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A Single Amino Acid Change in the Newcastle Disease Virus Fusion Protein Alters the Requirement for HN Protein in Fusion

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The role of a leucine heptad repeat motif between amino acids 268 and 289 in the structure and function of the Newcastle disease virus (NDV) F protein was explored by introducing single point mutations into the F gene cDNA. The mutations affected either folding of the protein or the fusion activity of the protein. Two mutations, L275A and L282A, likely interfered with folding of the molecule since these proteins were not proteolytically cleaved, were minimally expressed at the cell surface, and formed aggregates. L268A mutant protein was cleaved and expressed at the cell surface although the protein migrated slightly slower than wild type on polyacrylamide gels, suggesting an alteration in conformation or processing. L268A protein was fusion inactive in the presence or absence of HN protein expression. Mutant L289A protein was expressed at the cell surface and proteolytically cleaved at better than wild-type levels. Most importantly, this protein mediated syncytium formation in the absence of HN protein expression although HN protein enhanced fusion activity. These results show that a single amino acid change in the F_1 portion of the NDV F protein can alter the stringent requirement for HN protein expression in syncytium formation.

Enveloped viruses initiate infection with attachment to susceptible cells and subsequent membrane fusion, processes directed by the viral glycoproteins. Membrane fusion mediated by most paramyxoviruses, such as Newcastle disease virus (NDV), requires two virion-associated glycoproteins, the attachment protein or hemagglutinin-neuraminidase (HN) protein and the fusion (F) protein (reviewed in reference 13). The F protein is directly involved in membrane fusion, although in most systems, the HN protein has an undefined role in this step of infection that can be genetically separated from its attachment activity (25, 26). However, the simian virus 5 (SV5) and respiratory syncytial virus (RSV) attachment proteins are not absolutely required for fusion mediated by the F protein of these viruses (1, 8, 12). The reason for these different requirements for attachment proteins in fusion is not clear.

Paramyxovirus F protein is synthesized as an inactive precursor, F_0, which must be proteolytically cleaved to form a disulfide-linked heterodimer of F_1 and F_2 in order to direct membrane fusion (14). The F protein has several domains that are involved in fusion. The amino terminus of F_1, termed the fusion peptide or fusion sequence, is thought to insert into the target membrane (7). In addition, two heptad repeat (HR) regions in the F_1 polypeptide have been identified as important to fusion. One, HR1, is located just carboxyl terminal to the fusion peptide (4); the other, HR2, is located adjacent to the transmembrane domain (3). The HR2 domain has a leucine zipper motif. The importance of these domains for fusion has been shown by mutational analysis (2, 24, 27). In addition, peptides with sequences from either of these domains inhibit fusion (15, 23, 31–33), possibly because they mimic the respective domains in the intact protein and interfere with conformational changes in the molecule necessary for fusion. Indeed, it has been shown in three different paramyxovirus systems that peptides with sequences from the HR1 and HR2 domains form a complex which may mimic the interaction of these two domains during activation of the F protein (6, 11, 34).

Ghosh et al. (5) have recently noted that paramyxovirus F protein sequences contain a second leucine zipper-like motif located between HR1 and HR2. They suggested that this domain was important for fusion since a peptide with sequence from this region of the Sendai virus (SV) F protein inhibited hemolysis mediated by virions. Furthermore, they reported that this peptide formed complexes with peptides derived from the HR1 and HR2 domains of SV. These findings implicate this third domain in the activity of the F protein. Indeed, the NDV F protein has this leucine repeat motif which extends for four heptads. To explore the role of this domain in the NDV F protein maturation and fusion activity, we mutated each of the leucine residues in the motif. We report that single amino acid changes in this motif affect either folding of the molecule or the fusion activity as measured by syncytium formation. One alteration enhances the fusion activity of the protein in the presence of the HN protein. Surprisingly, this mutation also allows the NDV F protein to direct fusion in the absence of the HN protein. These results represent the first report of NDV syncytium formation in the absence of HN protein expression and suggest that this region of the F protein may play a role in determining the requirement of attachment protein in NDV fusion.

MATERIALS AND METHODS

Cells, vectors, and viruses. Cos-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco modified Eagle medium supplemented with nonessential amino acids, vitamins, penicillin-streptomycin, and 10% fetal calf serum.

NDV HN and F genes (derived from strain AV), characterized previously (26), were expressed in Cos cells by using pSVL, obtained from Pharmacia. Viral genes were inserted into *Sac*I- and *Xba*I-cut plasmid DNA.

