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Mutational Analysis of the Leucine Zipper Motif in the Newcastle Disease Virus Fusion Protein

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The paramyxovirus fusion proteins have a highly conserved leucine zipper motif immediately upstream from the transmembrane domain of the F_1 subunit (R. Buckland and F. Wild, Nature [London] 338:547, 1989). To determine the role of the conserved leucines in the oligomeric structure and biological activity of the Newcastle disease virus (NDV) fusion protein, the heptadic leucines at amino acids 481, 488, and 495 were changed individually and in combination to an alanine residue. While single amino acid changes had little effect on fusion, substitution of two or three leucine residues abolished the fusogenic activity of the protein, although cell surface expression of the mutants was higher than that of the wild-type protein. Substitution of all three leucine residues with alanine did not alter the size of the fusion protein oligomer as determined by sedimentation in sucrose gradients. Furthermore, deletion of the C-terminal 91 amino acids, including the leucine zipper motif and transmembrane domain, resulted in secretion of an oligomeric polypeptide. These results indicate that the conserved leucines are not necessary for oligomer formation but are required for the fusogenic ability of the protein. When the polar face of the potential alpha helix was altered by nonconservative changes of serine to alanine (position 473), glutamic acid to lysine or alanine (position 482), asparagine to lysine (position 485), or aspartic acid to alanine (position 489), the fusogenic ability of the protein was not significantly disrupted. In addition, a double mutant (E482A,D489A) which removed negative charges along one side of the helix had negligible effects on fusion activity.

Fusion between a viral membrane and a target cell membrane is an essential step in infection by paramyxoviruses such as Newcastle disease virus (NDV). The NDV membrane contains two types of glycoproteins which facilitate this process, the hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. The HN glycoprotein is responsible for attaching the virion to the target cell, whereas the fusion glycoprotein is believed to disrupt the target cell membrane and induce membrane fusion (reviewed reference 25). The fusion protein monomer is synthesized as a precursor designated F_0 and is cleaved during transport through the Golgi membranes into two disulfide-linked subunits, F_1 and F_2 (reviewed in reference 30). This processing exposes the hydrophobic domain at the amino terminus of the F_1 subunit and is essential for the biological activity of the mature protein (39, 40). This hydrophobic domain, called the fusion peptide, is highly conserved among paramyxovirus F proteins and is considered to be directly involved in mediating membrane fusion (25).

Paramyxovirus fusion proteins have several common structural features, including two regions which contain heptad repeats and have the potential to form alpha helices (25). The longest of the two repeats, heptad repeat A, lies adjacent to the hydrophobic fusion peptide at the amino terminus of F_1, while heptad repeat B lies immediately upstream of the transmembrane region. Heptad repeat B consists of a series of highly conserved leucine or isoleucine residues every seven residues and was first recognized by Buckland and Wild (6). When the amino acid sequence of the NDV heptad repeat B is displayed on an ideal alpha-helical wheel, the conserved leucine or isoleucine residues line up on one face of the helix for six helical turns while the charged residues line up on opposing sides of the helix. This type of leucine repeat within an alpha helix has been identified in numerous DNA-binding proteins and has been termed the leucine zipper motif (24, 26). Although this domain is expected to be distant from the site of fusion on a target cell, previous reports suggested that the zipper motif of the measles virus fusion protein is necessary for syncytium formation (5). Also, as previously suggested, heptad repeat B has properties similar to those of other proteins known to form coiled-coil structures (37). Thus, this motif may maintain an oligomeric form of the protein that may be necessary for membrane fusion.

To define the requirements for the leucine residues in this motif for the structure and biological activity of the NDV fusion protein, three of the highly conserved leucine residues were each replaced with an alanine. Although single leucine-to-alanine substitutions did not significantly affect the fusion activity, substitution of two or three leucines abolished the ability to cause syncytium formation. In contrast, nonconservative substitutions on polar sides of the potential helix either singly or in combination did not significantly affect the fusion activity. Results are also presented which show that the leucine zipper motif as well as the entire cytoplasmic and transmembrane domain are unnecessary for oligomer formation as detected on sucrose gradients.

MATERIALS AND METHODS

Cells and virus. Cos-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco modified Eagle medium supplemented with 5% complement-inactivated fetal calf serum, 4.5 g of glucose per ml, nonessential amino acids, vitamins, 2 mM glutamine, 0.17% $NaHCO_3$, and penicillin-streptomycin. Virus was NDV (strain AV) grown in eggs and purified by standard protocols.

Antibodies. Anti-Fu1a is a monoclonal antibody that was generated against a disrupted virion preparation and was a generous gift of Mark Peeples. This antibody has been shown to react with mature F protein but not with the nascent

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molecule (33). Anti-NDV antibody was raised in rabbits against UV-inactivated NDV (strain AV) virions (43); anti-F antibody was raised against a peptide consisting of the 29 carboxy-terminal residues of the protein (47) and was also a gift from Mark Peeples.

Site-directed mutagenesis. Positive-sense oligonucleotides were synthesized by the DNA Facility of University of Massachusetts Medical Center or DNA International. The oligonucleotides used for mutagenesis (and sequences starting with the 5′ nucleotide) were L3A (GATAAGGCAGAGGAAAG), L4A (CAGCAA AGCAGACAAAGTC), L5A (CAATGTCAAAGCGAACAG), dC49 (CATTA CCTAGATCGCTTT), S473A (CAACAACGCGATAAGTAATGC), N485K (GGAAAGCAACAGCAAAC), E482K (GGATAAGTTAAAGGAAAGC), D489A (CAAACTAGCCAAAGTCAATG), and E482A (GTTAGCGGAAAG-CAACAGC). Double and triple mutants were made by sequential mutagenesis. Oligonucleotide-directed in vitro mutagenesis of M13mp18 DNA was achieved by using methods and reagents from Amersham Co. The resulting phage DNAs were screened for the desired mutation by sequencing. A 564-bp *Ava*I-*Sac*I restriction fragment containing the desired mutation was ligated into the remainder of the wild-type F gene, and the entire fragment was sequenced. The final cDNAs were shuttled between M13, pSVL (Pharmacia), and pSP6 (Pharmacia) via the *Xba*I and *Sac*I sites positioned in the cloning cassette at the 5′ and 3′ ends of the F gene, respectively.

Plasmid constructions. Plasmid pSP6dC91 (without a leucine zipper motif) was constructed by deleting an 182-bp fragment between the *Eco*RV and *Sph*I restriction sites found at nucleotides 1438 and 1620, respectively. Plasmid SP6Fst (31) was digested with *Sph*I, and the site was blunted with T4 DNA polymerase and further digested with *Bgl*I. Ligation of the 1,478-nucleotide *Sph*I-*Bgl*I fragment to the 3,017-nucleotide pSP6Fst *Bgl*I-*Eco*RV fragment resulted in a frameshift and a premature translation termination. This deletion substituted 91 codons from the carboxy terminus of the parental plasmid, including the leucine zipper, the transmembrane domain, and the cytoplasmic domain, with a new sequence (following the *Eco*RV site) coding for amino acids LPNCQAKGAT KDLVMAWE. This cDNA insert was then subcloned into pSVL.

