Association of the Parainfluenza Virus Fusion and Hemagglutinin-Neuraminidase Glycoproteins on Cell Surfaces

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We previously observed that cell fusion caused by human parainfluenza virus type 2 or type 3 requires the expression of both the fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins from the same virus type, indicating that a type-specific interaction between F and HN is needed for the induction of cell fusion. In the present study we have further investigated the fusion properties of F and HN proteins of parainfluenza virus type 1 (PI1), type 2 (PI2), and type 3 (PI3), Sendai virus (SN), and simian virus 5 (SV5) by expression of their glycoprotein genes in HeLa T4 cells using the vaccinia virus-T7 transient expression system. Consistent with previous results, cell fusion was observed in cells transfected with homotypic F/HN proteins; with one exception, coexpression of any combination of F and HN proteins from different viruses did not result in cell fusion. The only exception was found with the closely related PI1 HN and SN HN glycoproteins, either of which could interact with SN F to induce cell fusion upon coexpression as previously reported. By specific labeling and coprecipitation of proteins expressed on the cell surface, we observed that anti-PI2 HN antiserum coprecipitated PI2 F when the homotypic PI2 F and PI2 HN were coexpressed, but not the F proteins of other paramyxoviruses when heterotypic F genes were coexpressed with PI2 HN, suggesting that the homotypic F and HN proteins are physically associated with each other on cell surfaces. Furthermore, we observed that PI3 F was found to coprecipitate with PI3 HN but not with PI2 HN, also indicating a specific association between the homotypic proteins. These results indicate that the homotypic F and HN glycoproteins are physically associated with each other on the cell surface and suggest that such association is crucial to cell fusion induced by paramyxoviruses.

Paramyxoviruses contain two surface glycoproteins: the fusion protein (F) and the hemagglutinin-neuraminidase (HN), which form spikes on the viral envelope and are expressed on the plasma membranes of infected cells. These two proteins are directly involved in virus entry and cytopathology. The HN protein provides an attachment function, allowing virus to bind to sialic acid-containing receptors on the cell surface, and also exhibits a fusion-promoting activity (21), whereas the F protein is known to mediate virus-cell fusion or cell-cell fusion (7, 8). The F protein is synthesized as an inactive precursor, F₀, which is subsequently cleaved into the F₁ and F₂ subunits by a cellular trypsin-like protease which activates its fusion activity (14, 39). Cleavage of F results in exposure of a hydrophobic amino terminus on the F₁ subunit (16, 19), which is thought to be directly involved in fusion activity (20).

A number of previous studies have indicated that both glycoproteins, F and HN, participate in the fusion process (10, 15, 17, 28–30, 43, 45, 49). We previously demonstrated that cell fusion caused by parainfluenza virus type 2 (PI2) or type 3 (PI3) glycoprotein requires expression of the F and HN proteins from the same virus type (17). No cell fusion was detected upon coexpression of F and HN proteins originating from different virus types. These results support the conclusion that the HN protein plays a specific role in cell fusion, in addition to its attachment function. A number of other recent reports also showed similar type-specific interactions among paramyxovirus glycoproteins (2, 6, 13, 15, 25).

Recently, evidence localizing a region in HN which is important for promoting cell fusion has been obtained by analysis of chimeric proteins (9, 42, 46). By constructing several different chimeras from the HN genes of PI3 and Newcastle disease virus (NDV), the region which contains the transmembrane domain and the first 82 residues of the ectodomain in PI3 HN was found to be important for its ability to interact with PI3 F proteins; the specificity of NDV HN for its homologous F is determined by a similar domain (9). In studies of PI2 and simian virus 41 (SV41) HN proteins, a similar region near the membrane-spanning domain and another region in the globular head of PI2 HN were reported to be involved in the cell fusion-promoting activity (46). A chimeric PI3-Sendai virus HN (SN HN) which contains only the 82-amino-acid region just outside the membrane-spanning segment of SN HN was sufficient for almost full fusion activity when coexpressed with the SN F protein (42). It is likely that these HN sequences could interact with sequences in the F protein which are located just external to the transmembrane region. Evidence has been obtained that a leucine zipper-like sequence near the transmembrane domain (heptad repeat B region) of the F protein is necessary for syncytium formation in measles virus and NDV (3, 37, 41). Furthermore, synthetic peptides corresponding to the heptad repeat region B of paramyxovirus F proteins have the potential to inhibit cell fusion (22, 33, 51).

