Identification of regions on the fusion protein of human parainfluenza virus type 2 which are required for haemagglutinin–neuraminidase proteins to promote cell fusion

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Using a plasmid expression system in HeLa cells, we have previously shown that the fusion (F) protein of simian virus 41 (SV-41) induces cell fusion when coexpressed with the haemagglutinin–neuraminidase (HN) protein of human parainfluenza virus type 2 (PIV-2), while the PIV-2 F protein does not induce cell fusion with the SV-41 HN protein. In the present study, we found that the PIV-2 F protein induced extensive cell fusion with the HN protein of mumps virus (MuV), whereas the SV-41 F protein did not. Chimaeric analyses of the F proteins of PIV-2 and SV-41 identified two regions (designated M1 and M2) on the PIV-2 F protein, either of which was necessary for chimaeric F proteins to show fusogenic activity with the MuV HN protein. Subsequently, two additional regions (P1 and P2) were identified on the PIV-2 F protein, both of which were necessary for chimaeric F proteins to prevent induction of cell fusion with the SV-41 HN protein. Consequently, it was proved that a given chimaeric F protein, harbouring regions P1 and P2 together with either of region M1 or M2, induced cell fusion specifically with HN proteins of PIV-2 and MuV, the same as the PIV-2 F protein. Region M2 was located at the membrane proximal end of the PIV-2 F1 ectodomain, while regions P1, M1 and P2 clustered together in the middle of the ectodomain. These regions on the PIV-2 F protein may be involved in a putative functional interaction with HN proteins, which is considered to be a prerequisite for cell fusion.

Introduction

Subfamily Paramyxovirinae of the family Paramyxoviridae contains three genera, Paramyxovirus, Rubulavirus and Morbillivirus, while subfamily Pneumovirinae contains one genus, Pneumovirus (for reviews see Lamb & Kolakofsky, 1995; Collins et al., 1995). Two kinds of transmembrane glycoproteins are inserted into the envelope of viruses from the genera Paramyxovirus and Rubulavirus: one of these proteins is designated haemagglutinin–neuraminidase (HN), which is responsible for attachment to and enzymatic cleavage of sialic acid-containing molecules on the cell surface; and the other one is the fusion (F) protein, which is involved in envelope-to-cell fusion and cell-to-cell fusion (cell fusion). The F protein is activated from a precursor (F0) by cellular protease(s) and forms a disulphide-bonded subunit structure consisting of F1 and F2. The well-conserved hydrophobic domain (fusion peptide) at the amino terminus of the F1 subunit is considered likely to be directly involved in the fusion event (Gething et al., 1978; Novick & Hoekstra, 1988).

Although the F protein seems to play a pivotal role in cell fusion, accumulated data suggest that a promoting function of the homologous HN protein is required for cell fusion by human parainfluenza virus type 2 (PIV-2) (Hu et al., 1992), PIV-3 (Ebata et al., 1991), bovine PIV-3 (Sakai & Shibuta, 1989), PIV-4A (Nishio et al., 1994), mumps virus (MuV) (Tanabayashi et al., 1992), Newcastle disease virus (NDV) (Morrison et al., 1991), simian virus 5 (SV-5) (Heminway et al., 1994), SV-41 (Tsurudome et al., 1995) and PIV-1/Sendai virus (Bousse et al., 1994). Therefore, it has been supposed that a virus typespecific functional interaction between homologous HN and F proteins takes place during the fusion process (Heminway et al.,
Fig. 1. Amino acid sequences around chimaeric junctions. Ten restriction enzyme sites were adopted in order to generate chimaeric F proteins. Numbers correspond to the amino acid positions of the PIV-2 F protein. Dots in the SV-41 F sequence are amino acids which are identical to those in the PIV-2 F sequence. Filled arrows between the amino acid sequences indicate the positions of chimaeric junctions at which the protein can be dissected by utilizing pre-existing restriction sites on the recombinant plasmid. Open arrows indicate the positions of chimaeric junctions which are created by newly introduced restriction sites by site-directed mutagenesis as described in Methods. In some cases, introduction of a restriction site was accompanied by substitution of amino acids, which were represented by open triangles together with the resulting amino acids.

