'-GATTTGGCATGCCTACTTGTATGATGTA for PI2F-d34. All right outside primers carried a stop codon and a *SphI* site. PCR amplifications were carried out by using the pGEM-3-PI2F plasmid as a template and 30 cycles with steps of 1 min at 94°C, 2 min at 65°C, and 3 min at 72°C. PCR products were isolated and purified by agarose gel electrophoresis and were digested with *AspI* and *SphI*. The resulting fragments containing the desired mutations were cloned into a pGEM-3-PI2F vector that had been digested with *AspI* and *SphI*. All constructs were sequenced by using a Sequenase sequencing kit (United States Biochemicals, Cleveland, Ohio) to confirm that the correct mutations were present.

Clones encoding PI3F cytoplasmic tail truncation mutants PI3F-d5, PI3F-d13, and PI3F-d22 (see Fig. 1B) were constructed by methods similar to those described above by introducing premature stop codons by mutations at nucleotide positions 1796 to 1797 (GT to TA), 1772 to 1773 (GT to TA), and 1747 (T to G) for PI3F-d5, PI3F-d13, and PI3F-d22, respectively. The left outside primer was 5'-CCAAGATATGCATTTGTCAATGGAGGAGTGGTT, which contained a *NsiI* site. The right outside primers were 5'-CTCTTTGAAGCTTGTACTACTAAC for PI3F-d22, 5'-CATATGGTTAAGCTTTTGTATCCTATCGATT for PI3F-d13, and 5'-CTGTCATTTGAAGCTTAATTAATATGG for PI3F-d5. All the right outside primers carried a stop codon and a *HindIII* site. PCR amplifications were performed, and the desired mutated fragments were cloned into plasmid pGEM-3-PI3F by procedures similar to those described above.

Construction of a PI3F-PI2F chimera. The extracellular and transmembrane domain sequences of the PI3F-PI2F chimera were from PI3F, while the cytoplasmic domain sequence of the PI3F-PI2F chimera was from PI2F. The chimera was constructed by the overlapping PCR technique described by Ho et al. (14) and Horton et al. (15). Briefly, PCR was performed to generate a fragment from the PI3F portion which had 15 bp homologous with PI2F at the 3' end and a fragment of PI2F which had 15 bp homologous with PI3F at the 5' end. A second PCR was carried out by mixing these two segments, allowing the 15-bp homologous parts to anneal with each other, and then extending to both sides to generate the PI3F-PI2F chimera. Five rounds after self-annealing and extension, two outer primers were added to the reaction mixture and PCR was carried out for 25 more cycles. The left outer primer was 5'-CCAAGATATGCATTTGTCAATGGAGGAGTGGTT, which had a *NsiI* site, and the right outer primer was 5'-GACATATATAAGCTTCTGATAGAT, which had a *HindIII* site. The chimeric fragment and PI3F plasmid were digested with *NsiI* and *HindIII*, and the chimeric fragment was then cloned into the enzyme-digested PI3F expression vector to generate the PI3F-PI2F chimera. The chimera sequence was confirmed by sequence analysis.

Antisera. Polyclonal rabbit antibodies against PI2 and PI3 viruses were generated by the method described by Hu et al. (18). Monoclonal antibodies against PI3F were kindly provided by K. Örvell (SBL Karolinska Institute, Stockholm, Sweden).

Radiolabeling and immunoprecipitation. To use the VV-T7 expression system, nearly confluent monolayers of HEp-2 cells grown in 35-mm-diameter dishes were infected with the VV-T7 recombinant (10) at a multiplicity of infection of 10 and incubated at 37°C for 1 h. Cells were carefully washed with phosphate-buffered saline (PBS) three times and then transfected with 5 μ g of plasmid DNA and 10 μ l of lipofectin (Gibco BRL Life Technologies) in 2 ml of DMEM. Cells were incubated for 16 h, starved in Dulbecco's modified Eagle's medium lacking methionine and cysteine for 30 min, and labeled with 100 μ Ci of [³⁵S]methionine and [³⁵S]cysteine per ml for 1 h at 37°C. For pulse-chase studies, cells were pulse-labeled with 100 μ Ci of [³⁵S]methionine and [³⁵S]cysteine per ml for 15 min and chased for various periods with DMEM containing 10% newborn calf serum. Immunoprecipitation was performed as described previously (33). Proteins were characterized by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-8% PAGE) and subsequent autoradiography.

Chemical cross-linking. Chemical cross-linking was performed by a method similar to that described by Paterson and Lamb (31). Sixteen hours after transfection, cells were labeled with 100 μ Ci of [³⁵S]methionine and [³⁵S]cysteine per ml for 2 h, chased for 1 h, and removed from the dish with 2 mM EDTA in PBS deficient in Mg²⁺ and Ca²⁺ (PBS⁻). Then cells were pelleted by centrifugation at 1,000 rpm (Eppendorf 5415C centrifuge) for 5 min and resuspended in 100 μ l of PBS⁻ (pH 8.5). Cells were solubilized in 0.5% Nonidet P-40, and cross-linking reactions were carried out with various concentrations (from 50 μ M to 4 mM) of 3,3'-dithiobis sulfosuccinimidylpropionate (DTSSP) (Pierce, Rockford, Ill.) at 4°C for 60 min and then quenched with 50 mM glycine for 5 min at 4°C. Immunoprecipitations were performed after cross-linking reactions. Proteins were analyzed by SDS-3.5% PAGE under nonreducing conditions.

Cell surface biotinylation assay. (i) **Radioactive cell surface biotinylation assay.** Cell surface proteins were identified by a procedure similar to that described by Le Bivic et al. (21). At 16 h posttransfection, cells were starved in cysteine- and methionine-deficient DMEM for 30 min. Then cells were pulse-

labeled with 100 μ Ci of [³⁵S]methionine and [³⁵S]cysteine per ml for 15 min and chased for various periods with DMEM containing 10% newborn calf serum. At the end of the pulse or chase period, cells were washed three times with ice-cold PBS-CM (PBS - containing 0.1 mM CaCl₂ and 1 mM MgCl₂) and incubated with 1 ml of a 0.5-mg/ml solution of sulfo-succinimidyl-2-(biotinamido) ethyl-1,3-dithiopyronate (NHS-SS-biotin; Pierce) in PBS-CM at 4°C for 30 min. Free biotin was blocked by being mixed with DMEM, and cells were washed three times with ice-cold PBS-CM. Then cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1% SDS, 1 mM EDTA, protease inhibitors [aprotinin, 2.0 mg/ml; E-64, 1 mg/ml; leupeptin, 0.5 mg/ml; pepstatin, 0.7 mg/ml; phosphoramidon, 10 mg/ml]). Then cell lysate samples were immunoprecipitated with PI3F-specific antisera and protein A-agarose beads (Pierce). Cell lysate beads were washed three times in lysis buffer and divided into two equal aliquots. One aliquot was used for immunoprecipitation. The other aliquot was boiled in 20 μ l of 10% SDS for 5 min and diluted with 1 ml of lysis buffer. Boiled beads were removed by centrifugation for 1 min in a microcentrifuge, and the supernatant was incubated with streptavidin-agarose beads (Pierce) at 4°C. Then beads were washed three times with lysis buffer. Proteins were characterized by SDS-8% PAGE and subsequent autoradiography.

