Oligomerization, Secretion, and Biological Function of an Anchor-Free Parainfluenza Virus Type 2 (PI2) Fusion Protein

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A number of studies indicate that the transmembrane domain, the cytoplasmic domain, or both regions of viral surface glycoproteins are involved in quaternary structure formation. In this report, the transmembrane domain and cytoplasmic tail coding sequence of the fusion (F) glycoprotein gene from parainfluenza type 2 virus was truncated by PCR and the resulting gene (PI2F′) was expressed in HeLa-T4 cells by using the vaccinia virus-T7 transient expression system. Pulse–chase experiments indicated that the anchor-free PI2F′ was expressed and processed into F_1 and F_2 subunits. Both the processed and the unprocessed anchor-free PI2F′ proteins were found to be efficiently secreted into the culture medium. Examination of the oligomeric form of the anchor-free PI2F′ by chemical cross-linking demonstrated that it assembles posttranslationally into dimers and trimers with a pattern similar to that of the wild-type PI2F protein. In an effort to better understand the biological properties of the truncated form of PI2F′, we anchored PI2F′ by a glycosyl-phosphatidylinositol (GPI) linkage. The GPI-anchored PI2F′ protein, when coexpressed with PI2HN, did not induce cell fusion seen as syncytium formation, but was found to initiate lipid mixing (hemifusion) as observed by transfer of R-18 rhodamine from red blood cells to the GPI-PI2F′/ PI2HN cotransfected cells. The results therefore indicate that the extracellular domain of the PI2 fusion protein contains not only the structural information sufficient to direct assembly into higher oligomers, but also is competent to initiate membrane fusion, suggesting that the anchor-free PI2F′ may be useful for further structural studies. © 2000 Academic Press

INTRODUCTION

Human parainfluenza viruses are negative-strand enveloped RNA viruses that belong to the family *Paramyxoviridae* (Lamb and Kolakofsky, 1996). They are important respiratory tract pathogens of infants and young children. Infection by paramyxoviruses proceeds by fusion of the viral envelope and cellular membrane, with entry of only the nucleocapsid into the cytoplasm of the cell. It is well known that the entry of enveloped viruses is determined by an interaction between specific receptors on the target cell surface and envelope glycoproteins on the virion. The parainfluenza viruses encode two envelope glycoproteins, the hemagglutinin-neuraminidase protein (HN) and fusion protein (F). The HN protein is a type II integral membrane protein with an amino-terminus located inside the virus and a carboxy-terminal ectodomain. The ectodomain binds to sialic acid residues that serve as viral receptors on the target cells. The F protein is a type I integral membrane protein that has a large ectodomain containing a cleavable signal sequence at the N-terminus, a hydrophobic transmembrane domain, and a short cytoplasmic tail. The F protein monomer is synthesized as an inactive larger F_o precursor molecule and is cleaved posttranslationally into the biologically

active forms by a trypsin-like host endoprotease as observed for Sendai virus or by intracellular furin as observed for most other paramyxoviruses (Lamb and Kolakofsky, 1996). This results in the production of F_1 and F_2 subunits that are covalently linked by disulfide bridges (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). The native F protein exists as homo-oligomeric trimers, which have been observed in cross-linking studies (Russell *et al.,* 1994). The cleavage of the F_o protein exposes a new hydrophobic domain designated the fusion peptide at the N-terminus of the F_1 subunit, which is believed to participate directly in the fusion process (Lamb, 1993).

The mechanism of paramyxovirus-induced membrane fusion has not been well defined. Previous studies in our laboratory showed that cell fusion caused by human parainfluenza virus type 2 and type 3 glycoproteins requires coexpression of F and HN proteins from the same virus type (Hu *et al.,* 1992; Yao and Compans, 1995). Other reports have shown similar type-specific interactions among paramyxovirus envelope glycoproteins (Horvath *et al.,* 1992; Cattaneo and Rose, 1993; Malvoisin and Wild, 1993; Bousse *et al.,* 1994; Heminway *et al.,* 1994; Wild *et al.,* 1994). It has been proposed that the HN and F proteins must communicate in a virus-specific manner, allowing a functional molecular interaction between homotypic HN and F proteins (Lamb, 1993). Studies with chimeric proteins have provided evidence that specific regions on F and HN proteins that are involved

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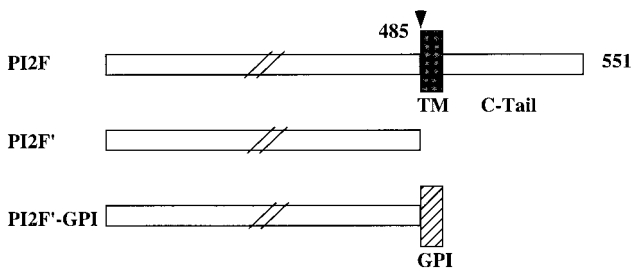
FIG. 1. Schematic structure of PI2F, PI2F′, and PI2F′-GPI. The PI2F transmembrane domain is indicated as a shaded box. The GPI anchor is shown as a cross-hatched box. The number on the right indicates the size (in amino acids) of the wild-type PI2F. The arrow and the number on the top indicate the site where the premature stop codons were introduced.

in this type-specific interaction reside in the extracellular domain (Sergel et al., 1993b; Tanabayashi and Compans, 1996).