To further understand the mechanism by which the paramyxovirus HN glycoprotein is involved in fusion activity, we have further investigated the interaction between F and HN. By using the vaccinia virus-T7 transient expression system, we investigated the requirements for cell fusion mediated by several paramyxoviruses, examined possible mechanisms which might be related to type-specific interactions between F and

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HN, and investigated the physical association between the F and HN proteins on cell surfaces.

MATERIALS AND METHODS

Cells and viruses. HeLa T4 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum (Hyclone Laboratories, Inc., Logan, Utah). The vaccinia virus-T7 recombinant virus (WR strain) was obtained from Bernard Moss (National Institutes of Health) (11).

Recombinant plasmids. PI2 F and HN cDNA genes were cloned in pGEM-3 and pGEM-3Zf(-) plasmids (Promega Biotech, Madison, Wis.), respectively, at the *Sma*I site as described previously (17). The PI3 F and HN cDNA clones were kindly provided by Mark Galinski. These two genes were subcloned into pGEM plasmid vectors. The PI1 HN cDNA gene was obtained from Yumiko Matsuoka and was subcloned in pGEM-4. The pGEM-4-SNF and pGEM-4-SNHN cDNA clones were kindly provided by Laurent Roux. The SV5 F and HN cDNA clones were kindly provided by Robert A. Lamb and were subcloned in pGEM plasmids.

Rabbit antisera. Rabbit antisera against PI2 or PI3 viruses and PI3 HN were produced as described previously (17). Rabbit antisera against PI2 HN were generated as described by Ray and Compans (35). Monoclonal antibodies specific for PI3 F and PI3 HN have been described previously (34, 36). Rabbit antiserum against Sendai virus was kindly provided by Laurent Roux. Rabbit antiserum against SV5 virus was kindly provided by Robert A. Lamb.

Transfection. Transfection of cells was performed as previously described (17). Briefly, confluent monolayers of cells grown in 35-mm-diameter dishes were infected with the vaccinia virus-T7 recombinant 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 µg of plasmid DNA and 10 µl of lipofectin (Gibco BRL Life Technologies) in 2 ml of DMEM. Cells were incubated at 37°C for 16 h. Immunoprecipitation assays were then performed.

Radiolabeling and immunoprecipitation. Sixteen hours posttransfection, cells were starved in DMEM lacking methionine and cysteine for 30 min and labeled with 100 µCi of [³⁵S]methionine per ml for 1 h at 37°C. Immunoprecipitation was performed as described previously (50) and the radiolabeled polypeptides were analyzed by 10% reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent autoradiography.

Biotinylation assay. Biotinylation of cell surface proteins was performed as described by Lisanti et al. (23). Briefly, transfected cells were labeled with 100 µCi of [³⁵S]methionine per ml at 16 h posttransfection, washed with cold PBS-CM [0.1 mM CaCl₂, 1 mM MgCl₂ in PBS-D (PBS lacking Mg²⁺ and Ca²⁺)] three times and agitated with PBS-CM on ice for 30 min. After addition of NHS-SS-Biotin (Pierce, Rockford, Ill.) at a concentration of 0.5 mg/ml, cells were incubated on ice with agitation for 30 min. Cells were then lysed with mild lysis buffer (10 mM lauryl maltoside, 100 mM NaCl, 50 mM HEPES, pH 7.3) and antibody was added. After addition of 10 µl of 50% protein A agarose beads and incubation at 4°C for 2 h, cell lysates were washed with the lysis buffer and 10 µl of 10% SDS was added, followed by boiling for 5 min. Supernatants were separated from agarose beads by addition of lysis buffer and centrifugation in a microcentrifuge. Ten microliters of 50% streptavidin agarose beads was added to the supernatant, followed by incubation at 4°C for 2 h. Beads were then washed and boiled, and biotinylated proteins were analyzed by 10% reducing SDS-PAGE and subsequent autoradiography. After scanning in the image, each band of interest was quantitated by using the IPLab gel program (Densitometry and Gel Analysis on the Macintosh; Signal Analytics Corporation).