(ii) **Nonradioactive cell surface biotinylation assay.** Sixteen hours after transfection, cells in 35-mm-diameter culture dishes were washed five times with PBS-CM and incubated with 0.5 mg of sulfo-NHS-biotin (Pierce) per ml for 30 min at 4°C. The reaction was then quenched for 10 min with DMEM and washed four times with PBS-CM containing 20 mM glycine. Then cells were lysed and immunoprecipitated. Samples were run on an SDS-PAGE gel, and the proteins were subsequently blotted onto a nitrocellulose membrane. Nitrocellulose was blocked with 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBS-T) at 4°C overnight. The nitrocellulose membrane was washed with PBS-T and probed with a 1:2,500 dilution of horseradish peroxidase-conjugated streptavidin in PBS-T for 1 h at room temperature. The blot was washed again in PBS-T before carrying out the enhanced chemiluminescence detection procedure (ECL Western blotting kit; Amersham). Densitometry was performed on the images after video capture. Each band of interest was quantitated by using the IPLab gel program (Densitometry and Gel Analysis on the Macintosh; Signal Analytics Corporation). The density of the negative control, which was the transfection of the vector only, was used as a background value. The density of each band was normalized for surface area, and the result was expressed as a percentage of the value obtained for wild-type PI2F or PI3F, which was taken as 100%.

Endo H treatment. Proteins were immunoprecipitated with PI2- or PI3F-specific antisera and protein A-agarose beads. The beads were washed with lysis buffer and treated with or without 5 mU of endoglycosidase H (endo H; Boehringer Mannheim, Indianapolis, Ind.) in 100 mM sodium acetate (pH 5.5) for 16 h at 37°C. Proteins were characterized by SDS-8% PAGE and subsequent autoradiography.

Cell fusion assay. A quantitative cell fusion assay was performed with some modifications of the method described by Nussbaum et al. (29). Cells were infected with VV-T7, transfected with wild-type HN and a series of F cytoplasmic tail truncation mutants, and then treated with neuraminidase to prevent cell fusion from occurring before the two cell types were mixed. Cytosine arabinoside (40 μ g/ml) was added to each plate. A second cell population was infected with wild-type VV strain IHD-J and transfected with plasmid pG1NT7 β -gal containing a T7 promoter. Each cell population was incubated separately overnight at 31°C. To determine that the amount of cells used was within the linear range of production of the enzyme, the β -galactosidase production abilities of different amount of cells and different ratios of the two cell populations were determined. One hundred microliters containing 10⁵ cells from each population mixed at a 1:1 ratio was chosen for fusion assays. At 16 h posttransfection, after neuraminidase had been removed, cells were aliquoted into individual wells of 96-well flat-bottom tissue culture plates. Plates were incubated in a 37°C tissue culture incubator, and cell fusion was allowed to occur. If two cells fused, the β -galactosidase gene was expressed by the T7 polymerase from the other cell population. Cell fusion was monitored at the indicated times by reporter gene activation assay for β -galactosidase, either a colorimetric lysate assay or an in situ staining assay.

For the colorimetric lysate assay, 5 h after cells had been mixed, 5 μ l of 20% (vol/vol) Nonidet P-40 was added to each well. Fifty microliters of each lysate was mixed with 50 μ l of 2 \times substrate solution (16 mM chlorophenol red- β -D-galactopyranoside [CPRG; Boehringer Mannheim], 0.12 M Na₂HPO₄ · 7H₂O, 0.08 M NaH₂PO₄ · H₂O, 0.02 M KCl, 0.002 M MgSO₄ · 7H₂O, 0.01 M β -mercaptoethanol). β -Galactosidase activities were quantitated at ambient temperatures on 96-well flat-bottom plates. The rate of substrate hydrolysis at ambient temperature was monitored by measuring the optical density at A₅₇₀ at various times with a microplate absorbance autoreader (EL 311; Bio-Tek Instruments). The quantity of β -galactosidase was calculated by comparing the hydrolysis rate for each sample with that of a standard commercial preparation of *Escherichia coli* β -galactosidase (Boehringer Mannheim). Data were analyzed by using the Delta Soft II Microplate analysis program. β -Galactosidase levels are reported in nanograms per well.

For the in situ staining assay, 10 h after cells had been mixed, 20 μ l of 10 \times fixative solution (20% formaldehyde-2% glutaraldehyde in PBS) was added to each well. The plate was then incubated at 4°C for 5 min. One hundred fifty microliters of medium was removed, and 150 μ l of 37°C equilibrated staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM

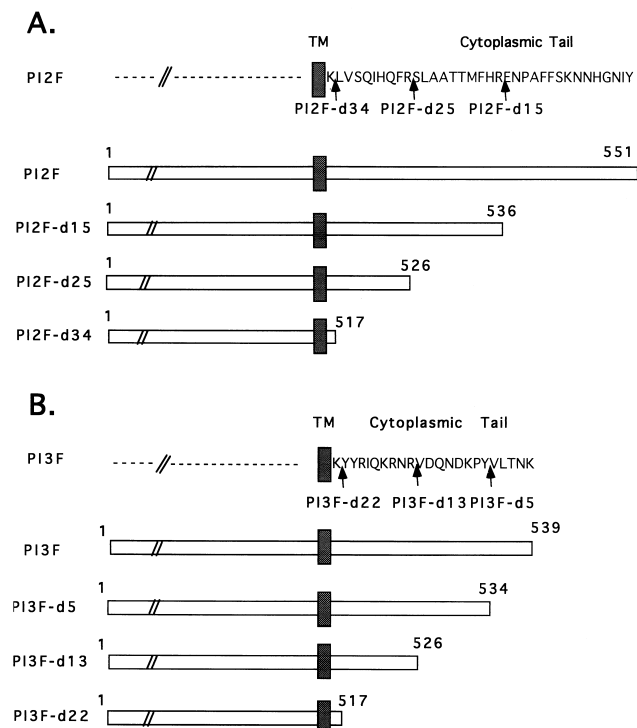


FIG. 1. Schematic diagrams of the full-length PI2F and PI3F proteins and cytoplasmic tail truncation mutants. (A) The 35 amino acids of the full-length PI2F cytoplasmic tail are shown. Each shaded box represents the transmembrane anchor (TM). Arrows indicate where the premature stop codons were introduced. The PI2F cytoplasmic tail truncation mutants PI2F-d15, PI2F-d25, and PI2F-d34 are aligned at the transmembrane and cytoplasmic domains with full-length PI2F. (B) The 23 amino acids of the full-length PI3F cytoplasmic tail are shown. The cytoplasmic tail truncation mutants PI3F-d5, PI3F-d13, and PI3F-d22 are aligned at the transmembrane and cytoplasmic domains with full-length PI3F.