It was previously observed that a cytoplasmic tail truncation mutant of the PI2F protein was similar to the wild-type (wt) PI2F protein with reference to biosynthesis, oligomerization, intracellular transport, fusogenic activity, and virus type-specific F and HN interaction (Yao and Compans, 1995). In this study, we have constructed a cytoplasmic tail- and transmembrane domain-truncated F protein mutant of parainfluenza type 2 virus (PI2F′) and compared its expression, processing, and oligomerization with that of the wild-type PI2F protein. We also constructed a GPI-anchored PI2F′ molecule and investigated the role of the ectodomain in the promotion of cell fusion.

RESULTS AND DISCUSSION

Construction and expression of soluble PI2F′ and GPI-anchored PI2F′

A segment of the PI2F cDNA in pGEM 3 (pGEM-3-PI2F) was used as template for oligonucleotide-directed mutagenesis by PCR to introduce a premature stop codon into the coding sequence as shown in Fig. 1. The resulting PCR product no longer contained the nucleotide sequence encoding the transmembrane domain and the cytoplasmic tail. The soluble PI2F′ protein was expressed by using the vaccinia virus-bacteriophage T7 RNA polymerase transient expression system (Fuerst et al., 1986) and compared to wild-type PI2F protein. As shown in Fig. 2, in the cell lysate, the anchor-free PI2F′ mutant protein (lane 2) was expressed at a level comparable to that of the wild-type PI2F (lane 1) and both the F_o precursor and the F_1 cleavage products were detected, although lower levels of the processed F_1 cleavage product were observed with the anchor-free PI2F′. The unprocessed anchor-free PI2F′ protein showed the expected decrease in molecular mass (estimated molecular mass of 54 kDa), compared with that of the wild-type

PI2F (estimated molecular mass of 60 kDa). The efficiency of proteolytic processing may be affected by the lack of anchoring to the membrane, resulting in a decreased level of the processed F_1 cleavage product. As expected, the anchor-free PI2F′ proteins were found to be efficiently secreted into the medium while the wild-type anchored PI2F proteins were primarily distributed on the cell surface.

To study the biological properties of the soluble PI2F′, the coding sequence for a chimeric GPI-PI2F′ was constructed by overlapping PCR as described under Materials and Methods. We substituted the transmembrane and cytoplasmic tail domains of PI2F with a 45-aa peptide of carboxy-terminal amino acids of CD16B. This small peptide constitutes a signal for GPI anchor addition (Low, 1989; Scallon et al., 1989; McHugh et al., 1995). During translocation into the endoplasmic reticulum, this peptide is cleaved and the GPI tail is added. The GPI-anchored PI2F′ was transfected into HeLa-T4 cells and expressed by the VV-T7 transient expression system. As shown in Fig. 2, the GPI-anchored PI2F′ (lane 3) was expressed and both the F_0 precursor and the F_1 cleavage products were detected. To address whether the GPI-anchored PI2F′ protein was expressed at the cell surface, cells expressing the GPI-anchored PI2F′ protein were radiolabeled with [^{35}S]methionine/cysteine and the proteins on cell surface were identified with a membrane-impermeable biotinylation reagent. As shown in Fig. 2, the GPI-anchored PI2F′ protein was found to be transported to the cell surface, although somewhat lower levels of the GPI-anchored PI2F′ protein were found both in the cell lysates and on the cell surface as compared to those of wt 2F. These results indicated that GPI-anchored PI2F′ is expressed, processed in HeLa-T4 cells, and efficiently transported to the cell surface, as also
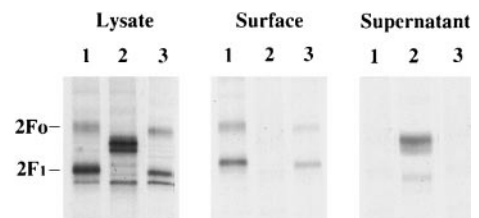
FIG. 2. Expression of the anchor-free PI2F′ (lane 2), wild-type PI2F (lane 1), and GPI-PI2F′ (lane 3) proteins expressed in HeLa-T4 cells using the vTF7-3 transient expression system. At 8 h posttransfection, cells were pulse-labeled with 100 μCi of [^{35}S]methionine/cysteine for 30 min at 37°C and chased with nonradioactive chase medium (DMEM containing 10% fetal calf serum) for a 2-h chase period. After being labeled, cells were biotinylated and lysed, and the chase medium were collected. The cell lysates (on the left) and the chase medium (on the right) were immunoprecipitated with rabbit or guinea pig anti-PI2 antiserum and protein A–agarose beads. The PI2-specific proteins on the cell surfaces (in the middle) were detected as described under Materials and Methods. Proteins were analyzed by SDS–8% PAGE under reducing conditions. The bands below both the wt $2F_1$ and GPI-anchored $2F'_1$ in the cell lysate were not observed using other PI2 antisera and are therefore thought to be nonspecific.