Methods

Cells and viruses. HeLa cells and African green monkey kidney-derived Vero cells were maintained in Eagle’s minimum essential medium (MEM) supplemented with 5% calf serum. PIV-2 (Toshiba/Chanock strain) and SV-41 (Toshiba/Chanock strain) were propagated in Vero cells and stored at −80 °C (Tsurudome et al., 1989, 1990).

Recombinant plasmids. A cDNA fragment encoding the HN or F protein of PIV-2 or SV-41 was inserted into the plasmid expression vector pcDL-SRα296 (SRα) as described previously (Takebe et al., 1988; Tsurudome et al., 1995). Similarly, a cDNA fragment encoding the HN protein of NDV (D26 strain) (Sato et al., 1987) or that encoding the F protein of NDV (Miyadera strain) (Toyoda et al., 1987) was inserted into plasmid SRα. Recombinant SRα plasmids harbouring HN and F genes of MuV (Miyahara strain) (Tanabayashi et al., 1992) were kindly donated by Akio Yamada and Kiyoshi Tanabayashi (National Institute of Infectious Diseases, Tokyo, Japan).

To create chimaeric recombinant plasmids, the desired restriction sites were introduced into the plasmids by the site-directed mutagenesis method described below. The amino acid sequences around each chimaeric junction are summarized in Fig. 1. The restriction sites for PvuII and AflII (situated between the SauII and BglII sites) were common to cDNAs for both PIV-2 F and SV-41 F genes and thus, could be used directly for chimaeric recombination. Most of the chimaeric junctions were located in the conserved amino acid stretches which were frequently observed between the sequences of PIV-2 F and SV-41 F proteins (Tsurudome et al., 1991). As a result of introducing restriction sites for PvuII, BstXI, ScaI, BglIII and VspI, an amino acid substitution was generated in PIV-2 F and/or SV-41 F sequences. For example, when a BstXI site was introduced, four amino acids (HKLQ) in the PIV-2 F
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Fig. 2. A middle region on PIV-2 F protein was necessary for MuV HN protein to promote cell fusion. Presented on the left is a diagram of the chimaeric proteins. Open boxes represent amino acids derived from the PIV-2 F protein, while filled boxes represent those from the SV-41 F protein. Numbers correspond to the amino acid positions of the PIV-2 F protein. At the top of the diagram is a schematic of chimaeric F protein structure, which is marked with the names of restriction enzymes in order to indicate the positions of chimaeric junctions shown in Fig. 1. Presented on the right is a table giving the quantitative data of cell fusion by F proteins. HeLa cells grown in 6-well culture plates were transfected with 1 µg DNA per well of recombinant plasmid encoding each F protein together with 1 µg DNA per well of plasmid encoding PIV-2 HN, SV-41 HN or MuV HN protein. After 24 h incubation at 37 °C, the fusion index (%) was estimated by measuring the areas occupied by syncytial cells as described in the Methods; –, cell fusion was not found even when the whole of the well was observed. Surface expression of the F proteins was detected by FACScan 24 h after transfection as described in Methods and standardized by the expression level of the PIV-2 F protein.

Site-directed mutagenesis. Introduction of a mutation-generating synthetic oligonucleotide into the target recombinant plasmid was performed as described previously (Tsurudome et al., 1995) with a Transformer Site-Directed Mutagenesis kit (Clontech). Since a Scal site was present in plasmid Srα (in the ampicillin-resistance gene) but not in cDNAs for SV-41 F and PIV-2 F genes, elimination of this site was a useful marker for selection of mutant plasmids in our present study. Thus, an oligonucleotide primer was chosen to eliminate the Scal site without affecting the amino acid sequence.

Induction of cell fusion in HeLa cells by transfection with recombinant plasmids. HeLa cells were seeded at 5 × 10^3 cells per well in 6-well culture plates (Costar) and incubated at 37 °C for 24 h in MEM containing 10% foetal calf serum (FCS). Each recombinant plasmid (1 µg) was added onto subconfluent HeLa cells by a calcium phosphate method with a commercial transfection kit (CellPhect; Pharmacia) according to the manufacturer's instructions. After 4 h incubation at 37 °C, cells were treated with 15% glycerol in HEPES-buffered saline (50 mM HEPES, 0.75 mM sodium phosphate, 140 mM NaCl) at room temperature for 3 min. After 24 h incubation at 37 °C in MEM supplemented with 10% FCS, cells were quickly dried, fixed with methanol, stained with Giemsa's solution and observed with an inverted microscope (Olympus).

