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Role of a Single Amino Acid at the Amino Terminus of the Simian Virus 5 F2 Subunit in Syncytium Formation

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The fusion (F) protein of simian virus 5 (strain W3A) induced extensive cell fusion in BHK cells when expressed alone, while that of strain WR did not. Mutational analysis demonstrated that the fusing activity can be transferred to the WR F protein by a proline residue at position 22 of subunit W3A F2.

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 (5, 15). The well-conserved hydrophobic domain (fusion peptide) at the amino terminus of F1 is exposed by the cleavage (7, 8) and is considered likely to be directly involved in the fusion event (2, 11).

The F protein of a paramyxovirus simian virus 5 (SV5) strain W3A is known to mediate syncytium formation when expressed alone in CV-1 cells by the SV40 vector system (6, 14). Intriguingly, however, when it is expressed in CV-1 cells by the recombinant vaccinia virus vector system or the vaccinia virus-T7 system, the W3A F protein cannot mediate cell fusion by itself and requires the coexpression of homologous hemagglutinin-neuraminidase (HN) protein (3, 6), as do other paramyxovirus F proteins. Although it has not been clarified why the fusogenic activity of the W3A F protein is dependent on the expression system that is used, this discrepancy could be explained either by the cytopathogenic effect of vaccinia virus or by an unknown function of SV40 viral proteins or SV40-induced unrecognized cellular protein(s). It is also not known whether HN-independent fusogenic activity is common to every SV5 strain.

To answer these questions, in the present study, we first examined the fusogenic activity of the F protein of another SV5 strain, 21004WR (WR) (21) by using our plasmid expression system in BHK cells in which no helper virus was employed. To obtain cDNA clones for the HN and F genes of strain WR, synthetic oligonucleotide primers were prepared on the basis of the sequence data of W3A glycoprotein genes (4, 13). Poly(A)$^+$ RNA was purified from virus-infected Vero cells with the aid of the QuickPrep Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden), and first-strand cDNA for each glycoprotein gene was synthesized by reverse transcriptase. Then the cDNA fragment was amplified by PCR and cloned in the plasmid expression vector pcDL-SRα296, in which expression is controlled by the SV40 early promoter and/or R-U5 sequence of human T-cell leukemia virus type 1 long terminal repeat (17). The nucleotide sequence of amino acid-coding regions was directly determined with a set of synthetic oligonucleotide primers. As shown in Table 1, five nucleotides in the HN gene and eight nucleotides in the F gene of strain WR proved not to be identical to the reported sequences of the glycoprotein genes of strain W3A (4, 13). It was also shown that amino acids at positions 22, 443, and 516 in the F proteins were not conserved between the two SV5 strains. None of these amino acids was involved in the potential N-glycosylation sites which were identified on the W3A F protein (13). On the basis of these findings, recombinant plasmids encoding mutant F proteins designated L22P, P443S, and A516V were constructed by site-directed mutagenesis (20) so that each one of the three amino acids in the WR F protein was replaced with its W3A F counterpart. Subsequently, a recombinant plasmid encoding the W3A F protein was constructed by chimeric recombination of the three mutant plasmids.

To examine whether the F proteins of the two SV5 strains could induce cell fusion by themselves, subconfluent cultures of BHK cells in 6-well culture plates were transfected with 2 μg of each recombinant plasmid by the calcium phosphate method (20). At appropriate times of incubation at 37°C, cells were stained with Giemsa solution and observed with an inverted microscope (Olympus, Tokyo, Japan). Then photomicrographs were subjected to morphometrical measurement of cell fusion (20). As shown in Fig. 1, W3A F protein induced cell fusion in BHK cells when expressed alone, which was first observed at 8 h posttransfection and reached its maximal level at 30 h posttransfection. In contrast, the WR F protein did not induce cell fusion even at 50 h posttransfection. Among three mutant F proteins, only one (L22P) mediated cell fusion, which was even more extensive than that mediated by W3A F protein (Fig. 1).