Transient gene expression. Two systems were used to express the cDNAs. DEAE-dextran transfection was performed by a modification of the method of Levesque et al. (27). Cos cells were plated on 35-mm-diameter plates (Falcon Primaria) at 2.5×10^5 cells 20 h prior to transfection. Two micrograms of DNA was mixed with 0.5 ml of a solution of DEAE-dextran (Pharmacia) suspended in Tris-buffered saline (TBS; 25 mM Tris base, 137 mM NaCl, 5.1 mM KCl, 0.7 mM Na_2HPO_4) at 0.5 mg/ml. The DNA was allowed to adsorb to the cells at room temperature for 20 min and then incubated at 37°C for 1 h. The DNA-dextran mix was replaced with 1 ml of 100 μM chloroquine in OptiMem (Gibco), and the cells were incubated at 37°C for an additional 5 to 6 h. The chloroquine was removed, and 2 ml of Cos medium was added. At 48 h posttransfection, the cells were washed in TBS and cells were lysed in 0.4 ml of lysis buffer (1% Triton X-100, 2 mg of iodoacetamide per ml, and 1 mM phenylmethylsulfonyl fluoride in reticulocyte standard buffer [RSB; 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris {pH 7.4}]). Plates were scraped with a rubber policeman; the extract was pulled through a 21-gauge needle five times and centrifuged for 30 s to remove nuclei. Extracts were stored at 4°C. For Lipofectin transfection, Cos cells were plated at 2×10^5 20 h prior to transfection. Ten microliters of Lipofectin (Gibco) in 200 μl of Opti-MEM (Gibco) was incubated with the DNA for 15 min, and then 0.3 ml of Opti-MEM was added. For fusion assays, optimal fusion required that 3 μg of F DNA and 1.5 μg of HN DNA be used. For pSVL alone, 4.5 μg of DNA was transfected. The plates were washed twice with TBS, and the DNA-Lipofectin mixture was applied. The plates were incubated for 6 h, and the DNA mixture was replaced with 2 ml of Cos medium.

Fusion assay. Following Lipofectin transfections, at 24, 48, and 72 h, the plates were systematically scanned for areas of fusion. In each field of view, all syncytia with three or more nuclei were counted, for a total of 20 syncytia. Two individuals scored the plates, and the numbers were averaged. Cos cells show background fusion at low frequency with an average of three to four nuclei per syncytia. This background value was also obtained from cells transfected with only the parental vector and was subtracted from each assay to obtain an index value. Each mutant protein was assayed three times, and the resulting values were averaged.

Radiolabeling. Labeling with [^{35}S]methionine (100 μCi/ml, 1,450 Ci/mmol; Amersham) was accomplished in methionine-free minimal essential medium supplemented with nonessential amino acids and 10% dialyzed fetal calf serum. For pulse-chase experiments, labeling was for the times indicated in figure legends and was followed by the addition of complete Cos medium.

Immunoprecipitations. Cell lysates were incubated with a 1:500 dilution of antibody for 1 h at room temperature. The immune complexes were precipitated with Immunobeads (Bio-Rad) to which goat anti-rabbit or anti-mouse immunoglobulin antibody was covalently coupled. Samples were resuspended in 80 μl of sample buffer and divided into two equal portions. The samples were denatured (with and without 1 μl of β-mercaptoethanol), 1 mg of iodoacetimide was added, the beads were pelleted, and the supernatants were loaded onto the gel.

Detection of cell surface molecules. Surface expression was quantitated by antibody binding as previously documented (32, 34, 44). After a pulse-label and a nonradioactive chase, monolayers were washed in ice-cold phosphate-buffered saline (PBS; 0.15 M NaCl, 7.7 mM K_2HPO_4, 2.4 mM KH_2PO_4) and incubated on ice with a 0.5 ml of a 1:50 dilution of anti-NDV antiserum (heat inactivated at 56°C for 30 min) in PBZ (1% bovine serum albumin and 0.02% sodium azide in PBS). After 60 min, the unbound antiserum was removed by extensive washing in ice-cold PBS. The cells were lysed in 0.5 ml of lysis buffer containing 1% Triton-X 100 and 0.5% sodium deoxycholate. The lysate was pulled through a 21-gauge needle several times, and the nuclei were removed from the lysate by a 30-s centrifugation. The immune complexes were precipitated with Immunobeads or *Staphylococcus aureus* cells as previously described (32, 44).

Sucrose gradients. To examine the oligomeric structure of the glycoproteins, sucrose gradients were used. A linear gradient of 25 to 10% (wt/vol) sucrose dissolved in RSB and 0.1% Triton X-100 was created by using a gradient maker to mix 5 ml of each solution into a polyallomer centrifuge tube (14 by 89 mm). A 200-μl cushion of 2 M sucrose was positioned at the bottom of the tube. Each 35-mm-diameter plate was lysed in 0.4 ml of RSB–1% Triton–10 mM iodoacetamide–1 mM phenylmethylsulfonyl fluoride. The lysate was homogenized by being pulled through a 21-gauge needle several times. Nuclei were removed by centrifugation. A total of 0.6 ml (from 1.5 plates) was carefully layered over the gradient. The lysates were fractionated by centrifugation at 38,000 rpm and 17°C for 18 h in an SW41 rotor. Fractions of 0.5 ml were collected starting from the top of the gradient. Fractions were numbered beginning with fraction 1 at the bottom and fraction 21 at the top. The pelleted material was dissolved in 90 μl of sodium dodecyl sulfate (SDS)-Tris sample buffer (6.25% SDS, 0.5 M Tris [pH 8]), and 30 μl was loaded on the gels. Fractions were trichloroacetic acid precipitated, and the pellets were dissolved by incubation in 30 μl of SDS-Tris sample buffer at 50°C for 15 min. Prior to loading, 15 μl of reduction mix (50% glycerol, 0.01% bromophenol blue) and 1 μl of β-mercaptoethanol were added, the samples were heated at 85°C for 2 min, and 0.2 mg of iodoactamide was added. A marker of 0.12 μl of NDV virions was loaded in the first lanes. In most cases, fractions 15, 17, and 19 were omitted, as any molecules that may have sedimented in those fractions were expected to be too small to be resolved. To calibrate the gradients, molecular weight protein markers were similarly fractionated three times, and the resulting gels were stained with Coomassie blue stain. Proteins used as markers were catalase (240 kDa), aldolase (158 kDa), and bovine serum albumin (68 kDa).

Polyacrylamide gel electrophoresis. Electrophoresis on SDS–10% polyacrylamide slab gels was carried out as previously described (33). Following electrophoresis, the gels were subjected to Western blot (immunoblot) analysis or fixed, dried, and subjected to autoradiography, using Kodak X-Omat AR X-ray film. For gels containing immunoprecipitated material, the gels were treated with 2,5-diphenyloxazole.

Western analysis. Following electrophoresis, the proteins were subjected to Western analysis as previously described (28). The blots were probed with the rabbit anti-F polyclonal antibody (1:5,000) for 90 min at room temperature, washed, and treated with a 1:40,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Boehringer Mannheim Corp.) for 1 h. The blot was thoroughly washed, treated with the luminol-based enhanced chemiluminescence reagents (Amersham) for 1 min, drained, wrapped in plastic wrap, and immediately detected by being placed against film (Kodak X-AR). To obtain adequate signals, exposures were varied from 1 to 120 s.

RESULTS

Mutagenesis of heptadic leucines. The amino acid sequence of the NDV fusion protein in the region of the leucine zipper motif is shown in Fig. 1A. To investigate the structural and functional roles of the conserved leucines, the third, fourth, and fifth repetitive leucines (L3, L4, and L5, respectively) at residues 481, 488, and 495, respectively, were changed to alanine. Mutations were introduced singly and in several combinations as indicated in Fig. 1A. The locations of mutations on the predicted helix are shown in Fig. 1B. To allow an examination of the function of the leucines and not the potential helical structure, alanine was chosen for the substitutions.