Site-specific mutagenesis. Mutations in the F gene were generated using a Chameleon double-stranded site-specific mutagenesis kit from Stratagene. The

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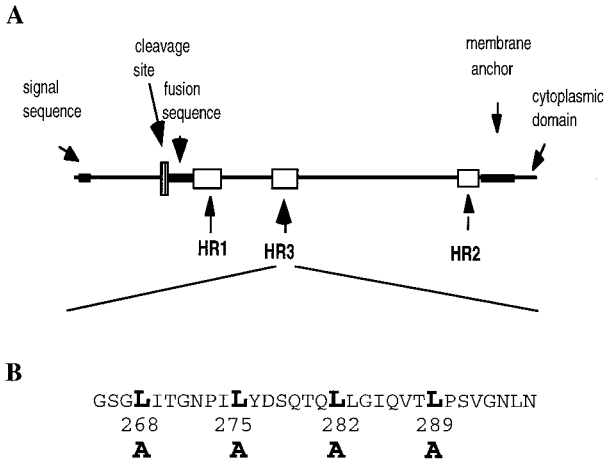

FIG. 1. Locations of HR3 mutations. (A) Linear representation of the sequence of the NDV fusion protein, with important domains indicated. HR3 is located from amino acids 268 to 289. (B) Amino acid sequence of HR3 in the NDV F_1 protein from amino acids 265 to 296. The mutated leucine residues are indicated in bold.

appropriate oligomer, at least 30 nucleotides in length, was used for each mutation. The entire gene of the mutant DNA was sequenced to verify that the rest of the gene remained unchanged by the mutagenesis reaction. The mutations isolated are shown in Fig. 1B. Mutants are named with the amino acid, in single-letter code, in the wild type, the position of the amino acid change, and the amino acid in the mutant.

Transfections. Cos cells were plated at 2×10^5 per 35-mm-diameter plate and transfected 20 to 24 h later. Transfections were accomplished using Lipofectamine (BRL/Gibco). For each 35-mm-diameter plate, a mix of 0.1 μg of DNA in 0.1 ml of OptiMEM (BRL/Gibco) and 10 μl of transfection reagent in 0.1 ml of OptiMEM was incubated at room temperature for 45 min, diluted with 0.7 ml of OptiMEM, and added to a plate previously washed with OptiMEM. Cells were incubated with the transfection reagent-DNA complexes for 5 to 6 h, and then 2 ml of complete Cos cell medium was added.

Antibodies. Antibodies used for detection of the fusion or HN protein were anti-Fu1a, anti-Ftail, anti-H antibody, and anti-NDV antibody. Anti-Fu1a, an F protein monoclonal antibody, was previously described (18) and obtained from

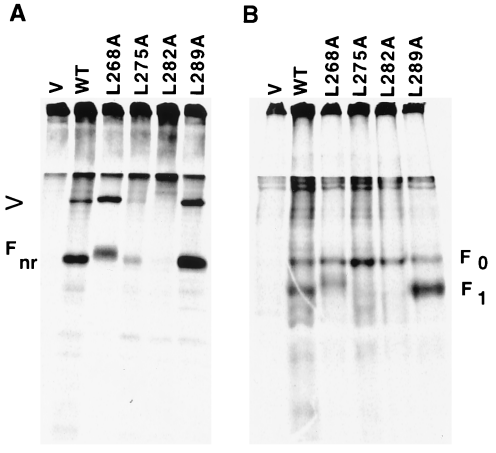

FIG. 2. Immunoprecipitation of radioactively labeled mutant F proteins. Cos cells transfected with pSVL-F DNA as well as each of the four HR3 mutants, L268A, L275A, L282A, and L289A, were radioactively labeled with ^{35}S as described in Materials and Methods. Proteins present in lysates from equivalent numbers of cells were immunoprecipitated with anti-Ftail, and the precipitated protein was electrophoresed in the absence (A) or presence (B) of reducing agent. Lane 1, vector (V) alone; lane 2, wild-type (WT) F; lane 3, L268A; lane 4, L275A; lane 5, L282A; lane 6, L289A. F_{nr}, nonreduced fusion protein; F_0, uncleaved fusion protein; F_1, cleaved form of the fusion protein. The arrowhead indicates SDS-resistant F protein species.

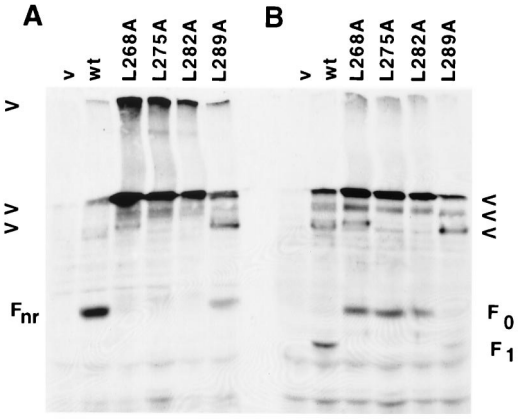

FIG. 3. SDS-resistant conformational forms of the F protein detected by Western analysis. Cos cells were transfected with wild-type and mutant F protein DNAs for 48 h as described in legend to Fig. 2. Proteins present in the resulting cell lysates were electrophoresed in the absence (A) or presence (B) of reducing agent. Proteins in sample buffer were incubated at 50°C for 5 min prior to loading onto the gel. Anti-Ftail was used to bind F protein in the blot. Lane 1, vector (v) alone; lane 2, wild-type (wt) F; lane 3, L268A; lane 4, L275A; lane 5, L282A; lane 6, L289A. F_{nr}, nonreduced fusion protein; F_0, uncleaved fusion protein; F_1, cleaved form of the fusion protein. Arrowheads on each side indicate SDS-resistant F protein species.