Cell surface immunofluorescence. Cell surface double immunofluorescence analysis was performed as described by Paterson et al. (32) with some modifications. In general, HeLa T4 cells were grown on glass coverslips in 24-well plates and transfected as described above. Sixteen hours posttransfection, cells were washed with ice-cold PBS three times before being fixed with 1% paraformaldehyde in PBS. Rabbit anti-PI2HN (in cells cotransfected with heterotypic glycoprotein genes) or rabbit anti-PI3 HN (in cells cotransfected with homotypic glycoprotein genes) antibody was added onto cell monolayers, and cells were then incubated at 4°C for 30 min. Cells were washed with ice-cold PBS three times, and then goat anti-rabbit rhodamine-conjugated antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala.) was added and cells were incubated at 4°C for 30 min. The cells were then incubated with mouse anti-PI3 F antibody at 4°C for 30 min. After PBS washing, goat anti-mouse fluorescein-conjugated antibody was added and cells were incubated at 4°C for another 30 min. Cell were then examined with a Zeiss LSM 410 invert confocal microscope.

Cocapping assay. HeLa cells were grown in 100-mm-diameter cell culture dishes in DMEM with 10% newborn calf serum. Antibody-induced capping was performed as described by Joseph and Oldstone (18) with modification. Briefly, confluent monolayers of cells were cotransfected with PI3 F and PI3 HN or PI3 F and PI2HN. Sixteen hours posttransfection, cells were washed with warm PBS-D three times and suspended with 2 mM EDTA at room temperature. After cells were washed with ice-cold PBS-D and pelleted by centrifugation at 1,000 rpm, primary antibody to the PI3 HN or PI2HN was added followed by incubation on ice for 30 min. Cells were then washed with ice-cold PBS-D and divided into two aliquots. After the secondary antibody conjugated with rhodamine (rhodamine isothiocyanate [RITC]) was added, the two aliquots of cells were

TABLE 1. Cell fusion after coexpression of paramyxovirus F and HN proteins^a

Protein	Cell fusion ^b after coexpression with:				
	PI1 HN	PI2 HN	PI3 HN	SN HN	SV5 HN
PI2 F	—	+	—	—	—
PI3 F	—	—	+	—	—
SN F	+	—	—	+	—
SV5 F	—	—	—	—	+

^a HeLa T4 cells were cotransfected with 5 µg of plasmid DNA following vaccinia virus-T7 infection at 37°C for 1 h. Cell fusion was examined 8 to 24 h posttransfection.

^b In positive experiments (+), 60 to 70% of cells were found to be involved in syncytium formation, whereas no cell fusion was observed in the negative experiments (—). Trypsin (4 µg/ml) was added to the medium of SN F-transfected cells.

incubated at 37 or 0°C, respectively, for 30 to 60 min. Then, primary antibody to the second antigen PI3 F and secondary antibody conjugated with fluorescein isothiocyanate (FITC) were added with each incubation interval for 30 min on ice. Cells were washed with cold PBS-D and pelleted at 200 × g after each incubation. Cells were finally fixed in 1% paraformaldehyde and resuspended in 200 µl of PBS-D, mounted, and examined with a Zeiss LSM 410 invert confocal microscope. The specificity of antibodies to PI2 or PI3 HN was confirmed by immunofluorescence using cells expressing F proteins or heterotypic HN proteins, which were found to be negative for fluorescence.