Expression of mutants. To characterize the mutant proteins, the mutated fusion protein cDNAs were expressed in Cos cells by using a simian virus 40-based expression vector as described in Materials and Methods. At 48 h posttransfection, proteins in the postnuclear extracts were separated on a nonreducing gel and subjected to Western analysis. Western blots were probed with a rabbit antibody generated against 29 residues at the C terminus of the F protein (47). The extract from cells expressing the wild-type protein resulted primarily in one novel band (Fig. 2A, lane 2). Expression of all of the mutant cDNAs resulted in products that migrated similarly to wild-type protein, and expression was at amounts comparable to wild-type amounts. Curiously, the proteins that have the L3A mutation

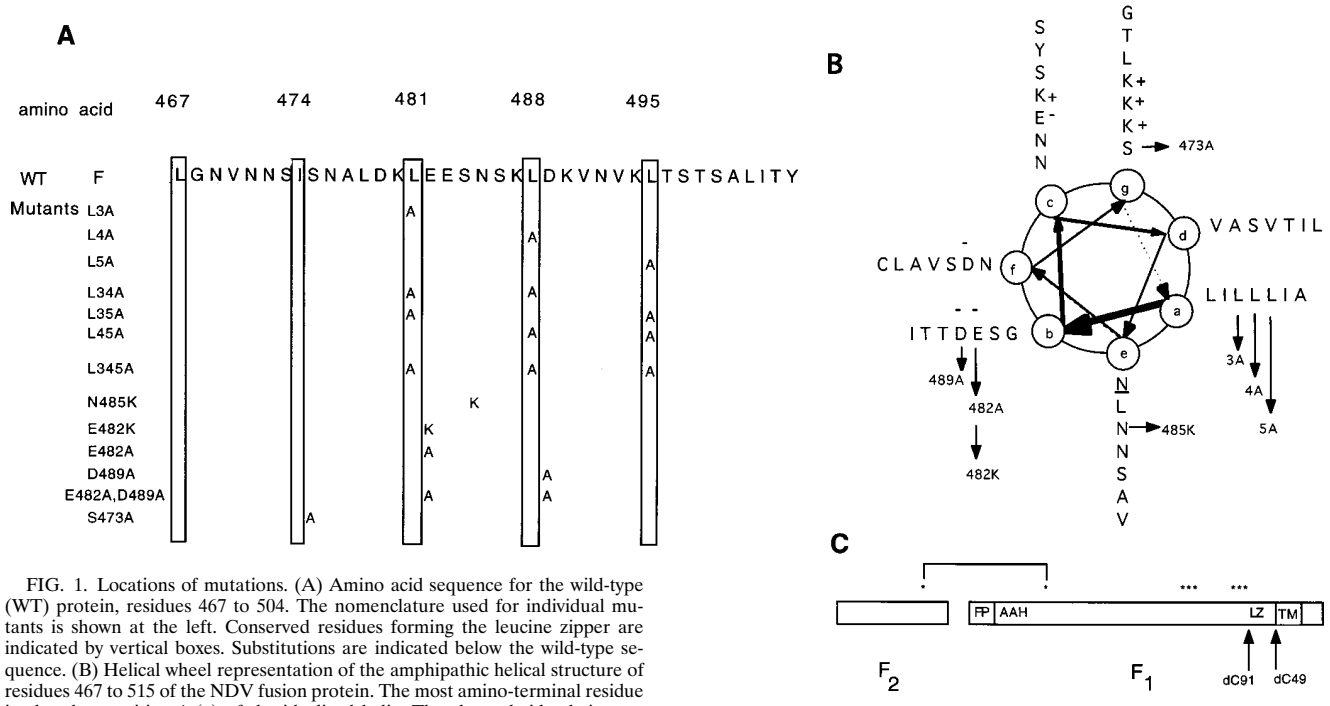

FIG. 1. Locations of mutations. (A) Amino acid sequence for the wild-type (WT) protein, residues 467 to 504. The nomenclature used for individual mutants is shown at the left. Conserved residues forming the leucine zipper are indicated by vertical boxes. Substitutions are indicated below the wild-type sequence. (B) Helical wheel representation of the amphipathic helical structure of residues 467 to 515 of the NDV fusion protein. The most amino-terminal residue is placed at position 1 (a) of the idealized helix. The charged side chains are indicated. Position of substitutions are marked by arrows. The glycosylated asparagine residue is underlined. (C) Structurally significant domains in the F_1 and F_2 subunits of the NDV fusion protein and the approximate location of the termination sites used to produce the dC49 and dC91 mutants. FP, fusion peptide; AAH, amphipathic alpha helix A; LZ, leucine zipper motif (heptad repeat B); TM, transmembrane domain; *, regions with cysteine residues; solid line indicates the likely disulfide linkage (23). Generation of the mutants is described in Materials and Methods.

ran slightly more slowly than the wild-type protein (lanes 3, 6, 7, and 9). This decreased mobility was also seen when the L3A mRNA was translated in a cell-free system in the absence of membranes and in the presence of a reducing agent, indicating it was not due to differential glycosylation or alteration in intramolecular disulfide bonds (not shown). In addition, all proteins resulted in various amounts of SDS-resistant oligomeric forms as previously reported (44).

Mutations in viral glycoproteins may result in proteins which are improperly folded. To examine the effect that these mutations may have on the folding of the molecules, the mutant proteins were radioactively labeled with [^{35}S]methionine in a 2-h pulse-label of transfected cells and immunoprecipitated with a monoclonal antibody, anti-Fu1A. This conformation-sensitive antibody has been shown to bind only mature fusion protein (33). Figure 2B shows that the antibody precipitated all of the mutant fusion proteins in approximately equivalent amounts.

Surface expression of wild-type and mutant proteins. For the fusion protein to be functional, it must be transported through the cell and properly expressed at the cell surface. To examine surface expression, we used an assay designed to quantitate surface expression of an antigen (32, 34, 44). The transfected cells were pulse-labeled with [^{35}S]methionine and chased with nonradioactive methionine to allow the labeled proteins to accumulate at the cell surface. The intact cells were incubated with a polyclonal antibody generated against virion proteins. The immune complexes were precipitated, and the cell surface proteins were electrophoresed under nonreducing conditions. The results from this assay (Fig. 3A) indicate that all leucine mutant fusion proteins are expressed at the cell surface at levels comparable to or higher than wild-type levels.

The autoradiogram from this experiment and one other comparable experiment were scanned with a microdensitom-

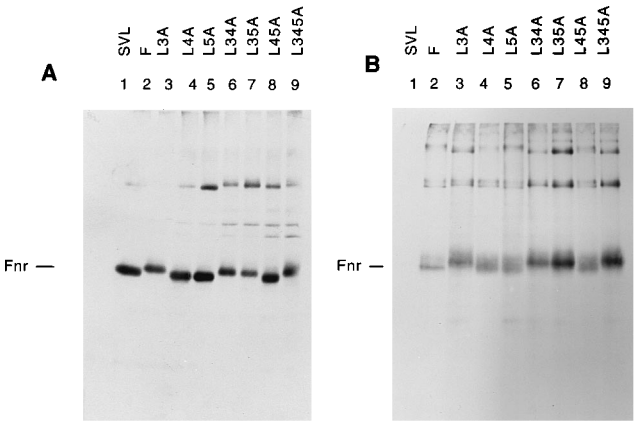

FIG. 2. Expression of leucine mutants. (A) Cells transfected with wild-type and mutant DNAs were lysed at 48 h posttransfection, and the postnuclear extracts were mixed with sample buffer, heated at 85°C for 2 min, electrophoresed under nonreducing conditions on a 10% polyacrylamide gel, and subjected to Western blot analysis as described in Materials and Methods. Blots were probed with an antibody raised against the C terminus of the NDV fusion protein. Lane 1, pSVL; lane 2, pSVL-F; lane 3, pSVL-L3A; lane 4, pSVL-L4A; lane 5, pSVL-L5A; lane 6, pSVL-L34A; lane 7, pSVL-L35A; lane 8, pSVL-L45A; lane 9, pSVL-L345A. (B) Metabolic labeling of F proteins expressed in Cos cells. At 48 h posttransfection, transfected cells were radioactively labeled for 2 h with [^{35}S]methionine, and proteins in the postnuclear extract were immunoprecipitated with an anti-F monoclonal antibody (Fu1a). The precipitated proteins were mixed with sample buffer and heated at 85°C for 2 min prior to loading of the gel. Lane 1, pSVL; lane 2, pSVL-F; lane 3, pSVL-L3A; lane 4, pSVL-L4A; lane 5, pSVL-L5A; lane 6, pSVL-L34A; lane 7, pSVL-L35A; lane 8, pSVL-L45A; lane 9, pSVL-L345A. Electrophoresis was in the absence of reducing agent. Fnr indicates the monomeric nonreduced form of the wild-type fusion protein.