Mark Peeples. Anti-Ftail was raised against a synthetic peptide with the sequence of the cytoplasmic tail of the fusion protein as described by Wang et al. (30) and prepared by the Peptide Core Facility of the University of Massachusetts Medical School. Antibody used to detect HN protein was anti-H, a polyclonal rabbit antibody raised against the carboxyl-terminal domain of the HN protein expressed in *Escherichia coli* as a TrpE fusion as previously described (26). Anti-NDV is a polyclonal antiserum raised in rabbits against UV-inactivated virions as previously described (16). This antiserum contains antibodies against both HN and F proteins.

Immunofluorescence. Cos cells were plated on 35-mm-diameter plates containing glass coverslips and transfected as described above. The cells were washed twice with phosphate-buffered saline (PBS) containing 1.5% bovine serum albumin (BSA) and 0.02% azide and incubated at 4°C in PBS containing 3% BSA, 0.02% sodium azide, and antibody (diluted 1:100) for 1 h. Cells were washed three times with ice-cold PBS containing BSA and azide and incubated on ice with PBS containing BSA, azide, and Alexa dye (Alexa 488)-conjugated anti-rabbit immunoglobulin G (IgG) or Alexa dye (Alexa 568)-conjugated anti-mouse IgG (Molecular Probes) for 1 h. Cells were washed with cold PBS containing BSA and azide and visualized in a Nikon fluorescence microscope using the appropriate filters. Photographs were taken using Kodak ASA 3200 film.

Western analysis of mutant proteins. Cell extracts were diluted in sample buffer and loaded onto 10% polyacrylamide gels without boiling. After electrophoresis, the gels were equilibrated in transfer buffer (25 mM Tris, 190 mM glycine, 5% methanol [pH 8.2]) and transferred to Immobilon-P (Millipore Corp.) membranes. The membrane was blocked in PBS containing 0.5% Tween 20 and 10% nonfat dried milk overnight at 4°C. Membranes were washed in PBS-Tween 20 and incubated with primary antibody diluted in PBS–Tween 20–0.5% nonfat milk for 2 h at room temperature. Membranes were washed in PBS-Tween 20 and then incubated for 1 h at room temperature in secondary antibody, anti-rabbit IgG coupled to horseradish peroxidase (Boehringer Mannheim) diluted in PBS–Tween 20–0.5% nonfat milk. Membranes were washed extensively, and bound antibody was detected using the ECL (enhanced chemiluminescence) Western blotting detection reagent system (Amersham).

Radiolabeling and immunoprecipitation of protein. Transfected cells were radiolabeled for 2 h at 37°C in Dulbecco modified Eagle medium lacking methionine but containing 100 mCi of [^{35}S]methionine (Amersham) per ml and then chased for 12 h in nonradioactive complete medium. At the end of the labeling period, cells were washed in PBS and lysed in reticulocyte standard buffer (0.01 M Tris-HCl [pH 7.4], 0.01 M NaCl) containing 1% Triton X-100, 0.5% sodium deoxycholate, and 2 mg of iodoacetamide per ml. Nuclei were removed by centrifugation. Immunoprecipitation of NDV proteins was accomplished as previously described (16).

Fusion assays. Cos cells were transfected with wild-type or mutant F protein genes or cotransfected with the wild-type HN protein gene. At 24 and 48 h posttransfection, the nuclei in 40 fusion areas were counted to determine the average size of syncytia at each time point as previously described (26). Values obtained after transfection of the vector alone were subtracted.