RESULTS

Specificity of the interaction between F and HN. We previously demonstrated that using the vaccinia virus-T7 expression system, cell fusion caused by PI2 or PI3 was only observed upon expression of F and HN proteins from the same virus type, which indicated that a type-specific interaction between the glycoproteins was involved in induction of cell fusion (17). To investigate the extent to which this type specificity extends to other paramyxoviruses, we have expressed the glycoproteins of three additional viruses, PI1, Sendai virus, and SV5 in HeLa T4 cells. By immunoprecipitation and/or indirect surface immunofluorescence staining, the proteins encoded by all nine clones presented in Table 1 were found to be expressed at high levels and transported to the surfaces of HeLa T4 cells (data not shown). In all F/HN coexpression combinations tested, expression of F and HN from the same virus resulted in cell fusion, whereas pairs of F and HN proteins originating from different viruses were not observed to induce cell fusion, with one exception. The single exception was observed in cells coexpressing SN F and PI1 HN, which is very closely related to SN HN (27). These results with PI1 HN are consistent with observations reported by Bousse and coworkers (2). These results demonstrate that the coexpression of HN and F proteins from the same virus, or from very closely related viruses, is required for cell fusion to occur, not only for parainfluenza virus type 2 and type 3 but also for the other paramyxoviruses examined.

Sialic acid receptors are essential for cell fusion. We previously observed that addition of the lectin wheat germ agglutinin to PI2 F-expressing cells did not result in cell fusion, indicating that the function of HN cannot be replaced by a lectin which promotes cell attachment (17). We therefore investigated whether the attachment activity of the HN protein to sialic acid is required for cell fusion by examining the effect of treatment of transfected cells with purified *Vibrio cholera* neuraminidase with four viruses: PI2, PI3, SNV, and SV5 as shown in Fig. 1. In the cells not treated with neuraminidase, transfection of F and HN resulted in extensive syncytium formation

FIG. 1. Effect of neuraminidase treatment of F/HN-cotransfected HeLa T4 cells on cell fusion. HeLa T4 cells were cotransfected with PI2 F and PI2 HN, PI3 F and PI3 HN, SN F and SN HN, and SV5 F and SV5 HN, as indicated. Cells were incubated at 37°C in the absence (top row) or presence (bottom row) of *V. cholera* neuraminidase (Gibco BRL Life Technology; protease content less than 0.1 mU/ml) at a concentration of 2.5 U/ml. Cell fusion was examined from 8 to 24 h posttransfection. Note that 4 µg of trypsin per ml was added to SN F-transfected cell medium.

(top photographs). In contrast, cell fusion was found to be almost completely inhibited when F/HN-transfected cells were treated with neuraminidase (bottom photographs). The results indicate that the attachment of HN to sialic acid receptors is critical to cell fusion, since removal of these receptors results in elimination of fusion activity. Moscona and Peluso (29, 30) have also reported evidence that interaction of the PI3 HN protein with sialic acid receptors on neighboring cells is one of the essential steps in the fusion process. It is likely that this interaction facilitates effective contact of the F protein with the target cell membrane.

An electrophoretic mobility change in F coexpressed with HN does not require a type-specific interaction. Our previous results showed that when PI2 F was expressed together with the HN protein, it always migrated faster than F expressed by itself, suggesting that sialic acid on the F protein was removed by the neuraminidase activity of HN (17). Since the neuraminidase activities of various paramyxoviruses could differ in their specificities, it was of interest to determine whether this phenomenon is related to the type-specific interaction between F and HN that is involved in cell fusion. HeLa T4 cells were transfected with the PI2 F gene together with the HN gene of PI2, SN, PI1, or SV5 as shown in Fig. 2. As observed previously, the PI2 F protein coexpressed with the PI2 HN protein (Fig. 2, lane 2) migrated faster than F expressed in the absence of HN (Fig. 2, lane 1). However, the PI2 F protein also migrated to the same position when coexpressed with other HN proteins, i.e., SN HN (lane 3), PI1 HN (lane 4), or SV5 HN (lane 5). To confirm that such a faster migration is caused by removal of sialic acid, PI2 F-transfected cells were treated with

