Fusion properties of cells constitutively expressing human parainfluenza virus type 4A haemagglutinin–neuraminidase and fusion glycoproteins

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We established HeLa cell lines that constitutively expressed the fusion (F) and/or haemagglutinin–neuraminidase (HN) glycoproteins of human parainfluenza virus type 4A (PIV-4A) and used them to analyse the roles of these glycoproteins in virus-induced cell fusion. No syncytium formation occurred, even in HeLa cells expressing both the F and HN proteins (HeLa-4aF + HN cells). Also no syncytium was found in a mixed culture of cells expressing the F protein (HeLa-4aF) and the HN protein (HeLa-4aHN). Syncytia were observed in HeLa-4aF cells transfected with the HN gene, but no syncytium formation was found in HeLa-4aHN cells transfected with the F gene. Co-cultivation of HeLa-4aF + HN cells with HeLa-4aF cells generated large polykaryocytes, whereas co-cultivation with HeLa-4aHN cells induced no cell fusion. Infection of HeLa-4aF cells with PIV-4A generated large syncytia and degenerated nuclei, whereas little or no polykaryocytes were found in HeLa-4aHN cells infected with PIV-4A. From the above findings, the following conclusions were drawn: (i) the expression of both the F and HN proteins in the same cell is necessary for cell fusion; (ii) the expression of the F protein alone enhances susceptibility to cell fusion; (iii) the constitutive expression of the HN protein promotes resistance to paramyxovirus-induced cell fusion.

Introduction

Human parainfluenza virus type 4A (PIV-4A), which belongs to the Paramyxoviridae, is an important respiratory tract pathogen in children and adults, but most of its biological and epidemiological properties remain unclarified. Paramyxoviruses have two envelope glycoproteins which play important roles in their life cycles. One of these, the fusion (F) protein, mediates fusion of the viral and the target cell plasma membranes, and of plasma membranes of virus-infected cells. The fusion proteins of paramyxoviruses are synthesized as a precursor, F₀, which is subsequently cleaved by a host cell protease into the biologically active form (F₁+₂) of the fusion protein. The amino acid sequence of the PIV-4A F protein cleavage site is Glu–Ile–Gln–Ser–Arg, that of an avirulent paramyxovirus (unpublished results; Bando et al., 1991). The haemagglutinin–neuraminidase (HN) protein has both neuraminidase and haemagglutination activities, and is believed to mediate the first and last steps in infection, the virus attachment to and the release from the surface of host cells. Recently a third function of the HN protein has been described, fusion promotion activity. The HN protein’s attachment function is not sufficient to promote fusion (Sergel et al., 1993). Several groups have investigated the induction of cell fusion using transient expression systems for the paramyxovirus F and/or HN proteins (Paterson et al., 1985; Sakai & Shibuta, 1989; Morrison et al., 1991; Ebata et al., 1991; Tanabayashi et al., 1992; Horvath et al., 1992; Hu et al., 1992) and there have been conflicting results concerning the requirement of the HN protein.

In this study, we established HeLa cell lines that constitutively express the F and/or HN glycoproteins of PIV-4A and have used these cells to analyse the roles of these glycoproteins in virus-induced cell fusion. We show that: (i) the expression of both the F and HN proteins in the same cell was necessary for cell fusion; (ii) the expression of the F protein alone enhanced the susceptibility to cell fusion; (iii) the constitutive expression of the HN protein conferred resistance to paramyxovirus-induced cell fusion.

Methods

Viruses and cells. The viruses used in this study were PIV-4A Toshiba strain, PIV-2 Toshiba strain, PIV-3 Toshiba strain and Newcastle disease virus (NDV) Sato strain. HeLa cells were grown in Eagle’s MEM supplemented with 5 % calf serum.

Immunofluorescent staining of the cell surface. HeLa cells, fixed with 10% paraformaldehyde for 15 min, were incubated with monoclonal
Fig. 1. Immunofluorescent staining of cell surfaces. HeLa-4aF (a, b), HeLa-4aHN (c, d) and HeLa-4aF+HN cells (e, f) were fixed with 10% paraformaldehyde for 15 min, incubated with anti-PIV-4A F protein MAb A161 (a, c, e) or anti-PIV-4A HN protein MAb A147 (b, d, f) for 1 h and then reacted with FITC-conjugated anti-mouse IgG goat serum. The scale bar represents 25 µm.

antibodies (MAbs; anti-PIV-4A HN or F protein MAb, or anti-PIV-1 HN MAb as a control) for 1 h and then were reacted with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG goat serum. Immunofluorescence-stained cells were examined under a fluorescence microscope.