Lysate

30' 60' 180' 300'

—2F'o

—2F'₁

Supernatant

30' 60' 180' 300'

—2F'o

—2F'₁
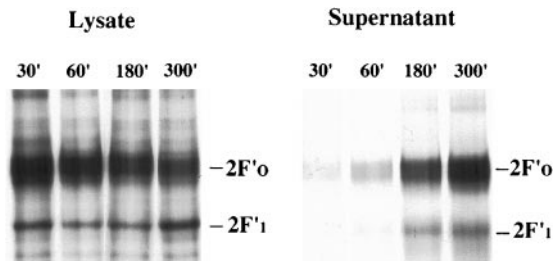
FIG. 3. Kinetics of secretion of the anchor-free PI2F′ protein into the medium. HeLa-T4 cells expressing the anchor-free PI2F′ protein were labeled with 100 μCi of [^{35}S]methionine/cysteine at 37°C at 8 h posttransfection for 30 min and incubated in chase medium (DMEM containing 10% fetal calf serum) for 30-, 60-, 180-, or 300-min chase periods. The cells were lysed and the culture supernatants were also collected at the indicated time point. PI2F′ polypeptides were immunoprecipitated with PI2-hyperimmune rabbit serum and analyzed by SDS–PAGE on an 8% gel under reducing conditions.

reported for other chimeric molecules containing GPI anchors in place of their normal transmembrane domains (Crise *et al.,* 1989; Lin *et al.,* 1990; Singh *et al.,* 1990; Duval *et al.,* 1992; Gilbert *et al.,* 1993; Weiss and White, 1993; Zhou *et al.,* 1997).

Kinetics of secretion of soluble PI2F′

To determine the kinetics by which the anchor-free PI2F′ protein was transported through the secretory pathway, a time course analysis was performed. As depicted in Fig. 3, the PI2F′ protein is discernible in the culture supernatant as early as 30 min after chases. As the time of chase was increased, the level of the secreted PI2F′ increased and it was found to accumulate in the medium, whereas the level of PI2F′ detected in cell lysate was correspondingly reduced. We found that at 180 min about 80% of the soluble PI2F remained in cell lysates, while about 50% of the wt PI2F remained in cell lysates (Fig. 4). This indicates that the kinetics of secretion for the anchor-free PI2F′ protein mutant were slower than those for the transport of wt PI2F protein to the cell surface as shown in Fig. 4. This result is consistent with previous results showing that transport from the ER to the Golgi and from the Golgi to the plasma membrane is slower for anchor-free molecules than for membrane-anchored molecules (Copeland *et al.,* 1986; Singh *et al.,* 1990).

Analysis of the oligomeric state of the PI2F′ protein

Previous mutational studies have shown that proper assembly of envelope glycoproteins is necessary for their biological functions (Gething *et al.,* 1986; Kreis and Lodish, 1986; Thompson *et al.,* 1988; Parks and Lamb, 1990a; Takimoto *et al.,* 1992; Doms *et al.,* 1993). The paramyxovirus fusion protein is directly involved in the process of fusion of the viral envelope and cell membrane. A trimeric envelope glycoprotein structure has

previously been proposed for the wild-type PI2F protein and other paramyxovirus fusion proteins that were analyzed via cross-linking chemically (Walsh *et al.,* 1985; Sechoy *et al.,* 1987; Arumugham *et al.,* 1989; Collins and Mottet, 1991; Anderson *et al.,* 1992; Russell *et al.,* 1994; Yao and Compans, 1995; Tong and Compans, 1999). To investigate the assembly of the soluble form and GPI-anchored form of PI2F′ in comparison with the wt PI2F glycoprotein, we compared the pattern of oligomer formation of the proteins after cross-linking with the reagent 3,3-dithiobis(sulfosuccinimidyl propionate (DTSSP). As shown in Fig. 5, after cross-linking the soluble form and GPI-anchored form of the PI2F′ proteins with DTSSP in NP-40-solubilized cell extracts, we consistently observed three prominent PI2F′ and GPI-anchored 2F′ protein bands migrating at the positions of homotrimers, homodimers, and monomers. No additional larger bands were detected even in the presence of a high concentration of DTSSP. In this respect, the soluble form of the PI2F′ proteins did not show any significant difference from the wt PI2F protein in PI2 virus-infected or PI2F-transfected cells (Yao and Compans, 1995). These results indicate that the transmembrane domain and cytoplasmic domain of the PI2F protein are not essential for the formation of the PI2F homotrimers. Previous studies have shown that some cytoplasmic domain mutants, transmembrane domain mutants, or transmembrane and cytoplasmic domain truncation mutants were unable to assemble into oligomeric forms (Rose and Bergmann, 1983; Doms and Helenius, 1988; Rose and Doms, 1988; Parks and Lamb, 1990b; Singh *et al.,* 1990; Sergel *et al.,* 1993a; Sergel and Morrison, 1995; Yao and Compans, 1995; Center *et al.,* 1997). We believe that this is the first report of oligomer formation solely between ectodomain subunits of paramyxovirus fusion proteins. It indicates that oligomerization of the PI2F protein might have less stringent structural requirements and that structural features sufficient for oligomerization of the protein must reside in the extracellular domain. As expected, the GPI-