magnesium chloride, 1 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside [X-Gal; Boehringer Mannheim] per ml) was added. The plate was incubated at 37°C overnight. Photomicrographs were taken with an inverted tissue culture microscope (magnification of $\times 100$).

RESULTS

Construction and expression of cytoplasmic tail truncation mutants of parainfluenza virus F proteins. To determine whether the cytoplasmic tail of the parainfluenza virus F protein plays a role in protein transport or function, a series of cytoplasmic tail truncation mutants was constructed for the PI2F and PI3F proteins by using PCR to introduce premature stop codons into the coding sequences. Figure 1 shows a schematic diagram of the full-length F protein and truncated F mutants. As shown in Fig. 1A, wild-type PI2F has 35 amino acids in the cytoplasmic domain, whereas mutants PI2F-d15, PI2F-d25, and PI2F-d34 have C-terminal truncations in their cytoplasmic domains of 15, 25, and 34 amino acids, respectively. The PI2F-d34 mutant has the entire cytoplasmic tail deleted except for one positively charged lysine at the junction of the cytoplasmic and transmembrane domains. PI2F-d25 and PI2F-d15 retain 10 and 20 amino acids of their cytoplasmic domains, respectively. The cytoplasmic tail truncation mutants of PI3F were constructed in a similar pattern. As depicted in Fig. 1B, wild-type PI3F has 23 amino acids in the cytoplasmic domain, while mutants PI3F-d5, PI3F-d13, and PI3F-d22 have C-terminal truncations of 5, 13, and 22 amino acids, respectively. Thus, mutant PI3F-d22 has the entire cytoplasmic tail deleted except for one remaining positively charged lysine,

while PI3F-d13 and PI3F-d5 retain 10 and 18 amino acids of their cytoplasmic domains, respectively.

To compare the synthesis and processing of wild-type and mutant F proteins, the VV-T7 transient expression system was used; the expression of the wild type and truncation mutants was detected by immunoprecipitation. As shown in Fig. 2A, wild-type PI2F (lane 1) and truncated mutant proteins PI2F-d15 (lane 4), PI2F-d25 (lane 3), and PI2F-d34 (lane 2) were expressed and both the F_0 precursor and F_1 cleavage products were detected. The diffuse bands above F_0 appear to be differently glycosylated forms of F_0 , since they disappeared after endo H treatment of samples (data not shown). The sizes of the truncation mutants decreased progressively compared with that of the full-length protein (Fig. 2A). In Fig. 2B, both F_0 and F_1 were detected for PI3F, PI3F-d5, and PI3F-d13, and the two truncation mutants (PI3F-d5 and PI3F-d13) showed decreased molecular weights, compared with that of wild-type PI3F, when the proteins were run under reducing conditions. However, for PI3F-d22 (Fig. 2B, lane 2), F_1 was not detected while F_0 was detected at a level similar to those of the other constructs. In Fig. 2C, when the proteins were run under nonreducing conditions, a high-molecular-mass band (estimated molecular mass of about 180 kDa) was detected for PI3F, PI3F-d13, and PI3F-d5, but not for PI3F-d22. It therefore seemed possible that PI3F, PI3F-d13, and PI3F-d5 formed stable oligomers while PI3F-d22 was not assembled into an oligomeric form.

To further investigate the nature of the high-molecular-weight band detected by SDS-PAGE, the chemical cross-linking reagent DTSSP was used to compare PI3F and mutant PI3F-d22 for the ability to be cross-linked by the chemical reagent. In Fig. 3A, three predominant species (1, 2, and 3) were detected for PI3 virus-infected cells (lane 1) and PI3F-transfected cells (lane 2), but only species 1 and 2 were detected for mutant PI3F-d22 (lane 3) when a 1 mM concentration of the cross-linking reagent DTSSP was used. In Fig. 3B, in the absence of DTSSP, species 1 was detected for both PI3 virus-infected cells (lane 1) and PI3F-transfected cells (lane 7). With increasing amounts of DTSSP (from 50 μ M to 4 mM), the amounts of species 2 and especially species 3 increased progressively (Fig. 3B, lanes 2 to 6 for PI3 virus-infected cells and lanes 8 to 10 for PI3F-transfected cells). The molecular masses for these three species are estimated as follows: species 1, 60 kDa; species 2, 120 kDa; and species 3, 180 kDa. These results are consistent with those of a previous report (37) that paramyxovirus F proteins form trimers. The position of the high-molecular-weight band in Fig. 2 is similar to the position of species 3 (180 kDa) when the cross-linker DTSSP was used in either PI3 virus-infected cells or PI3F-transfected cells. Since PI3F-d22 failed to show species 3 even when the cross-linking reagent was present, it may be defective in assembly into the oligomeric form required for its transport to the cell surface. Sequence analysis of the entire gene confirmed that no second-site mutation occurred during the construction of this mutant.