Flow cytometry. HeLa cells (5 x 10^6) were incubated with various MAbs for 1 h and were then reacted with FITC-conjugated anti-mouse IgG goat serum. Immunofluorescence-stained cells were analysed on a FACScan (Becton Dickinson) using Consort 30 software (Becton Dickinson).

ELISA. HeLa cells, cultured in 96-well plates, were fixed with 10% paraformaldehyde for 20 min and were incubated with various MAbs for 1 h. They were then reacted with peroxidase-conjugated anti-mouse IgG goat serum.

Haemadsorption assay. Cells were overlaid with a 0.4% suspension of guinea pig erythrocytes and incubated for 1 h at room temperature. The cells were washed vigorously three times with MEM and then examined for the presence of bound red blood cells under a light microscope.

Measurement of sialic acid on the cell surface. Sialic acid on the cell surface was assayed by a modification of the thiobarbituric acid method of Warren (Bando et al., 1990). HeLa cells (1 x 10^6) were treated with 0.167 M-sulphuric acid at 80°C for 1 h and then the cell suspension was centrifuged at 3000 r.p.m. for 10 min. The pH of supernatant was adjusted to 5.0 and A_540 was measured.

Fig. 2. Expression levels of viral glycoproteins on the cell surfaces of HeLa cells and HeLa cells infected/co-cultured with PIV-4A, HeLa-4aF, HeLa-4aHN and HeLa-4aF+HN cells. The expression levels of virus-specific antigen and proportions of expressing cells were investigated by a flow cytometer; (---), cells stained with anti-F or HN MAb; (----), cells stained with control MAb; (-----), normal HeLa cells stained with anti-F or HN MAb.

Construction of recombinant plasmids

(i) PIV-4A F gene clone (4aF-pkan2). A cDNA clone of the PIV-4A F gene was inserted into the pCDL-SRα 296 plasmid between the PstI site and the KpnI site. Plasmid pDS-4aF5, donated by Dr H. Bando (Hokkaido University, Japan) was cut with Clal and SalI. The cDNA fragment was inserted into the SalI site of the pkan-2 vector. The promoter in the pkan-2 vector is identical to the SRα promoter [simian virus 40 (SV40) plus human T cell leukaemia virus type 1 promoters].

(ii) PIV-4A HN gene clone (4aHN-pkan2). A cDNA clone of the PIV-4A HN gene was inserted into the pCDL-SRα 296 plasmid between the PstI and KpnI sites. Plasmid pDR-4aH5, donated by Dr H. Bando, was cut with Sali. The cDNA fragment was inserted into the Sali site of the pkan-2 vector.

Transfection. HeLa cells were grown in 90 mm dishes at 37°C in MEM containing 5% calf serum. The cells were washed twice with warm MEM and then transfected with the plasmids 4aF-pkan2 and/or
4aHN-pkan2 with Lipofectin (Gibco) in 4 ml of MEM. After incubation for 8 h at 37 °C, MEM with 10% calf serum was added. After 2 days of further incubation the culture medium was changed to MEM containing 10% calf serum, 1 mg/ml Geneticin and 0.1% agarose and then the cells were cultured for about 3 weeks. Since cell selection may occur in the colony isolation procedure used to isolate cell lines expressing the viral proteins, two to four transfectant cells were used in this study.

Cell lines that constitutively express the PIV-4A F protein. HeLa cells transfected with the plasmid 4aF-pkan2 were purified by the colony isolation procedure to give HeLa-4aF cells, constitutively expressing the PIV-4A F protein. This was detected by ELISA using the F-specific MAb, A161 (Komada et al., 1989). The surface expression of the F protein was confirmed using indirect immunofluorescent staining of paraformaldehyde-fixed cells (Fig. 1 and 2).

Cell lines that constitutively express the PIV-4A HN protein. We also isolated HeLa-4aHN cell lines that were transfected with 4aHN-pkan2. The expression of the HN protein was detected by ELISA using the HN-specific MAb A147 (Komada et al., 1989). The surface expression of the HN protein was examined by a haemadsorption assay and indirect immunofluorescent staining of paraformaldehyde-fixed cells. The HeLa-4aHN cells were positive for haemadsorption and surface fluorescence (Fig. 1 and 2).

Cell lines that constitutively express both PIV-4A F and HN proteins. HeLa cells were transfected with recombinant plasmids 4aHN-pkan2 and pDS-4aF5 together and HeLa-4aF+HN cells were isolated. The expression of the HN and F protein was detected by ELISA using anti-PIV-4A HN and F MAbs. To confirm the expression of these proteins on the cell surface, we carried out a haemadsorption assay and surface immunofluorescent staining (Fig. 1 and 2).