Quantification of cell surface expression of F proteins. The amount of F proteins expressed on the cell surface was measured by flow cytometric analysis as described previously (Tabata et al., 1994; Yuasa et al., 1995) with some modifications. We have shown previously that an anti-PIV-2 F MAb (117-1A) cross-reacted with and was able to immunoprecipitate the SV-41 F protein (Tsurudome et al., 1989, 1990). Subsequently, the developed X-ray film, produced as in a previous study (Tsurudome et al., 1990) (Fig. 2, lanes 3 and 4), was subjected to densitometric analysis using graphics software (NIH-Image, version...
1.52), revealing that the amount of SV-41 F1 band precipitated by 117-1A was approximately 87% of that precipitated by anti-SV-41 F1 MAb (31A-2) (data not shown). However, the amount of SV-41 F1 band precipitated by a mixture of cross-reactive MAbs 117-1A and 144-1A (Tsurudome et al., 1989, 1990) was almost the same as that precipitated by MAB 31A-2 (data not shown). Hence, we decided to use the mixture of MAbs to quantify expression levels of F proteins. Briefly, HeLa cells transfected with recombinant plasmid encoding each F protein were suspended in 0.02% EDTA in PBS; after 24 h incubation, they were washed with PBS and successively stained with a mixture of anti-PIV-2 F MAbs (culture fluid of hybridomas 117-1A and 144-1A diluted 1:2 in PBS) and fluorescein-conjugated goat IgG specific for mouse immunoglobulins (Cappel) which was used at 1:100 dilution in PBS. Then, the mean fluorescence intensity of 5 x 10⁶ cells in each sample was measured on a FACScan (Becton Dickinson), subtracted from that of control cells transfected with plasmid SRS and normalized by the value given by the PIV-2 F protein. The normalized mean fluorescence intensity was regarded as the relative surface expression.

Quantification of cell fusion. Cell fusion was quantified as described previously (Tsurudome et al., 1995). Briefly, a monochrome photograph was taken, which corresponded to an area containing approximately 25 x 10⁶ single cells. The photograph on developed negative film was introduced into a Macintosh computer with the aid of a film scanner (Nikon). With graphics software (NIH-Image), the extent of cell fusion was estimated as the percentage of syncytial area in the total area (approximately 250,000 pixels) of the photograph. Three randomly taken photographs were assessed for each sample and the mean fusion index (%) was calculated. Even in untransfected HeLa cells we observed multinucleated cells with (at most four) large nuclei. However, such spontaneously generating multinucleated cells were morphologically distinguishable from the ‘syncytial cells’ induced by viral proteins in terms of the number and size of nuclei and thus, were not included in the syncytial areas. By taking this step, we could demonstrate undetectable cell fusion as being negative (−) and quantify very weak cell fusion as precisely as possible.

Results

Cell fusion induced in HeLa cells by expressing various combinations of HN and F proteins of viruses belonging to genus Rubulavirus

In order to find a suitable system for identifying region(s) on the F protein which could be involved in the putative functional interaction with the HN protein, the glycoproteins of viruses of the genus Rubulavirus were coexpressed in HeLa cells in various HN/F combinations with a plasmid expression system. As shown in Table 1, all F proteins induced cell fusion most extensively when coexpressed with their homologous HN proteins. In addition, it was revealed for the first time that the PIV-2 F protein induced extensive cell fusion when coexpressed with the MuV HN protein. As reported previously (Tsurudome et al., 1995), the SV-41 F protein induced cell fusion when coexpressed with the PIV-2 HN protein, while the PIV-2 F protein did not cause cell fusion with the SV-41 HN protein (Table 1). On the other hand, the SV-41 F protein was unable to induce cell fusion with the MuV HN protein (Table 1). Thus, it was revealed that the PIV-2 F protein and the SV-41 F protein exhibited a different preference for HN proteins of PIV-2, SV-41 and MuV as partners in the induction of cell fusion. Since the F proteins of PIV-2 and SV-41 showed the most similarity with each other among the F proteins of viruses of the genus Rubulavirus (Kusagawa et al., 1993; Tsurudome et al., 1991), we decided to construct chimaeric F proteins of PIV-2 and SV-41 in order to determine regions on the PIV-2 F protein which could be responsible for its preference for HN proteins in induction of cell fusion; i.e. PIV-2 F protein induced cell fusion upon coexpression with PIV-2 F and SV-41 HN and MuV HN proteins but it did not induce cell fusion with the SV-41 HN protein.

