JOURNAL OF VIROLOGY, Mar. 1989, p. 1293–1301
0022-538X/89/031293-09$02.00/0

Vol. 63, No. 3

Analysis of the Relationship between Cleavability of a Paramyxovirus Fusion Protein and Length of the Connecting Peptide

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Received 11 August 1988/Accepted 17 November 1988

The relationship between the length of the connecting peptide in a paramyxovirus F_0 protein and cleavage of F_0 into the F_1 and F_2 subunits has been examined by constructing a series of mutant F proteins via site-directed mutagenesis of a cDNA clone encoding the simian virus 5 F protein. The mutant F proteins had one to five arginine residues deleted from the connecting peptide. The minimum number of arginine residues required for cleavage-activation of the simian virus 5 F_0 protein by host cell proteases was found to be four. F proteins with two or three arginine residues in the connecting peptide were not cleaved by host cell proteases but could be cleaved by exogenously added trypsin. The mutant F protein possessing a connecting peptide consisting of one arginine residue was not cleaved by trypsin. The altered F proteins were all transported to the infected-cell plasma membrane as shown by cell surface immunofluorescence or cell surface trypsinization. However, the only mutant F protein found to be biologically active as detected by syncytium formation was the F protein which has four arginine residues at the cleavage site. The results presented here suggest that in the paramyxovirus F protein the number of basic amino acid residues in the connecting peptide is important for cleavage of the precursor protein by host cell proteases but is not the only structural feature involved. In addition, the data indicate that cleavage of F_0 into F_1 and F_2 does not necessarily result in biological activity and that the connecting peptide may affect the local conformation of the F polypeptide.

Simian virus 5 (SV5) is a prototype member of the paramyxovirus family of negative-strand RNA viruses. The paramyxovirus particle possesses an envelope which consists of a membrane derived from the host cell plasma membrane with two virus-encoded integral membrane glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins, forming spikelike projections on the outer surface (6). Paramyxoviruses are widely known for their ability to cause cell fusion and hemolysis, and extensive studies on the surface glycoproteins have shown that the F protein is involved in virus penetration, hemolysis, and cell fusion and is also required for the intracellular spread of the virus (7, 45). The F protein of SV5 has been expressed in eucaryotic cells from cDNA to the F mRNA (32) by using a late-region SV40 replacement vector and has been shown to be capable of causing cell fusion in the absence of the other SV5 proteins (34). The F glycoprotein is synthesized as an inactive precursor, F_0, that is cleaved by a host cell protease (or proteases) to produce the biologically active form of the protein, consisting of the disulfide-linked F_1 and F_2 subunits, and cleavage is a prerequisite for virus infectivity (16, 30, 36, 45, 46). Upon cleavage of F_0, the NH_2 terminus of F_1 is generated with a concomitant conformational change in the molecule (19).

Direct amino acid sequencing of the NH_2 terminus of the F_1 subunit from SV5, Sendai virus, and Newcastle disease virus indicated that this region of the F glycoprotein is highly conserved, and it has been suggested that the NH_2 terminus of F_1 may be directly involved in mediating membrane fusion (12, 42, 47). More recently, the nucleotide sequences of the F genes from SV5, respiratory syncytial virus, measles virus, Newcastle disease virus (NDV), human parainfluenza virus 3, Sendai virus, mumps virus, canine distemper virus, rinderpest virus, and bovine parainfluenza virus type 3 have been elucidated (1, 2, 5, 8, 11, 25, 33, 41, 48, 50–53). The predicted amino acid sequences indicate that in addition to conservation of the NH_2 terminus of F_1, cleavage of the F_0 precursor protein occurs at a stretch of basic amino acid residues (connecting peptide or cleavage-activation site) preceding the F_1 NH_2 terminus. The number of basic residues in the connecting peptide varies from one in the F_0 protein of Sendai virus (2, 15) to six in respiratory syncytial virus (8). There is an apparent correlation between the cleavability of the F_0 precursor protein and the length of the basic connecting peptide, since the Sendai F_0 protein is cleaved in only a few cell types, such as the cells in the chorioallantoic membrane of embryonated hen eggs, whereas the F_0 protein of respiratory syncytial virus is cleaved in many cell types. Thus, the paramyxovirus F protein would appear to be analogous to the hemagglutinin (HA) of influenza viruses, in which cleavability of the HA protein appears in part to be a function of the length of the connecting peptide between HA_1 and HA_2 (3, 4, 21), but the relationship is complicated in that other structural features of HA may be involved (22). The similarity is extended further because virulence and pathogenicity of both influenza virus and Newcastle disease virus in the host have been shown to be a function of the cleavability of the HA and F proteins, respectively (3, 21, 30).

The SV5 F protein has five arginine residues in the connecting peptide (33), and SV5 virions grown in tissue culture cells (e.g., CV-1) always contain a cleaved F protein (36, 44). As described above, we have previously reported the expression in eucaryotic cells of cleaved, biologically active SV5 F protein from cloned cDNA (34). Therefore, this provides a system in which the relationship between the length of the connecting peptide in a paramyxovirus F protein and cleavability can be examined experimentally. In this report we describe the construction and expression of a series of mutants with mutations in the gene encoding the SV5 F protein, in which the arginine residues of the connecting peptide have been deleted, resulting in F proteins

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possessing cleavage-activation sites ranging in length from five to zero arginine residues. The cell surface expression, cleavage, and biological activity of each mutant has been examined.