Lysate

30' 60' 180' 300'

—2F₀

—2F₁

Surface

30' 60' 180' 300'

—2F₀

—2F₁
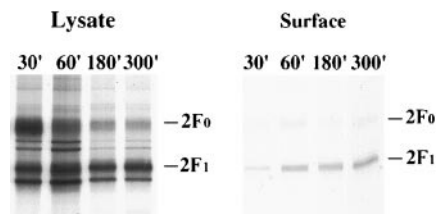
FIG. 4. Kinetics of cell surface transport of the wt PI2F. HeLa-T4 cells expressing the anchor-free PI2F protein were labeled with 100 μCi of [^{35}S]methionine/cysteine at 37°C at 8 h posttransfection for 30 min and incubated in chase medium (DMEM containing 10% fetal calf serum) for 30-, 60-, 180-, or 300-min chase periods. The cells were lysed and PI2F polypeptides were immunoprecipitated with PI2-hyperimmune guinea pig serum. The cell surface wt 2F proteins were detected via biotinylation analysis as described under Materials and Methods. The PI2F polypeptides were analyzed by SDS–PAGE on an 8% gel under reducing conditions.
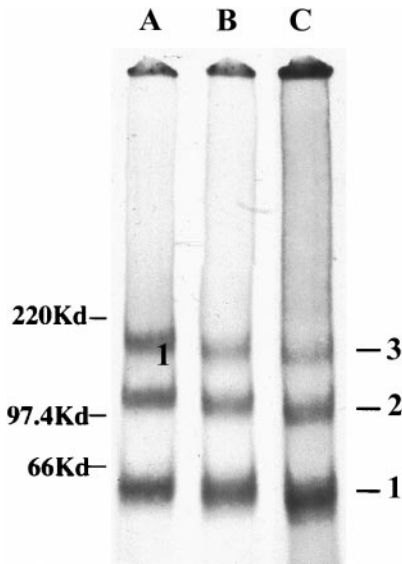
FIG. 5. Chemical cross-linking of wild-type PI2F, anchor-free PI2F′, and GPI-anchored 2F′ proteins. HeLa-T4 cells were infected with vTF7-3 at a m.o.i. of 10 for 1 h at 37°C and then transfected with wild-type PI2F (lane A), GPI-anchored PI2F′ (lane B) or anchor-free PI2F′ clones (lane C). At 8 h posttransfection, transfected cells were labeled with 100 μCi of [³⁵S]methionine/cysteine for 60 min at 37°C. After chasing in nonradioactive chase medium for 2 h, cross-linking reagent (1 mM DTSSP) was added to 0.5% NP-40-solubilized cells for 60 min at 4°C and then free DTSSP was quenched with 50 mM glycine for 5 min at 4°C. Immunoprecipitations were performed after the cross-linking reaction. Proteins were analyzed by 3.5% SDS–PAGE gel under nonreducing conditions. 1, 2, and 3 represent monomer, dimer, and trimer, respectively. The positions of protein size markers are shown on the left.

anchored form of 2F′ was able to be normally oligomerized as was also reported for other GPI-anchored viral envelope proteins (Singh et al., 1990; Gilbert et al., 1993; Weiss and White, 1993).

To determine the rate of oligomerization of the anchor-free PI2F′ protein in comparison to wt PI2F protein, HeLa-T4 cells expressing the truncated and wt PI2F at 8 h posttransfection were pulse-labeled for 3 min with 100 μCi/ml of Trans [³⁵S]methionine/cysteine and then chased with an excess of nonradioactive chase medium for 3-, 5-, 7-, 10-, or 15-min periods. Cell extracts were cross-linked using a protocol described by Paterson and Lamb (1993). PI2F and PI2F′ molecules were then immunoprecipitated and analyzed by nonreducing low-percentage SDS–PAGE and autoradiography. As shown in Fig. 6, within the 3-min pulse some of the labeled anchor-free PI2F′ was already found to form cross-linked oligomers (dimers and trimers). The maximum extent of cross-linking into trimers was attained after approximately 10 min of chase. Similar results were obtained with the wt PI2F protein. These data establish that wt PI2F is assembled into trimers within a few minutes of completion of synthesis of the polypeptide chain, and truncation of the cytoplasmic tail and transmembrane domain did not significantly alter the rate of oligomer

formation, although the initial rate of soluble form 2F′ oligomerization was seen to be slower.