Analysis using chimaeric proteins of PIV-2 F and SV-41 F proteins

Besides the highly hydrophobic domains in subunit F1 (the fusion peptide and TM domain), Chambers et al. (1992) indicated previously that the F1 subunit contained two typical heptad repeat (HR) domains which were conserved among the F proteins of viruses of the genera Paramyxovirus and Rubulavirus. Furthermore, a characteristic cysteine-rich (Cys-rich) domain was identified in between the two HR domains (Chambers et al., 1992). Accordingly, chimaeric junctions used in this study were arranged taking these regions into consideration. As shown in Fig. 2, one of the HR domains

Table 1. Cell fusion induced in HeLa cells by coexpressing various combinations of HN and F proteins of viruses belonging to genus Rubulavirus

<table>
<thead>
<tr>
<th>F</th>
<th>HN</th>
<th>PIV-2</th>
<th>SV-41 (62.3)*</th>
<th>MuV (40.5)*</th>
<th>NDV (32.8)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIV-2</td>
<td></td>
<td>56.6</td>
<td>—</td>
<td>42.6</td>
<td>—</td>
</tr>
<tr>
<td>SV-41 (63:1)†</td>
<td></td>
<td>6.2</td>
<td>17.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MuV (40:5)†</td>
<td></td>
<td>—</td>
<td>—</td>
<td>93.3</td>
<td>—</td>
</tr>
<tr>
<td>NDV (32:6)†</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>20.1</td>
</tr>
</tbody>
</table>

* Amino acid identity (%) with PIV-2 HN protein was determined by comparing each sequence with PIV-2 HN sequence independently of the other HN proteins.
† Amino acid identity (%) with PIV-2 F protein was determined by comparing each sequence with PIV-2 F sequence independently of the other F proteins.
Chimaeric analysis of PIV-2 F protein

Fig. 3. A region including the HR2 domain on the PIV-2 F protein is another determinant for MuV HN protein to promote cell fusion. Open boxes in the diagram represent the amino acids derived from the PIV-2 F protein, while filled boxes represent those from the SV-41 F protein. The fusion index (%) and relative surface expression were estimated 24 h after transfection as described in the legend for Fig. 2; —, cell fusion was not found even when the whole of the well was observed. Data for CF128–551 are also shown in Fig. 2.

following the fusion peptide was designated HR1 and the other one preceding the TM domain was named HR2 (Lambert et al., 1996). It has been demonstrated previously that amino acid sequences of the PIV-2 F and SV-41 F proteins could be aligned without introducing gaps (Tsurudome et al., 1991). Therefore, the positions of the characteristic domains on subunit F1, shown in Fig. 2, were common to both of the F proteins, except that the SV-41 F protein had a longer cytoplasmic tail. It should be pointed out here that none of the chimaeric F proteins used in this study induced cell fusion when expressed alone (data not shown).

Determination of a region on the PIV-2 F protein required for the MuV HN protein to promote cell fusion