MATERIALS AND METHODS

Cells. Monolayer cultures of the TC7 clone of CV-1 cells and LLC-MK_2 cells (ATCC CCL7) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Primary African green monkey kidney cells were purchased from Whittaker Bioproducts, Walkersville, Md., and passaged once in DMEM supplemented with 10% fetal calf serum.

Construction of the F arginine deletion mutants. Construction of the SV40/WT F recombinant virus has been described previously (34). Preparation of the F-(arginine 0) mutant protein (no arginines in the F_0 connecting peptide) by oligonucleotide-directed mutagenesis of F cDNA has been reported previously (35). The remaining mutants, except for F-(arginine 4), were constructed essentially as described for F-(arginine 0), except that the template for mutagenesis was prepared by excising from the SV40/WT F recombinant plasmid an approximately 1,100-base-pair *Asp*718–*Bam*HI fragment that contains the 5′ 737 nucleotides of the F cDNA. This fragment was cloned into *Asp*718–*Bam*HI-digested replicative form of the bacteriophage M13mp19. Following mutagenesis, DNA containing the desired mutation was ligated to the large *Asp*718–*Bam*HI fragment containing the remainder of the SV40/WT F recombinant plasmid. Construction of the F-(arginine 4) mutant could not be carried out with wild-type (WT) F DNA as the template. Attempts with two different 24-mer mutagenic oligonucleotides gave rise to DNA insertions both upstream and downstream of the region encoding the F connecting peptide as a result of regions of homology between the template DNA and the mutagenic oligonucleotides. Therefore, the F-(arginine 4) mutant was prepared by oligonucleotide-directed insertion of six nucleotides encoding two arginine residues into template DNA derived from the F-(arginine 2) mutant. Following reconstruction of the SV40/F arginine deletion mutant plasmids, the entire region of the F cDNA used in the mutagenesis was sequenced by the dideoxy-chain termination sequencing method (43) with double-stranded plasmid DNA and an F-specific oligonucleotide primer.

Transfection, radioisotopic labeling of polypeptides in infected cells, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Transfection of recombinant DNA molecules into CV-1 cells, preparation of virus stocks, and radioisotopic labeling of infected-cell polypeptides were carried out as previously described (34), except that Tran-[^{35}S]-label (ICN Radiochemicals, Irvine, Calif.) was used instead of [^{35}S]methionine. Immunoprecipitation was carried out as described previously (23) with monospecific antibodies to purified SV5-F protein (26, 27). Samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed on 15% polyacrylamide gels (23).

Treatment of infected cells with *N*-acetyl trypsin. CV-1, LLC-MK_2, and secondary African green monkey kidney cell monolayers were infected with the SV40/WT F and F arginine deletion mutant recombinant viruses. At 48 h postinfection, infected monolayers were labeled for 2 h with 100 μCi of Tran-[^{35}S]-label per ml in methionine- and cysteine-free DMEM, the label was removed and replaced with DMEM supplemented with 0.3 μg of *N*-acetyl trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml, and the monolayers were incubated for a further 2 h. Control monolayers were incubated in the absence of exogenous trypsin. Following trypsin treatment, monolayers were washed twice with phosphate-buffered saline containing 0.1 μg of pepstatin A per ml, 0.1 μg of chymostatin per ml, 0.1 μg of leupeptin per ml, 0.1 μg of antipain per ml, and 10 kallikrein units of aprotinin per ml (all from Sigma), and the labeled proteins were prepared for immunoprecipitation and polyacrylamide gel electrophoresis as described above.

Indirect immunofluorescence. Cover slips of infected CV-1 cells were prepared for surface fluorescence by being fixed for 5 min at room temperature in a freshly prepared solution of 0.1% paraformaldehyde in phosphate-buffered saline. Indirect immunofluorescence with a monoclonal antibody to native SV5 F protein, kindly provided by Rick Randall (40), as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (Organon Teknika-Cappel, Malvern, Pa). as the secondary antibody was carried out as previously described (10).

Syncytium formation. Mutant F proteins were assayed for their ability to cause cell fusion as described previously (34), except that at 2 days postinfection, 0.3 μg of *N*-acetyl trypsin (Sigma) per ml was added to one monolayer from each set of duplicate samples.

Direct amino acid sequencing of the F_1 amino terminus. Monolayers of CV-1 cells in 6-cm dishes were infected with P1 stocks of the SV40/WT F and F arginine deletion mutant recombinant viruses. At 40 to 48 h postinfection, cell monolayers were labeled with 625 μCi of [^{3}H]valine (Amersham Corp., Arlington Heights, Ill.) per ml in valine-free DMEM for 2 h. At the end of the labeling period, infected-cell monolayers were treated as described above for treatment with *N*-acetyl trypsin. Following immunoprecipitation with anti-F IgG and polyacrylamide gel electrophoresis, the labeled proteins were electroblotted onto activated glass fiber paper as described previously (54), the transferred proteins were located by autoradiography of the blot, and the F_1 bands were excised. Protein sequencing of the blotted F_1 polypeptides was carried out by the Northwestern University Biotechnology Facility on a model 477A/120A protein sequencer (Applied Biosystems, Foster City, Calif.). The material from each cycle of Edman degradation was dried in a scintillation vial and then counted in a model LS 6800 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.).