Cell fusion activities of F truncation mutants. To compare the cell fusion activities of the F protein cytoplasmic tail truncation mutants with that of the wild-type F protein, we used a quantitative cell fusion assay as well as an in situ staining assay. Figure 4 shows the results of a colorimetric assay that quantitated the β -galactosidase activities resulting from cell fusion after transfection with different combinations of wild-type HN and various truncation mutants of F protein. In Fig. 4A, when coexpressed with PI2HN, the β -galactosidase level detected for PI2F was 21.57 ng per well and those detected for mutants PI2F-d15, PI2F-d25, and PI2F-d34 were 17.33, 19.51, and 20.98 ng per well, respectively. The corresponding cell fusion

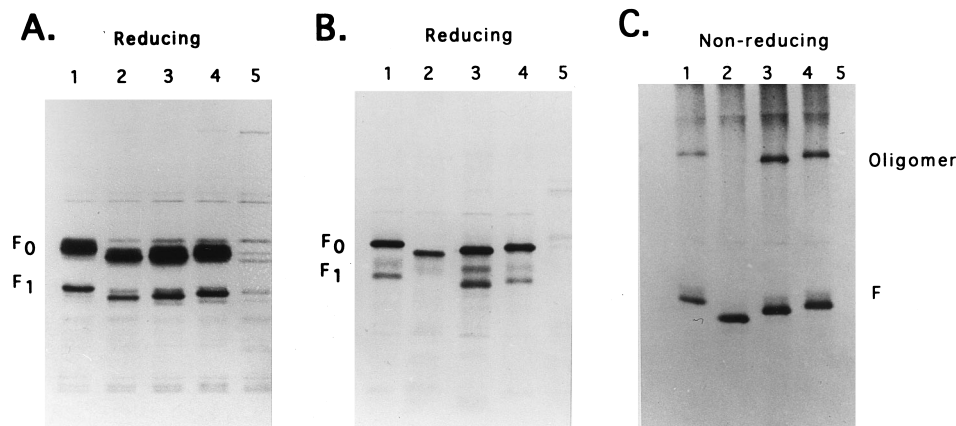


FIG. 2. Immunoprecipitation of the proteins expressed by full-length and cytoplasmic tail truncation mutants of PI2F and PI3F by using the VV-T7 transient expression system in HEp-2 cells. (A) Cells were infected with VV-T7 at a multiplicity of infection of 10 for 1 h at 37°C and then transfected with plasmids expressing PI2F (lane 1), PI2F-d34 (lane 2), PI2F-d25 (lane 3), PI2F-d15 (lane 4), and the vector (lane 5). At 16 h posttransfection, cells were labeled with 100 μ Ci of [³⁵S]methionine and [³⁵S]cysteine per ml for 1 h at 37°C. Then cells were lysed and immunoprecipitated with rabbit anti-PI2 antiserum and protein A-agarose beads. Proteins were analyzed by SDS-8% PAGE under reducing conditions. (B) Cells were transfected with plasmids expressing PI3F (lane 1), PI3F-d22 (lane 2), PI3F-d13 (lane 3), PI3F-d5 (lane 4), and the vector (lane 5) (negative control). Immunoprecipitation was done with mouse anti-PI3F-specific antibody, and proteins were analyzed by SDS-8% PAGE under reducing conditions. (C) Cells were transfected with plasmids expressing PI3F (lane 1), PI3F-d22 (lane 2), PI3F-d13 (lane 3), and PI3F-d5 (lane 4). Immunoprecipitation was done as described for panel B, and proteins were analyzed under nonreducing conditions.

levels for mutants PI2F-d15, PI2F-d25, and PI2F-d34 were calculated to be 81, 90, and 97% of the wild-type level, respectively (Table 1). The results indicate that these cytoplasmic tail truncation mutants of the PI2F protein are able to induce cell fusion to an extent similar to that of wild-type PI2F protein.

In contrast to the results with PI2F, when PI3F-d22 was coexpressed with PI3HN, no β -galactosidase activity was detected (Fig. 4B). For mutants PI3F-d5 and PI3F-d13, β -galactosidase activities were detected at levels of 17.98 and 12.52 ng were well, respectively, while the level detected for wild-type PI3F was 23.55 ng per well. The cell fusion activities of PI3F-d22, PI3F-d5, and PI3F-d13 were 0, 76, and 53%, respectively, relative to that of wild-type PI3F (Table 1). These findings indicate that in contrast to the results with PI2F, the removal

of the cytoplasmic tail of PI3F (mutant PI3F-d22) totally abolished cell fusion.

These observations were also confirmed by in situ staining in which fused cells were directly stained by X-Gal in the monolayer (Fig. 5). Figure 5B through E show that when PI2F, PI2F-d15, PI2F-d25, and PI2F-d34 were coexpressed with PI2HN, the extents of cell fusion were similar. As shown in Fig. 5G, I, and J, when PI3F, PI3F-d13, and PI3F-d5 were coexpressed with PI3HN, extensive cell fusion was observed. However, when PI3F-d22 and PI3HN were coexpressed (Fig. 5H), no fusion was observed. Therefore, these results again confirmed that cell fusion activity was abolished by the cytoplasmic tail truncation mutant of PI3F.

The cytoplasmic tail is necessary for cell surface expression of PI3F but not PI2F. To explore whether the cytoplasmic tail truncation mutants had been transported to the cell surface, a cell surface biotinylation assay was performed. As shown in Fig. 6A, all of the PI2F truncation mutants were efficiently transported to the cell surface. By comparison with the surface expression level of wild-type PI2F, the surface expression levels of mutants PI2F-d15, PI2F-d25, and PI2F-d34 were 102, 102, and 75%, respectively. When we normalized the cell fusion activities to the surface expression levels, the fusogenic activities for mutants PI2F-d15, PI2F-d25, and PI2F-d34 were determined to be 80, 88, and 125%, respectively (Table 1). This implies that the PI2F protein needs only its extracellular and transmembrane domains to undergo transport to the cell surface. Once this fusion protein has been transported to the surface, it can induce cell fusion to a similar extent regardless of whether it has a full-length or truncated cytoplasmic domain.

As shown in Fig. 6B, the F₁ of mutant PI3F-d22 was not detected on the cell surface, nor was it secreted into the medium (data not shown). The F₁ surface expression levels for PI3F-d13 and PI3F-d5 (compared with that of the wild type set at 100%) were 61 and 98%, respectively (Fig. 6B). The cell fusion activity levels for PI3F-d13 and PI3F-d5 were 53 and 76%, respectively (Table 1). The normalized fusogenic activities for mutants PI3F-d13 and PI3F-d5 were 87 and 78%, respectively. These results indicate that mutants PI3F-d5 and

TABLE 1. Cell surface expression and fusogenic activity of F constructs

F construct	Cell surface expression (%) ^a		Cell fusion (%) ^b	Fusogenic activity ^c
	F ₀	F ₁		
PI3F	100	100	100	1
PI3F-d22	11	0.9	0	0
PI3F-d13	83	61	53	0.87
PI3F-d5	99	98	76	0.78
PI2F	ND	100	100	1
PI2F-d34	ND	75	97	1.25
PI2F-d25	ND	102	90	0.88
PI2F-d15	ND	102	81	0.80
PI3F-PI2F chimera	71	49	30	0.61

^a On the basis of the cell surface biotinylation gels shown in Fig. 6 and 8, cell surface expression levels were quantitated and analyzed by using the IPLab gel program. The cell surface expression of PI2F and PI3F was taken as 100%. ND, not done.