First of all, the F1 subunit (except for the fusion peptide) of SV-41 F protein was replaced with its counterpart (residues 128–551) from the PIV-2 F protein. Then, the resulting chimaeric protein (CF128–551), was examined for its fusogenic activity by coexpression with the HN proteins of PIV-2, SV-41, and MuV. As shown in Fig. 2(a, b), CF128–551 exhibited similar properties to the PIV-2 F protein in that it induced extensive cell fusion when coexpressed with PIV-2 HN or MuV HN proteins but not with SV-41 HN protein. This finding suggested that the PIV-2 F1 subunit (except for the fusion peptide) could represent the preference of the PIV-2 F protein for the HN proteins of PIV-2 and MuV. However, since the reverse chimaera of CF128–551 (i.e. CF1–127) was not active either antigenically or biologically, involvement of the PIV-2 F2 subunit and fusion peptide could not be fully excluded (data not shown). To investigate this in more detail, PIV-2 F-derived amino acids in CF128–551 were further replaced with SV-41 F counterparts stepwise from the carboxyl terminus. It was proved that chimaéra CF128–496 did not induce cell fusion when coexpressed with MuV HN protein, whereas it exhibited much higher fusogenic activity with the HN proteins of PIV-2 and SV-41 than chimaera CF128–325 (Fig. 2f, g). Since CF128–325 was able to induce cell fusion with MuV HN protein (Fig. 2f), it was suggested that a region (residues 227–325) on the PIV-2 F protein contained some sequences important for its fusogenic activity with MuV HN protein. Alternatively, an amino-terminal region (residues 128–226) on the PIV-2 F1 subunit did not seem to contain essential sequences for MuV HN protein.
Fig. 4. Determination of regions on the PIV-2 F protein responsible for its inability to induce cell fusion with the SV-41 HN protein. Open boxes in the diagram represent amino acids derived from the PIV-2 F protein, while filled boxes represent those from the SV-41 F protein. UD, Surface expression was not detectable; −, cell fusion was not found even when the whole of the well was observed. The fusion index (%) and relative surface expression were estimated 24 h after transfection as described in the legend for Fig. 2. Data for CF227–551 and CF128–370 are also shown in Fig. 2 and Fig. 1, respectively.

To verify the possible importance of amino acids 227–325 of the PIV-2 F protein, the corresponding part of the SV-41 F protein was replaced with these amino acids. Although the resulting chimaeric protein CF227–325 was expressed on the cell surface, it was not fusogenic with any of the HN proteins (Fig. 2 h).

A middle region of the PIV-2 F protein was necessary for MuV HN protein to promote cell fusion

We then created another chimaera CF254–370, which contained a PIV-2 F-derived middle region (residues 254–370). Since CF254–370 efficiently induced cell fusion with MuV HN protein (Fig. 2 i), the PIV-2 F-derived middle region (designated M1) was assumed to contain sequences involved in the fusogenic activity with MuV HN protein. Further production of chimaeras resulted in only two fusogenic proteins (CF304–370 and CF254–297; Fig. 2 j, k). However, their fusogenicity with MuV HN protein was considerably lower than that of CF254–370 (Fig. 2 i, j, k). These findings indicated that replacement of either the amino- or carboxyl-terminal part of region M1 with its SV-41 F counterpart resulted in drastic reduction in fusogenic activity with MuV HN protein. Selective reduction of fusogenicity with MuV HN protein was clearly shown with CF204–376, because it exhibited efficient fusogenic activity with HN proteins of PIV-2 and SV-41, the same as CF254–370 (Fig. 2 i, j). Taken together, region M1 on the PIV-2 F protein proved to be a minimal determinant required for MuV HN protein in order to efficiently promote cell fusion. Region M1 consisted of the amino-terminal half of the Cys-rich domain and about 70 amino acids neighbouring the amino terminus of the Cys-rich domain (Figs 2 and 5).

Analysis of the carboxyl-terminal region on PIV-2 F protein

Next, to investigate whether the carboxyl-terminal region on the PIV-2 F1 subunit also contained some sequences involved in fusogenic activity with MuV HN protein, PIV-2 F-derived amino acids in CF254–370 were replaced with their SV-41 F counterparts stepwise from the amino terminus of the PIV-2 F1 subunit (Fig. 3). MuV HN protein promoted cell fusion even when a large portion of the amino-terminal part (including region M1) on the PIV-2 F1 subunit was replaced with its SV-41 F counterpart as represented by chimaera CF254–370 (Fig. 3 c), which contained PIV-2 F-derived HR2, TM and cytoplasmic domains. Further replacement of the PIV-2 F-derived HR2 domain, however, resulted in an evident loss of cell fusion with MuV HN protein as exhibited by chimaera CF254–370, which still contained the PIV-2 F-derived cytoplasmic domain and most of the TM domain (Fig. 3 d). These findings suggested that a PIV-2 F-derived region (residues 453–496), containing the HR2 domain, could be involved in fusogenic activity with MuV HN protein. On the other hand, it seemed unlikely that PIV-2 F-derived amino acids (residues 497–551) which contained the TM domain and most of the cytoplasmic domain were significantly involved. To verify these assumptions, the cytoplasmic domain and most of the TM domain on CF453–551 were further replaced with their SV-41 F counterparts. Since the resulting chimaera (CF453–496)
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Chimaeric analysis of PIV-2 F protein