Results
The levels of expression and proportions of expressing cells

The levels of virus-specific antigen expression and the proportions of expressing cells were investigated by three methods, analyses of immunofluorescent-stained cells using a fluorescent microscope and a flow cytometer, and ELISA. Almost all cells of types HeLa-4aHN, HeLa-4aF and HeLa-4aF+HN were positive for surface immunofluorescence (Fig. 1). When HeLa cells were transfected with the plasmid pcDL-SRc containing cDNA of PIV-4A HN or F gene, 1 to 2% of cells transiently expressed antigen (data not shown). Fig. 2 shows the results of flow cytometry of immunofluorescent cells. The expression levels of PIV-4A HN and F proteins were highest in virus-infected cells (Fig. 2). ELISA titres of these cells measured using anti-HN or anti-F MAbs are shown in Table 1. The expression levels of HN protein were almost the same in HeLa-4aHN, HeLa-4aF+HN and virus-infected HeLa cells, whereas the F protein was expressed at a higher level in HeLa-4aF and HeLa-4aF+HN cells than in virus-infected cells.

PIV-4A possesses neuraminidase, although it shows a low activity using fetuin and neuraminilactose as substrates (Bando et al., 1990). The amounts of sialic acid on the surface of HeLa-4aHN and HeLa-4aF+HN cells were approximately 75% of that on HeLa-4aF or untreated HeLa cells (Table 2).

No induction of syncytium formation in HeLa-4aHN, HeLa-4aF and HeLa-4aF+HN cells

The cleavage site of PIV-4A F corresponds to that of an avirulent type, so in PIV-4A-infected HeLa cells the F protein is not cleaved and consequently no cell fusion is induced. Therefore, to analyse the fusion-related function of F or HN proteins, acetylated trypsin (0.1 μg/ml) was added to serum-free culture fluids of HeLa-4aHN, HeLa-4aF or HeLa-4aF+HN cells. After 24 h of incubation, the cells were stained with Giemsa and observed with a microscope. No syncytium formation occurred in these cells, even in HeLa cells expressing both F and HN proteins (HeLa-4aF+HN cells; Fig. 3c). Syncytium formation was also not found in the mixed culture of HeLa-4aF and HeLa-4aHN cells with or without untreated HeLa cells (Fig. 3a, b, d and e).

Induction of syncytium formation in HeLa-4aF cells transfected with the PIV-4A HN gene

Subsequently, HeLa-4aF and HeLa-4aHN cells were transfected with the PIV-4A HN and F genes, respectively, and acetylated trypsin was added to the medium at 24 h after transfection. After further incubation for 8 h, large syncytia were observed in HeLa-4aF cells transfected with the HN gene (Fig. 3g), but no syncytium formation was found in HeLa-4aHN cells transfected with the F gene (Fig. 3h) or in HeLa-4aF cells with vector (SRc) alone (Fig. 3f).

Induction of syncytium formation in HeLa-4aF+HN cells co-cultured with HeLa or HeLa-4aF cells

HeLa-4aF+HN cells, which constitutively expressed both the HN and F proteins, showed no cell fusion even when treated with trypsin. When HeLa-4aF+HN cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>ELISA titre</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Anti-F MAb</td>
</tr>
<tr>
<td>HeLa-4aF</td>
<td>1.82</td>
</tr>
<tr>
<td>HeLa-4aHN</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>HeLa-4aF + HN</td>
<td>1.74</td>
</tr>
<tr>
<td>HeLa infected with PIV-4A*</td>
<td>0.67</td>
</tr>
</tbody>
</table>

* At a m.o.i. of 1 for 24 h.
Table 2. Levels of sialic acid on different cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Sialic acid content (A540)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa-4aF</td>
<td>1.10</td>
</tr>
<tr>
<td>HeLa-4aHN</td>
<td>0.91</td>
</tr>
<tr>
<td>HeLa-4aF + HN</td>
<td>0.92</td>
</tr>
<tr>
<td>HeLa</td>
<td>1.22</td>
</tr>
<tr>
<td>HeLa transiently expressing HN protein</td>
<td>1.14</td>
</tr>
</tbody>
</table>

* Per 10 μg of total protein.