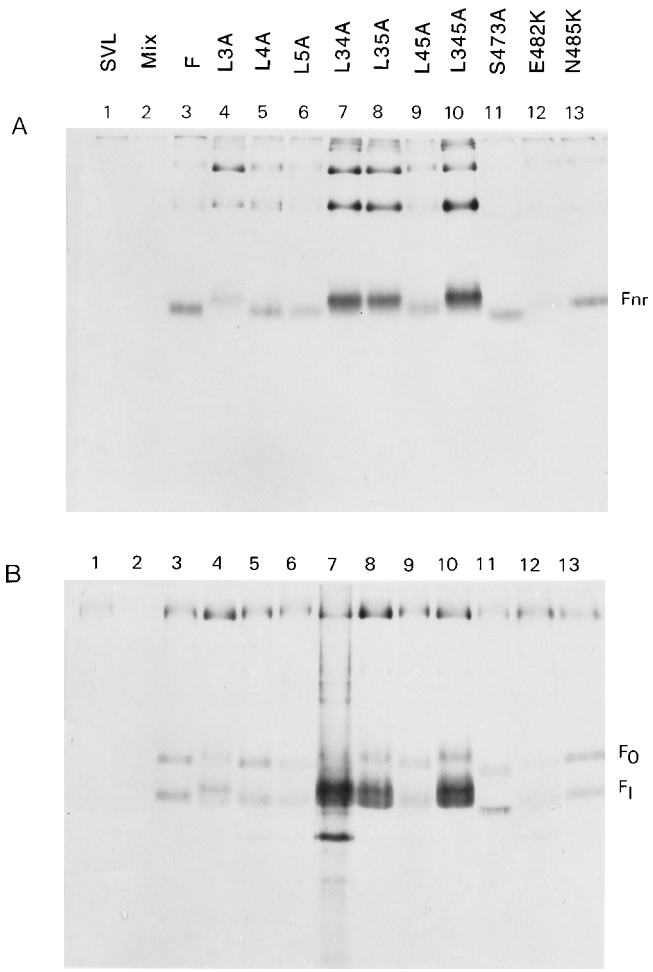

FIG. 3. Cell surface expression and precursor processing of mutant proteins. At 40 h posttransfection, the cells were radioactively labeled for 2 h and chased with nonradioactive methionine for 4 h. The cells were washed in ice-cold PBS and incubated with an anti-NDV polyclonal antibody as described in Materials and Methods. Immune complexes containing cell surface proteins were precipitated and electrophoresed in the absence of a reducing agent (A) or under reducing conditions (B). (A) Lane 1, pSVL; lane 3, pSVL-F; lane 4, pSVL-L3A; lane 5, pSVL-L4A; lane 6, pSVL-L5A; lane 7, pSVL-L34A; lane 8, pSVL-L35A; lane 9, pSVL-L45A; lane 10, pSVL-L345A; lane 11, pSVL-S473A; lane 12, pSVL-E482K; lane 13, pSVL-N485K. Lane 2 shows a mixing control for the experiment as described previously (32). Extracts from cells radioactively labeled but not incubated with antibody were mixed with extracts from unlabeled cells which had antibody bound to the cell surface. Immune complexes were isolated as described above. (B) Lanes are the same as for panel A; samples were electrophoresed in the presence of β-mercaptoethanol. Fnr, nonreduced wild-type form; F_0, precursor form; F_1, F_1 subunit.

eter to quantitate the amount of surface protein immunoprecipitated. The results shown in Table 1 indicate that the proteins with multiple substitutions or the L3A mutation are expressed at the cell surface at levels exceeding that of the wild-type protein.

Precursor processing. For the mutant fusion proteins to have biological activity, the precursor must become cleaved and the disulfide-linked F_1 and F_2 subunits must exist at the cell surface (39, 40). To evaluate the form of the proteins at the cell surface, the precipitated proteins shown in Fig. 3A were electrophoresed in the presence of a reducing agent. Under these conditions, the subunits should dissociate and the F_1 subunit should be detected. The results (Fig. 3B) indicate that all proteins become cleaved and result in the faster-migrating F_1 species. In all cases, the efficiency of cleavage was at least comparable to that of the wild type.

TABLE 1. Quantitative analysis of surface expression of mutant proteins

DNA	Cell surface expression (% of WT level)[a]	Fusion activity[b]	
		Size of syncytia (No. of nuclei)	% of WT level
Set A			
WT	100	24.6	100.0
L3A	277	19.7	79.9
L4A	84	16.5	67.1
L5A	93	14.2	57.8
L34A	315	2.2	8.7
L35A	571	0.4	1.7
L45A	310	2.0	8.0
L345A	446	0.6	2.6
N485K	63	16.9	68.8
E482K	36	18.1	73.7
S473A	91	9.9	68.8
Set B			
WT	100	21.0	100.0
E482A	90	16.8	80.0
D489A	92	17.1	81.4
E482A,D489A	100	23.7	112.8

[a] Average of two separate experiments. WT, wild type.
[b] Average of three experiments.

Fusion activity of proteins with leucine mutations. To measure fusion activity of mutant proteins, the fusion and HN protein cDNAs were cotransfected into subconfluent Cos cells (43). When the wild-type HN and F proteins are coexpressed, multinucleated cells or syncytia increase in size with time for over 72 h (Fig. 4). To quantitate this activity, the plates were scanned for syncytia, and the number of nuclei in 40 syncytia were counted at 24, 48, and 72 h posttransfection for each sample.

To define the requirements for the individual leucine residues in fusion, the activities of mutants L3A, L4A, and L5A were determined. When any of these mutant proteins were expressed alone, they had near-background levels of fusion, again demonstrating the requirement for the presence of the HN protein (not shown). As shown in Fig. 4A, when these mutant fusion proteins were coexpressed with HN protein, all showed fusion activity. Curiously, there appeared to be a gradient effect on the fusion activity with an increasing effect from the third to the fifth leucine. Although the fifth leucine is least conserved among the other paramyxovirus fusion proteins, substitution of this leucine resulted in the greatest inhibition of the syncytium-forming ability of any of the single substitutions. Although the three mutants showed a slight decrease in fusion activity, these results suggested that a single leucine substitution does not significantly alter the fusion ability of the protein.

To determine the effects of mutations in two or three leucine residues, the double and triple leucine mutants were cotransfected with HN protein cDNA. Results are shown in Fig. 4B. When two or more of the conserved leucines were changed, the ability of the protein to induce fusion was abolished. These results suggested that the leucines do not have an individual function and that the fusion activity of the protein depends on a repetitive leucine structure.

Mutations in other regions of the helix. The lack of fusion activity of proteins with multiple leucine substitutions indicated that this region of the fusion protein is required for its