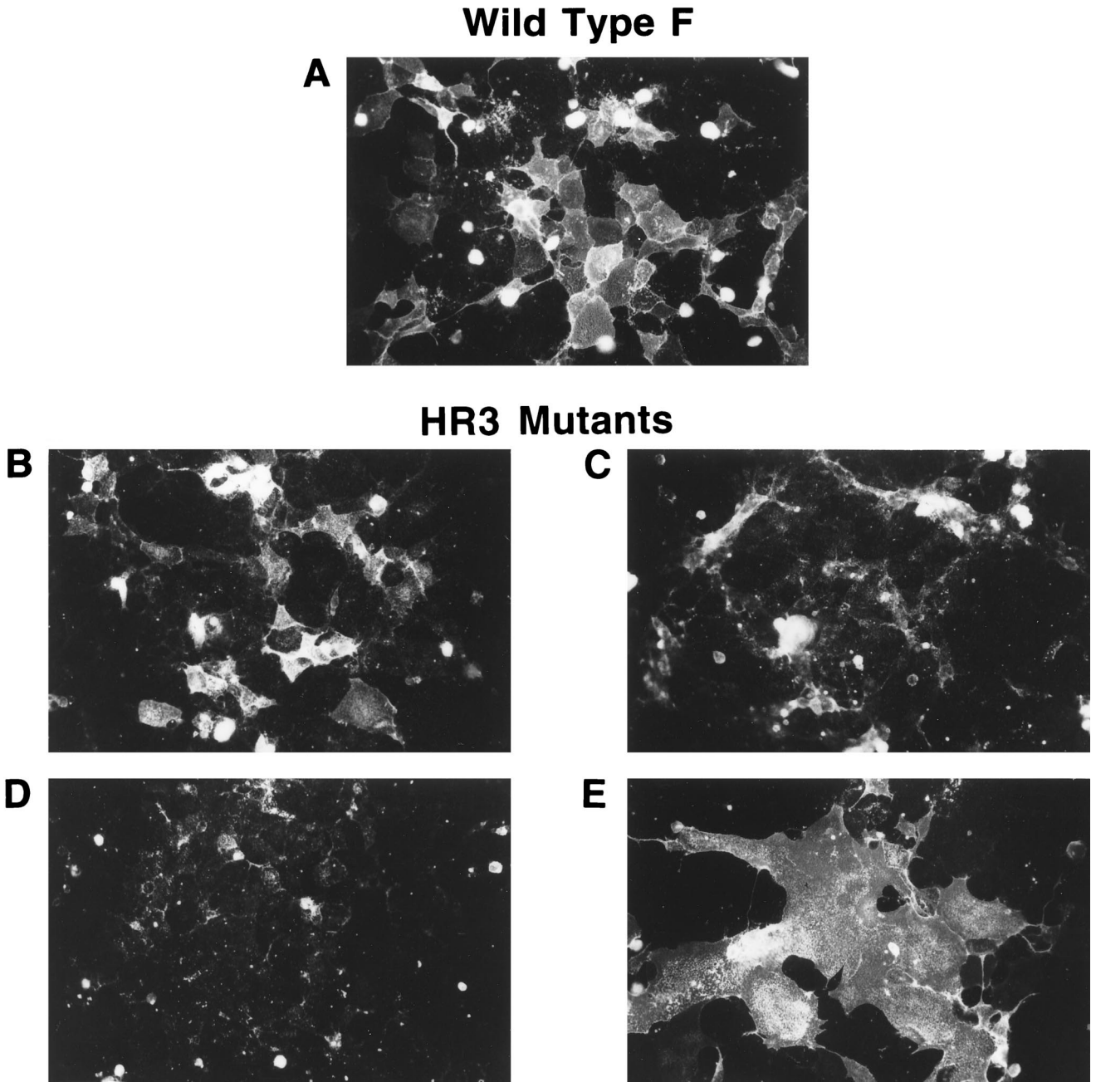

FIG. 4. Surface expression of mutant proteins detected by immunofluorescence. Intact confluent monolayers of Cos cells growing on coverslips and transfected with wild-type (A) or mutant F protein cDNAs were incubated with anti-NDV antisera and then with fluorescence-labeled goat anti-rabbit antisera as described in Materials and Methods. (B) L268A protein; (C) L275A protein; (D) L282A protein; (E) L289A protein. All fields shown were exposed for 2 s using a 25× objective.

RESULTS

Synthesis, stability, and processing of mutant proteins. A leucine repeat motif, first noted by Ghosh et al. (5) in the SV F protein sequence, is located between HR1 and HR2 at amino acids 268 to 296 (Fig. 1A) in the NDV F protein sequence. Because this sequence represents the third heptad repeat motif characterized in the NDV F protein sequence, it is labeled HR3 in Fig. 1. The amino acid sequence in this region of the F protein is shown in Fig. 1B, with the leucine residues in the repeat motif shown in bold. Each of these residues was changed to alanine individually to generate four mutants, L268A, L275A, L282A, and L289A.

To characterize the synthesis and processing of each mutant protein, F proteins were immunoprecipitated from radioactively labeled Cos-7 cells transfected with each mutant cDNA. The proteins present in the immunoprecipitate are shown in Fig. 2. In the absence of reducing agent (Fig. 2A), cleaved as well as the disulfide-linked cleaved fusion protein comigrated with an approximate molecular mass of 66 kDa. As previously reported, wild-type F protein preparations also contained small amounts of a higher-molecular-weight form (24, 27).