Fig. 5. Comparison of amino acid sequences of F proteins. The amino acid sequences of F proteins of SV-41 (Tsurudome et al., 1991) and MuV (Takeuchi et al., 1989) were aligned with that of the PIV-2 F protein (Kawano et al., 1990). Numbers correspond to amino acid positions of the PIV-2 F protein. The dots in SV-41 F and MuV F sequences indicate amino acids identical to those of the PIV-2 F protein. Potential N-glycosylation sites are boxed. Open triangles in regions M1 and M2 indicate the positions of amino acids which are conserved only between the F proteins of PIV-2 and MuV. Closed triangles in regions P1 and P2 indicate the positions of amino acids which are not conserved between the F proteins of PIV-2 and SV-41. Asterisks show the positions of eight cysteines in the Cys-rich domain which were conserved among the F proteins of viruses of genera Rubulavirus and Paramyxovirus (Chambers et al., 1992).

induced very weak cell fusion when coexpressed with MuV HN protein (Fig. 3c), further analysis was performed as follows.

A region including the HR2 domain of PIV-2 F protein is another determinant for MuV HN protein to promote cell fusion

As described above, chimaera CF153–496 containing the PIV-2 F-derived HR2 domain and part of the TM domain, induced a low extent of cell fusion with MuV HN protein (Fig. 3c). As shown in Fig. 3(f), a new chimaera (CF128–190), which contained the PIV-2 F-derived HR1 domain, did not fusogenic with MuV HN protein, whereas it induced efficient cell fusion with PIV-2 HN or SV-41 HN protein. It was proved, however, that a doubly chimaeric protein CFI28–190:143–496 which was generated with these two chimaeras, was able to induce evident cell fusion with MuV HN protein (Fig. 3g). In contrast, another doubly chimaeric protein CFI28–190:453–496 induced a much lower level of cell fusion with MuV HN protein (Fig. 3h). Since CF128–190:453–496 and CFI28–190:493–551 induced cell fusion with the PIV-2 HN and SV-41 HN proteins at levels similar to those of CFI28–190 (Fig. 3f, g, h), the ability of CFI28–190:453–496 to be efficiently promoted by MuV HN protein could be due to a PIV-2 F-derived region (residues 453–496) which included the HR2 domain. To further investigate this region, a doubly chimaeric protein CFI28–190::145–482: Containing PIV-2 F-derived HR1 and HR2 domains, was created (Fig. 3i). As expected, CFI28–190:145–482 induced evident cell fusion with MuV HN protein, confirming the importance of the HR2 domain. On the other hand, since another doubly chimaeric protein CFI28–190:182–551 induced weak cell fusion with MuV HN protein, PIV-2 F-derived amino acid residues 482–551 also seemed to be involved in fusogenic activity with MuV HN protein (Fig. 3j). However, PIV-2 F-derived amino acid residues 497–551 did not seem to be significantly involved in this activity as described above. Therefore, it was suggested that a PIV-2 F-derived short region (residues 482–496) neighbouring the carboxyl terminus of HR2 domain was involved in fusogenic activity with MuV HN protein.

CIF
Determination of regions on the PIV-2 F protein responsible for the inability to induce cell fusion with SV-41 HN protein

