An amino acid in the heptad repeat 1 domain is important for the haemagglutinin–neuraminidase-independent fusing activity of simian virus 5 fusion protein

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A canine isolate (strain T1) of simian virus 5 (SV-5) performed multiple replication in BHK cells but did not induce cell fusion for up to 3 days. In contrast, a prototype strain (WR) provoked extensive cell fusion within 2 days during the course of its replication. Accordingly, the fusion (F) protein of the T1 strain did not cause cell fusion even when co-expressed with the SV-5 haemagglutinin–neuraminidase (HN) protein, whereas the WR F protein induced cell fusion in the presence of the HN protein. Differences in the predicted amino acid sequences of the T1 and WR F proteins were found at 12 positions and it was proved that the T1 F protein had a longer cytoplasmic tail than the WR F protein. By reducing the length of the cytoplasmic tail or by replacing the tail with the WR F counterpart, the T1 F protein partly restored its HN-dependent fusing activity. Chimeric and mutational analyses between the T1 F protein and the mutant F protein (L22P) suggested that Glu-132 in the heptad repeat 1 domain was involved in the HN-independent fusing activity in addition to the previously identified Pro-22 at the F2 N terminus. It was also shown that Ala-290 in the heptad repeat 3 domain contributed to the HN-independent fusing activity to some extent.

Introduction

The fusion (F) protein of paramyxoviruses is activated from a precursor (F0) by cellular protease(s) and forms a disulfide-bonded subunit structure consisting of F1 and F2, which is a prerequisite for the fusion process (Homma & Ohuchi, 1973; Scheid & Choppin, 1974). The well-conserved hydrophobic domain (fusion peptide) at the N terminus of F1 is exposed by the cleavage (Hsu et al., 1981; Kohama et al., 1981) and is considered likely to be directly involved in the fusion event (Gething et al., 1978; Novick & Hoeckstra, 1988). In addition, the F1 subunit has three characteristic heptad repeat domains (Chambers et al., 1992; Ghosh et al., 1997). Mutational analyses indicate that the leucine residues in the heptad repeat 2 (HR2) domain near the transmembrane anchor are required for cell fusion, whereas they are not critical for oligomer formation (Buckland et al., 1992; Reitter et al., 1995).

It is known that the F protein of strain W3A of the paramyxovirus simian virus 5 (SV-5) mediates cell fusion when expressed alone in CV-1 cells using the SV-40 vector system (Horvath et al., 1992; Paterson et al., 1985). Intriguingly, however, when expressed in CV-1 cells by using recombinant vaccinia virus vector system or the vaccinia virus T7 system, the W3A F protein cannot mediate cell fusion by itself and requires co-expression of homologous haemagglutinin–neuraminidase (HN) protein, similarly to other paramyxovirus F proteins (Heminway et al., 1994; Horvath et al., 1992). Recently, by using a plasmid expression system in BHK cells, we have confirmed that the W3A F protein exhibits a remarkable fusing activity on its own, independently of SV-5 HN protein and SV-40 infection (Ito et al., 1997). In contrast, the F protein of another SV-5 strain (WR) required co-expression of the HN protein in order to induce cell fusion. By mutational analysis of the three amino acids which were not conserved between the F proteins of strains W3A and WR, a critical amino acid (Pro-22) was identified in W3A F2 as being responsible for the HN-independent fusing activity (Ito et al., 1997). Accordingly, a mutant F protein (L22P), in which a leucine residue at position 22 of the WR F protein was replaced with proline, induced extensive cell fusion in the absence of the HN protein. It seems very likely, however, that there are other
important amino acids involved that were not studied in our previous investigation since we evaluated only three (unconserved) amino acids.

In order to tackle this problem, we used an SV-5 strain (T1) isolated from a dog with kennel cough complex (Azetaka & Konishi, 1988). On the basis of the difference in the predicted amino acid sequences between the T1 F protein and the mutant L22P, chimeric and mutational analyses were performed, identifying additional amino acids important for the HN-independent fusing activity of the SV-5 F protein.