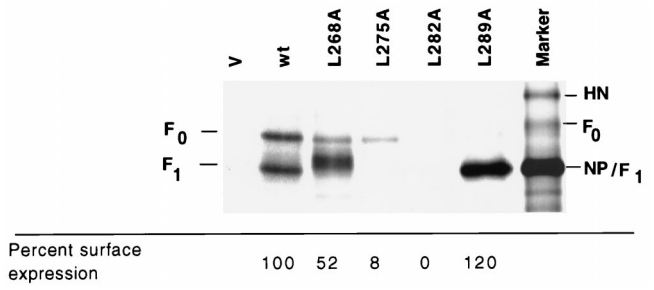

FIG. 5. Quantitation of surface expression of mutant proteins. Cells transfected with wild-type and mutant F protein DNAs for 30 h were labeled with [^{35}S]methionine for 2 h and then incubated in complete medium for 12 h. Cells were incubated with anti-NDV antibody on ice as previously described, unbound antibody was removed, and immune complexes in cell lysates were electrophoresed in polyacrylamide gels. The resulting autoradiograph was scanned with a microdensitometer, and the amount of radioactively labeled protein present in each lane, relative to the wild-type (wt) level, which is set at 100%, is shown at the bottom. V, vector, F_0, uncleaved fusion protein; F_1, cleaved form of the fusion protein; Marker, infected cell lysate; NP, nucleocapsid protein.

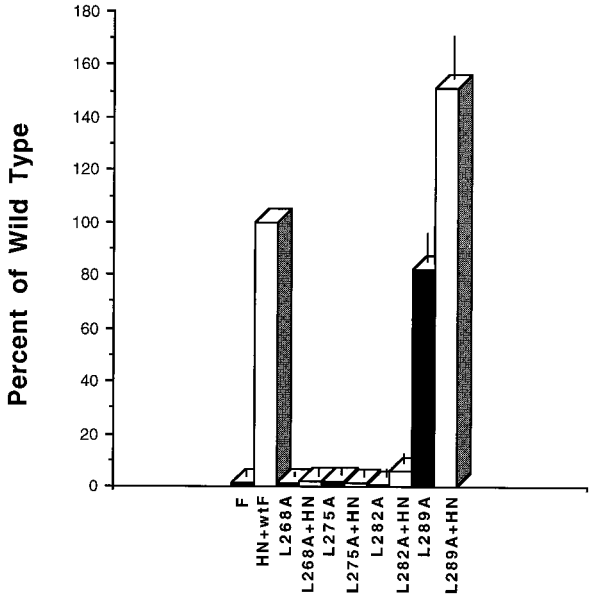

FIG. 6. Fusion activity of mutant proteins in the presence and absence of HN protein expression. Sizes of syncytia formed in monolayers of Cos cells transfected with wild-type (wtF) or mutant F protein cDNAs in the presence or absence of HN protein cDNA were determined as described in Materials and Methods. The results are the average of three different experiments.

Expression of L289A mutant DNA resulted in both monomer and oligomer forms of the protein which comigrated with the wild-type protein and which were expressed at levels comparable to wild-type levels. L268A mutant protein was also present in amounts roughly comparable to those of wild-type protein. However, both the monomer and oligomeric forms of the protein migrated slightly slower than the wild-type forms of the protein. Only small amounts of L275A and L282A mutant proteins entered the gel, although densitometer analysis showed that there was more radioactively labeled material at the top of the gel and in the stacking gel than in other lanes.

When the precipitated fusion proteins were electrophoresed in the presence of reducing agent, wild-type F_0 and the cleaved form, F_1, were resolved. L289A protein was clearly cleaved at least as efficiently as wild-type F protein. L268A protein was also cleaved. Furthermore, the L268A F_1 migrated slightly slower than wild-type F_1, while the uncleaved form of the protein comigrated with wild-type F_0. This result suggested that either the conformation, cleavage, or, more likely, the oligosaccharide processing of the F_1 protein was abnormal. Interestingly, in contrast to results shown in Fig. 2A, under reducing conditions, the monomeric forms of L275A and L282A F_0 proteins were resolved but no F_1 was seen, suggesting that these proteins were not cleaved. Furthermore, these results suggested that these two mutant proteins form large sodium dodecyl sulfate (SDS)-resistant aggregates which did not enter the gel in the absence of reducing agent.

To assess the relative steady-state levels of these proteins in transfected cells and to characterize SDS-resistant species formed by these mutant proteins, the proteins were detected by Western analysis (Fig. 3). Because NDV F protein is not well detected by Western analysis after boiling of the protein (27), samples were incubated in sample buffer at 50°C. In the absence or presence of reducing agent, material reactive to the F antibody could be detected at levels comparable to wild-type levels, suggesting that these proteins were relatively stable in transfected cells. However, the forms of the protein seen in the absence of boiling were quite different from wild-type forms, suggesting conformational differences in the mutant proteins. In the absence of reducing agent, all mutant proteins formed large complexes that electrophoresed at the top of the gel or in the stacking buffer. In the presence of reducing agent, a fraction of L268A, L275A, and L282A mutant proteins resolved as monomeric forms, although only monomeric F_0 was resolved for the three mutants, a result again suggesting that these mutant proteins were not efficiently cleaved to F_1 and F_2. However, the F289A mutant protein contained very little monomeric material. Thus, while F289A mutant protein appeared to be cleaved as shown in Fig. 2, it may exist in conformational forms somewhat different from wild-type forms.