Membrane fusion activity of the GPI-anchored PI2F′

The cell fusion process involves several steps including the formation of a membrane stalk, mixing of both the outer membrane leaflet lipids normally called hemifusion, and pore formation or mixing of inner leaflet membrane lipids with concomitant mixing of the aqueous contents of donor and target cells (Hernandez et al., 1996). Since the soluble form of PI2F′ that we constructed was capable of forming a homotrimer, we investigated whether the cytoplasmic tail- and transmembrane domain-truncated PI2F′ protein still remained biologically active. We constructed GPI-anchored PI2F′, which has shown cell surface expression and biologically active trimer formation, and we compared its fusogenic activity with that of the wt F protein. Coexpression of the GPI-PI2F′ with homotypic HN was not observed to
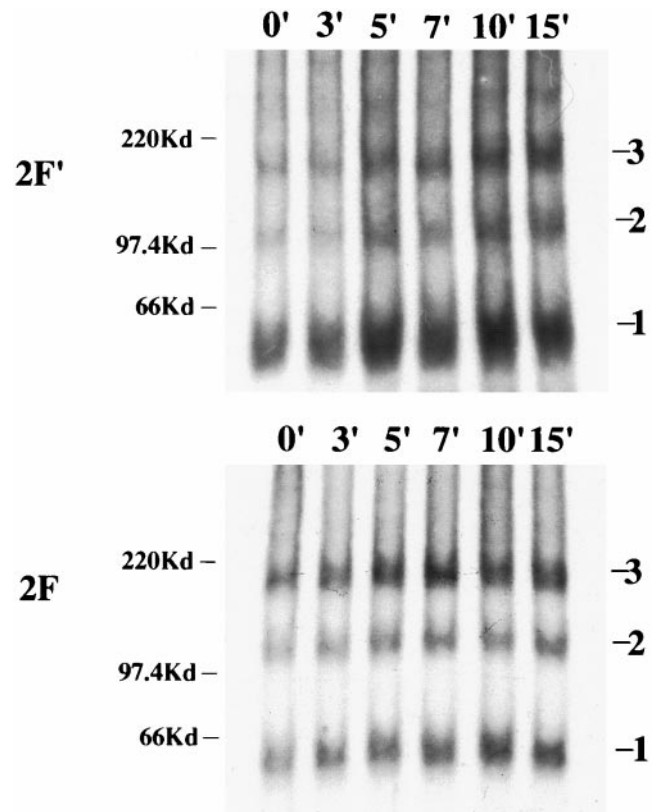
FIG. 6. Time course of oligomer formation of wild-type PI2F and anchor-free PI2F′ proteins. At 8 h posttransfection, HeLa-T4 cells expressing wild-type PI2F or anchor-free PI2F′ proteins were labeled with 100 μCi of [³⁵S]methionine/cysteine for 3 min at 37°C and then chased in nonradioactive chase medium (DMEM containing 10% fetal calf serum) for 3-, 5-, 7-, 10-, or 15-min chase periods. The labeled HeLa-T4 cells then were cross-linked as described for Fig. 5. PI2F and PI2F′ molecules were immunoprecipitated and analyzed by nonreducing 3.5% SDS–PAGE gel and autoradiography. The positions of protein size markers are shown on the left.

cause cell fusion as judged by syncytium formation (data not shown) and by a content mixing assay (shown in Fig. 7). We employed the octadecyl rhodamine B (R18) assay to measure whether GPI-PI2F′ mediates lipid mixing of the outer leaflet membrane. The lipophilic probe R18 was incorporated into guinea pig red blood cells (RBCs), which were then mixed with HeLa-T4 cells that expressed various combinations of viral membrane glycoproteins. When cells coexpressing the wt PI2F or GPI-anchored PI2F′ protein with the homotypic HN proteins were mixed with R18-labeled erythrocytes, transfer of fluorescence from the RBCs to the expressing cells was detected as shown in Fig. 8. However, when HeLa-T4 cells coexpressing the homotypic HN protein with anchor-free PI2F′ or expressing the homotypic HN alone were incubated with R18-labeled RBCs, no transfer of fluorescence from the RBCs to the expressing cells was detected. Thus, we found that the GPI-anchored PI2F′ mediated the transfer of the lipophilic fluorescent probe R18 from R18-labeled erythrocyte membranes to expressing cells, but failed to mediate complete fusion seen as syncytium formation. These results indicate that the ectodomain of PI2F is able to mediate hemifusion. It has been demonstrated that after the fusion peptide inserts into a target cell membrane, the protein forms a hairpin structure that draws the two membranes into close proximity, thereby facilitating their union, hemifusion, and complete fusion (Weissenhorn et al., 1997; Montefiori and Moore, 1999; Lamb et al., 1999). In paramyxoviruses, a conformational change of the fusion protein during the fusion process has been suggested (Lamb, 1993). Our results suggest that a GPI anchor penetrating through only the outer leaflet is sufficient for PI2F′ to promote hemifusion while a transmembrane domain is required for PI2F to form a stable fusion pore that can subsequently dilate, leading to complete fusion. From these experiments we can conclude that the ectodomain remains biologically active in the absence of the transmembrane and cytoplasmic domains. A similar study of GPI-anchored influenza hemagglutinin has shown promotion of hemifusion, but not complete fusion (Kemble et al., 1994).