RESULTS

Site-directed mutagenesis of the F_0 connecting peptide. The paramyxovirus F protein is synthesized as an inactive pre-

	F_2	F_1
WT	Thr– Arg– Arg– Arg– Arg– Arg–	Phe
4	Thr– – – – Arg– Arg– Arg– Arg–	Phe
3	Thr– – – – – – – Arg– Arg– Arg–	Phe
2	Thr– – – – – – – – – – Arg– Arg–	Phe
1	Thr– – – – – – – – – – – – Arg–	Phe
0	Thr– – – – – – – – – – – – – –	Phe

FIG. 1. Amino acid sequence at the cleavage site of the SV40/F recombinant viruses. Thr is the presumptive C terminus of the F_2 subunit. Phe is the NH_2-terminal amino acid of the F_1 subunit.

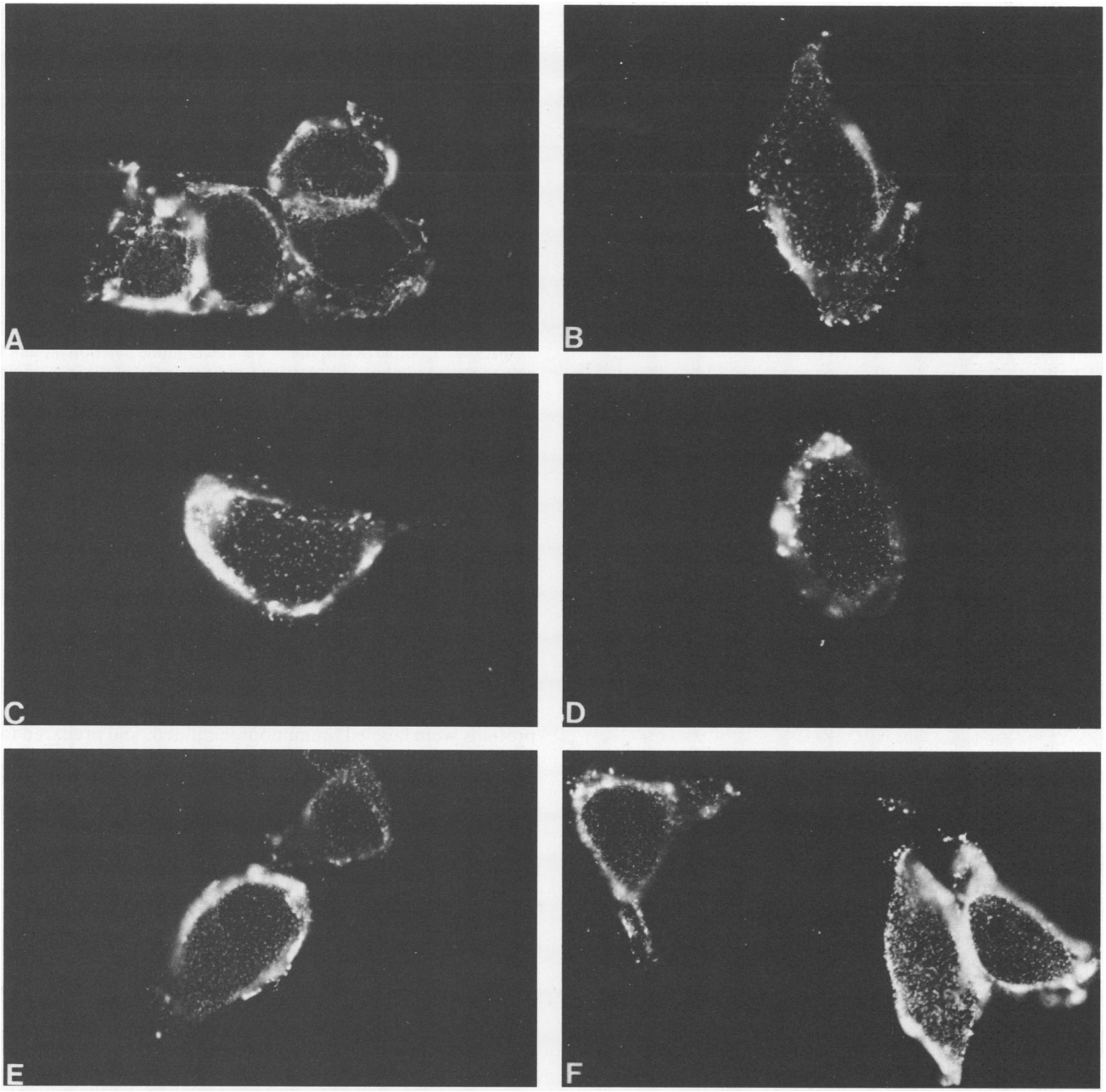

FIG. 2. Indirect immunofluorescent staining of the WT and mutant F proteins expressed at the cell surface. CV-1 cells infected with the recombinant SV40 expressing the WT and mutant F proteins were fixed by using 0.1% paraformaldehyde and incubated sequentially with phosphate-buffered saline containing 1% bovine serum albumin, F monoclonal antibody (40), and fluorescein-conjugated goat anti-mouse IgG. (A) WT F. (B) F-(arginine 4). (C) F-(arginine 3). (D) F-(arginine 2). (E) F-(arginine 1). (F) F-(arginine 0).