V. cholera neuraminidase as shown in Fig. 2, lane 6. After neuraminidase treatment, PI2 F migrated to a position similar to that of the PI2 F protein which was coexpressed with HN. These results indicate that the sialic acid of the F protein can be removed by coexpression with any of the HN proteins tested, demonstrating that this phenomenon is unlikely to be involved in the type-specific F/HN interactions required for cell fusion. Further, the removal of sialic acid by HN demonstrates that the F protein is at least transiently present in close

FIG. 2. Effect of neuraminidase activity of the HN protein on the electrophoretic mobility of coexpressed F. HeLa T4 cells were transfected with PI2 F alone (lane 1), PI2 F with PI2 HN (lane 2), PI2 F with SN HN (lane 3), PI2 F with PI1 HN (lane 4), PI2 F with SV5 HN (lane 5), and PI2 F plus neuraminidase (lane 6). Sixteen hours posttransfection, cells were labeled with [³⁵S]methionine at 37°C for 1 h, lysed with radioimmunoprecipitation assay buffer, and immunoprecipitated with rabbit anti-PI2 virus antiserum.

FIG. 3. Coprecipitation of F and HN proteins is type specific. (A) Specificity of antiserum to the PI2 HN protein. HeLa T4 cells were transfected with PI2 F (lanes 2 and 3) or PI2 HN (lane 4) or infected with vaccinia virus-T7 (lane 1). Cells were then lysed and immunoprecipitated with rabbit anti-PI2 virus antiserum (lane 2) or rabbit anti-PI2 HN antiserum (lanes 1 and 3 to 4). PI2 HN protein was precipitated by the anti-PI2 HN antiserum (lane 4), whereas no PI2 F protein was precipitated by this antibody (lane 3). (B) Biotinylation and coprecipitation of the F and HN proteins on the cell surface. HeLa T4 cells were cotransfected with PI2 F and PI2 HN (lanes 1 and 2), SN F and SN HN (lane 3), SN F and PI2 HN (lane 4), SV5 F and SV5 HN (lane 5), or SV5 F and PI2 HN (lane 6) or transfected with PI2 F (lane 7), SN F (lane 8), or SV5 F (lane 9). At 16 h posttransfection cells were labeled with [³⁵S]methionine for 3 h at 37°C, surface proteins were biotinylated with NHS-SS-Biotin, and then the biotinylated proteins were immunoprecipitated with rabbit anti-PI2 virus antiserum (lane 1), rabbit anti-PI2 HN antiserum (lanes 2, 4, 6 to 9), rabbit anti-Sendai virus antiserum (lane 3), or rabbit anti-SV5 antiserum (lane 5). Trypsin (4 μg/ml) was added to SN F-transfected cell medium during the labeling. Biotinylated proteins were finally precipitated with streptavidin agarose beads and analyzed by SDS-PAGE. Double bands in the middle of the gel in all lanes are the background proteins from vaccinia virus infections.

physical proximity to homotypic as well as heterotypic HN proteins during intracellular transport or after arrival on the cell surface.

Coprecipitation of the F protein by anti-HN antibody. The type-specific interaction of F and HN in induction of cell fusion suggests that these two glycoproteins could be physically associated with each other, forming a functional complex which is critical for syncytium formation. To investigate the interactions of the F and HN proteins, a surface biotinylation assay was employed to selectively identify the proteins expressed on the cell surface (Fig. 3). The results in Fig. 3A demonstrate the specificity of the anti-PI2 HN antibody in immune precipitation assays. In Fig. 3B, HeLa T4 cells were cotransfected with PI2 F and PI2 HN (lanes 1 and 2), SN F and SN HN (lane 3), SN F and PI2 HN (lane 4), SV5 F and SV5 HN (lane 5), and SV5 F and PI2 HN (lane 6) or singly transfected with PI2 F (lane 7), SN F (lane 8), or SV5 F (lane 9). The biotinylated

surface proteins were immune precipitated with specific antibodies, solubilized, and then precipitated with streptavidin agarose beads. The F₁ protein of PI2 was found to be coprecipitated by anti-PI2 HN antiserum when coexpressed with PI2 HN (lane 2). In contrast, although F₁ proteins of SN F (lanes 3) or SV5 F (lanes 5) were shown to be expressed on cell surfaces when precipitated with antisera to the respective virus, there was no coprecipitation of SN F₁ (lane 4) or SV5 F₁ (lane 6) when they were coexpressed with PI2 HN and precipitated by PI2 HN. In other experiments we observed that PI3 HN antibody was also able to coprecipitate the PI3 F protein but not PI2 F (not shown).