In summary, a soluble form of the extracellular domain of PI2F is stably expressed in HeLa-T4 cells. Characterization of this anchor-free PI2F′ glycoprotein demonstrated that it was efficiently secreted and was folded spontaneously into oligomers. The observation of three prominent cross-linked bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) that closely resemble those found with wt PI2F indicated that the ectodomain of PI2F′ protein preserves its native trimeric structure. Additionally the GPI-anchored PI2F′ glycoprotein was found to mediate lipid mixing, suggesting that the GPI-anchored PI2F′ is biologically active. Studies of the structure of the water-soluble ectodomain of influenza virus hemagglutinin (HA) has established a paradigm for virus glycoprotein-mediated membrane fusion. Similarly, expression of a soluble F domain, devoid of the cytoplasmic domain and transmembrane domain, should facilitate studies of the structure of the fusion protein of paramyxoviruses.

MATERIALS AND METHODS

Cells, viruses, and plasmids

Monolayer cultures of HeLa-T4 cells and CV-1 cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (HyClone Laboratories, Inc., Logan, UT). The recombinant vaccinia virus vTF7-3 and wild-type vaccinia virus strain IHD-J were kindly provided by Bernard Moss (National Institutes of Health, Bethesda, MD). Both vaccinia virus stocks were propagated and titrated on CV-1 cells. All recombinant bacterial plasmids were propagated in an Escherichia coli DH5a strain. The plasmid pGEM-3-PI2F encoded the PI2F fusion protein and was described previously (Hu et al., 1990). The plasmid pCDM8-CD16B containing a DNA fragment encoding the signal for GPI-anchor attachment of CD16B (McHugh et al., 1995) was a gift from Periasamy Selvaraj (Emory University, Atlanta, GA).

Construction of DNAs encoding soluble and GPI-anchored PI2F′ proteins

To PCR amplify a soluble PI2F′ coding sequence from plasmid pGEM-3-PI2F, two synthetic oligonucleotides were used. The sense primer was the T7 primer, located upstream of the PI2F coding sequence. The antisense primer corresponded to the 3′-terminal 24 nucleotides of the coding sequence for the PI2F ectodomain and carried a stop codon and an EcoRI site. The resulting PCR product encoding the entire ectodomain was digested with SalI and EcoRI and then ligated into vector pGEM-3, which was digested with SalI and EcoRI.

The GPI-anchored PI2F′ was constructed by the overlapping PCR technique described by Ho et al. (1989) and Horton et al. (1989). The ectodomain sequence was PCR amplified from the PI2F clone. The signal sequence for GPI-anchor attachment was PCR amplified from the CD16B clone. The antisense primer for the PI2F ectodomain fragment contained a 12-nucleotide overhang at the 3′ end complementary to the 5′ end of the sense primer for the signal sequence for GPI-anchor attachment. The two amplified gene sequences were annealed to each other and were further extended to generate the chimeric GPI-anchored PI2F′ by five cycles of PCR. Finally, the chimeric GPI-anchored PI2F′ was used as template and amplified by PCR using two outer primers. The resulting chimera flanked with SalI/EcoRI sites was cloned into vector pGEM-3 at the SalI/EcoRI sites. All end products were sequenced to confirm that the correct sequence
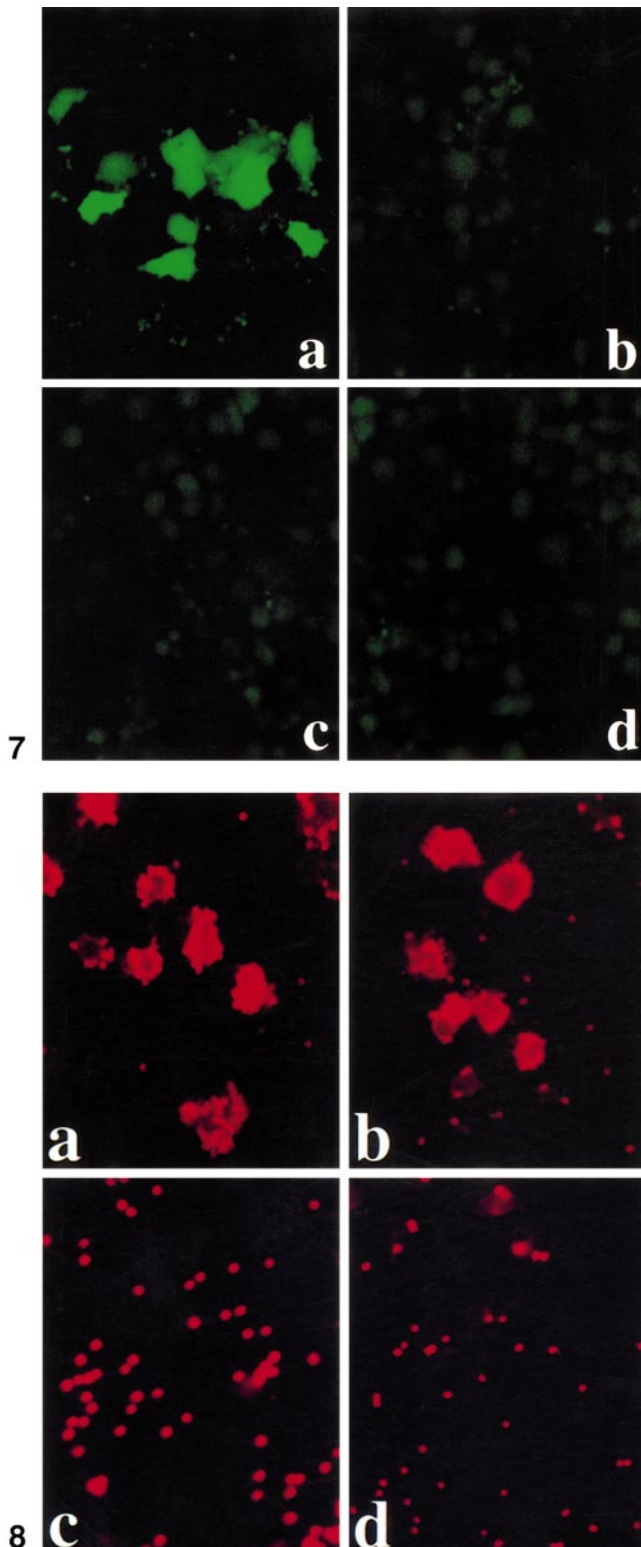
FIG. 7. Content mixing of calcein-labeled RBCs with HeLa-T4 cells expressing wild-type PI2F/PI2HN or GPI-PI2F′/PI2HN. At 16 h posttransfection at 32°C, the calcein-labeled RBCs were overlaid onto HeLa-T4 cell monolayers expressing (a) PI2F/PI2HN, (b) GPI-PI2F′/PI2HN, (c) PI2HN alone, or (d) anchor-free PI2F′/PI2HN and first incubated at 4°C for 30 min and then incubated at 37°C for 60 min. After incubation, calcein transfer from RBCs to cells was visualized and photographed with a Nikon fluorescence microscope using a "fluorescein" filter set.