Shown in Table 2 are the quantitative data for cell fusion measured at 24 h posttransfection. When expressed alone, all the F proteins were successfully expressed on the cell surface, as measured by flow cytometry (16, 20) with anti-SV5 rabbit serum (Denka Institute of Biological Science, Niigata, Japan) and fluorescein-conjugated goat anti-rabbit immunoglobulins (Cappel Laboratories, Durham, N.C.) (Table 2) and efficiently cleaved into F1 (52 kDa) and F2 (14 kDa) as analyzed by radioimmunoprecipitation assay (data not shown). The WR F protein, P443S, and A516V did not mediate cell fusion when expressed alone as described above. However, these F proteins induced cell fusion when coexpressed with the WR HN protein (Table 2), indicating that they are fusogenic in the presence of HN protein, as are other paramyxovirus F proteins. Similar results were obtained when the F proteins were coexpressed with W3A HN protein, which was expressed from the plasmid

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TABLE 1. Differences in the primary structures of HN and F proteins between strains WR and W3A of SV5

Protein[a,b]	Nucleotide no. (nucleotides for WR and W3A, respectively)	Amino acid no. (amino acids for WR and W3A, respectively)
HN	191 (G, A)	42 (Arg, Gln)
	692 (C, T)	209 (Ser, Phe)
	862 (C, T)	266 (His, Tyr)
	900 (T, C)	278 (Pro, Pro)
	1647 (T, C)	527 (Thr, Thr)
F	41 (T, A)	4 (Ile, Ile)
	94 (T, C)	22 (Leu, Pro)
	167 (G, A)	46 (Ser, Ser)
	326 (A, G)	99 (Arg, Arg)
	404 (A, G)	125 (Val, Val)
	1356 (C, T)	443 (Pro, Ser)
	1382 (G, A)	451 (Gln, Gln)
	1576 (C, T)	516 (Ala, Val)

[a] W3A HN sequences were reported previously by Hiebert et al. (4).
[b] W3A F sequences were reported previously by Paterson et al. (13).

created by site-directed mutagenesis of the plasmid encoding WR HN protein (data not shown).

On the other hand, it was revealed for the first time that mumps virus (MuV) HN protein was able to promote cell fusion when coexpressed with WR F protein (Table 2), whereas the HN proteins of Newcastle disease virus, SV41, and parainfluenza virus types 2 and 4A were not (data not shown). As shown in Table 2, the MuV HN protein also efficiently promoted cell fusion when coexpressed with P443S or A516V. The cell fusion-promoting activity of MuV HN protein was apparently higher than that of WR HN protein. However, it should be pointed out that the fusion given by the single expression of L22P was not significantly promoted, even upon coexpression with the MuV HN protein (Table 2, Fig. 2). This observation suggests that the fusogenic activity of L22P is almost maximally displayed in BHK cells when it is expressed alone.

Taken together, the results of our present study indicate that the proline residue at position 22 (Pro-22) is involved in the HN-independent fusogenic activity of W3A F protein. Pro-22 is located in one of the hydrophobic regions of W3A F2, and after removal of the signal peptide, it is the third amino acid from the amino terminus (13). In the case of the WR F protein, the change from leucine to proline at position 22, as represented by L22P, may result in either a decrease in local hydrophobicity or an alteration in local conformation at the F2 amino terminus. It was previously shown that Sendai virus F2 cannot be released from the virion, even after the splitting of interchain disulfide bond under denaturing conditions (1), while the HA1 of the influenza virus can be released under similar conditions (12). Thus, it seems possible that Sendai virus F2 has a noncovalent but tight association with the F1 molecule in addition to the covalent association via the intermolecular disulfide bridge. Interestingly, in this context, studies on neutralization escape mutants of Newcastle disease virus have suggested a topological interrelationship between F1 (heptad repeat A domain or cysteine-rich region) and F2 (10, 19). Undoubtedly, further investigation is needed to elucidate the possible role of F2 in the fusogenic activity.