Methods

**Cells and viruses.** BHK, MDCK and Vero cells were maintained in Eagle’s minimum essential medium (MEM) supplemented with 5% foetal calf serum (FCS). The T1 (Azetaka & Konishi, 1988) and WR (Nishikawa et al., 1974; Ito et al., 1997) strains of SV-5 were propagated in MDCK cells and stored at −80 °C. The virus titre (TCID50) was determined by the haemadsorption method using Vero cells and sheep erythrocytes.

**Antipeptide antibody.** A synthetic peptide, 5F2, which corresponded to the C-terminal sequence (KLLQPGLENTRNLQPQI) of the WR F subunit was prepared by Savady Technology. Rabbit antiserum specific for 5F2 was also provided by Savady Technology.

**SDS–PAGE and Western blot analyses.** BHK cells were infected with virus strains WR (m.o.i. = 0.001) or T1 (m.o.i. = 0.1) and incubated for 26 or 72 h, respectively. Infected cells were then lysed with lysis buffer (1% Triton X-100, 137 mM NaCl, 3 mM β-glycerophosphate, 3 mM EDTA, 1 mM PMSE, 25 mM HEPES, pH 7.6) on ice for 20 min and clarified by centrifugation (13,000 g for 5 min). Aliquots of the lysates were subjected to SDS–PAGE using a Tris/Tricine buffer system (Schägger & von Jagow, 1987) under non-reducing or reducing conditions and blotted onto a PVDF membrane (Hybond-P; Amersham Pharmacia Biotech). The membrane was treated successively with anti-5F2 rabbit serum (diluted 1:50 in PBS), biotinylated horse immunoglobulin to rabbit IgG (heavy and light chains; Vector Laboratories) and 5F2 rabbit serum (diluted 1:50 in PBS), biotinylated horse immunoglobulin to rabbit IgG (heavy and light chains, Vector Laboratories) and avidin–biotin–peroxidase complex (Vector Laboratories) as described previously (Tsurudome et al., 1989). The membrane was then treated with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and exposed to an X-ray film (Konica).

**Recombinant plasmids.** To obtain a full-length cDNA clone for the F gene of strain T1, synthetic oligonucleotide primers were prepared on the basis of the sequence data of the W3A F gene (Paterson et al., 1984). Poly-A(+) RNA was purified from virus-infected Vero cells with the aid of QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech), and cDNA was synthesized by using a First-strand cDNA Synthesis kit (Amersham Pharmacia Biotech). Then, the cDNA fragment was amplified by PCR and cloned in the plasmid expression vector pcDL-SRα290 (SRα), in which the expression is under control of the SV-40 early promoter and/or R-U5 sequence of human T-lymphotropic virus-1 LTR (Takebe et al., 1988). The nucleotide sequence of amino acid-coding regions was determined by using a set of synthetic oligonucleotide primers.

**Site-directed mutagenesis.** Introduction of mutation-generating synthetic oligonucleotide to the target recombinant plasmid was performed by using the U. S. E. Mutagenesis kit (Amersham Pharmacia Biotech) according to the manufacturer’s instruction. Since an FspI site was present in the SRα plasmid (in the ampicillin-resistance gene) but absent in the cDNAs for T1 F and L22P genes, its elimination was a useful marker for the selection of the mutant plasmids in our present study. Thus, an oligonucleotide primer was arranged so that the FspI site was eliminated with a silent mutation in the ampicillin-resistance gene.

