

Truncation of the COOH-Terminal Region of the Paramyxovirus SV5 Fusion Protein Leads to Hemifusion but Not Complete Fusion

Sangeeta Bagai and Robert A. Lamb

Howard Hughes Medical Institute, and Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208-3500

Abstract. The role of the simian virus 5 (SV5) fusion (F) protein 20 residue COOH-terminal region, thought to represent the cytoplasmic tail, in fusion activity was examined by constructing a series of COOH-terminal truncation mutants. When the altered F proteins were expressed in eukaryotic cells, by using the vaccinia virus-T7 transient expression system, all the F proteins exhibited similar intracellular transport properties and all were expressed abundantly on the cell surface. Quantitative and qualitative cell fusion assays indicated that all of the F protein COOH-terminal truncation

mutants mediated lipid mixing with similar kinetics and efficiency as that of wild-type F protein. However, the cytoplasmic content mixing activity decreased in parallel with the extent of the deletion in the F protein COOH-terminal truncation mutants. These data indicate that it is possible to separate the presumptive early step in the fusion reaction, hemifusion, and the final stage of fusion, content mixing, and that the presence of the F protein COOH-terminal region is important for the final steps of fusion.

MEMBRANE fusion is an essential biochemical process critical for intracellular membrane trafficking, fertilization, and synaptic transmission. Enveloped virus-induced fusion has been one of the best characterized membrane fusion reactions. The entry of enveloped viruses into cells occur by fusion of the viral membrane with either the host cell plasma membrane or with the membrane of an endocytic vesicle. With many enveloped viruses, membrane fusion is induced by a fusogenic spike glycoprotein which is also responsible for attachment of viral particles to the target cell. With the paramyxoviruses, however, the functions of attachment and fusion are mediated by two different spike glycoproteins (Scheid et al., 1972; for review see Stegmann et al., 1989; White, 1990).

Simian virus 5 (SV5)¹ is a member of the family paramyxoviridae and contains two integral membrane spike glycoproteins, the hemagglutinin-neuraminidase protein (HN), and the fusion protein (F) (Paterson et al., 1984b, 1985; Scheid et al., 1972; Scheid and Choppin, 1974). The HN protein provides the attachment function allowing the virus particle to bind to sialic acid containing receptors on

the cell surface, whereas the F protein is directly involved in virus-induced membrane fusion. The F protein is a type I integral membrane protein and computer-assisted analysis indicates that it contains a 484-residue ectodomain, a 25-residue transmembrane domain, and a 20-residue cytoplasmic tail (Paterson et al., 1984b, Lamb, 1993). It is synthesized as an inactive precursor (F₀) which is cleaved by a cellular protease in the *trans*-Golgi apparatus (Klenk and Garten, 1994; Scheid and Choppin, 1974) to form the biologically active protein consisting of the disulfide linked chains F₁ and F₂.

It has become increasingly clear that there is a difference in the requirements for fusion among different paramyxoviruses (Bagai and Lamb, 1995b; Horvath et al., 1992). Whereas most of the paramyxoviruses require the coexpression of homotypic F and HN proteins in order to induce cell-cell fusion (Ebata et al., 1991; Hu et al., 1992; Morrison et al., 1991; Sergel et al., 1993; Tanabayashi et al., 1992; Wild et al., 1991), a homotypic HN protein is not essential for SV5 F protein-mediated cell fusion (Bagai and Lamb, 1995b). Further evidence for the sufficiency of SV5 F protein in cell fusion comes from the finding that F mutants containing single Gly to Ala changes in the fusion peptide cause a remarkable increase in the syncytia formation compared to the wild-type (wt) F protein (Horvath and Lamb, 1992; Ward et al., 1995). To understand the mechanism of F protein-induced membrane fusion, it is important to understand the role of different domains of the F protein in mediating fusion. While it is known that the fusion peptide and adjacent heptad repeat regions have important roles in fusion (Horvath and Lamb, 1992;