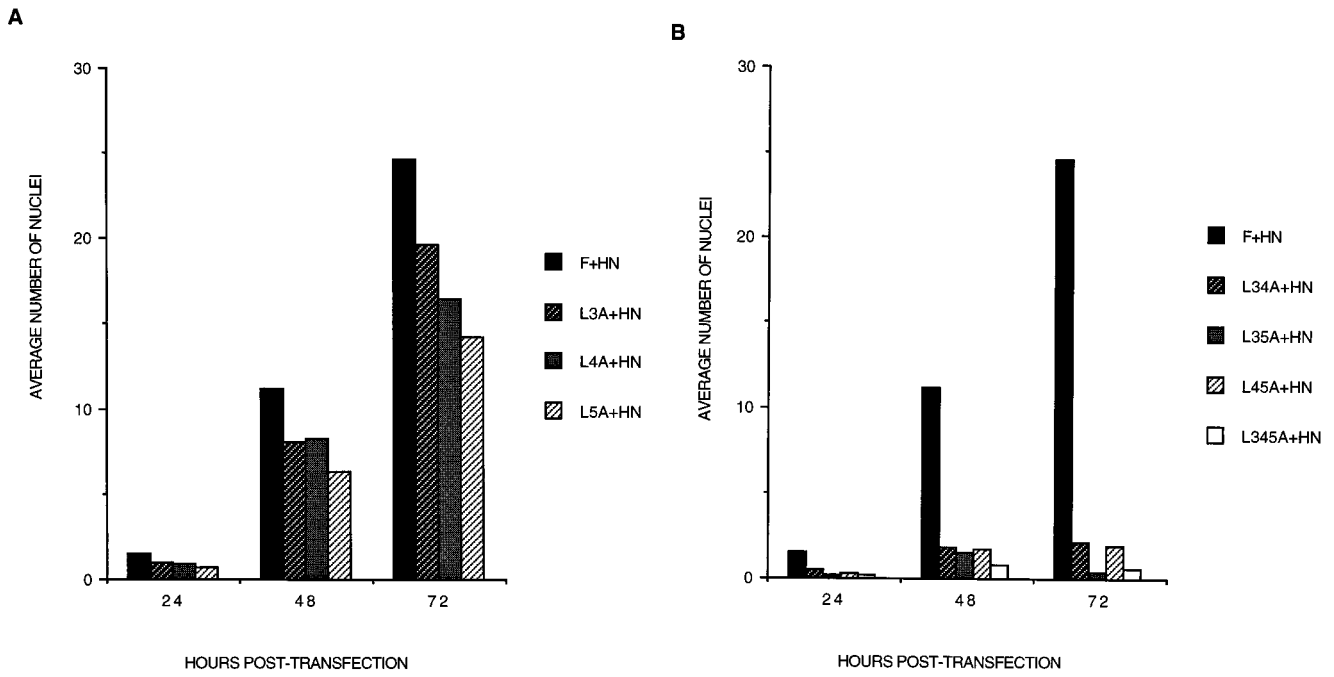

FIG. 4. Quantitation of fusion activity. The number of nuclei in 40 fusion areas was counted at 24, 48, and 72 h posttransfection to determine the average size of syncytia at each time point. The value obtained from pSVL alone has been subtracted. The results are averages from three separate experiments. (A) Fusion activity with single leucine-to-alanine mutations. Values shown were obtained from cotransfections of pSVL-HN and pSVL-F, pSVL-L3A, pSVL-L4A, or pSVL-L5A as indicated. (B) Fusion activity with multiple leucine mutations obtained from cotransfection pSVL-HN and pSVL-F, pSVL-L34A, L35A, L45A, or L345A.

biological activity. To assess further the sensitivity of this region to mutations, nonconservative substitutions (Fig. 1A) were introduced into the polar face of the potential helix. The asparagine at position 485, which falls between leucines 3 and 4, is predicted to be on a polar, noncharged face of the alpha helix as shown in Fig. 1B. This residue was changed to a basic residue, lysine, to create the N485K mutant. The predicted alpha helix has acidic residues in the b positions on the helix. To alter this region, a basic residue replaced the acidic glutamic acid at residue 482 to produce the E482K mutant. In addition, this amino acid was changed to an alanine to produce E482A. The other negatively charged amino acid on this face of the helix, an aspartic acid, was also changed to an alanine to produce D489A. Furthermore, since mutations of leucine residues exerted their effects on fusion only in combination, we made a double mutant which removed both negative charges on the b face of the helix to produce E482A,D489A.

An additional mutant was created to test the functional requirements of the partially conserved N-linked glycosylation consensus sequence that falls between the first and second heptadic leucine residues (Fig. 1A). This mutant, S473A, abolished the potential for asparagine-linked glycosylation at position 471 by replacing the polar serine residue with the nonpolar alanine residue.

The expression of these mutant genes was characterized by Western analysis or immunoprecipitation. The E482K and N485K proteins had an electrophoretic mobility similar to that of the wild-type protein (Fig. 5A, lanes 4 and 5), while the S473A protein had a slight increase in mobility compared with the wild-type protein, suggesting that the N-linked glycosylation site at residue 471 is normally used (lane 3). The three mutants were found to be present at the cell surface and proteolytically cleaved when the surface antigens were immunoprecipitated with a conformation-sensitive antibody as described above (Fig. 3, lanes 11 to 13). Quantitation of the surface expression, expressed as a percentage of the wild-type protein level, is shown in Table 1. Mutants D489A and E482A, as well as the double mutant E482A,D489A, were precipitated by a conformation-specific antibody (Fig. 5B), proteolytically cleaved (Fig. 5C), and expressed at the cell surface at levels comparable to wild-type levels (Fig. 5D and Table 1).

When the single mutants N485K, E482K, E482A, D489A, and S473A were subjected to the fusion assay, all had fusion activity as shown in Fig. 6. While levels were lower than wild-type levels, none of the mutations abolished fusion to the extent of the double or triple leucine mutations. Furthermore, the double mutant E482A,D489A had normal levels of fusion activity (Fig. 6B). These results, along with the finding that the fusion activity is abolished by multiple leucine substitutions, suggested that only the nonpolar side of the helix involving the conserved leucine residues is specifically required for the function of the protein. The biologically active structure of the protein appears to tolerate single amino acid substitutions along the hydrophobic face or alterations that disrupt the charge distribution or glycosylation on other sides of the helix.

Oligomer formation. The lack of fusion activity of proteins with multiple leucine mutations could not be accounted for by any obvious defect in processing, steady-state level, or cell surface expression. Since the leucine zipper motif has been implicated in the oligomerization of proteins (26), it follows that the leucine mutants might be defective in fusion activity because of failure to oligomerize or an instability of an oligomeric structure.

To examine the oligomeric structure of the wild-type fusion protein, sucrose gradients were used. Cos cells expressing the F protein were lysed in a nonionic detergent and fractionated on a 10 to 25% linear sucrose gradient. Molecular weight markers were used to calibrate the gradient and indicated that

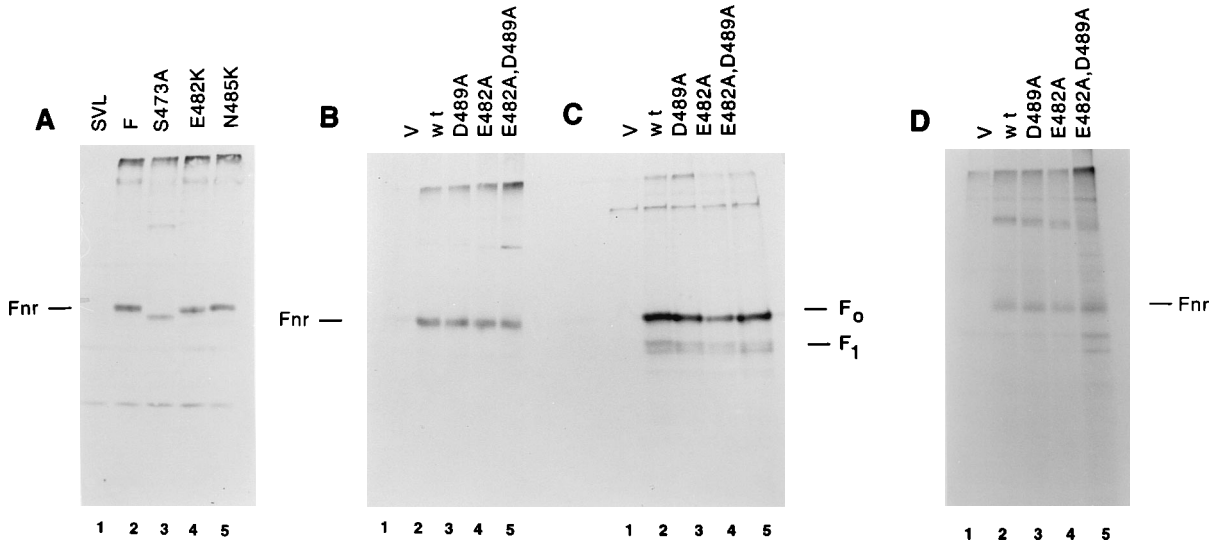

FIG. 5. Expression of fusion proteins with nonconservative substitutions. (A) Western analysis of mutant proteins. Proteins in transfected cells were prepared, electrophoresed, and detected as indicated in the legend to Fig. 2. Lane 1, pSVL; lane 2, pSVL-F; lane 3, pSVL-S473A; lane 4, pSVL-E482K; lane 5, pSVL-N485K. Fnr indicates the nonreduced monomeric form of the fusion protein. (B and C) Immunoprecipitation of radioactively labeled proteins precipitated as described in the legend to Fig. 2B. Samples were electrophoresed in the absence (B) or presence (C) of reducing agent. V, vector; wt, wild type. (D) Immunoprecipitation of cell surface protein prepared as described in the legend to Fig. 3.