**Induction of cell fusion in BHK cells by transfection with recombinant plasmids.** BHK cells were seeded at 5 × 104 cells per well in 6-well culture plates (Costar) and incubated at 37 °C for 24 h in MEM containing 10% FCS. Each recombinant plasmid (2 µg) was added onto subconfluent BHK cells by the calcium phosphate method. After 4 h incubation at 37 °C, the cells were treated with 15% glycerol in HEPES-buffered saline (0.75 mM sodium phosphate, 140 mM NaCl, 50 mM HEPES) at room temperature for 3 min. After 24 h incubation at 37 °C in MEM supplemented with 10% FCS, the 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 protein expressed on the cell surface was measured by flow cytometric analysis as described previously (Ito et al., 1997; Tabata et al., 1994; Tsurudome et al., 1995). Briefly, BHK cells transfected with 2 µg recombinant plasmid encoding each F protein were suspended in 0.02% EDTA in PBS after 12 h incubation at 37 °C. Cells were then immunostained with anti-5F2 rabbit serum (1:100) and fluorescein-conjugated goat anti-rabbit immunoglobulins (1:800) (Cappel Laboratories). Then, the mean fluorescence intensity of control cells transfected with SRα plasmid was subtracted from this value and it was normalized by the value given by the T1 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, 1998). Briefly, subconfluent cultures of BHK cells in 6-well culture plates were transfected with 2 µg of each recombinant plasmid by the calcium phosphate method and treated with glycerol as described above. After incubation at 37 °C for 24 h, cells were stained with Giemsa’s solution and observed by using an inverted microscope (Olympus). Then, photomicrographs were subjected to morphometric measurement of cell fusion, and the average fusion index (%) and SD were calculated (Tsurudome et al., 1995).

Results

A canine isolate of SV-5 replicates in cells without causing cell fusion

Strain T1 of SV-5 was previously isolated from a dog with kennel cough complex (Azetaka & Konishi, 1988). We prepared virus stock by propagating it in MDCK cells for 7 days, which yielded 106.6 TCID50 per ml. Since neither cell fusion nor apparent cytopathogenic effect (CPE) was observed in MDCK cells, titration of the virus was performed in Vero cells but CPE was also hardly detected. Therefore, the virus titre was judged by using the haemadsorption method. In contrast, strain WR caused extensive cell fusion in Vero cells as well as in MDCK.
Cell fusion by SV-5 F protein

Fig. 1. A canine isolate (strain T1) of SV-5 does not induce cell fusion in BHK cells. Cells were inoculated with strains T1 or WR (m.o.i. = 0.005), fixed with methanol at times indicated after infection and stained with Giemsa’s solution (magnification, ×99).

Fig. 2. Strain T1 performs multiple-step replication in BHK cells. Cells were inoculated with strains T1 (○) or WR (■) (m.o.i. = 0.005) and the culture fluids were collected at indicated times after infection. After clarification, the released virus titre was determined by the haemadsorption method using sheep erythrocytes.

Comparison of amino acid sequences of F proteins

To understand the molecular basis of the non-fusion phenotype of strain T1, cDNA for the T1 F mRNA was cloned in the plasmid vector SRα. As shown in Fig. 5, comparison of predicted amino acid sequences revealed a 12 amino acid difference between the F proteins of T1 and WR. In particular, the F subunit of the T1 F protein migrated slightly slower than that of the WR F protein under the non-reducing conditions (Fig. 4b), whereas the F subunit of the T1 F protein migrated at the same speed or even faster than the WR F (Fig. 4a), indicating that the F subunit of the T1 F protein might be larger than that of the WR F protein. This conclusion seemed to reflect the finding that the F subunit of the T1 F protein is longer by 22 amino acids than that of the WR F protein; this is described below.

The WR F and T1 F proteins were clearly detected by anti-5F2 rabbit serum whereas uncleaved precursor (F₀) of either of the F proteins was hardly detectable (Fig. 4a). This result indicated that the T1 F protein was cleaved into F₁ and F₂ subunits as efficiently as that of the WR F protein. In particular, the F₁₀₂ of the T1 F protein migrated slightly slower than that of the WR F protein under the non-reducing conditions (Fig. 4b), whereas the F₁₀ of the T1 F protein migrated at the same speed or even faster than the WR F₁₀ (Fig. 4a), indicating that the F₁₀ subunit of the T1 F protein might be larger than that of the WR F protein. This conclusion seemed to reflect the finding that the F₁₀ subunit of the T1 F protein is longer by 22 amino acids than that of the WR F protein; this is described below.
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Fig. 3. Spread of infection of strain T1 in BHK cells. Cells were inoculated with strain T1 (m.o.i. = 0.005) and after indicated times of incubation they were overlaid with sheep erythrocyte suspension (0.4%, v/v) in MEM at room temperature for 30 min. After extensive washing with MEM, photomicrographs of unstained cells were taken (magnification, x 180).