Please address all correspondence to R.A. Lamb, Department of Biochemistry, Molecular and Cell Biology, Northwestern University, 2153 North Campus Drive, Evanston, Illinois 60208-3500. Tel: (847) 491-5433. Fax: (847) 491-2467. E-Mail: ralamb@nwu.edu

1. *Abbreviations used in this paper:* endo H, endo- β -N-acetylglucosaminidase; F, fusion; GPI, glycosyl phosphatidyl inositol; HA, hemagglutinin; HN, hemagglutinin-neuraminidase protein; RBC, red blood cell; SV5, simian virus 5.

Sergel-Germano et al., 1994; Wang et al., 1992), the role of the cytoplasmic tail of the F proteins is less clear. Studies with human immunodeficiency virus type 2 and simian immunodeficiency virus envelope glycoproteins (env) have shown that cytoplasmic tail truncation mutants significantly enhance syncytia formation and alter the conformation of the external domain of env (Mulligan et al., 1992; Ritter et al., 1993). For the paramyxoviruses, examination of the role of the F protein cytoplasmic tail in fusion has led to disparate results: for Newcastle disease virus, F protein cytoplasmic tail deletions greatly reduced syncytia formation (Sergel and Morrison, 1995), for human parainfluenza virus type 3, the F protein cytoplasmic tail mutants showed reduced cell surface expression due to a defect in oligomerization, and for human parainfluenza virus type 2, F protein cytoplasmic tail deletions did not affect fusion (Yao and Compans, 1995). However, in all of these studies, the effect of mutations in the cytoplasmic tail on the final extent of fusion was examined and the early steps in fusion were not investigated.

Recent studies with a glycosyl phosphatidyl inositol (GPI)-linked form of influenza virus hemagglutinin (HA), where the HA lacks its normal transmembrane and cytoplasmic domains, have shown that GPI-anchored HA is competent in mediating fusion of the outer leaflets of the lipid bilayer (hemifusion) but there was no mixing of the aqueous contents (content mixing) (Kemble et al., 1994; Melikyan et al., 1995). It has been suggested for influenza virus HA that separate domains of HA may control individual steps in fusion (Kemble et al., 1994; Melikyan et al., 1995). The present study was undertaken to define the requirement of the cytoplasmic domain of the SV5 F protein in the membrane fusion process, using quantitative biophysical assays to measure the kinetics of membrane fusion and the extent of content mixing and employing qualitative fluorescence measurements to follow the movement of membrane and aqueous dyes. The results indicate that hemifusion but not complete fusion occurs in the absence of the cytoplasmic domain of the SV5 F protein. We interpret these data to indicate that hemifusion is an intermediate step common to virus-mediated membrane protein fusion.

Materials and Methods

Cells and Recombinant Plasmid Vectors

Monolayer cultures of the TC7 subclone of CV-1 cells and HeLa-T4 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% NU Serum IV (Collaborative Research Laboratories, Inc., Bedford, MA) as described previously (Paterson et al., 1985).

The SV5 F and HN cDNAs described previously (Horvath et al., 1992; Paterson et al., 1984a, b) were subcloned into pGEM2X, a derivative of pGEM2 containing an XhoI site (Bagai and Lamb, 1995b). The plasmid pTF7.5 containing the hemagglutinin (HA) cDNA of influenza virus A/Udorn/72 was described previously (Simpson and Lamb, 1992). The plasmid pGINT7 β -gal (Nussbaum et al., 1994) containing the β -galactosidase cDNA was kindly provided by Drs. Edward Berger and Bernard Moss (National Institutes of Health, Bethesda, MD). All the cDNAs were cloned in pGEM2X in an orientation such that mRNA-sense RNA transcripts could be synthesized by using bacteriophage T7 RNA polymerase.