cursor (F_0), which is cleaved by a trypsinlike host cell protease at a stretch of basic residues, known as the connecting peptide or cleavage-activation site, to produce the active form of the protein, the disulfide-linked F_1 and F_2 subunits (16, 30, 36, 45, 46). The SV5 connecting peptide consists of five arginine residues (33). A series of mutant F proteins were constructed by using site-directed mutagenesis, as described in Materials and Methods, such that they contained connecting peptides consisting of four, three, two, one, or no arginine residues. The amino acid sequences of the connecting peptides are illustrated in Fig. 1. The DNAs containing the desired deletions were inserted in place of the equivalent fragment encoding the WT F protein in the SV40/F recombinant plasmid, in which the F cDNA is under the control of the SV40 late-region promoter and polyadenylation signals (34). To verify that the only nucleotide changes introduced into the mutant DNAs during the mutagenesis procedure were those leading to deletions in the connecting peptide, the entire region from each SV40/F arginine deletion mutant recombinant plasmid that had been

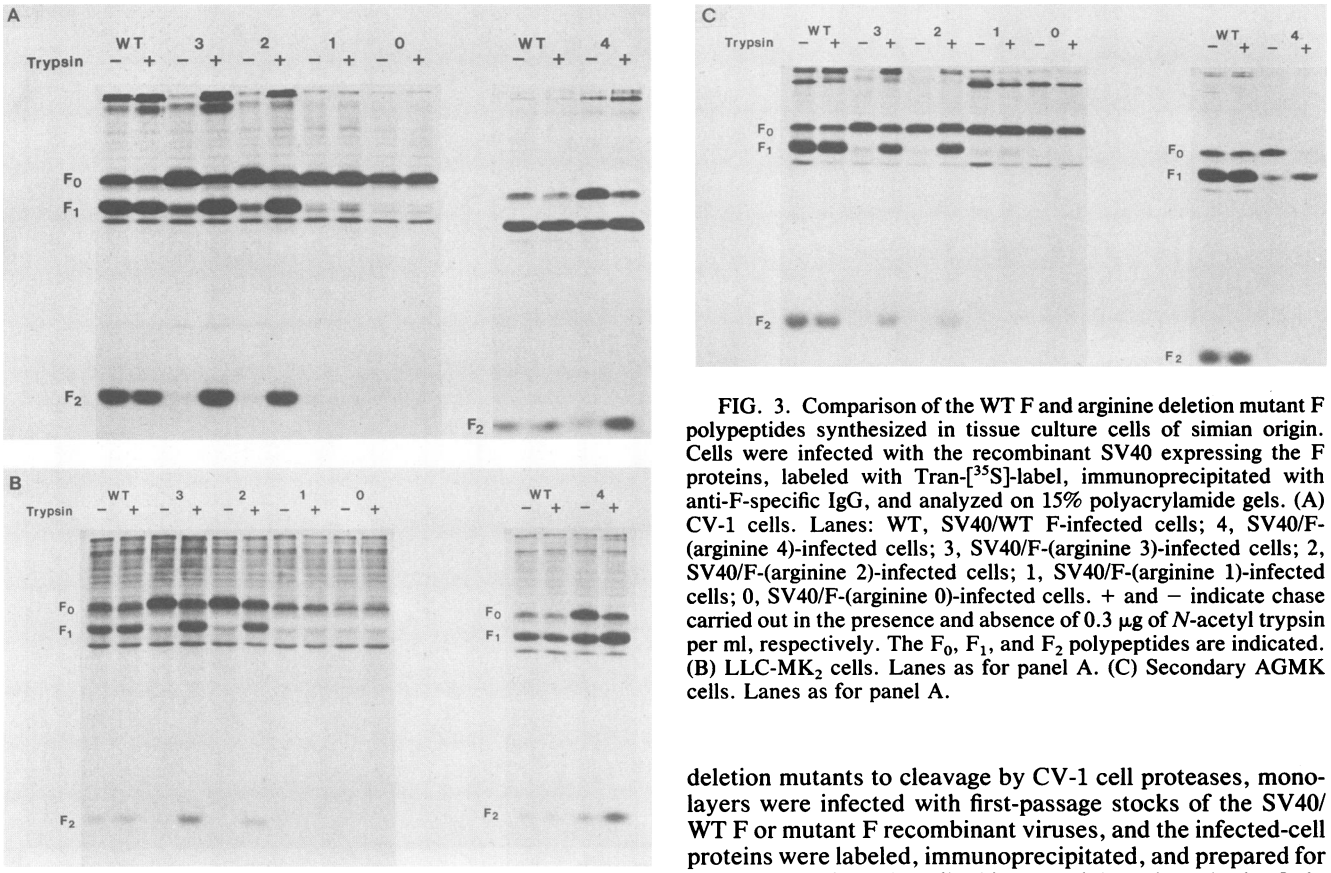

FIG. 3. Comparison of the WT F and arginine deletion mutant F polypeptides synthesized in tissue culture cells of simian origin. Cells were infected with the recombinant SV40 expressing the F proteins, labeled with Tran-[^{35}S]-label, immunoprecipitated with anti-F-specific IgG, and analyzed on 15% polyacrylamide gels. (A) CV-1 cells. Lanes: WT, SV40/WT F-infected cells; 4, SV40/F-(arginine 4)-infected cells; 3, SV40/F-(arginine 3)-infected cells; 2, SV40/F-(arginine 2)-infected cells; 1, SV40/F-(arginine 1)-infected cells; 0, SV40/F-(arginine 0)-infected cells. + and − indicate chase carried out in the presence and absence of 0.3 μg of *N*-acetyl trypsin per ml, respectively. The F_0, F_1, and F_2 polypeptides are indicated. (B) LLC-MK$_2$ cells. Lanes as for panel A. (C) Secondary AGMK cells. Lanes as for panel A.

subcloned from the replicative form of M13mp19 was sequenced by the dideoxy-chain termination procedure (43). The only differences detected between the nucleotide sequences of the WT F and mutant F cDNAs were in the regions encoding the F_0 connecting peptides. Thus, any observed difference in the properties of the WT and mutant F proteins can be attributed to the alteration in the connecting peptide.

Expression and cell surface localization of mutant proteins by immunofluorescence. The SV40/WT and mutant F recombinant DNAs, together with DNA from an SV40 early-region deletion mutant (38), were introduced into CV-1 cells by means of DEAE-dextran-mediated transfection (24), and virus stocks were made. To determine whether the mutant F proteins were transported to the plasma membrane, we examined infected cells by indirect immunofluorescence. A monoclonal antibody to the native SV5 F protein (40) and fluorescein-conjugated goat anti-mouse IgG were used as described in Materials and Methods. Figure 2 shows the pattern of cell surface fluorescence displayed by cells infected with the SV40/WT F and mutant F recombinant viruses. The cell surface fluorescence observed for all the mutant F proteins (Fig. 2B to F) was similar in distribution and intensity to that observed for the WT F protein (Fig. 2A). These data indicate that at least a proportion of each mutant F protein, possessing a deletion in the connecting peptide, is competent for transport to the cell surface.