To further determine whether the coprecipitation of HN and F requires interaction of the two proteins, cell lysates expressing PI2 F alone and lysates expressing PI2 HN alone were mixed and immunoprecipitated with PI2 HN antiserum. No coprecipitation of PI2 F₁ was observed with PI2 HN antiserum (not shown). To determine what fraction of the surface F protein was coprecipitated with the anti-HN antibody, the amount of PI2 F protein detected on cell surfaces by antibody against PI2 virus and the amount coprecipitated by the PI2 HN antibody were quantitated and compared. The results indicated that about 80% of the PI2 F protein expressed on the cell surface was coprecipitated with PI2 HN antiserum. In order to exclude the possibility that failure to precipitate a heterotypic F is due to low levels of surface expression in the heterotypic F and HN combination, a cell surface immunofluorescence assay was performed. The levels of PI3 F on cell surfaces in cells coexpressing homotypic PI3 HN or heterotypic PI2 HN were found to be similar (data not shown).

These results indicate that the PI2 and PI3 F and HN proteins are physically associated with each other on cell surfaces, whereas heterotypic combinations of F and HN do not show a similar association. This physical association is likely to reflect their functional interaction in the induction of cell fusion.

Cocapping of homotypic F and HN glycoproteins. Many viral glycoproteins have the potential to undergo antibody-induced lateral redistribution on plasma membranes to form a discrete cap on the cell surface (24). To further study the interactions of the F and HN proteins on the cell surface, capping and cocapping assays were performed. If under conditions facilitating capping by specific antibody to HN the F proteins also migrate to the same position (cocapping) on the cell surface, this would indicate that these two proteins are physically associated with each other. In contrast, if HN is capped by specific anti-HN antibody while F still remains evenly distributed around the cell surface (no cocapping), this would indicate that the two proteins are not associated. Cocapping was examined by double staining with RITC labeling for the HN proteins and FITC labeling for the F proteins, respectively. After cells were cotransfected with PI3 F and PI3 HN for 16 h, cells were incubated with rabbit anti-PI3 HN antiserum (at 0°C) for 30 min and RITC-conjugated goat anti-rabbit immunoglobulin G (IgG) (at 37°C) for 45 min, respectively, followed by incubation for 30 min with monoclonal antibody to PI3 F (at 0°C) and FITC-conjugated goat anti-mouse IgG (at 0°C). Figure 4a shows the red cap formed by PI3 HN, while Fig. 4b shows a green cap formed by PI3 F. On the surface of the doubly stained cells (Fig. 4c), it can be seen that the two membrane proteins are co-redistributed to form a unipolar cap in the same position. In contrast, we observed a lack of association of heterotypic combinations of F and HN proteins on the cell surface by the cocapping assay. As shown in Fig. 4d, most of the RITC-stained PI2 HN formed a cap, while FITC-stained PI3 F remained uniformly distributed on the cell surface (Fig. 4e). Figure 4f shows the lack of colocal-