^b On the basis of the cell fusion colorimetric assay results shown in Fig. 4 and 9, the percentage of cell fusion for wild-type PI2F and PI3F were set at 100%. For each mutant, the quantity of β -galactosidase for each sample divided by the quantity of β -galactosidase for the respective wild-type protein was used to calculate the percent fusion.

^c Fusogenic activity of each sample = cell fusion/F₁ cell surface expression.

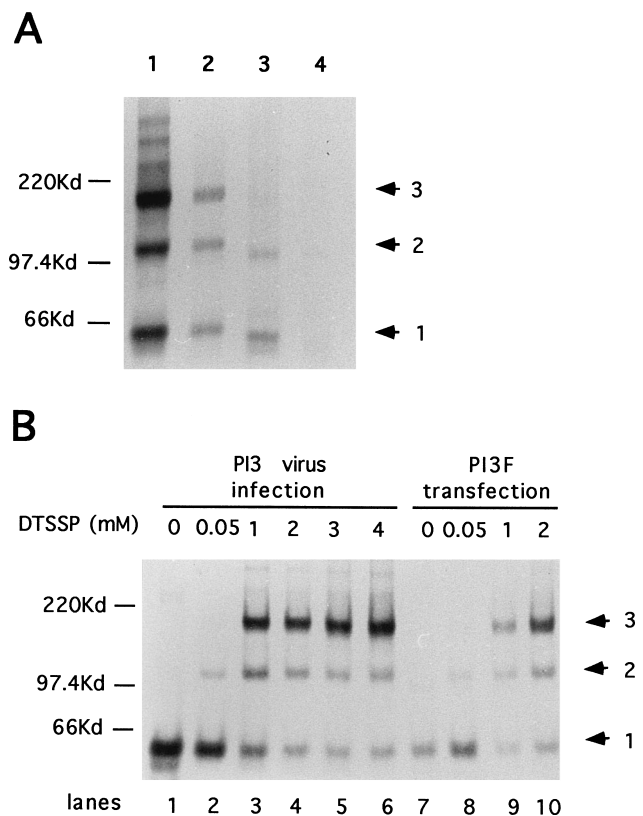


FIG. 3. Cross-linking of wild-type PI3F and mutant PI3F-d22. (A) Cells were infected with PI3 virus (lane 1) or transfected with wild-type PI3F (lane 2), mutant PI3F-d22 (lane 3), or the vector (lane 4). At 16 h posttransfection, cells were labeled with 100 μ Ci of [35 S]methionine and [35 S]cysteine for 2 h and incubated in chase medium for 1 h. Cross-linking reactions were performed by using 1 mM DTSSP on cell suspensions in the presence of 0.5% Nonidet P-40 as described in Materials and Methods. (B) Cells were infected with PI3 virus (lanes 1 to 6) or transfected with wild-type PI3F (lanes 7 to 10). After cells had been labeled as described above, the following concentrations of DTSSP were used: 0 (lanes 1 and 7) 50 μ M (lanes 2 and 8), 1 mM (lanes 3 and 9), 2 mM (lanes 4 and 10), 3 mM (lane 5), and 4 mM (lane 6). After immunoprecipitation, polypeptides were analyzed by SDS-3.5% PAGE under nonreducing conditions. The positions of the three predominant species (1, 2, and 3) are indicated on the right. Kd, kilodaltons.

PI3F-d13 are successfully transported to the cell surface and retain fusogenic activities similar to that of wild-type PI3F.

To further investigate the processing and transport of the PI3F-d22 mutant, a pulse-chase study was used to compare the processing and expression of both wild-type PI3F and mutant PI3F-d22 at different time points in cell lysates and on the cell surface. The F_0 of both PI3F (Fig. 7A) and PI3F-d22 (Fig. 7B) could easily be detected in the cell lysate but not on the surface (lanes P) after a 15-min pulse. For PI3F with chases of 1, 3, and 5 h, increasing amounts of F_0 and F_1 could be detected both in the cell lysate and on the cell surface. In contrast, for PI3F-d22, F_0 but not F_1 could be detected in the cell lysate. The PI3F-d22 F protein was never detected on the cell surface, even with a 5-h chase. These results indicate that the cytoplasmic tail truncation mutant PI3F-d22 is transport deficient. A cell surface immunoprecipitation assay (25) also confirmed that PI3F was detected on the cell surface while PI3F-d22 was not detected on the cell surface (data not shown).

Construction and expression of a chimeric PI3F-PI2F protein. To further investigate the possible sequence specificity of the requirement for a cytoplasmic tail in PI3F, we constructed

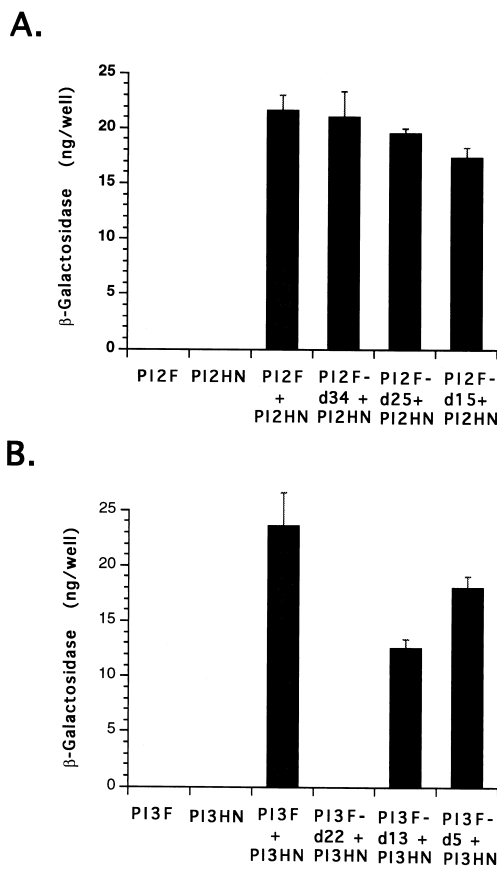


FIG. 4. Quantitative colorimetric assay of the fusogenic activities of full-length PI2F and PI3F and cytoplasmic tail truncation mutants of PI2F and PI3F. One cell population was transfected with the indicated combinations of the following plasmids which contain the T7 promoter: PI2F, PI2HN, PI2F + PI2HN, PI2F-d34 + PI2HN, PI2F-d25 + PI2HN, and PI2F-d15 + PI2HN (A) or PI3F, PI3HN, PI3F + PI3HN, PI3F-d22 + PI3HN, PI3F-d13 + PI3HN, and PI3F-d5 + PI3HN (B). They were also incubated with cytosine arabinoside (40 μ g/ml) and neuraminidase (2 U/ml). The other cell population was transfected with plasmid pG1NT7 β -gal. All cells were incubated separately at 31°C overnight. The two cell populations were then mixed at a ratio of 1:1 at 16 h posttransfection. Five hours after cells had been mixed at 37°C, samples were analyzed by colorimetric lysate assay. The data are presented as histograms of the A_{570} nm, with CPRG as the substrate and β -galactosidase quantitated in nanograms per well. Each datum point is the mean of triplicate samples. Error bars denote sample standard deviations.