In the absence of the HN protein, a conformational change of W3A F protein, which is considered a prerequisite for the induction of cell fusion, may be triggered upon contact with the target membrane or upon docking with an unrecognized receptor on the target membrane (9, 22). The presence of Pro-22 on the F2 may somehow contribute to the destabilization of the F1 so that the conformational change should be triggered. Alternatively, it is conceivable that Pro-22 is involved in the attachment to the putative cellular receptor.

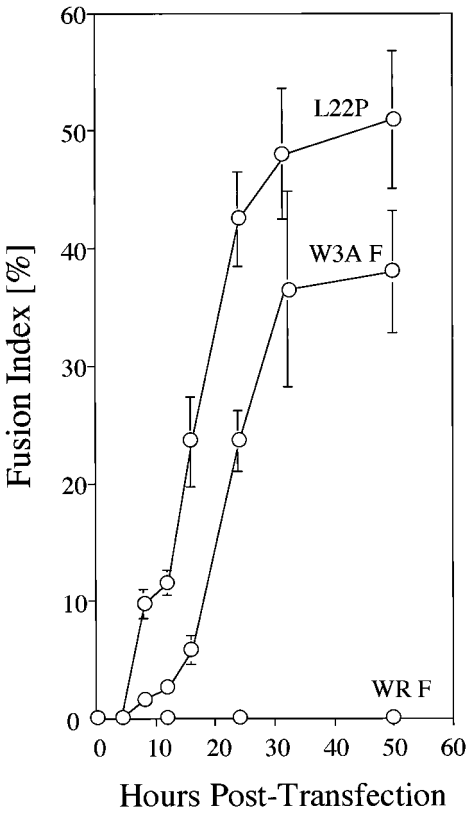

FIG. 1. Time course of syncytium formation induced by expressed F proteins. BHK cells grown in 6-well culture plates were transfected with 2 μg of plasmid encoding each F protein per well. At indicated times after transfection, cells were stained with Giemsa solution and photographed, and the fusion index was calculated morphometrically as described previously (20). Error bars represent standard deviation of the mean.

In conclusion, we have confirmed the HN-independent fusogenic activity of W3A F protein, identified a critical amino acid in the F2, and excluded the possibility of the involvement of SV40 in the fusion induction. Since WR F protein requires

TABLE 2. Fusogenic activities of F proteins expressed alone or coexpressed with HN proteins in BHK cells

F protein	Relative expression[a]	% Cell fusion when coexpressed with[b]:		
		None	WR HN	MuV HN
WR F	1.00	0	17.7 ± 2.2	37.9 ± 1.5
L22P	0.94	39.6 ± 2.4	44.0 ± 2.3	46.4 ± 5.9
P443S	0.88	0	3.4 ± 1.2	19.7 ± 1.8
A516V	0.86	0	20.7 ± 1.3	36.3 ± 1.0
W3A F	0.53	17.4 ± 2.0	35.3 ± 0.7	42.5 ± 5.0

[a] Cell surface expression was measured by flow cytometry (16, 20) with anti-SV5 rabbit serum at 8 h posttransfection and normalized by that of WR F protein.
[b] Fusion index (mean ± standard deviation) was morphometrically estimated (20) at 24 h posttransfection.