FIG. 4. Cocapping of F and HN proteins. By using the transient vaccinia virus-T7 expression system, HeLa cells were cotransfected with homotypic PI3 F and PI3 HN (a through c) or heterotypic PI3 F and PI2 HN (d through f). At 16 h posttransfection, cotransfected PI3 F and PI3 HN HeLa cells were first incubated with rabbit anti-PI3 HN antiserum (a through c); PI3 F and PI2 HN expressing HeLa cells were incubated with rabbit anti-PI2 HN antibody (d through f) at 0°C for 30 min and then with RITC-conjugated goat anti-rabbit IgG at 37°C for 45 min. Cells were then incubated with monoclonal antibody to PI3 F at 0°C for 30 min and subsequently with FITC-conjugated goat anti-mouse IgG at 0°C for 30 min. Cells were examined with a Zeiss LSM confocal microscope. PI3 F and PI3 HN cotransfected cells showed rhodamine staining of a red cap formed by PI3 HN (a), fluorescein staining showing cocapping of PI3 F (b), and an image corresponding to PI3 F and PI3 HN in the same focal plane is shown in panel c. The yellow cap shows that both the PI3 F and PI3 HN proteins cocap in the same position. PI3 F and PI2 HN cotransfected cells showed rhodamine staining of a red cap formed by PI2 HN (d), fluorescein staining of PI3 F uniformly distributed on the cell surface (e), and an image corresponding to PI3 F and PI2 HN in the same focal plane is shown in panel f. There was no cocapping observed with heterotypic PI3 F and PI2 HN on the cell surface.

ization of the two images. These results indicate that F and HN proteins of the same virus type laterally move together on the cell surface under conditions of antibody-induced capping, whereas heterologous F and HN proteins fail to show a similar association.

DISCUSSION

Our previous finding that the HN protein is not only required for cell fusion but that coexpressed F and HN proteins also need to be derived from the same virus type demonstrated that the F and HN glycoproteins are involved in a type-specific interaction which promotes cell fusion. Therefore, the mechanism of cell fusion seems to be more complex than had been previously believed because of the nature of the specific involvement of the HN protein in the fusion process. On the basis of our previous findings, several alternative mechanisms of paramyxovirus F-HN interaction were considered. These include the role of the binding of HN to sialic acid receptors, the effect of HN neuraminidase on removal of sialic acid from F, and the possible formation of an F/HN glycoprotein complex. The present results provide evidence for the formation of such a complex on cell surfaces.

We demonstrated by coexpression of homotypic and heterotypic F/HN proteins that the type-specific F-HN interaction is not only required for parainfluenza virus types 2 and 3 but also occurs with other paramyxoviruses, including Sendai virus and SV5. These results are consistent with our previous conclusion that an interaction between F and HN of the same virus serotype is crucial to cell fusion induced by paramyxoviruses and indicate that they share a common mechanism in the fusion process. The finding that PI1 HN can interact with SN F to

induce cell fusion is an exception, but it can be explained by the fact that the HN proteins of PI1 and SN viruses are the most closely related among paramyxoviruses with primary sequence identity of 83.1% (27). The structure of HN of the two viruses would be predicted to be very similar, as shown by a nearly identical hydropathicity profile and by conservation of structural elements, including all 17 cysteines, 26 of 27 prolines, and 29 of 31 glycines (12). Recently, it was reported in several studies that certain heterotypic glycoprotein combinations could also induce cell fusion (1, 2, 46). Our result with PI1 is in agreement with the report by Bousse and coworkers (2). These results are thought to reflect a high level of sequence similarity in the HN proteins or differences in requirements of the F proteins for induction of cell fusion.

In the present study we found that the F protein of PI2 was coprecipitated by antibody to PI2 HN, indicating that the two proteins are tightly associated with each other. Furthermore, the anti-PI2 HN antibody was only found to coprecipitate the homotypic F protein, indicating that the homotypic F and HN proteins are physically associated on the cell surface. The results suggest that a type-specific fusion complex may form on the cell surfaces in which the two proteins physically contact each other. In contrast, no such interaction was detected between the heterotypic F and HN combinations on the cell membrane. Similarly, there is no coprecipitation of homotypic F and HN by HN antibody when cell lysates expressing the individual proteins were mixed together. Malvoisin and Wild (25) reported that measles virus F and HA proteins expressed in HeLa cells infected with vaccinia virus recombinants can be coimmunoprecipitated in the presence of the cross-linking reagent DSP. We found that the coprecipitation of the F and HN

proteins of PI2 virus was detected even without the addition of a cross-linking reagent. Additional evidence for homotypic F and HN association was obtained by a cocapping assay, showing that combinations of F and HN proteins which interact to induce cell fusion laterally move together when redistributed on the cell surface by bivalent antibody. Only if the two proteins are physically linked together would migration of one protein be expected to cause the other protein to redistribute to the same position. Consistent with the coprecipitation results, the cocapping of the two glycoproteins was found to occur only with F and HN of the same virus type; heterotypic combinations of F and HN were not found to laterally move together.