Surface expression of mutant proteins in the absence of HN protein. Immunofluorescence detection of F protein on the surfaces of intact cells transfected mutant F protein genes was used to determine if the mutant proteins were expressed at cell surfaces. Shown in Fig. 4A, is the fluorescence observed on cells expressing wild-type F protein. The mutant L268A was detected at the surface, although the signal was much less intense than for wild-type protein (Fig. 4B). L275A (Fig. 4C) was minimally detected at the cell surface, while L282A was undetectable (Fig. 4D). Significant levels of the L289A mutant protein were detected at the surfaces of transfected cells (Fig. 4E). Surprisingly, these L289A-expressing cells formed syncytia. Furthermore, the vast majority of L289A-expressing cells were in syncytia.

Surface expression of these mutants was quantitated by surface immunoprecipitation as previously described (16, 27). Cells transfected with wild-type and mutant proteins were subjected to a radioactive label followed by a 12-h chase to minimize differences due to different kinetics of intracellular transport. These cells were incubated with anti-NDV antibody, and complexes formed were resolved on polyacrylamide gels as shown in Fig. 5. Interestingly, L268A and L289A proteins detected at the surface were cleaved much more efficiently than wild-type protein. The small amount of L275A protein detected was uncleaved, consistent with results shown in Fig. 2 and 3.

Fusion activity of mutant proteins in the presence and absence of HN protein expression. Syncytium formation after L289A transfection shown in Fig. 4 suggested that this mutant protein could direct fusion in the absence of HN protein ex-