Susceptibility of the mutant F proteins to cleavage by host cell proteases and exogenously added trypsin. To assay for the susceptibility of the cleavage-activation site in the F arginine deletion mutants to cleavage by CV-1 cell proteases, monolayers were infected with first-passage stocks of the SV40/WT F or mutant F recombinant viruses, and the infected-cell proteins were labeled, immunoprecipitated, and prepared for electrophoresis as described in Materials and Methods. Only the WT F_0 protein and the mutant F_0 protein containing four arginine residues at the cleavage site [F-(arginine 4)] (Fig. 3A, lanes WT − and 4 −, respectively) were cleaved by CV-1 cell proteases to give rise to significant amounts of the F_1 and F_2 subunits. On addition of 0.3 μg of *N*-acetyl trypsin per ml to the surface of infected cells, the mutant F_0 proteins containing either three or two arginine residues at the cleavage site [F-(arginine 3) and F-(arginine 2), respectively] (Fig. 3A, lanes 3 + and 2 +, respectively) were cleaved into F_1 and F_2 subunits. In addition, an increase in the amount of cleaved F-(arginine 4) protein was observed after treatment with exogenous trypsin relative to that seen without trypsin. Cleavage of nearly all the F_0 protein of these mutants by exogenously added protease also provides a simple biochemical assay for cell surface expression. Although the mutant F protein possessing a cleavage site consisting of one arginine residue [F-(arginine 1)] was shown by immunofluorescence to be expressed at the cell surface, it was resistant to cleavage by both exogenous trypsin and CV-1 cell proteases (Fig. 3A, lanes 1 + and 1 −, respectively). As expected, the F protein lacking a cleavage site [F-(arginine 0)] was resistant to digestion by both CV-1 cell proteases and exogenously added trypsin (Fig. 3A, lanes 0 − and 0 +, respectively). Because the F-(arginine 3) and F-(arginine 2) mutants could be cleaved by trypsin but were resistant to CV-1 cell proteases, different host cells were examined for the presence of proteases capable of cleaving these proteins. As the mutant proteins were expressed from SV40 recombinant viruses, only cells of simian origin which can support SV40 replication were tested. The data obtained from infection of LLC-MK$_2$ cells (rhesus monkey kidney cells) and secondary African green monkey kidney cells infected with the recom-

binant viruses are shown in Fig. 3B and 3C, respectively, and the pattern of F_0 cleavage with the mutants was found to be identical to that obtained with CV-1 cells.

In addition to analyzing the mutant F proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of the reducing agent dithiothreitol, the trypsin-cleaved samples were also electrophoresed under nonreducing conditions to check that disulfide bond formation was occurring between the F_1 and F_2 subunits. In the absence of reducing agent, the cleaved mutant F proteins were found to migrate on sodium dodecyl sulfate-polyacrylamide gels as $F_{1,2}$ (data not shown), thus indicating the presence of disulfide bonds.