Oligonucleotide-directed Mutagenesis and Generation of F Protein COOH-Terminal Truncation Mutants

pGEM2X-F was used as the template for mutagenesis. Clones encoding

the SV5 F protein COOH-terminal truncation mutants $\Delta 5$, $\Delta 10$, $\Delta 15$, and $\Delta 19$ were constructed by oligonucleotide-directed unique site elimination (U.S.E) mutagenesis (Deng and Nickoloff, 1992) by introducing premature translational stop codons into the coding sequence to obtain COOH-terminal deletions of 5, 10, 15, and 19 residues, respectively. In addition to the COOH-terminal truncation mutants, a revertant BWT (back wt) was also constructed by replacing the translational stop codon in mutant $\Delta 19$ with the codon for leucine. U.S.E mutagenesis was performed using the U.S.E™ mutagenesis kit following the instructions of the supplier (Pharmacia Biotech, Inc., Piscataway, NJ).

Oligonucleotides were synthesized by the Northwestern University Biotechnology Facility on a DNA synthesizer (model 380B, Applied Biosystems, Inc. Foster City, CA). The nucleotide sequence of the F protein COOH-terminal truncation mutants was determined by dideoxynucleotide chain-terminating sequencing (Sanger et al., 1977).

Expression of F Protein COOH-Terminal Truncation Mutants and Metabolic Labeling of Cells

The mutant F proteins were expressed by using the vaccinia virus-bacteriophage T7 RNA polymerase transient expression system (Fuerst et al., 1986). Subconfluent monolayers of HeLa-T4 or CV-1 cells were infected with recombinant vaccinia virus vTF7-3 at a multiplicity of infection of 10 pfu/cell and incubated at 37°C for 45 min. The virus inoculum was removed and cells were transfected with wt and mutant plasmid DNA using cationic liposomes made in our laboratory as described previously (Rose et al., 1991). Transfection was done according to the protocol for using TransfectACE™ (GIBCO BRL, Gaithersburg, MD) essentially as described (Rose et al., 1991). Typically 2.5 μ g of plasmid DNA was used for a 35-mm tissue culture dish and the volume was adjusted to 0.5 ml with OPTI-MEM (GIBCO BRL). At 5 h posttransfection, cells were washed twice in phosphate-buffered saline and incubated in cysteine- and methionine-deficient DMEM (DMEM Cys⁻ Met⁻) for 30 min. For pulse-chase experiments, cells were labeled with Tran-³⁵S-label (100 μ Ci per ml) (ICN Radiochemicals, Irvine, CA) in DMEM Cys⁻ Met⁻ for the pulse period indicated. The chase period was initiated by removal of the labeling medium, the cells were washed with prewarmed chase medium (DMEM supplemented with 2 mM unlabeled cysteine and methionine and 2% NU serum IV), and then additional chase medium was added. The cells were then incubated for varying periods after which they were lysed in ice-cold RIPA buffer containing protease inhibitors (Paterson and Lamb, 1987) and 25 mM iodoacetamide.

Chemical Cross-Linking

HeLa-T4 cells expressing the SV5 wt F and COOH-terminal truncation mutant proteins were labeled with Tran-³⁵S-label (100 μ Ci per ml) in DMEM Cys⁻ Met⁻ for 30 min at 5 h posttransfection and incubated in chase medium for 2 h. Chemical cross-linking analysis was performed as described previously (Russell et al., 1994) using the cross-linking reagent 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP) (Pierce Chemical Co., Rockford, IL).