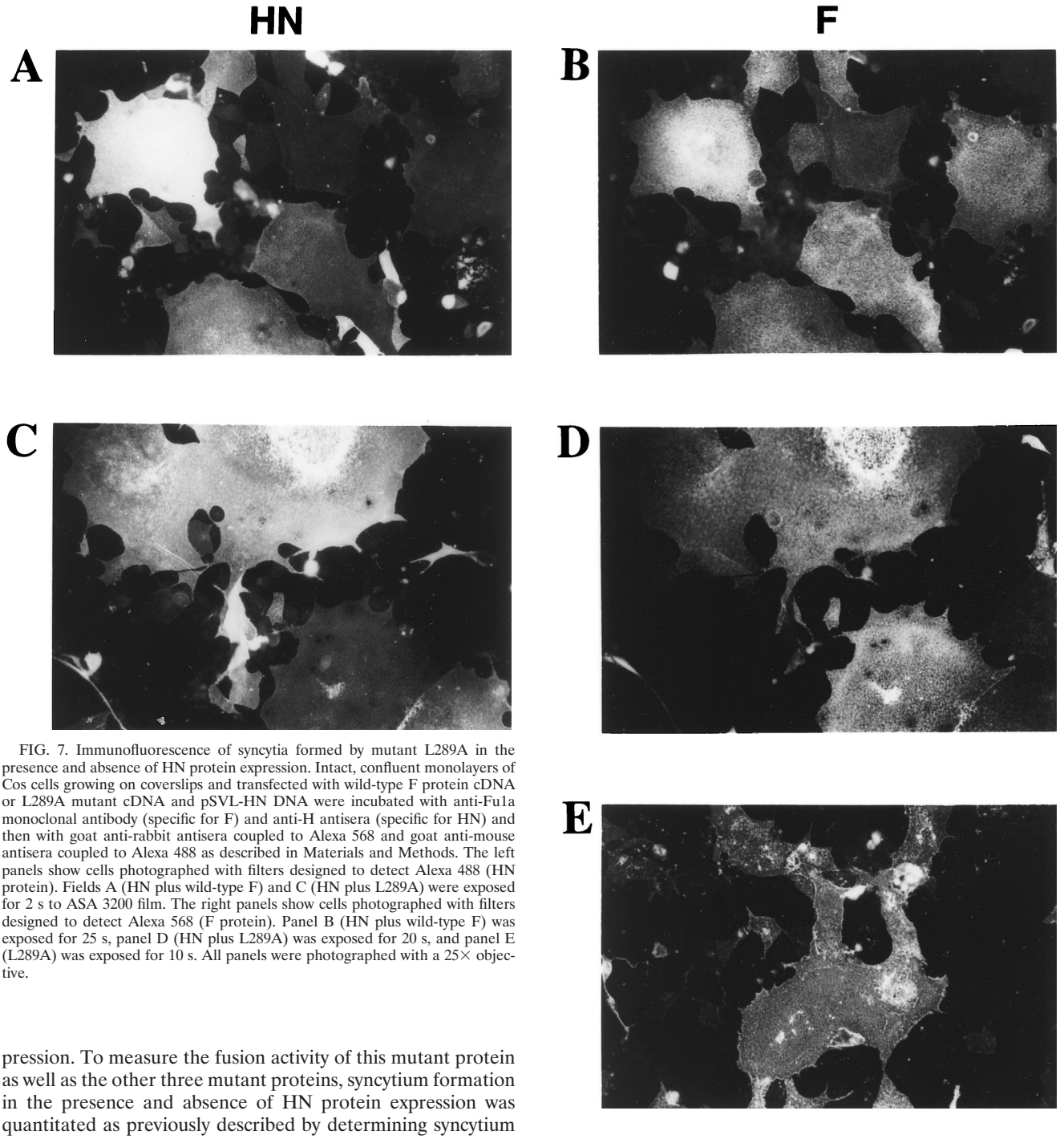

FIG. 7. Immunofluorescence of syncytia formed by mutant L289A in the presence and absence of HN protein expression. Intact, confluent monolayers of Cos cells growing on coverslips and transfected with wild-type F protein cDNA or L289A mutant cDNA and pSVL-HN DNA were incubated with anti-Fu1a monoclonal antibody (specific for F) and anti-H antisera (specific for HN) and then with goat anti-rabbit antisera coupled to Alexa 568 and goat anti-mouse antisera coupled to Alexa 488 as described in Materials and Methods. The left panels show cells photographed with filters designed to detect Alexa 488 (HN protein). Fields A (HN plus wild-type F) and C (HN plus L289A) were exposed for 2 s to ASA 3200 film. The right panels show cells photographed with filters designed to detect Alexa 568 (F protein). Panel B (HN plus wild-type F) was exposed for 25 s, panel D (HN plus L289A) was exposed for 20 s, and panel E (L289A) was exposed for 10 s. All panels were photographed with a 25× objective.

pression. To measure the fusion activity of this mutant protein as well as the other three mutant proteins, syncytium formation in the presence and absence of HN protein expression was quantitated as previously described by determining syncytium size (Fig. 6). Mutants L268A, L275A, and L282A had no fusion activity in the presence or absence of HN protein expression. However, L289A mutant protein in the absence of HN protein clearly directed syncytium formation nearly as well as the wild-type F protein in the presence of HN protein. Interestingly, the expression of HN protein enhanced fusion directed by L289A protein, significantly increasing syncytium size over that observed with wild-type F protein and HN protein expression.

These results were visualized and confirmed by immunofluorescence detection of syncytia formed in the presence and absence of HN protein expression (Fig. 7). Monolayers of cells expressing L289A only (Fig. 7E) contained syncytia which were somewhat smaller than those found in monolayers of cells expressing both wild-type F and HN proteins (Fig. 7A and B). Coexpression of both L289A and wild-type HN proteins (Fig. 7C and D) resulted in syncytia which were considerably larger than those seen after coexpression of wild-type F and HN.

DISCUSSION

The report that a peptide, consisting of amino acids 269 to 307 in the SV F protein, inhibited fusion as measured by

hemolysis implicated this region of the SV F protein in the fusion activity of the protein (5). This SV F protein sequence contains a leucine repeat motif in which a leucine residue is present every seven residues for a span of 28 amino acids. Such heptad repeat sequences are often found in α-helical structures that participate in intermolecular or intramolecular coiled-coil interactions. Analysis of this sequence using several different secondary structure prediction programs predicted only short stretches of α helix within this region of the SV F protein. However, this repeated leucine motif is conserved in comparable regions in several paramyxovirus F protein sequences (5), suggesting that this region may have some importance in the structure or function of these proteins. To analyze the role of this region in the structure and function of the NDV F protein, single-point mutations were introduced into the repeated leucine residues. Interestingly, mutations in this region affected either folding of the protein or the fusion activity of the protein.

Mutation of the two middle leucine residues, at positions 274 and 281, affected the folding of the molecule, and expression of these two mutant proteins resulted in little to no detection of the molecules at the cell surface. Mutation of the first leucine in the motif, at amino acid 268, also resulted in a conformationally abnormal protein that was nevertheless proteolytically cleaved and expressed at the cell surface, although at a slightly lower level than the wild-type protein. This mutant protein had no fusion activity in the presence of the HN protein, a result that suggests that the HR3 region may be involved in fusion activity in some way. However, the properties of the protein mutated in the last leucine in this motif, at amino acid 289, indicate even more clearly that this region is important in fusion.