Our chimaeric analyses shown in Figs 2 and 3 also indicated that a given chimaeric protein which contained the PIV-2 F-derived middle region (residues 227–370) did not induce cell fusion when coexpressed with SV-41 HN protein irrespective of fusogenic activity with PIV-2 HN or MuV HN protein. This is shown by results with CF\textsubscript{227–370} and CF\textsubscript{128–370} (Fig. 4a, b). Thus, the corresponding part on SV-41 F protein was replaced with the PIV-2 F-derived middle region, but the resulting chimaeric protein CF\textsubscript{227–370} was inactive either antigenically or biologically (Fig. 4c). However, a doubly chimaeric protein CF\textsubscript{227–370: 133–496} which contained the middle region and region M2, was expressed on the cell surface and proved to be fusogenic with the HN proteins of PIV-2 and MuV but unable to induce cell fusion with SV-41 HN protein (Fig. 4d). Since additional chimaeric proteins CF\textsubscript{254–370: 133–496} and CF\textsubscript{128–325: 133–496} induced cell fusion with SV-41 HN protein (Fig. 4e, f), it was shown that the inability of CF\textsubscript{227–370: 133–496} to induce cell fusion with SV-41 HN protein was due to a combination of the two PIV-2 F-derived regions, designated P1 and P2 (Fig. 4), but not due to region M2. As shown in Fig. 5, region P1 (residues 227–253) was next to the amino terminus of region M1, while region P2 (residues 326–370) corresponded to the amino-terminal half of the Cys-rich domain and was included in region M1. Thus, regions P1, M1 and P2 clustered together in the middle of PIV-2 F1 subunit (also shown in Fig. 4).

Finally, our present chimaeric analyses proved that a given chimaeric F protein, which contained at least both regions P1 and P2 plus either of region M1 or M2, exhibited clear specificity for the HN proteins of PIV-2 and MuV as partners in the induction of cell fusion, the same as the PIV-2 F protein.

Discussion

In the present study, we have identified four regions (M1, M2, P1 and P2) on the PIV-2 F protein which could determine its preference for HN proteins. Either region M1 or M2 seemed necessary for the PIV-2 F protein to induce cell fusion with MuV HN protein, while both regions P1 and P2 were required for the PIV-2 F protein to prevent induction of cell fusion with SV-41 HN protein. Alternatively, regions P1 and P2 were responsible for the specificity of the PIV-2 F protein for PIV-2 HN protein.

Amino acid sequences of the F proteins of PIV-2, SV-41 and MuV were compared in terms of the four regions (Fig. 5). Twenty-one amino acids in regions P1 and P2 were not conserved between the F proteins of PIV-2 and SV-41, which might be responsible for the specificity of the PIV-2 F protein for PIV-2 HN protein in the induction of cell fusion. In region P2, a potential N-glycosylation site was present in the corresponding SV-41 F sequence but not in the PIV-2 F sequence, suggesting that absence of a sugar chain might also contribute to the specificity. As for regions M1 and M2, only seven conserved amino acids were identified between PIV-2 HN and MuV HN sequences. Since the MuV F protein could not induce cell fusion with the PIV-2 HN protein, it seemed that the fusogenic activity of PIV-2 F protein with MuV HN protein was not simply attributable to these common amino acids. In addition to them, 59 amino acids in regions M1 and M2 on the PIV-2 F protein, which were not conserved in the SV-41 F protein, might somehow contribute to its fusogenic activity with MuV HN protein, possibly shaping a conformation compatible with the MuV F protein.

As recently demonstrated by Yao \textit{et al.} (1997), the PIV-2 F protein may physically interact with PIV-2 HN or MuV HN protein in the induction of cell fusion. Previously, by chimaeric analyses of the HN proteins of PIV-2 and SV-41, we identified a 58-amino-acid region (designated I′) on the PIV-2 HN protein located at the membrane proximal end of the ectodomain that was responsible for specificity for the PIV-2 F protein as the partner in the induction of cell fusion (Tsurudome \textit{et al.}, 1995). Thus, given that the identified regions (P1 and P2) on the PIV-2 F protein, both of which were required to determine specificity for the PIV-2 HN protein, are involved in the physical interaction with region I′ on the PIV-2 HN protein, they should also be located adjacent to the membrane to which both the HN and F proteins are anchored. Since region M1 forms a cluster with regions P1 and P2 (Fig. 5), and region M2 is the membrane proximal end of the ectodomain, it seems likely that all four regions (P1, M1, P2 and M2) may gather together in the vicinity of the membrane. Although the roles played by the four regions were not the same in terms of the preference for the HN proteins of MuV and PIV-2, they may be, more or less, involved in the interaction with the HN proteins and subtle conformational difference within these regions may influence the preference of the F protein for the HN proteins. However, we have no direct evidence supporting these speculations at present. The site-specific photocross-linking method (High \textit{et al.}, 1993; Martoglio \textit{et al.}, 1995) may help further investigations on the physical interaction between the identified domains on the HN and F proteins of PIV-2.