had been obtained in the PCRs. A schematic diagram of the constructs is shown in Fig. 1.

Transfection, radiolabeling, and immunoprecipitation

The mutant and the wild-type PI2F proteins were expressed by using the vaccinia virus-bacteriophage T7 RNA polymerase transient expression system (Fuerst *et al.,* 1986). Briefly, 35-mm dishes of subconfluent cells were infected for 1 h with vTF7-3 at an m.o.i. of 10 and then transfected with 5 μg plasmid DNA mixed with 10 μl of Lipofectin (Gibco BRL, Gaithersburg, MD) in 1 ml of DMEM. At 8 or 16 h posttransfection, the transfected cells were starved in DMEM lacking methionine and cysteine for 30 min, pulse-labeled with 100 μCi/ml of [^{35}S]methionine/cysteine for 30 min at 37°C, and then chased with nonradioactive chase medium (DMEM containing 10% fetal calf serum) for 30-, 60-, 180-, or 300-min chase periods. The cells were washed and then lysed with cell dissociation buffer [10 mM Tris–HCl (pH 8.0), 250 mM NaCl, 0.5% Triton X-100, and 0.5% sodium deoxycholate] at the indicated time point. The culture supernatants, containing proteins that were secreted during the expression period, were also collected. The culture supernatants and the cell lysate were analyzed for anchor-free PI2F′ expression by incubation with PI2-hyperimmune rabbit or guinea pig serum for 2 or more h at 4°C followed by precipitation with protein A–agarose (Immunopure; Pierce Chemical Co., Rockford, IL) for 2 h. Proteins were characterized by SDS–8% PAGE and by subsequent autoradiography. Each band of interest was quantified by densitometry analysis using the National Institutes of Health image program.

Cell surface biotinylation assay

Cell surface protein expression was detected by a biotinylation assay as described by Le Bivic *et al.* (1989). Eight hours posttransfection, cells expressing GPI-anchored PI2F′ or wt PI2F proteins were starved in DMEM lacking methionine and cysteine for 1 h and pulse-labeled with 100 μCi/ml of [^{35}S]methionine/cysteine for 30 min at 37°C. After chasing with DMEM containing 10% fetal calf serum for the indicated period, cells were washed three times with ice-cold PBS containing 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$ (PBS-CM) and incubated with 1