Examination of the biological activity of the F protein cleavage site mutants. We have previously shown that the SV5 WT F protein expressed by a recombinant SV40 is capable of causing cell fusion (34). The F mutant proteins with cleavage sites consisting of four, three, or two arginine residues were examined for their ability to cause cell fusion as described in Materials and Methods. Briefly, CV-1 cell monolayers were infected with P1 stocks of the SV40/WT and mutant F recombinant viruses. At 2 days postinfection, the medium was removed, fresh DMEM was added with or without 0.3 μg of *N*-acetyl trypsin per ml, and the monolayers were incubated for a further 2 days. Surprisingly, syncytium formation was observed, with or without trypsin treatment, only in monolayers infected with the WT F (Fig 4A) and the F-(arginine 4) mutant (Fig. 4B) recombinant viruses. Syncytia were not observed in monolayers infected with the F-(arginine 3) and F-(arginine 2) recombinant viruses (data not shown), even after treatment of the cell surfaces with trypsin under conditions which resulted in the cleavage of the mutant F_0 proteins into the F_1 and F_2 subunits (Fig. 3, Lanes 3 + and 2 +, respectively). Although paramyxovirus F proteins cause fusion at neutral pH, in the unlikely event that the pH optimum of fusion had been altered the effect of exposing cells expressing the F-(arginine 3) and F-(arginine 2) proteins to low pH was examined. Briefly, following treatment of the cell surface with trypsin, the monolayers were incubated at 37°C for 1 min at pH 5.0 (in phosphate-buffered saline, 10 mM morpholineethanesulfonic acid [MES], 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 5.0]) and subsequently incubated for a further 2 h at 37°C in DMEM. Exposure to low pH did not confer the ability to cause syncytium formation upon the F-(arginine 3) and F-(arginine 2) proteins.

Direct amino acid sequencing of the F_1 amino terminus. The lack of fusion activity observed with cleaved F-(arginine 3) and F-(arginine 2) mutant proteins was an unanticipated result, since the only sequence difference between the WT F_0 protein and the mutant F_0 proteins was the number of arginine residues present in the connecting peptide. In addition, the F_1 and F_2 subunits produced from the mutant F_0 proteins by the action of exogenous trypsin had electrophoretic mobilities indistinguishable from those of the F_1 and F_2 subunits resulting from cleavage of the WT F_0 protein (Fig. 3A to C, lanes WT +, WT −, 3 +, 2 +). One possible explanation for the observed results is that the F_1 NH_2 terminus produced upon cleavage of the mutant F_0 proteins by trypsin is not identical to that of the WT. To examine this possibility, monolayers infected with the WT F and the F-(arginine 4), F-(arginine 3), and F-(arginine 2) recombinant viruses were labeled with [^{3}H]valine and the protein sequence of the F_1 subunits was obtained as described in Materials and Methods. The F_1 polypeptides were subjected to 20 cycles of Edman degradation, and the products of each cycle were assayed for the presence of ^{3}H radioactivity. Peaks of radioactivity were found in cycles 4, 5, 13, and 19 (Fig. 5, Val), as would be expected from the predicted amino acid sequence. The results obtained for the mutant F proteins were identical to those obtained for the WT F_1 polypeptide, indicating that the F_1 NH_2 termini produced upon