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Fig. 3. Induction of syncytium formation in HeLa-4aF, HeLa-4aHN and HeLa-4aF + HN cells. Acetylated trypsin (0.1 mg/ml) was added to serum-free culture fluids of HeLa-4aF (a), HeLa-4aHN (b) and HeLa-4aF + HN (c) cell monolayers. After 24 h incubation, the cells were stained with Giemsa. A mixed culture of HeLa-4aF and HeLa-4aHN cells without (d) or with (e) untreated HeLa cells was incubated for 24 h in the presence of acetylated trypsin. HeLa-4aF cells transfected with vector sRα (f) or the PIV-4A HN gene (g) and HeLa-4aHN with the PIV-4A F gene (h) were incubated for 8 h in the presence of acetylated trypsin. The scale bar represents 200 μm.

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Fig. 4. Induction of syncytium formation in HeLa-4aF + HN cells co-cultured with HeLa, HeLa-4aF or HeLa-4aHN cells. Mixed cultures of HeLa-4aF + HN cells with untreated HeLa (a), HeLa-4aF (b) or HeLa-4aHN (c) cells (ratio 1:1) were incubated for 24 h in the presence of acetylated trypsin. The scale bar represents 200 μm.

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Fig. 5. Induction of syncytium formation by PIV-4A infection. HeLa (a, b), HeLa-4aF (c), HeLa-4aHN (d) and HeLa-4aF + HN (e) cells were infected with PIV-4A at a m.o.i. of approximately 0.01 and acetylated trypsin (0.1 μg/ml) was added to the culture fluids 24 h p.i. After further incubation for 5 h (a, c, d, e) or 24 h (b), these cells were stained. The scale bar represents 200 μm.

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large syncytia (Fig. 4b), whereas co-cultivation with HeLa-4aHN cells induced no cell fusion (Fig. 4c).

**Induction of syncytium formation by PIV-4A infection**

HeLa cells were infected with PIV-4A and acetylated trypsin was added to the culture medium 24 h post infection (p.i.). None, or very small polykaryocytes appeared after 5 h of further incubation (Fig. 5a). Thereafter polykaryocyte formation increased and intermediate-sized syncytia were found after 24 h of...
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Fig. 6. Induction of syncytium formation by PIV-2 infection. PIV-2 was used to infect HeLa (a) or HeLa-4aF cells (b) at a m.o.i. of 0.01 and 16 h after infection, the cells were fixed and stained. The scale bar represents 200 μm.

further incubation (Fig. 5 b). When HeLa-4aF cells were used, extensive syncytia and degenerated nuclei were found at 5 h after addition of acetylated trypsin (Fig. 5 c). Infection of HeLa-4aF + HN cells with PIV-4A generated large syncytia (Fig. 5 e). In contrast, when HeLa-4aHN cells were infected with PIV-4A with trypsin, none or few polykaryocytes were found even at 24 h after treatment with trypsin, although the cells were damaged (Fig. 5 d). Virus yields in these cultures were almost the same when virus titres were measured at 48 h p.i., indicating that virus–cell membrane fusion occurs in HeLa-4aHN cells infected with PIV-4A. Furthermore, other paramyxoviruses such as PIV-2, PIV-3 and NDV induced large, intermediate, small or no syncytia in HeLa-4aF, HeLa-4aF + HN, untreated HeLa or HeLa-4aHN cells, respectively, when observed 16 h p.i., though yields of these viruses were almost the same in these cells (shown for PIV-2 in Fig. 6; data not shown). This indicated that the expression of the PIV-4A F protein enhanced the susceptibility to virus-induced cell fusion and expression of the PIV-4A HN protein suppressed the susceptibility.

Discussion

The functions of paramyxovirus glycoproteins have recently been analysed using transient expression systems (Paterson et al., 1985; Sakai & Shibuta, 1989; Morrison et al., 1991; Ebata et al., 1991; Tanabayashi et al., 1992; Horvath et al., 1992; Hu et al., 1992) and both HN and F glycoproteins have been found to be required for fusion in most cases. However, the expression of the simian virus 5 (SV5) F cDNA in CV-1 cells using an SV40-derived vector has been shown to cause syncytium formation (Paterson et al., 1985). In this study, we established cell lines constitutively expressing PIV-4A F and/or HN proteins and investigated the fusion properties of these cells (summarized in Table 3). This system has several advantages, namely (i) more stable and reproducible conditions compared with transient expression; (ii) the glycoproteins are expressed in all cells whereas in transient expression systems, other than those using a virus vector, few cells express the proteins; (iii) transfection causes some amount of cell damage, whereas established cells constitutively expressing the glycoproteins are stable and their growth is indistinguishable from untreated cells.