an ideal monomer, dimer, trimer, and tetramer of F would sediment in fractions 15, 11, 7, and 2, respectively. However, the molecular weight positions were intended only to serve as a reference point for the oligomeric structure of the fusion protein, which sedimented in fractions 8 to 11 at approximately 190 kDa (Fig. 7A). Very little protein sedimented in the monomer region or toward the bottom of the gradient.

To examine the stability of this oligomer, a lysate of Cos cells expressing the F protein was supplemented with either 0.5% sodium deoxycholate or 5% SDS prior to centrifugation. Sodium deoxycholate had no effect on the sedimentation pattern of the protein (not shown), whereas a majority of the protein dissociated into a monomer and sedimented in fractions 14 to 16 in the presence of SDS (Fig. 7B). This result indicated that the mature F protein expressed in Cos cells forms a Triton X-100-insensitive oligomeric structure that can be disrupted by solubilization in SDS and, as expected, is not covalently associated.

To examine the oligomeric structure of the triple leucine mutant, cells expressing the L345A mutant protein were lysed and fractionated on a sucrose gradient. As shown in Fig. 7C, this protein formed a Triton X-100-insensitive structure that sedimented similarly to the wild-type protein. Again, very little protein sedimented in the monomer region or pellet. Thus, the conserved leucines are not required for the maintenance of the Triton X-100-insensitive oligomeric structure of the fusion

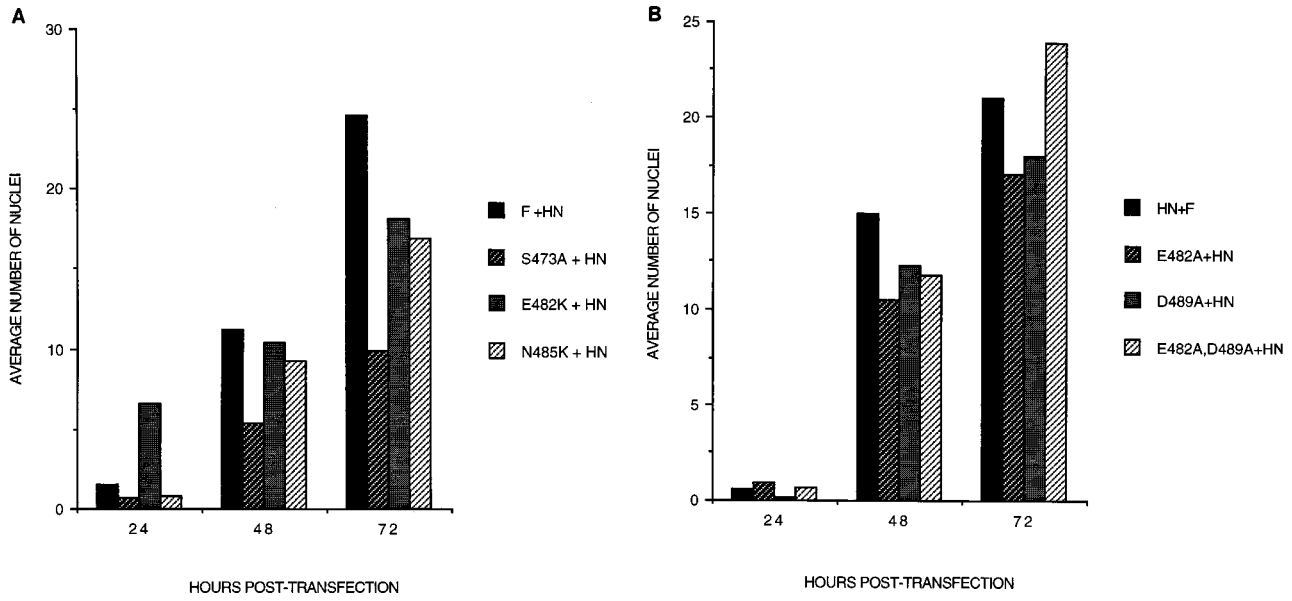

FIG. 6. Fusion activities of mutants with nonconservative changes. The fusion activity of each mutant was quantitated as described in the legend to Fig. 4.

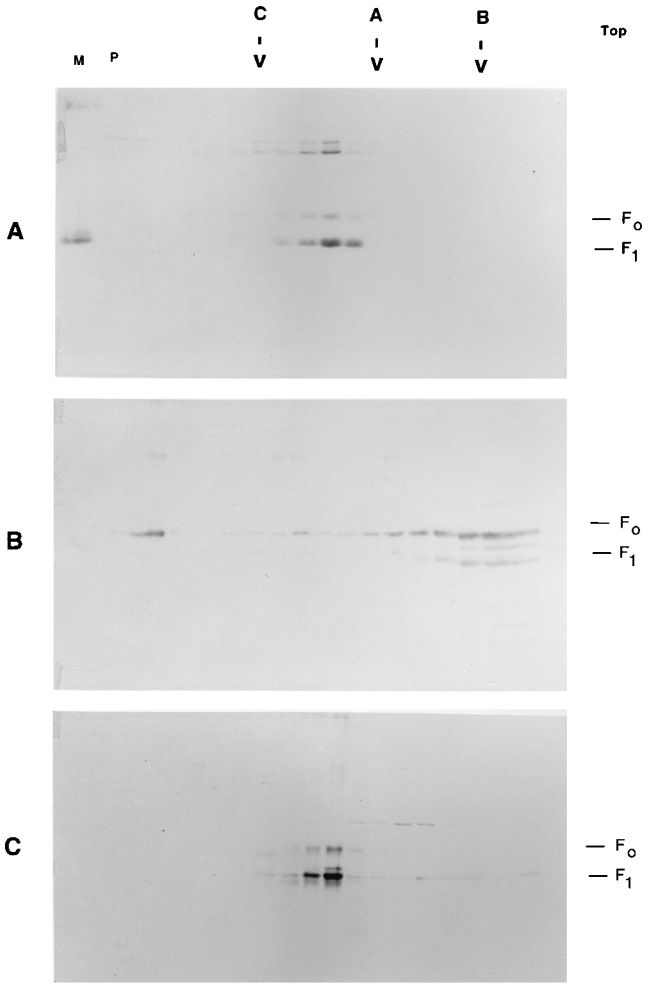

FIG. 7. Sucrose gradient analysis of wild-type and L345A fusion proteins. At 48 h posttransfection, cells were lysed in 1% Triton-X100, and the extracts were layered on a 10 to 25% sucrose gradient without further treatment (A) or after incubation in 5% SDS for 5 min (B). Protein in each fraction was trichloroacetic acid precipitated, solubilized, and electrophoresed as described in Materials and Methods. Blots were probed with an antibody generated against the C terminus of F. (C) Fractionation of extracts from Triton X-100-solubilized cells expressing the L345A mutant. Lanes M and P represent AV virion proteins and one-third of the total solubilized pellet, respectively; remaining lanes represent fractions 1 to 15, 17, 19, and 21. Positions of marker proteins (C, catalase; A, aldolase; B, bovine serum albumin) are indicated at the top.