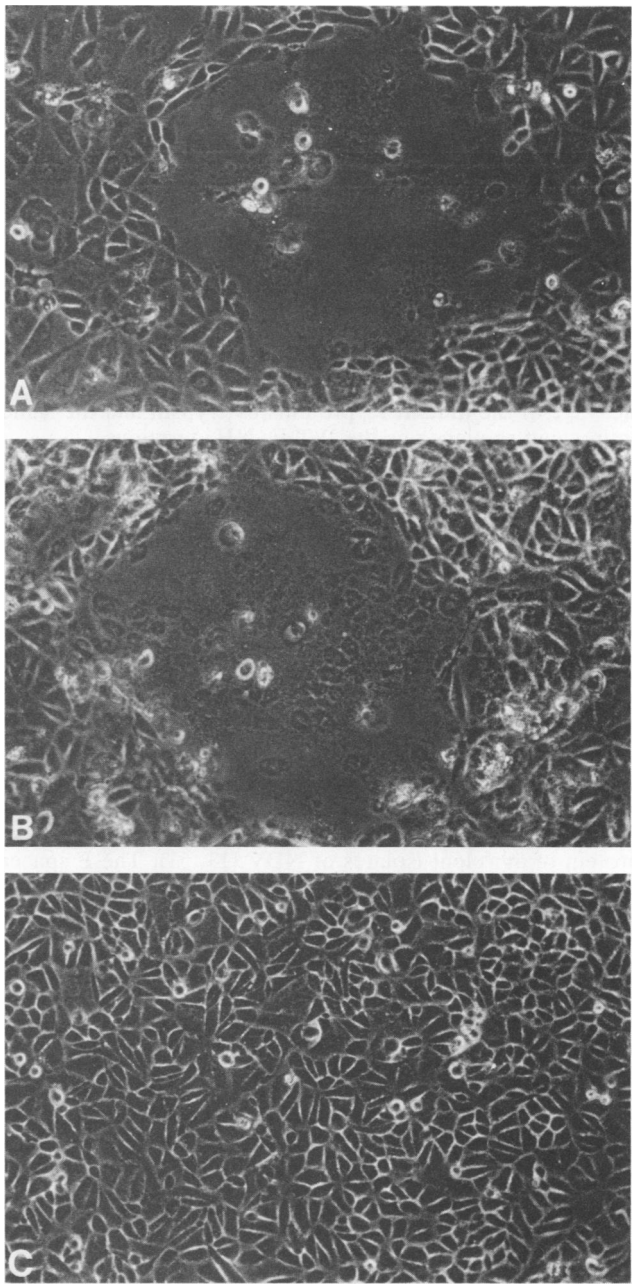

FIG. 4. Functional assay for the biological activity of the expressed F proteins. CV-1 cells were infected with the SV40/WT F, F-(arginine 4), and F-(arginine 0) recombinant viruses, and the infected cells were examined for syncytium formation. (A) Cell-cell fusion induced by the SV5 WT F protein (without trypsin). (B) Cell-cell fusion induced by the SV5 F-(arginine 4) protein (without trypsin). (C) Cells infected with the SV40/F-(arginine 0) recombinant virus (without trypsin).

	1				5				10				15						20
WT Sequence	Phe	Ala	Gly	Val	Val	Ile	Gly	Leu	Ala	Ala	Leu	Gly	Val	Ala	Thr	Ala	Ala	Gln	Val	Thr
Edman Cycles	X	X	X	Val	Val	X	X	X	X	X	X	X	Val	X	X	X	X	X	Val	X

FIG. 5. Amino-terminal sequence analysis of the F arginine deletion mutant proteins. CV-1 cells were infected with the SV40/WT F, F-(arginine 4), F-(arginine 3), and F-(arginine 2) recombinant viruses, and the F proteins were labeled with [^{3}H]valine and prepared for NH_2-terminal sequence analysis as described in Materials and Methods. The F_1 polypeptides were subjected to 20 cycles of Edman degradation, and the material released at each cycle was analyzed for ^{3}H radioactivity by scintillation counting. The deduced amino acid sequence of the SV5 WT F_1 polypeptide is shown. The numbers indicate the amino acid position in F_1 with the NH_2-terminal phenylalanine designated 1. The Edman cycles giving rise to a peak of ^{3}H radioactivity are indicated by Val in the lower line. The results for the F-(arginine 4), F-(arginine 3), and F-(arginine 2) mutant proteins were identical to WT, with peaks of radioactivity occurring on cycles 4, 5, 13, and 19 as indicated.

cleavage of the mutant F_0 proteins by trypsin had the same amino acid sequence as the WT F_1 NH_2 terminus.

Cleavage site spacer mutant. As mentioned above, in constructing the F-(arginine 0) through F-(arginine 4) mutants, arginine residues were deleted from the cleavage site of the SV5 F protein without being replaced by alternative amino acids, resulting in a reduction in the number of residues occurring between the C-terminal threonine of F_2 and the N-terminal phenylalanine of F_1 (Fig. 1). To investigate whether the total number of amino acids between the C terminus of F_2 and the N terminus of F_1 is important in retaining the ability of the F protein to cause syncytium formation upon cleavage, we constructed a cleavage site "spacer" mutant. The spacer mutant was constructed by inserting two amino acid residues (Gln and Gly) into the cleavage site of the F-(arginine 3) protein (Fig. 6, bottom), thus making the cleavage site similar to that found in the F protein of avirulent isolates of NDV (13, 51). The F spacer protein was not cleaved by host cell proteases but was susceptible to cleavage by exogenous protease (Fig. 6, lanes S − and S +). In addition, the F spacer protein was not capable of causing cell fusion following cleavage by trypsin (data not shown), similar to the finding with the F-(arginine 3) mutant protein from which it was derived.

DISCUSSION

Studies concerning the differences between virulent and avirulent strains of influenza virus led to the proposal that virulence was related to the cleavability of the influenza virus HA (4). A similar observation has also been made for the paramyxovirus Newcastle disease virus, for which it was noted that there was a correlation between pathogenicity and susceptibility of the viral glycoproteins to proteolytic cleavage (31). It was suggested that cleavability was a function of the number of basic residues in the connecting peptide between HA_1 and HA_2 (3). Further support for this suggestion has been obtained by comparing the sequences of the HAs from virulent and avirulent H5 and H7 influenza A viruses (21, 29, 39) and, for paramyxoviruses, the sequences of the F_1 proteins from virulent and avirulent strains of Newcastle disease virus (13, 51). However, when the properties of a series of mutant H5 HAs possessing site-directed mutations in the cleavage site were examined, the data suggested that although the length of the basic connecting peptide is the most important factor in determining the cleavability of HA and hence the pathogenicity of the virus, other structural features of the HA molecule are also involved (22).