In most paramyxovirus systems, expression of the HN protein as well as F protein is strictly required for fusion (13). Indeed, wild-type NDV F protein shows no fusion activity in the absence of the HN protein even at low frequency (26). There are, however, two exceptions to this observation. The SV5 F protein can direct fusion in the absence of HN protein expression although the HN protein enhances the fusion activity of the F protein (1, 8). Similarly, several reports indicate that the RSV F protein can mediate fusion independent of expression of the attachment protein, G (12, 22). Since the structural determinants of the paramyxovirus F protein are quite highly conserved across the family (17), it is not clear why the SV5 and RSV F proteins have requirements for fusion different from those of other paramyxovirus F proteins. However, the report of Ito et al. (10) that a single amino acid difference in the F_2 polypeptide of SV5 can account for different requirements for HN protein of different strains of SV5 may indicate that conformational alterations affect the HN protein requirement. Our results show that a single amino acid change in the NDV F_1 polypeptide results in an F protein, F-L289A, which mediates fusion in the absence of HN protein expression. As in the SV5 system (1), coexpression of the HN protein significantly enhances the fusion activity of F-L289A.

As noted by Ghosh et al. (5), the repeated leucine motif, HR3, is conserved in several paramyxovirus F proteins. It was therefore of interest to compare the HR3 sequences of SV5 and RSV F proteins with other paramyxovirus F proteins to determine if variation in this region could account for the different requirements for the HN protein. Figure 8 shows a comparison of the primary amino acid sequence of the HR3 regions from a number paramyxovirus F proteins. As noted by Ghosh et al. (5), the leucine repeat motif is found in F proteins of the paramyxoviruses SV, parainfluenza virus 1 (PIV1), and PIV3, as well as NDV. However, the sequences of the SV5 and RSV F proteins do not have a leucine repeat motif in this region.

Paramyxoviruses

275	DVD**L**ERYMVT**L**SVKIPI**L**SEVPGV**L**IHKASS**I**SY	**SV**
268	DVD**L**NDYSIA**L**QVRLPL**L**TRLLNT**Q**IYRVDS**I**SY	**PIV3**
271	DVD**L**EKYMVT**L**LVKIPI**L**SEIPGV**L**IYRASS**I**SY	**PIV1**

Rubulaviruses

265	GSG**L**ITGNPI**L**YDSQTQ**L**LGIQVT**L**PSVGNL**N**NM	**NDV**
261	SVL**L**DEMQMI**V**KINIPT**I**VTQSNA**L**VIDFYS**I**SS	**MuV**
251	MAS**G**LIKGQI**I**AVDIPT**M**TLVLMV**Q**IPSISP**L**RQ	**PIV4**
265	SIS**P**MYMQML**I**QINVPT**F**IMQPGA**K**VIDLIA**I**AN	**PIV2**
261	GLD**L**TYMQMV**I**KIELPT**L**TVQPAT**Q**IIDLAT**I**SA	**SV5**

Morbilliviruses

274	HVD**I**ESYFIV**L**SIAYPT**L**SEIKGV**I**VHRLEG**V**SY	**MV**

Pneumoviruses

249	TYM**L**TNSELL**S**LINDMP**I**TNDQKK**L**MSNNVQ**I**VRQ	**RSV**
263	DMP**I**TNDQKK**L**MSNNVQ**I**VRQSYS**I**MSIIKE**E**VLA	

FIG. 8. Comparisons of sequences of HR3 regions in F proteins of different paramyxoviruses. The leucine residues or hydrophobic residues in the heptad repeat motif are in bold type. Amino acid positions are indicated by numbers at the left. Two different sequences are shown for RSV. MuV, mumps virus; MV, measles virus.

The absence of a leucine repeat in the HR3 region of a paramyxovirus F protein does not, however, indicate that the F protein can mediate fusion in the absence of HN protein. As shown in Fig. 8, the PIV4 and PIV2 F proteins do not have this motif. In addition, the measles virus and mumps virus F protein sequences have only two heptadic leucine residues, although these sequences contain heptad repeats of hydrophobic residues (L, V, I, or V) which extend for 35 amino acids. All of these F proteins are reported to require the presence of HN for fusion (9, 21, 28, 29). Thus, there is not an absolute correlation between the presence of a leucine repeat motif and the requirement for the attachment protein in fusion. Perhaps it is the conformation of the domain or the interactions of this domain with other protein domains rather than the leucine repeat motif per se that is important for the HN protein requirement for fusion. Additional mutational analysis of this region in several F proteins may clarify the properties of this domain.

The results reported here as well as the properties of the SV5 and RSV F proteins raise the question of the role of attachment in membrane fusion (20). Based on the finding that syncytium formation is inhibited after neuraminidase treatment of cells, which destroys the receptor for HN protein, it has been argued that HN protein attachment is required for membrane fusion in cells coexpressing the HN and F proteins. However, syncytium formation by F proteins or mutant F proteins in the absence of attachment protein clearly shows that attachment mediated by the HN protein is not required in all circumstances. Perhaps F proteins also have an attachment activity that can function in these situations.

In summary, we report that a single amino acid change in the NDV F protein sequence can alter the requirement for the HN protein expression in syncytium formation.

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