a chimeric protein whose extracellular and transmembrane domains were derived from PI3F and whose cytoplasmic domain was derived from PI2F (Fig. 8A). The expression of this construct was confirmed by SDS-PAGE (data not shown); it was found to have a molecular weight similar to that of wild-type PI3F. The cell surface expression of the PI3F-PI2F chimera was detected by cell surface biotinylation assay (Fig. 8B) and quantitated by IPLab gel software (Table 1). The F_1 subunit of this chimeric protein was expressed on the cell surface at 49% of the level of PI3F. Therefore, the cytoplasmic tail of PI2F can partially restore the transport of PI3F-d22 to the cell surface.

Chimeric PI3F-PI2F protein has reduced fusogenic activity. The colorimetric lysate assay was used to quantitate the cell fusion induced by the PI3F-PI2F chimera. When normalized to surface expression, the PI3F-PI2F chimera coexpressed with PI3HN had 61% of the fusogenic activity of wild-type PI3F (Fig. 9). This indicates that an alternate cytoplasmic domain with a different sequence is sufficient for PI3F to exhibit fusion

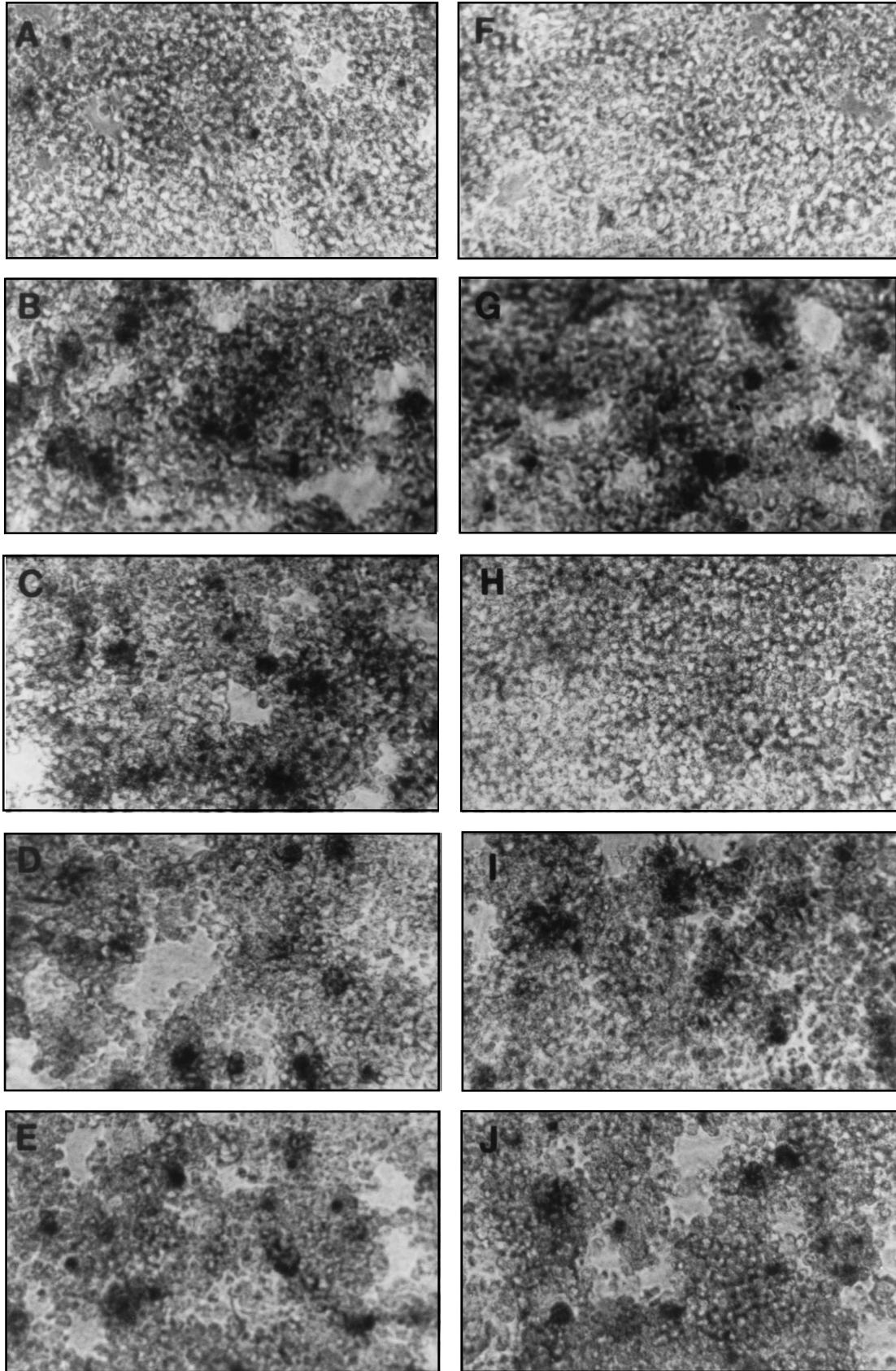


FIG. 5. In situ X-Gal staining fusion assay of full-length PI2F and PI3F and cytoplasmic tail truncation mutants of PI2F and PI3F. Two cell populations were treated as described in the legend to Fig. 4. Ten hours after cells had been mixed at 37°C, samples were analyzed by the in situ staining method. (A) PI2F; (B) PI2F + PI2HN; (C) PI2F-d34 + PI2HN; (D) PI2F-d25 + PI2HN; (E) PI2F-d15 + PI2HN; (F) PI3F; (G) PI3F + PI3HN; (H) PI3F-d22 + PI3HN; (I) PI3F-d13 + PI3HN; (J) PI3F-d5 + PI3HN.