FIG. 8. Hemifusion of R18-labeled RBCs with HeLa-T4 cells expressing wild-type PI2F/PI2HN or GPI-PI2F′/PI2HN. Guinea pig RBCs were labeled with the lipid probe R18 using a protocol described by Bagai and Lamb(1995). At 16 h posttransfection, the R18-labeled RBCs were overlaid onto HeLa-T4 cell monolayers expressing (a) PI2F/PI2HN, (b) GPI-PI2F′/PI2HN, (c) PI2HN alone, or (d) anchor-free PI2F′/PI2HN and first incubated at 4°C for 30 min and then incubated at 37°C for 60 min. After incubation, R18 transfer from RBCs to cells was visualized and photographed with a Nikon fluorescence microscope using a "rhodamine" filter set.

ml of a 0.5 mg/ml solution of sulfosuccinimidyl-2-(biotin-amido)ethyl-1,3-dithiopropionate (NHS-SS-biotin; Pierce) in PBS-CM at 4°C for 30 min. Free biotin was removed by addition of DMEM and three washes with PBS-CM. Biotinylated cell surface PI2F-specific proteins were identified by immunoprecipitation with PI2-specific antisera and protein A–agarose beads (Pierce). Cell lysate beads were washed three times and divided into two aliquots. One aliquot was used for immunoprecipitation. The other aliquot was boiled in 10 μl of 10% SDS and diluted with 0.5 ml of lysis buffer. The supernatant from the protein A–agarose beads was incubated with streptavidin–agarose beads and proteins were characterized by SDS–8% PAGE and autoradiography.

Chemical cross-linking

Chemical cross-linking analysis was performed as described previously (Russell et al., 1994) using the cross-linking reagent DTSSP (Pierce Chemical Co.). In brief, subconfluent monolayers of HeLa-T4 cells in 35-mm-diameter dishes were infected with recombinant vaccinia virus vTF7-3 at an m.o.i. of 10. After 1 h of incubation at 37°C, cells were transfected with 5 μg of plasmid DNA and 10 μl of Lipofectin (Gibco BRL Life Technologies) in 1 ml of DMEM. At 8 h posttransfection, cells were starved in DMEM lacking methionine and cysteine for 1 h, pulse-labeled with 100 μCi/ml [^{35}S]methionine/cysteine for the indicated time at 37°C, and then chased with DMEM containing 10% fetal calf serum for the indicated period. The radiolabeled cells were detached from the dish with 5 mM EDTA in PBS, washed, and resuspended in 100 μl of PBS deficient in Mg^{2+} and Ca^{2+} (PBS−). The cells were solubilized in 0.5% Nonidet-P40, treated with 2 mM DTSSP at 4°C for 60 min, and inactivated with 50 mM glycine for 5 min at 4°C. Immunoprecipitations were performed as described above and the precipitates were analyzed by SDS–3.5% PAGE under nonreducing conditions.

Fusion of R18-labeled RBCs with cells expressing GPI-anchored PI2F′

Guinea pig erythrocytes were labeled with the hydrophobic fluorescent dye R18 (Molecular Probes, Eugene, OR) based on the procedure described previously (Bagai and Lamb, 1995). Fifteen microliters of 1 mg/ml R18 in ethanol was added to 10 ml of 1% fresh guinea pig erythrocyte suspension in PBS while vortexing. After incubation at room temperature in the dark for 30 min, 30 ml of DMEM with 10% fetal calf serum was added to the suspension and further incubated for 20 min in order to absorb the unbound probe. After labeling, the RBC suspension was washed five times with 40 ml of PBS to remove the unincorporated R18. The last washing step was in PBS with the addition of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS++). The R18-labeled RBCs could be stored in PBS++ at 4°C for up to 24 h before use. To determine membrane fusion, monolayer HeLa-T4 cells expressing PI2F/PI2 HN, GPI-anchored PI2F′/PI2 HN, PI2 HN, or soluble PI2F′/PI2 HN at 16 h posttransfection at 32°C were washed twice with DMEM and then incubated for 1 h at 37°C with 50 mU per milliliter of neuraminidase in DMEM. The neuraminidase-treated cells were washed twice with PBS and first incubated with 1 ml of R18-labeled RBCs (0.2% hematocrit) in PBS++ at 4°C for 30 min in the dark with intermittent gentle agitation and then incubated at 37°C for 1 h. Unbound RBCs were removed by two washes with PBS. Fusion was monitored and photographed by using an inverted fluorescence microscope.

Fusion of calcein-labeled RBCs with cells expressing GPI-anchored PI2F′

Calcein AM (Molecular Probes, Leiden, The Netherlands) dissolved in dimethylsulfoxide was added to a 1% erythrocyte suspension at a final concentration of 5 μM, immediately mixed, and incubated at room temperature for 30 min in the dark. After incubation, 30 ml of DMEM with 10% fetal calf serum was added to the suspension and further incubated for 20 min in order to absorb the unbound probe. Calcein-filled erythrocytes were washed five to six times and resuspended in PBS. Cell–erythrocyte fusion was performed as described for the experiments with R18-labeled erythrocytes. Permeation of calcein from calcein-labeled RBCs to PI2F/HN, GPI-2F′/2HN, PI2F′/2HN or PI2HN expressing HeLa-T4 cells was monitored by fluorescence microscopy and photographically recorded.

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