Immunoprecipitation, SDS-PAGE, and Quantification of Autoradiograms

F proteins were immunoprecipitated from cell lysates by using a rabbit antiserum raised against a mixture of three synthetic peptides specific for F₂ residues 22 to 39, 31 to 46, and 77 to 94 (Horvath and Lamb, 1992). Immunoprecipitation was performed as described previously (Lamb et al., 1978). Polypeptides were analyzed by SDS-PAGE. Cross-linked polypeptides were analyzed on 3.5% acrylamide gels (Gething et al., 1989; Russell et al., 1994), nonreduced F polypeptides on 10% acrylamide gels and reduced F polypeptides on 15% acrylamide gels, and gels were processed for fluorography as described previously (Lamb and Choppin, 1976; Paterson and Lamb, 1993). Autoradiograms were quantified by using a laser densitometer (LKB Ultrascan; Pharmacia-LKB, Inc., Piscataway, NJ) equipped with LKB Gelscan SL software.

Endo- β -N-acetylglucosaminidase Digestions

To test for the conversion of N-linked carbohydrate chains from high mannose to the complex form, immune complexes were digested with 1 mU of endo- β -N-acetylglucosaminidase (endo H) (Boehringer-Mannheim Biochemicals, Indianapolis, IN) as described previously (Ng et al., 1989). Pep-

tide *N*-glycosidase F and deoxymannojirimycin (DMJ, 0.5 mM) treatments were done as described previously (Melikyan et al., 1995; Paterson and Lamb, 1993).

Quantification of Surface Expression: FACS Analysis

For flow cytometry, HeLa-T4 cells were grown on culture dishes (6 cm) to 70% confluence. At 5 h posttransfection, cells were chilled on ice and treated for flow cytometric analysis as described previously (Horvath and Lamb, 1992) using monoclonal antibody F1a specific for the SV5 F glycoprotein (Randall et al., 1987) as the primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG as the secondary antibody. Fluorescence intensity of 10,000 cells was measured by a FACSCAN flow cytometer (Becton Dickinson, Mountain View, CA).

Incorporation of Fluorescent Probes into Erythrocytes

Fresh human red blood cells (RBCs) were labeled either singly or colabeled with membrane and aqueous dyes. Labeling of RBCs with the lipid probe octadecyl rhodamine B (R18) was carried out as described previously (Bagai and Lamb, 1995b). Entrapment of the aqueous dye NBD-aurine was done as described previously (Sarkar et al., 1989). RBCs were colabeled with R18 and NBD-aurine by injecting 15 μ l of 1 mg/ml R18 in ethanol to the RBCs suspended in 10 mM NBD-aurine in PBS at 37°C as described (Sarkar et al., 1989). After labeling, the RBC suspension was washed six times with 50 ml PBS to remove the unincorporated R18 and NBD-aurine. The double-loaded erythrocytes could be stored for 2–3 d without significant leakage of dyes. RBCs were loaded with either the fluorescent aqueous dye ethidium bromide (EtBr) or HOECHST 33258 by mild hypotonic lysis as described previously (Ellens et al., 1989). The hypotonic solution contained either 1 mM ethidium bromide or HOECHST 33258 in 10 mM Tris-HCl, 0.5 mM EGTA, pH 7.5. More than 90% of the cells trapped the dyes.

Binding and Fusion of Loaded RBCs with Cells Expressing Viral Envelope Proteins: Fluorescence Microscopy

CV-1 cells grown on coverslips and expressing wt or mutant F and wt HN proteins at 5 h posttransfection were washed twice and incubated overnight at 32°C in DMEM containing 10% NU serum IV. The cells were washed with PBS, incubated for 1 h at 37°C with 50 mU per ml of neuraminidase (*Vibrio cholerae*) (Boehringer Mannheim, Indianapolis, IN) in DMEM and washed twice with PBS. 3 ml of (co)labeled RBCs (0.1% hematocrit) in PBS were added to the monolayer and incubated at 4°C for 30 min with occasional gentle agitation. Unbound RBCs were removed by six washes with PBS. Fusion was triggered by transferring the coverslips to 37°C for varying time periods and coverslips were then placed on microscope slides and cells photographed with a Nikon Microphot FXA microscope system (Nikon Corp., Tokyo, Japan). NBD fluorescence was visualized under a “fluorescein” filter set, R18 and EtBr fluorescence was observed with a “Texas red” filter set, and HOECHST 33258 fluorescence under a “DAPI” filter set.