mRNA and, importantly, if the WR F mRNA was translated to this (downstream) termination codon, the amino acid sequence of the WR F protein between these termination codons would be identical to the T1 F protein sequence (from residue 531–551; Fig. 5).

Next, to investigate the fusing activity of the T1 F protein, the protein was expressed in BHK cells together with the SV-5 (strain W3A) HN protein, but no cell fusion was induced (Fig. 6). In contrast, co-expression of the WR F protein with the W3A HN protein resulted in typical cell fusion (Fig. 6) as reported previously (Ito et al., 1997). The inability of recombinantly expressed T1 F protein to induce cell fusion seemed to simply reflect the above-described observation that strain T1 did not cause cell fusion upon infection of BHK cells despite the fact that the F protein was efficiently cleaved. The amino acid sequence of the mutant F protein, L22P, was also included in Fig. 5. The L22P sequence differed from the WR F sequence only at residue 22 (Pro vs Leu) and Pro-22 did define the HN-independent fusing activity of L22P (Ito et al., 1997). Intriguingly, the T1 F protein shared Pro-22 with L22P (Fig. 5), in spite of its inability to induce cell fusion even upon co-expression with the HN protein.

Chimeric analysis of T1 F protein and L22P

We then decided to map those amino acids on L22P, which could be required for its HN-independent fusing activity in addition to the Pro-22. Since the N-terminal 19 amino acids were considered to be the signal peptide (Paterson et al., 1984) and should be removed by the signal peptidase, the amino acid sequence of the mature T1 F protein may differ from that of L22P at nine positions, disregarding the difference in the length of the cytoplasmic tails as described above. Firstly, to verify the possibility that the long cytoplasmic tail had some deleterious effect on the fusing activity, a termination codon was introduced to the T1 F cDNA so that the mutated T1 F protein had a truncated cytoplasmic tail as short as L22P. This truncated T1 F protein, designated T1-F(TR), showed a weak

Fig. 4. The F1+2 of strain T1 migrates slower than that of strain WR. BHK cells were inoculated with strains T1 or WR and the cell lysates were prepared as described in the Methods. The lysates were then analysed by SDS–PAGE under (a) reducing (9% and 14% discontinuous separation gel) or (b) non-reducing (9% separation gel) conditions and processed for ECL Western blotting by using the anti-5F2 rabbit serum as described in the Methods. Asterisks indicate the position of an uncharacterized cellular protein that is also immunostained by the anti-5F2 peptide antiserum.
Cells were stained with Giemsa's solution after 24 h (magnification, Fig. 6). ORF WR F protein together with the plasmid encoding W3A HN protein. BHK cells were transfected with plasmid encoding the T1 F protein does not induce cell fusion even when co-expressed with the W3A HN protein. Therefore, it seemed that shortening the cytoplasmic tail contributed towards restoring the HN-dependent fusing activity of the T1 F protein and that substituting Asn-529 with Lys has an additive effect on the fusing activity of the T1 F protein with the short cytoplasmic tail. Interestingly, a reverse chimera of CF(L22P) designated CF76-135, induced cell fusion on its own (Fig. 7). Furthermore, another chimera, CF76-310, also caused cell fusion when expressed alone, whereas its reverse chimera, CF128–529, did not. When co-expressed with the W3A HN protein, however, CF128–529 induced cell fusion to a similar extent as CF76-438 (Fig. 7). These observations suggest that the HN-independent fusing activity of L22P could be defined by some of the amino acids at residues 76, 92, 132, 135, 290 and 310.