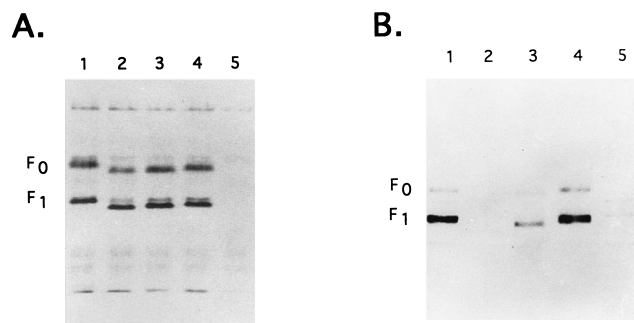


FIG. 6. Cell surface biotinylation assay of full-length PI2F and PI3F and cytoplasmic tail truncation mutants of PI2F and PI3F. HEp-2 cells were transfected with the plasmids indicated below. (A) Lane 1, PI2F; lane 2, PI2F-d34; lane 3, PI2F-d25; lane 4, PI2F-d15; lane 5, vector. (B) Lane 1, PI3F; lane 2, PI3F-d22; lane 3, PI3F-d13; lane 4, PI3F-d5; lane 5, vector. At 16 h posttransfection, cells were incubated with sulfo-NHS-biotin for 30 min at 4°C. Immunoprecipitation was done with rabbit anti-PI2 virus antibody (A [lanes 1 to 4]) and monoclonal antibody to PI3F for (B [lanes 1 to 4]). Samples were run on an SDS-8% PAGE gel and subsequently blotted onto a nitrocellulose membrane. Membranes were probed with horseradish peroxidase-conjugated streptavidin and detected by using a chemiluminescence kit.

activity. When the PI3F-PI2F chimera was coexpressed with PI2HN in the cell fusion assay, no cell fusion was observed (data not shown). This indicates that the homologous extracellular domains of F and HN are needed for cell fusion to occur. The finding of reduced fusion activity for the PI3F-PI2F chimera indicates that changes in the sequence of the cytoplasmic tail can influence the fusion activity of PI3F.

DISCUSSION

We have demonstrated here that PI2F proteins with truncated cytoplasmic tails show unimpaired transport, cleavage, and membrane fusion activities. The use of a quantitative cell fusion assay made it possible to accurately compare the different fusion activities of a series of deletion mutants. Cell fusion was carried out between two cell populations, one infected with VV-T7 and then transfected with different plasmid combinations and the other transfected with the β -galactosidase gene under the control of the T7 promoter. Neuraminidase treatment was employed to prevent the fusion of cells expressing paramyxovirus proteins prior to the addition of the second cell population. When the two cell types fused, the β -galactosidase gene was expressed by the T7 polymerase present in the other cell population. The ability to quantitate cell fusion by enzymatic assays eliminates ambiguity in the determination of the relative degree of cell fusion.

The intracellular transport properties of the PI2F cytoplasmic tail truncation mutants are similar to those of several other type I glycoprotein deletion mutants. In studies of the Semliki forest virus E2 glycoprotein and the H-2 transplantation antigen, partial removal of the cytoplasmic domain (11, 28, 51) did not affect transport or surface expression, and the deletion of cytoplasmic domains from the vesicular stomatitis virus G protein (35), the influenza virus HA protein (12, 45), Rous sarcoma virus envelope glycoprotein (32), and Friend murine leukemia virus envelope glycoprotein (19) did not block transport to the cell surface. We observed that the biosynthesis and intracellular transport of the cytoplasmic domain-truncated proteins of PI2F were not significantly different from those of the wild-type glycoproteins, nor was the fusogenic activity reduced when truncated proteins were coexpressed with PI2HN. These results indicate that any protein signals for intracellular transport of PI2F and for the type-specific interaction with

PI2HN must reside in the transmembrane domain or extracellular domain of the F protein.

In contrast to the studies with PI2F described above, cytoplasmic tail truncation mutations of the simian virus 5 and NDV HN proteins (30, 41) significantly reduced or eliminated surface expression of these proteins. In addition, defined alterations in the cytoplasmic domain of the vesicular stomatitis virus G protein reduced or eliminated its transport to the cell surface (36). An NDV HN cytoplasmic tail deletion mutant was found at the cell surface in reduced amounts; it was inefficient in fusion promotion but was not completely defective (41). In this study PI3F-d22, a PI3F mutant with the entire cytoplasmic tail deleted, showed no cell surface expression or cell fusogenic activity. Our cross-linking results suggest that this mutant protein does not assemble into the oligomeric form which may be required for efficient transport to the cell surface. It is interesting that although PI2 and PI3 belong to the same parainfluenza genus, the PI2F cytoplasmic tail is not needed for glycoprotein transport or cell fusion activity, while the PI3F cytoplasmic tail is necessary for the transport of the protein to the cell surface. Comparisons of the sequences of paramyxovirus F proteins across this virus family show that the cytoplasmic domain is among the least conserved regions (17, 26). This may reflect the different roles that the cytoplasmic tails of the parainfluenza virus F proteins play in glycoprotein

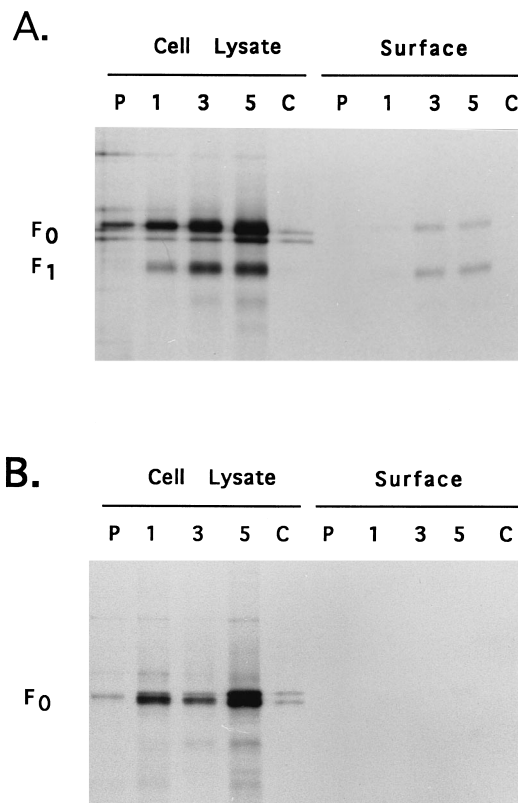


FIG. 7. Pulse-chase analysis of PI3F and mutant PI3F-d22. HEp-2 cells were infected with VV-T7 at a multiplicity of infection of 10 for 1 h at 37°C and then transfected with plasmids expressing PI3F (A) and PI3F-d22 (B). At 16 h posttransfection, cells were pulse-labeled (lanes P) with [35 S]methionine and [35 S]cysteine for 15 min and chased with cold methionine and cysteine for 1, 3, and 5 h. At the end of the chase period, cell surface proteins were biotinylated and immunoprecipitated with PI3F-specific antiserum. F proteins on the cell surface were detected by precipitating half of the cell immunoprecipitate with streptavidin-agarose beads as described in Materials and Methods. Lanes C, samples from VV-T7-infected control cells with transfection of the vector only.