The cleavability of the F proteins from different members of the paramyxovirus family grown in tissue culture is found to vary, with the F protein from Sendai virus rarely being cleaved, whereas the F proteins from viruses such as SV5 and measles virus are always cleaved. The sequences of the F proteins from these viruses also support the hypothesis that cleavability is related to the length of the basic connecting peptide, since Sendai virus is found to possess one arginine residue, whereas SV5 has five arginines and measles virus has three arginines and a lysine (2, 18, 33, 41). The protease(s) responsible for the cleavage-activation of the paramyxovirus F protein has not yet been identified. However, endopeptidases exist in the Golgi apparatus that cleave many cellular precursor proteins at pairs of dibasic amino acids (for reviews, see references 14, 20, and 49), and it is

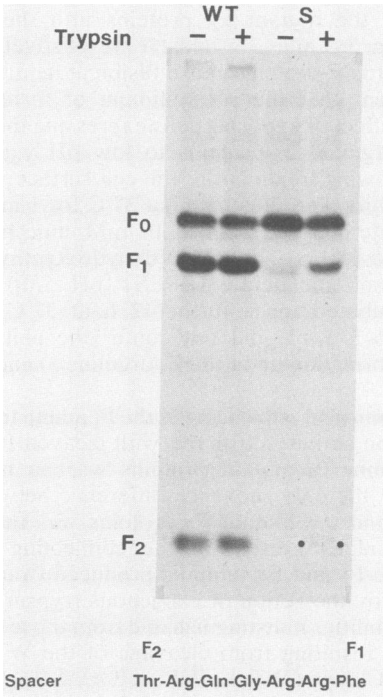

FIG. 6. Comparison of the WT F and spacer mutant F protein synthesized in CV-1 cells. Cells were infected with the recombinant SV40 expressing the F proteins and labeled and analyzed as described in the legend to Fig. 3. Lanes: WT, SV40/WT F-infected cells; S, SV40/F-spacer-infected cells. Symbols: + and −, chase carried out in the presence and absence of 0.3 μg of *N*-acetyl trypsin, respectively; ●, positions of the F_2 subunit of the F spacer protein.

possible that such an enzyme is involved in the proteolytic cleavage of F_0.

We examined the relationship between the cleavability of the paramyxovirus F protein and the length of the basic connecting peptide experimentally by constructing a series of mutant SV5 F proteins having connecting peptides varying in length from five (WT) to no amino acids. All these F proteins were competent for transport to the cell surface as determined by cell surface immunofluorescence with a monoclonal antibody, thus indicating that cleavage of the SV5 F protein is not required for its cell surface expression. A similar conclusion has been drawn with mutants of the Rous sarcoma virus glycoprotein, which were transported to the cell surface in the uncleaved precursor form (37). Since it is now widely presumed that for cell surface expression of integral membrane proteins to be obtained they must fold properly (9), it is reasonable to assume that the mutant F proteins must be properly folded at the gross level. Our data with the SV5 F protein mutants indicate that the minimum number of basic amino acids in the connecting peptide required for cleavage by host cell proteases is four. This fits with the observation that the minimum number of basic residues at the cleavage site of HA from a virulent influenza virus strain is four and that the F protein from virulent strains of Newcastle disease virus have two pairs of dibasic residues, whereas avirulent strains have two basic residues separated by two other amino acids (13, 51). The SV5 mutant F proteins in which the cleavage site consists of two or three arginine residues were not cleaved by host cell proteases but were found to be susceptible to cleavage by exogenous trypsin. However, the F-(arginine 1) mutant was found to be resistant to trypsin cleavage and therefore differs from the F protein of Sendai virus, which also has one arginine at the cleavage site but is susceptible to trypsin. A possible explanation for this finding is that there has been a local conformational change in the F protein in the region encompassing the cleavage site such that this part of the molecule is no longer accessible to trypsin. Presumably, in the Sendai F protein other structural features compensate for the short connecting peptide and allow the cleavage site to be in an exposed position in the molecule and hence to be susceptible to cleavage by proteases present in the chorioallantoic fluid of embryonated chicken eggs (16, 28) and exogenous trypsin (17, 45).

Cleavage of the paramyxovirus F_0 into the F_1 and F_2 subunits is a prerequisite for the molecule to be biologically active and capable of causing cell fusion (16, 45, 46). Of the mutants examined here, only the F-(arginine 4) mutant caused syncytium formation, although both the F-(arginine 2) and F-(arginine 3) mutants were cleaved by trypsin into the F_1 and F_2 subunits. Although it is possible that a minimum number of F molecules are required for fusion to occur and therefore only cells expressing large amounts of F on their surface can induce fusion (34), the number of cells expressing the mutant F proteins was found by immunofluorescence to be equivalent and the total synthesis of the F proteins as measured by ^{35}S incorporation was similar. Therefore, this possibility does not explain the differences observed. Cleavage of F_0 is accompanied by a conformational change in the molecule, with exposure of a new hydrophobic region presumed to be the NH_2 terminus of F_1 (19). It is possible that reducing the length of the SV5 cleavage site from five arginine residues to fewer than four arginines puts a structural constraint on the molecule so that the protein is unable to undergo the correct conformational change on cleavage. The results obtained with the F spacer mutant protein indicate that replacing the arginines with alternative amino acids does not function to abrogate the putative structural constraint on the SV5 F protein molecule and that the sequence of the cleavage site rather than the length per se is of primary importance in determining biological activity. Thus, these data suggest that during evolution of the F proteins of different paramyxoviruses, coincident with the critical changes in the length of the connecting peptide, there must have been changes in structural features elsewhere in the molecule such that biological activity is maintained.

ACKNOWLEDGMENTS

We thank Rick E. Randall, St. Andrews University, St. Andrews, Scotland, for kindly providing the monoclonal antibody to F, and John R. Tozer for construction and expression of the spacer mutant, which was carried out as part of a C99 project for undergraduates at Northwestern University.

This research was supported by Public Health Service research grants AI-23173 and AI-20201 from the National Institutes of Health.

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