Spectrofluorometric Measurements

For measurement of kinetics of lipid mixing, R18 labeled erythrocytes bound to cells as described above were removed from the dish using either trypsin-EDTA solution or 50 mM EDTA in PBS at 4°C. The R18-labeled RBC-acceptor cell complexes were washed with cold PBS and placed on ice until further use. Fluorescence changes as a result of fusion of R18-labeled erythrocytes with acceptor cells were measured continuously using a spectrofluorimeter (Alpha Scan, Photon Research Inc., South Brunswick, NJ) with 1 s time resolution at 560/590 nm excitation and emission, respectively. A 570-nm cut off filter was placed in the emission optical pathway to reduce scattering. 50 μ l of the R18-labeled RBC and acceptor cell suspension was placed into a cuvette containing 3 ml PBS prewarmed to 37°C. To normalize the data, percent fluorescence dequenching (% FDQ) at any time point was calculated according to the following equation: % FDQ = 100. $(F_t - F_0) / (F_i - F_0)$ where F_0 and F_t are the fluorescence intensities at time 0 and at a given time point. F_i is the fluorescence intensity in the presence of 0.1% Triton X-100 and defined as fluorescence at “infinite” dilution of the probe (Bagai et al., 1993).

Content Mixing Assay

The content mixing assay based on the cytoplasmic activation of the reporter gene β -galactosidase was performed essentially as described (Nussbaum et al., 1994) with slight modifications. The cells expressing viral envelope proteins at 5 h posttransfection were trypsinized, suspended in DMEM containing 2.5% NU serum IV to a density of 2×10^5 /ml, and incubated overnight at 32°C in a near horizontal position. The cells were washed twice in OPTI-MEM (containing 2.5 mM CaCl_2), suspended to a density of 2×10^7 /ml, and treated with neuraminidase (50 mU/ml) for 1 h at 37°C. The cells were then washed and suspended in OPTI-MEM at 10^6 cells/ml. A second population of CV-1 cells was infected with wt vaccinia virus (strain WR) for 45 min at 37°C and transfected with plasmid pGINT7 β -gal. At 5 h posttransfection, cells were washed and incubated overnight as described above. The cells were washed with OPTI-MEM. The two cell populations were mixed in triplicate by adding 100 μ l of each cell population in a 96-well tissue culture plate. The plates were incubated at 37°C for 4 h. Cell fusion was measured at the indicated times by using a colorimetric lysate assay for β -galactosidase as described previously (Nussbaum et al., 1994). The quantity of β -galactosidase was calculated by comparing the rates of hydrolysis for each sample with that obtained for a standard preparation of *E. coli* β -galactosidase (Boehringer Mannheim) and was expressed as ng/well. The in situ assay involving X-gal staining of cells was done as described previously (Nussbaum et al., 1994).

Results

Construction and Expression of F Protein COOH-Terminal Truncation Mutants

The transmembrane domain of a type I integral membrane protein is defined as the carboxyl-terminal hydrophobic domain, usually bounded by two charged residues, which acts during synthesis to stop the transfer of the nascent polypeptide chain across the bilayer and brings its stable integration in the membrane. In the case of the paramyxovirus SV5 F protein, computer-assisted analysis of the hydrophobicity of the F protein suggests that the transmembrane domain begins at F residue 485 after a series of serine and threonine residues and ends before a lysine residue at F residue 510, yielding a 25-residue transmembrane domain. Thus, the cytoplasmic tail is presumed to consist of 20 COOH-terminal residues. To examine the role of the paramyxovirus SV5 F protein cytoplasmic tail in membrane fusion, translational stop codons were introduced into the cytoplasmic tail. Four deletion mutants were constructed and they were designated $\Delta 5$, $\Delta 10$, $\Delta 15$, and $\Delta 19$ and their cytoplasmic tails contained deletions of 5, 10, 15, and 19 amino acids, respectively (Fig. 1). The $\Delta 19$ mutant has the entire cytoplasmic tail deleted except for one positively charged lysine that immediately follows the transmembrane domain and marks the presumptive junction of the transmembrane and the cytoplasmic domain.