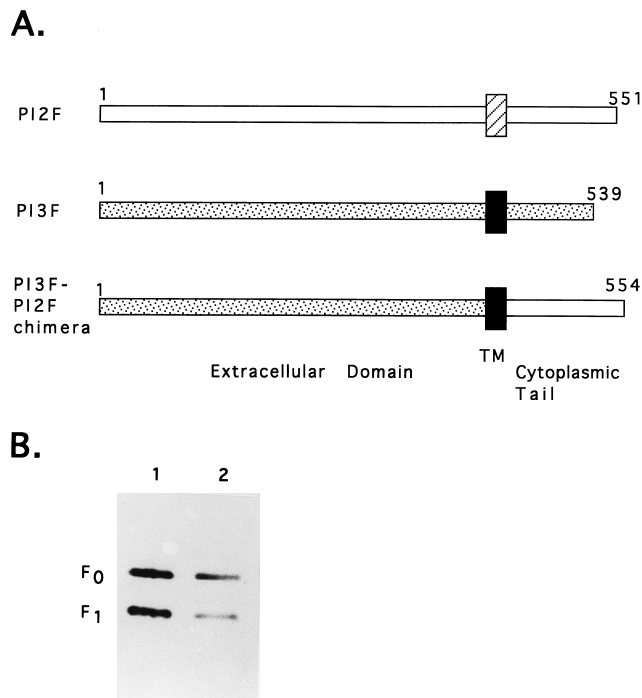


FIG. 8. Construction and detection of the PI3F-PI2F chimera. (A) Schematic diagrams of the chimeric PI3F-PI2F protein, wild-type PI2F, and the PI3F construct. ▨, transmembrane domain (TM) of PI2F; ■, TM of PI3F. (B) Cell surface expression of the PI3F-PI2F chimera, as determined by the cell surface biotinylation assay described in the legend to Fig. 6. Lane 1, PI3F; lane 2, PI3F-PI2F chimera.

transport. This feature is different from some other viruses, such as influenza virus, in which the amino acid sequence of the cytoplasmic domain of the HA protein shows several conserved residues (43).

We observed that the transport-deficient truncation mutant of the PI3F cytoplasmic tail could be partially rescued by adding the entire PI2F cytoplasmic tail. This chimeric protein was transported to the cell surface and induced cell fusion, but at a reduced level compared with that of wild-type PI3F. Thus, the requirement of the cytoplasmic tail for cell surface transport is not entirely sequence specific. The altered cytoplasmic sequence of this chimera may affect the conformation of its external domain, which results in reduced fusogenic activity. Although the mechanism of the membrane fusion process is not well defined, it is generally believed that a conformational change in the viral glycoprotein results in increased exposure of the fusion peptide. The insertion of the fusion peptide into the target cell membrane promotes the fusion of the two membranes (20, 24). It has been reported that cytoplasmic tail truncation mutants of the simian immunodeficiency virus Env glycoprotein alter the conformation of the external domain (44), but the detailed mechanisms have not been defined. An altered conformation may affect the oligomerization of the F protein, its transport, or its fusogenic activity. There is some evidence that the transmembrane domain, the cytoplasmic domain, or both regions of several paramyxovirus HN proteins are involved in the tetrameric structure of these proteins since the elimination of both of these domains results in dimers, not tetramers, as seen by sucrose gradient analysis (30, 46, 47).

A number of studies indicate that the correct folding and oligomerization of viral surface glycoproteins are prerequisites for transport through the exocytotic pathway. Molecules which

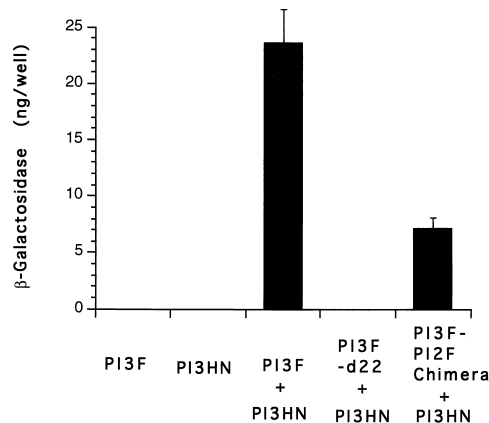


FIG. 9. Cell fusion activity of the PI3F-PI2F chimera. The quantitative colorimetric assay was done as described in the legend to Fig. 4. HEP-2 cells were transfected with plasmids expressing PI3F, PI3HN, PI3F + PI3HN, PI3F-d22 + PI3HN, and PI3F-PI2F chimera + PI3HN. Each datum point is the mean of triplicate samples. Error bars denote sample standard deviations.

are malformed or not oligomerized are blocked in transport in the endoplasmic reticulum (9). Oligomeric forms of several paramyxovirus F proteins, including those of Sendai virus (39), respiratory syncytial virus (1, 2, 8, 48), simian virus 5, and NDV and PI3 (37), have been reported. Although the oligomerization of the paramyxovirus HN proteins often involves disulfide bonds, paramyxovirus F oligomers generally exhibit noncovalent association (2). In this report, an F oligomer was detected in PI3F as well as in the PI3F-d13 and PI3F-d5 mutants under nonreducing conditions without any cross-linking reagent. This high-molecular-weight band was observed even when samples were boiled at 100°C for various time intervals in 10% SDS under nonreducing conditions (data not shown), but it was not observed in samples prepared at pH 8.5. Even though a definitive statement concerning the nature of the interactions which stabilize the F oligomer cannot yet be made, the results suggest that the F oligomer we observed is SDS resistant and alkaline pH sensitive.

We conclude that the cytoplasmic tail of PI2F is dispensable for glycoprotein transport, cleavage, and cell fusion. In contrast, the cytoplasmic tail of PI3F is necessary for the transport of this protein to the surface. The availability of sufficient quantities of cleaved protein on the surface when coexpressed with the HN protein is an important parameter for cell fusion activity. The PI2F cytoplasmic tail can partially substitute for the PI3F-d22 deletion by partially restoring transport to the cell surface as well as fusogenic activity. However, the reduced fusion activity of the chimeric F protein indicates that the sequence of the F cytoplasmic domain can affect paramyxovirus-induced cell fusion.

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