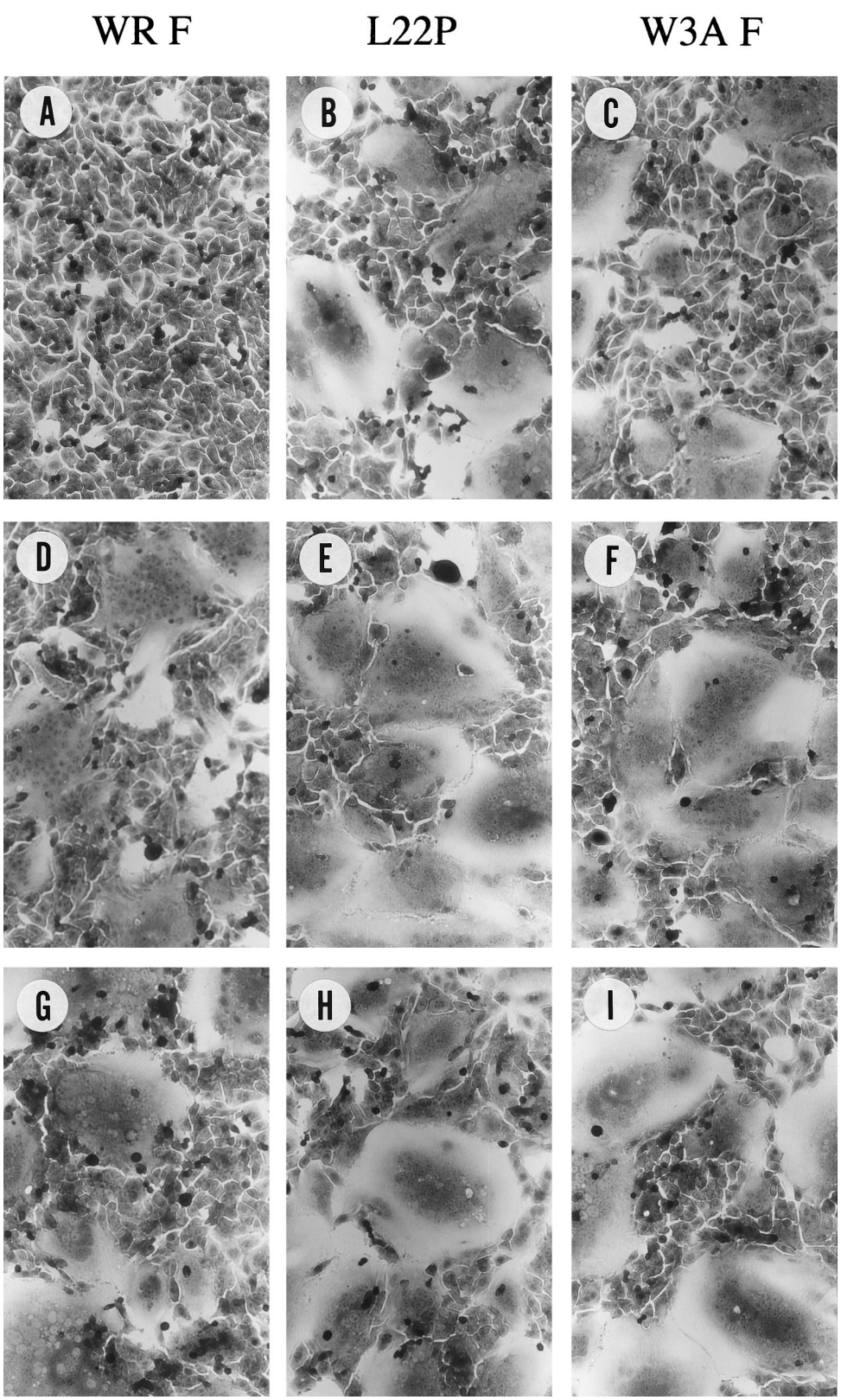

FIG. 2. Syncytium formation induced by F proteins expressed alone or coexpressed with HN proteins. BHK cells grown in 6-well culture plates were transfected independently or cotransfected with 2 μg of each plasmid per well, stained with Giemsa solution at 24 h posttransfection, and photographed. Magnification, ×180. The WR F protein (A, D, and G), L22P (B, E, and H), and W3A F protein (C, F, and I) were expressed in BHK cells either alone (A, B, and C) or coexpressed with WR HN protein (D, E, and F) or MuV HN protein (G, H, and I).

HN protein to mediate cell fusion, W3A F protein is still exceptional among the paramyxovirus F proteins because of its unique fusogenic activity.

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