Important amino acids for HN-independent fusing activity

To identify such amino acid(s) which could be involved in the HN-independent fusing activity, we created additional chimeras CF76-135 and CF76-92 but they were not expressed on the cell surface (data not shown). As shown in Fig. 8, however, combining the L22P-derived cytoplasmic domain with these chimeras resulted in creation of functional chimeric proteins CF76-135:529 and CF76-92:529; they were successfully expressed on the cell surface and induced cell fusion upon co-expression with the W3A HN protein. Furthermore, CF76-135:529 mediated cell fusion by itself whereas CF76-92:529 did not. Thus, it was suspected that amino acid(s) at residues 132 and/or 135 in the HR1 domain of L22P played an important role in the HN-independent fusing activity. Therefore, these amino acids were further evaluated by mutational analysis of CF76-92:529. As shown in Fig. 8, the resulting mutant CF76-92:132:529 mediated cell fusion by itself (5.8%) but the other mutant CF76-92:135:529 did not, whereas these mutant proteins induced cell fusion upon co-expression with the W3A HN protein (Fig. 7). This result suggested that the HR1 domain of L22P played an important role in the HN-independent fusing activity. On the other hand, it was also shown that the HN-independent fusing activity of CF76-135:529 (3.9%) was apparently lower than that of CF76-310:529 (11.9%), whereas the extents of cell fusion with the W3A HN protein were similar (23.1% and 27.3%, respectively). Since the mutant CF76-290:529 exhibited even higher HN-independent fusing activity than CF76-310:529 (13.2% vs 11.9%) with lower fusing activity with the W3A HN protein (22.6% vs 27.3%), the L22P-derived Met-310 did not seem to be significantly involved. Taken together, it could be postulated that L22P-
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Fig. 7. N-terminal half of L22P is involved in HN-independent fusing activity. On the left is a diagram of the chimeric proteins. Open boxes represent amino acid regions derived from the T1 F cDNA; closed boxes represent those derived from the L22P cDNA. Numbers on the boxes indicate the presence and position of L22P-derived amino acids which differ from the T1 F counterparts. At the top of the diagram is a schematic of F protein structure, which is marked with the names of restriction enzymes in order to indicate the positions of chimeric junctions on cDNA (also shown in Fig. 5). On the right is a table giving the quantitative data of cell fusion by the F proteins. BHK cells grown in 6-well plates were transfected with recombinant plasmid encoding each F protein together with (HN+) or without (HN-) the recombinant plasmid encoding the W3A HN protein. After 24 h, the fusion index (%) was morphometrically estimated as described in the Methods; -- indicates that cell fusion was not found even when the whole well was observed. Surface expression of the F proteins was measured by FACScan 12 h after transfection as described in the Methods and standardized by the expression levels of the T1 F protein.

Discussion

Our present study has indicated that Glu-132 in the HR1 domain is involved in the HN-independent fusing activity of the SV-5 F protein. As reported recently, Pro-22 at the N terminus of F₂ is also involved in the HN-independent fusing activity (Ito et al., 1997). It was previously shown that Sendai virus F₂ cannot be released from the virion even after splitting the interchain disulfide bond under denaturing conditions (Asano et al., 1983), whereas the HA of influenza virus can be released under similar conditions (Ozawa et al., 1979). It seems thus possible that Sendai virus F₂ is non-covalently but tightly associated with some location on the F₁ molecule in addition to the covalent association via the intermolecular disulfide bridge. Interestingly, in this context, a topological interrelationship between the F₁ (HR1 domain or cysteine-rich region) and F₂ of Newcastle disease virus has been suggested by studies on neutralization escape mutants (Neyt et al., 1989; Toyoda et al., 1988) or on temperature-sensitive mutants and the revertants (Wang et al., 1992). Taken together, it is conceivable that tight interactions between F₂ and HR1 may play an important role in stabilizing the paramyxovirus F protein. The presence of Pro-22 and Glu-132 may destabilize the SV-5 F protein to be easily triggered for conformational change even in the absence of the HN protein; the conformational change of the F protein is considered to be a prerequisite for fusion induction (Baker et al., 1999; Lamb, 1993). Probably, an amino acid change from lysine to glutamic acid at position 132 results in drastic conversion of local charge in the HR1 domain which may influence an electrostatic interaction with some location in the F₂. On the other hand, the change from leucine to proline at position 22 in the F₂ may result in a decrease in local hydrophobicity or an alteration in local conformation at the F₂ N terminus that is unfavourable for its putative interaction with the HR1 domain.