Intriguingly, among the four regions on the PIV-2 F protein, region P2 corresponds to the amino-terminal half of the Cys-rich domain and region M2 contains the HR2 domain; these two domains have long been considered to be involved in the fusogenic activity of the F protein. Previously, the importance of the Cys-rich domain of the F proteins of NDV
and Sendai virus was suggested by studies using fusion-inhibiting MAbs (Neyt et al., 1989; Portner et al., 1987; Toyoda et al., 1988). Subsequently, Wild et al. (1994) reported that the F protein of canine distemper virus (CDV) did not induce cell fusion when coexpressed with the haemagglutinin (HA) protein of measles virus (MV); thus, they performed chimaeric analysis of the F proteins of MV and CDV, which indicated that the amino-terminal half of the Cys-rich domain on the MV F protein might be involved in its functional interaction with the MV HA protein in the induction of cell fusion. Subsequently, however, Stern et al. (1995) demonstrated that the CDV F protein was interchangeable with the MV F protein in the induction of cell fusion with CDV HA protein, and vice versa, which made the interpretation of the former result somewhat complicated. We cannot explain the discrepancy between the two studies, but our present results seem to reinforce the view that the amino terminus of the Cys-rich domain on the F protein may be involved in the functional interaction with the attachment protein in the induction of cell fusion.

The remarkable importance of the HR2 (or leucine zipper) domain for the fusogenic activity has been demonstrated using synthetic peptides corresponding to the HR2 domains of the F proteins of PIV-2, PIV-3, MV, Sendai virus and respiratory syncytial virus (Lambert et al., 1996; Rapaport et al., 1995; Wild & Buckland, 1997; Yao & Compan, 1996). Several mutational analyses further pointed out that leucines in the HR2 domain of the F proteins of MV and NDV were required for cell fusion, while they were not critical for oligomer formation (Buckland et al., 1992; Reitter et al., 1995). As described above, we speculate that the HR2 domain is a candidate for the site involved in the functional interaction with the attachment protein as well as the Cys-rich domain.

On the one hand, mutations in the HR1 domain, in the fusion peptide or in the cytoplasmic domain of NDV F protein could abolish its fusogenic function (Sergel-Germano et al., 1994; Sergel & Morrison, 1995). However, on the other hand, mutating Ser-195 (located about 20 amino acids from the carboxyl terminus of the HR1 domain) of the MuV F protein into amino acids with aromatic side chains resulted in reduction of fusogenic activity (Tanabayashi et al., 1994). Our present findings suggest that these regions on the F protein are not involved in the interaction with the HN protein, although they may still contribute to fusogenic activity through different mechanisms. It should be remembered, however, that a combination of HR1 and TM/cytoplasmic domains of PIV-2 F protein resulted in very weak fusion with MuV HN protein (Fig. 3h). Thus, we cannot fully exclude the possibility that HR1 or TM/cytoplasmic domains may somehow be involved in the functional interaction with MuV HN protein.

The tertiary structure of the F protein is not known at present. Hence, it seems very difficult to imagine what kind of conformational changes will take place in the F protein during the fusion process. Epitope mapping analyses by Neyt et al. (1989) and Toyoda et al. (1988) suggested a close topological interrelationship between the F2 subunit, HR1 domain, an amino-terminal part of the Cys-rich domain and the fusion peptide. This assumption, taken together with our speculation on the topological relationship between the four identified regions, suggests that the ectodomain of the F protein seems to adopt a highly folded conformation where the F2 subunit, fusion peptide, HR1 domain and the amino-terminal half of the Cys-rich domain are gathered together around the HR2 domain. The fusion peptide may be surrounded by some of the other regions or buried in the oligomer interface, the same as that of the influenza HA protein in prefusion conformation (Wilson et al., 1981). It may be that once the F protein interacts physically with the HN protein via either of the identified four regions, then the ectodomain of the F protein may undergo drastic conformational changes, relocating the fusion peptide toward the target membrane and resulting in induction of cell fusion.

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