Recently, an alternative explanation of the type specificity of F-HN interaction was proposed by Tanaka and coworkers (44). They reported that PI3 F-KDEL, a mutant F protein containing a signal for retention in the endoplasmic reticulum, can down-regulate PI3, SV5, and SN HN and measles virus H expression, suggesting that the down-regulation of heterotypic HN expression may result in the serotype-specific restriction of syncytium formation. They proposed that a transient F-HN interaction could induce a conformational change in the F protein. We previously observed, however, that coexpression of a heterotypic PI3 F protein in cells coexpressing PI2 F and PI2 HN did not block the cell fusion induced by the homotypic proteins (17). Therefore, it seems unlikely that the heterotypic F proteins have a dominant negative effect on fusion activity.

Paramyxoviruses also encode a matrix protein which forms a dense layer which underlies the viral membrane. It has been reported that in Sendai virus-infected cells, the M protein binds independently to either the F or the HN protein, which is considered to be an important step in virus assembly (38). An association with M could thus play an important role in HN and F association. However, the present results support the conclusion that such an association can occur in the absence of M, and many studies have obtained evidence that expression of M is not required for paramyxovirus fusion activity. We have also observed that PI2 F cytoplasmic domain truncation mutants promote efficient cell fusion when coexpressed with PI2 HN (50). It will be of interest to determine whether the association of the M protein differs in the case of homotypic or heterotypic F or HN proteins and in truncated forms of the F protein.

Recently, some progress has been made toward defining the regions involved in the type-specific interaction of F and HN. Sergel and coworkers (40) reported that the HN fusion promotion activity can be separated from its hemagglutinating activity. Several studies on determination of the regions of the proposed F and HN interactions (9, 42, 46) have concluded that a region in the extracellular domain adjacent to the transmembrane domain of the HN protein is important for interaction with the respective F protein in promoting cell fusion. Using chimeric proteins, a 45-amino-acid cysteine-rich segment has been identified in the measles virus F protein that can transfer specificity for its attachment protein to the F protein of canine distemper virus (48). These results all support the proposal that type-specific association between the homotypic F and HN plays an important role in the cell fusion process.

Taken together, the present study further demonstrates the requirement of F and HN in paramyxovirus-induced cell fusion, the requirement that both glycoproteins have to be from the same or a very closely related virus type, and the requirement for attachment of HN to sialic acid in addition to its specific role in cell fusion. In most enveloped viruses, a single fusion protein is sufficient for fusion activity; however, the same molecule is also responsible for receptor binding (47). In

contrast, paramyxovirus fusion proteins do not have receptor binding activity. However, our data indicate that cell fusion induced by paramyxovirus also involves formation of a complex in which F and HN are physically associated with each other. It will be of interest to determine whether the formation of such a complex may result in a conformational change of the F protein, similar to the conformational changes of fusion proteins of other enveloped viruses such as influenza virus, vesicular stomatitis virus, or Semliki Forest virus, which are triggered by a low-pH environment (26, 31). The resulting change may lead to further exposure of the amino terminus of the F₁ subunit, as occurs in the influenza virus HA protein (4, 5). Alternatively, the functional interaction of the two proteins may depend on specific lateral interactions between F and HN, leading to assembly into a patch-like structure on the plasma membrane. Such a domain may lead to the effective contact of F with target membranes, which results in membrane fusion, whereas individual F subunits may be unable to mediate fusion efficiently because they are randomly distributed on the cell surface.

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