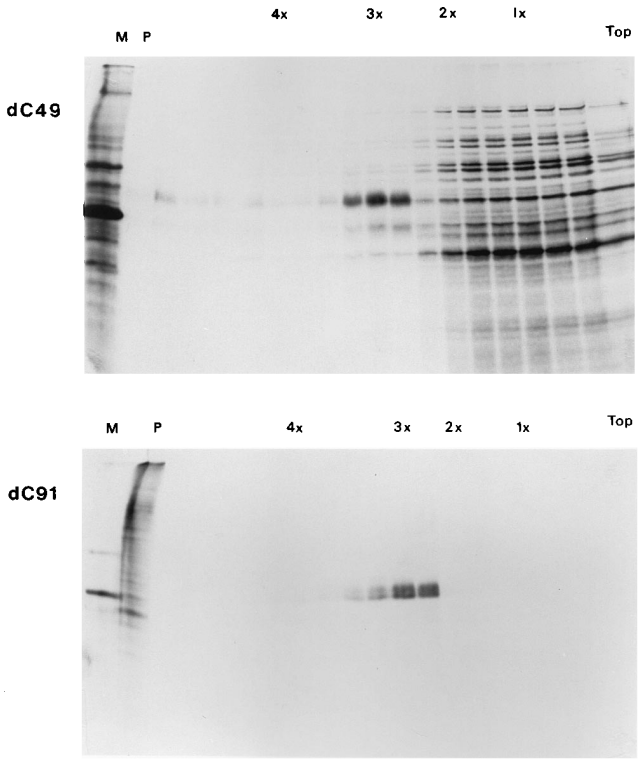

FIG. 8. Sucrose gradient analysis of truncated mutant proteins. (A) at 48 h posttransfection, cells expressing the dC49 mutant protein were radioactively labeled for 2 h and chased with nonradioactive methionine for 6 h. The medium was removed, centrifuged to remove debris, layered over a gradient, and fractionated as described in Materials and Methods. The resulting fractions were immunoprecipitated with a monoclonal antibody directed against mature F protein. Lanes M and P represent total extract from AV-infected, radiolabeled cells and immunoprecipitate from the solubilized pellet, respectively; remaining lanes represent immunoprecipitates of fractions 1 to 15, 17, 19, and 21. (B) At 48 h posttransfection, cells expressing the dC91 mutant were radioactively labeled for 2 h. The cells were lysed, and the postnuclear fraction was layered over a gradient as described in the text. The resulting fractions were immunoprecipitated as for panel A. Lanes are identical as those described for panel A. As determined by sedimentation of marker proteins (Fig. 7), the relative positions expected for an ideal monomer, dimer, trimer, and tetramer (1x, 2x, 3x, and 4x) are shown for reference.

protein. Furthermore, the lack of biological activity of the multiple leucine mutant cannot be accounted for by a disruption of this apparent oligomeric structure as detected on sucrose gradients.

Oligomerization of truncation mutants. Given this finding, we attempted to identify the region of the protein that is responsible for the oligomerization. A termination codon was inserted immediately upstream of the coding sequence of the transmembrane region to delete the cytoplasmic tail and the transmembrane region while retaining the leucine zipper (dC49; Fig. 1C). Also, the protein was truncated to delete the transmembrane domain, the cytoplasmic domain, and the leucine zipper motif (dC91). Since neither of these mutant proteins has the membrane anchor region, they were secreted from the cell (not shown). Neither mutant was able to induce fusion when coexpressed with HN protein (not shown). To examine the oligomeric structure of these mutants, cells expressing these mutant cDNAs were radioactively labeled, and the cell lysate or supernatants were fractionated on gradients previously described. The protein in the resulting fractions was immunoprecipitated with a monoclonal antibody generated against mature virion F protein. As shown in Fig. 8, both of the truncation mutant proteins sedimented on the gradients consistent with having an oligomeric structure. No fusion protein was detected in the monomer region. The sedimentation patterns of proteins derived from the cell lysate and the supernatant were similar, and only one gradient of each mutant is shown. These results indicated that the carboxy-terminal 91 amino acids of the fusion protein molecule are not necessary for the formation of this oligomeric structure detected on sucrose gradients. This result suggests that the ectodomain of the protein amino terminal to the leucine zipper motif is sufficient for oligomer formation and stability as determined by sucrose gradient sedimentation.

DISCUSSION

A leucine zipper-type motif is found adjacent to the transmembrane region in paramyxoviruses (6), in the ectodomain of

the TM protein of retroviruses (9, 13, 16), and in the coronavirus S protein (4). When Buckland and coworkers replaced four of the heptadic leucines in the measles virus fusion protein, fusion was abolished (5). We have also shown here that fusion activity of the NDV fusion protein containing two or more leucine substitutions in this region is abolished.

The leucine zipper motif is spatially placed well away from the presumed site for the fusion inducing regions of the proteins, the fusion peptide, and adjacent heptad repeat (25, 44). It was expected, therefore, that this region does not directly participate in membrane fusion. However, mutations of conserved leucine residues resulted in proteins whose transport and folding were similar to those of the wild-type protein, as indicated by detection with the available conformation-sensitive monoclonal antibody. The lack of fusion activity for the double and triple leucine mutants also cannot be accounted for by a lack of precursor processing. Thus, the leucines are not conserved for these functions. Indeed, the helix and all subsequent residues can be deleted with no apparent defect in transport or antibody reactivity, suggesting that this region has little role in the actual folding of the molecule.

The prediction that a leucine zipper motif may function in oligomer formation (5) suggested that failure of the double and triple leucine mutants to direct fusion might be due to failure of the protein to form oligomers. When the wild-type F protein was sized on sucrose gradients, the mature protein consistently sedimented as a 190-kDa species, between the aldolase (158 kDa) and catalase (240 kDa) molecular weight markers. The sedimentation profile would suggest that the F oligomer is a trimer. The gradients gave no indication that the mature protein exists as a 68-kDa monomer, dimer (136 kDa), or tetramer (272 kDa). This result is in agreement with the recent report from Russell and coworkers (38) suggesting that the NDV fusion protein forms a trimeric structure. The oligomeric species of the fusion protein was not stable in 5% SDS, indicating that it is held together by noncovalent forces. In addition, the existence of a single homogeneous species of the F protein following centrifugation through sucrose gradients suggested that the larger forms of F detected following electrophoresis in SDS represent incompletely solubilized monomers (Fig. 2 and 3).

The sedimentation profile of the triple leucine mutant as well as the sedimentation of the truncation mutants indicated that the leucine zipper motif and all subsequent residues are not necessary for assembly of the Triton X-100-insensitive oligomeric structure. These results are comparable to those of Singh et al. (46) and Crise et al. (11), who found that truncation mutants of the fusogenic influenza virus HA and vesicular stomatitis virus G proteins, respectively, lacking the membrane anchor and cytoplasmic domains, could form trimers. Cellular transport of many viral glycoproteins has been shown to be dependent on prior oligomerization (for a review, see reference 15). Since the truncated fusion protein is secreted as an oligomer, the portion of the NDV F protein required for oligomerization, and therefore cellular transport, depends on regions upstream from the leucine zipper. Thus, no role for oligomer formation could be attributed to the leucine zipper. Furthermore, the stability of the oligomer as detected on sucrose gradients is unaffected by the absence of the leucine zipper.

An alternative hypothesis for the absence of fusogenic activity of the double and triple leucine mutants may be that the mutant proteins cannot properly interact with the NDV HN glycoprotein. Reports from our laboratory, as well as others, suggested that a specific interaction between F and HN proteins must occur to promote membrane fusion (22, 43). One way that these two proteins could associate is by hydrophobic interactions near the transmembrane domain. Indeed, the HN proteins have a hydrophobic sequence in this region (29). Therefore, an attempt was made to detect the association between the two glycoproteins. However, efforts to cross-link or coimmunoprecipitate the two wild-type proteins were unsuccessful (unpublished observations). Thus, any potential defects in interaction of mutant F proteins with the HN protein could not be assessed.