HeLa cells expressing the PIV-4A F or HN protein showed no cell fusion. Syncytium formation was induced when HeLa-4aF cells were transfected with the recombinant plasmid carrying the HN gene, showing that both the F and HN proteins of PIV-4A are required for cell fusion. PIV-4A is therefore similar to bovine and human PIV-3 (Sakai & Shibuta, 1989; Ebata et al., 1991; Moscona & Peluso, 1992), PIV-2 (Hu et al., 1992), mumps virus (Tanabayashi et al., 1992) and NDV (Morrison et al., 1991) in that HN is required for cell fusion. Furthermore, mixed cultures of HeLa-4aF and

Table 3. Summary of syncytium formation

<table>
<thead>
<tr>
<th>Cells</th>
<th>Trypsin alone</th>
<th>HeLa-4aF + HN cells</th>
<th>PIV-4A F gene</th>
<th>PIV-4A HN gene</th>
<th>PIV-4A</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>*</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HeLa-4aF</td>
<td></td>
<td>7.2 (4–14)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa-4aHN</td>
<td></td>
<td>66.8 (28–113)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa-4aF+HN</td>
<td></td>
<td></td>
<td>118.4 (65–191)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>293.6 (156–360)</td>
<td></td>
</tr>
</tbody>
</table>

* Multinuclear cells not found.
† Average number of nuclei per multinucleate cell.
‡ Range of number of nuclei.
HeLa-4aHN cells did not cause cell fusion, indicating that cell fusion requires both the F and HN proteins expressed in the same cells. Morrison et al. (1991) reported that mixing of NDV F-expressing chicken embryo cells with HN-expressing cells did not result in cell fusion, whereas cell fusion occurred when the two glycoproteins were expressed in the same cells. In contrast Hu et al. (1992) have recently found that cell fusion is observed when PIV-2 F-transfected HeLa cells are mixed with PIV-2 HN-transfected cells. The level of expression of the glycoproteins in our system is sufficient for induction of cell fusion since (i) transfection of the HN gene into HeLa-4aF cells generated large syncytia and (ii) HeLa-4aHN cells showed resistance to virus-induced cell fusion. In addition, the cells used in our study were normal HeLa cells but Hu et al. (1992) used HeLa-T4 cells. Therefore, the difference may be due to a difference in the vector system and the vaccinia virus system may have some unknown function(s).

We also showed that the expression of the F protein alone enhanced susceptibility to virus-induced cell fusion. When HeLa-4aF + HN cells were added to untreated HeLa cells, small syncyta were induced. However, mixing of HeLa-4aF + HN and HeLa-4aF cells generated extensive cell fusion. Similarly, infection of untreated HeLa cells with PIV-4A resulted in small polykaryocytes, whereas infection of HeLa-4aF cells resulted in extensive polykaryocyte formation. This indicated that the expression of F protein influences the function and/or integrity of cell membranes and induces ‘a prefusion state’. Many investigations have been reported in which paramyxovirus F protein reconstituted in liposomes mediates haemolysis of erythrocytes and fusion with other liposomes (Novick & Hoekstra, 1988). The enhanced susceptibility found in cells expressing the F protein alone may be related to cases in which expression of F cDNA alone causes cell fusion, for example SV5 F cDNA in CV-1 cells using an SV40-derived vector (Paterson et al., 1985; Horvath et al., 1992).

HeLa-4aF + HN cells do not fuse with each other, yet they fuse with untreated HeLa cells. Co-culture of HeLa-4aF + HN cells with HeLa-4aF cells resulted in extensive cell fusion, whereas HeLa-4aF + HN cells were unable to fuse with HeLa-4aHN cells, indicating that the inability of HeLa-4aF + HN cells to fuse with each other is due to the constitutive expression of the HN protein. Moscona & Peluso (1992) reported that cells persistently infected with PIV-3 were completely resistant to fusion with each other and the resistance was due to a lack of cell surface neuraminic acid. PIV-3 can infect cells that have been treated with neuraminidase but these cells do not fuse, suggesting that there is a different neuraminic acid requirement for a virus particle to infect a cell by fusion of the viral envelope with the cell membrane than for fusion of an infected cell with neighbouring cells. Neuraminic acid was found to be the receptor for the HN glycoprotein of PIV-4A (Bando et al., 1990). Little neuraminidase activity was detected in PIV-4A using fetuin and neuraminilatedose as substrates, even though the receptor-destructing activity was present in the virions (Bando et al., 1990). Treatment of cells with 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, an inhibitor of neuraminidase (Waxham & Wolinsky, 1986) did not induce cell fusion in HeLa-4aF + HN cells (unpublished data). This study showed that the amounts of sialic acid on the surface of HeLa-4aHN and HeLa-4aF + HN cells were approximately 75% of that of HeLa-4aF or untreated HeLa cells. It will be important to determine the relative density of PIV-4-specific receptors on the cells used in this study.

References


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