Recent reports have suggested that, in the course of fusion induction, the SV-5 F protein undergoes conformational changes in which the HR1 domain of the F trimer forms a triple-stranded coil and three HR2 domains stably associate to the coil in an antiparallel orientation (Baker et al., 1999; Dutch et al., 1999). The possible interaction between the HR1 domain and F₂ subunit may thus somehow interfere with the conformational change of the F protein in the fusion process. The putative tight HR1–F₂ interaction could be liberated through interaction with the HN protein or destabilized depending on the amino acids at residues 22 and 132 as discussed above.
The HR3 domain seems not to firmly associate with the above HR1–HR2 complex (Dutch et al., 1999). However, our observation that Ala-290 in the HR3 domain partly contributes to the HN-independent fusing activity suggests a subtle but significant role of the HR3 domain played in the course of fusion induction.

On the other hand, our present study does not exclude the possibility that some chimeric F proteins, showing no HN-independent fusion activity, may be efficiently cleaved only when co-expressed with the HN protein which may result in exhibition of HN-dependent cell fusing activity, since our Western blot analysis hardly detected recombinantly expressed F proteins (not shown). However, to our knowledge, so far there has been no study supporting this possibility (HN-dependent cleavage of the F protein). It should be pointed out, in this context, that the WR F protein was efficiently cleaved and cell surface-localized, but did not exhibit HN-independent fusing activity (Ito et al., 1997).

Our present study also proved that the cytoplasmic tail of the dog-derived T1 F protein was longer than that of monkey-derived WR F or W3A F protein. No difference was found between the F proteins in the number of potential glycosylation sites (Fig. 5, and data not shown). Thus, the observation that the F1 subunit of strain T1 migrated slower than that of strain WR should mainly reflect the difference in the number of amino acids in the cytoplasmic tails of their F1 subunits. Previously, Randall et al. (1987) reported that the F1 subunit of a canine isolate of SV-5 migrated slower than that of a simian isolate. Intriguingly, the F1 of four human isolates migrated to the same position as that of the canine isolate, whereas the F1 of another human isolate co-migrated with that of the simian isolate (Randall et al., 1987). Although the primary structures of these F proteins are not known, this observation may reflect a possible difference in the length of the cytoplasmic tails. Therefore, given this and the previous assumption that the natural host of SV-5 is the dog, from which human and then monkey were successively infected (Baty et al., 1991), it is conceivable that the prototype, dog SV-5, possessed an F protein with a long cytoplasmic tail. After infecting humans, the cytoplasmic tail of the F proteins of some SV-5 populations might become short, whereas those of others might not. Monkeys might then be infected only with the SV-5 population that has the short-tailed F protein. To certify this hypothesis, a phylogenetic investigation of the F proteins derived from a number of SV-5 isolates is required.

Whether the length of the tail has some relevance to the cytopathogenicity remains an open question, although our present study suggests that the long cytoplasmic tail of the T1 F protein is partly involved in the non-fusion phenotype of strain T1 in vitro. It should be pointed out, in this context, that the length of the cytoplasmic tail of the T1 F protein is comparable to that of the SV-41 F protein which is the longest among the paramyxovirus F proteins (Tsurudome et al., 1991; unpublished data) but SV-41 F protein was able to induce cell
fusion in the presence of the HN protein (Tsurudome et al., 1995).

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