Another alternative hypothesis for the role of the leucine zipper in membrane fusion is suggested by studies of the interaction of amphipathic α helices with membranes. These protein sequences may spontaneously associate with membranes (12, 41), with the hydrophobic face of the helix embedded in the bilayer. For example, the fusogenic and hemolytic peptide melittin has been reported to lie parallel to the membrane surface by virtue of a hydrophobic face of an alpha helix (1, 14), as have portions of the apolipoproteins and apolipoprotein analogs (42). The induction of membrane fusion by amphipathic helices such as melittin has been attributed to a local disruption of the bilayer (14). The hydrophobic photolabeling studies of Harter and coworkers suggested that the influenza virus HA fusion peptide may also have this topology (19). In addition, the protein components of plant storage lipid bodies, the oleosins, are predicted to have domains with this structure and membrane topology (35).

Similarly, evidence suggesting that the hydrophobic face of the heptad repeat region of the influenza virus HA2 becomes embedded in the bilayer of the target membrane as fusion proceeds has been recently presented (18). Indeed, the paramyxovirus fusion protein has a heptad repeat region just past the fusion sequence that may function similarly. During membrane fusion with a target cell, the fusion peptide is believed to insert into the target cell bilayer (3, 21, 36). At this point, the two membranes are tethered together by the two hydrophobic domains on the F_1 polypeptide, the fusion peptide, and the transmembrane domain. The heptad repeat adjacent to the fusion peptide may then serve to disrupt the target membrane. However, fusion requires disruption of both target and attack membranes. The proximity of the leucine zipper motif to the attack or host cell membrane suggests that this region may serve to penetrate the attack membrane during fusion in response to any conformational changes in the ectodomain of the protein as diagramed in Fig. 9. The transmembrane domains themselves have also been proposed to destabilize the bilayer and may contribute to the process (45). The concomitant disruption of both the infected cell and target cell membranes should assist in the fusion process (20). The bulky leucine side chains may be required for interactions with the hydrophobic milieu of the membrane. Also, the charged residues on the polar sides of the helix would interact with the polar extracellular solvent and the polar head groups of the membrane. It is likely that these interactions with the polar face of the helix would not be specific and therefore could tolerate substitutions as was suggested for the E482K, N485K, and E482A,D489A mutants. However, Anantharamaiah and coworkers have used an 18-residue peptide to show that positively charged residues at the hydrophobic-hydrophilic interface of an amphipathic helix assists the helix in binding to the phospholipid and promoting lipid hydration (2, 17). Thus, the lysine-rich strip (in g positions; Fig. 1B) may be less able to tolerate substitutions, and multiple mutations of these residues should address this point. In addition, the residues between the transmembrane domains and leucine zipper motifs of the paramyxoviruses are rich in serines and glycines, and these residues may allow the protein to bend between the two seg-

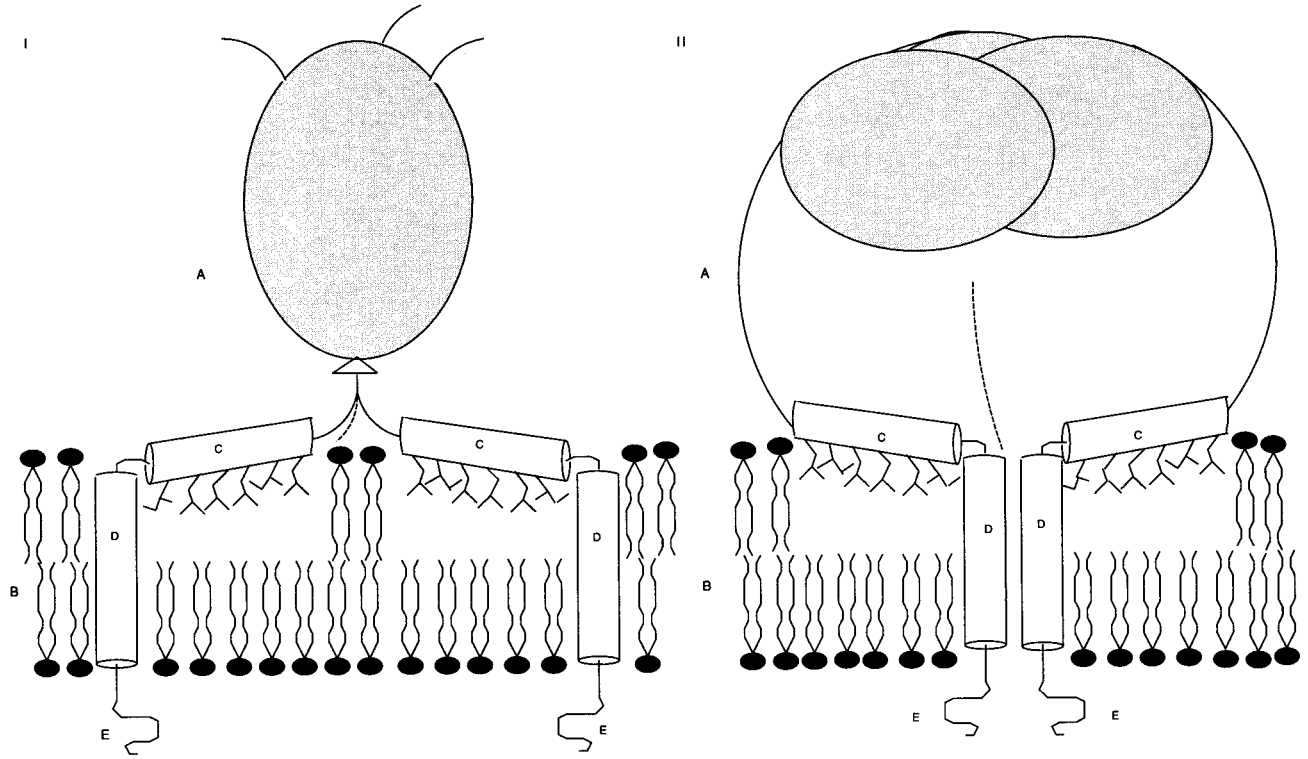

FIG. 9. Hypothetical model of the role of the leucine zipper motif. A, the oligomeric structure of the ectodomain of the mature fusion protein along with the exposed fusion peptides of the F1 subunits. B, the milieu of the bilayer of the infected cell membrane. C, the amphipathic helix and the conserved leucines. The hydrophobic face of each helix is predicted to embed in the membrane. The heptadic leucine and isoleucine residues are shown in contact with the hydrophobic portion of the monolayer. The lysines in position g may reside at the hydrophobic and hydrophilic interface. D, the hydrophobic transmembrane domain. E, the cytoplasmic tail. Only two subunits of the proposed oligomer are shown. The helices would be oriented as shown by either form I or form II.

ments. Both residues have a high bend potential and frequently occur at β-turn regions in a polypeptide chain (10).

Such an interaction of the fusion protein with membranes could potentially occur only after activation of the fusion protein. Studies of the influenza virus HA protein have clearly shown a conformational change in the HA protein upon exposure to acid pH (49). Recent results have show that the conformational change includes a rearrangement of the fusion peptide and adjacent sequences (7, 8). The activation of paramyxovirus fusion proteins, which mediate fusion at neutral pH (48), is thought to be caused by an interaction with the HN protein (25, 43). If such an activation includes conformational changes, then the leucine zipper in the fusion-active form may be in a very different conformation with respect to membranes and/or adjacent proteins. For example, association of the leucine zipper with the attack membrane, as proposed above, may not occur until activation and concomitant conformational change induced by the HN protein.

In sum, mutational analysis of the leucine zipper region of the NDV fusion glycoprotein has shown that it is the leucine residues in combination that are important to the fusion activity of the protein. Elimination of charged residues or a glycosylation site have little effect on the activity of the protein. The leucine residues are, however, not essential for oligomer formation.

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