The F protein COOH-terminal truncation mutants were expressed in HeLa-T4 cells by using the recombinant vaccinia virus-T7 RNA polymerase system. To analyze the expression and cleavage of F_0 to F_1 and F_2 , the cells were labeled with Tran- ^{35}S -label for 15 min and were either lysed immediately or incubated in chase medium for 3 h. Proteins were immunoprecipitated and polypeptides were analyzed by SDS-PAGE. As shown in Fig. 2 A, all the proteins were expressed and a significant fraction of the F proteins was cleaved to F_1 and F_2 after a 3-h chase period. In the case of mutant $\Delta 19$, only a weak band corresponding to F_1 polypeptide could be identified but a diffuse band of higher mobility than that expected for F_1 was also ob-

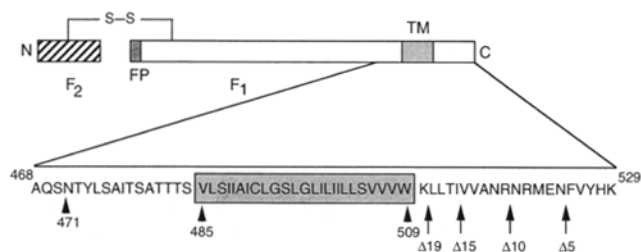


Figure 1. Schematic diagram of SV5 F protein and the COOH-terminal truncation mutants. The amino acid sequence of the residues around the presumptive SV5 wt F protein transmembrane domain are shown. As a working hypothesis it is assumed that the demarcation between the transmembrane domain and the cytoplasmic tail is between residues 509 and 510. Arrows indicate the positions where the premature translational stop codons were introduced to shorten the COOH-terminal region of the F protein. The designation of the truncation mutants ($\Delta 5$, $\Delta 10$ etc.) is shown, where $\Delta 5$ represents a mutant for which five amino acids from COOH-terminal end had been deleted and so forth. The positions of the fusion peptide (FP) and the presumptive transmembrane domain (TM) are shown.

served. This diffuse band is thought to be a heterogeneously glycosylated form of F_1 as on treatment of cells with deoxymannojirimycin, an inhibitor of α -mannosidase I, a discrete F_1 band was observed (data not shown).

Oligomerization of SV5 F Protein COOH-Terminal Truncation Mutants

We have shown that the F protein forms a homo-oligomer shortly after its synthesis in the ER (Russell et al., 1994) and the data is consistent with the formation of a homotrimer. As some cytoplasmic tail mutants have the propensity to cause profound alterations to native protein folding and oligomerization, and in turn affect intracellular transport of the glycoproteins (Parks and Lamb, 1990; Rose and Bergmann, 1983; Sergel and Morrison, 1995), it was important to examine the effect of the F protein cytoplasmic tail mutations on oligomerization and transport properties of the protein. To assess the competence of altered F proteins to form oligomers (trimers), chemical cross-linking studies were done. HeLa-T4 cells expressing the truncation mutants were labeled with Tran- ^{35}S -label at 5 h posttransfection for 30 min at 37°C and cultures were incubated in chase medium for 2 h to allow for intracellular transport to the plasma membrane. Cross-linking reactions were performed using the homobifunctional reagent DTSSP on either intact cells, to react with the cell surface F molecules, or on detergent solubilized (NP-40) cell extracts to render all F molecules available for cross-linking. As shown in Fig. 2 B all the F protein COOH-terminal truncation mutants could be cross-linked to homotrimers (species 3) both in the presence and absence of the detergent, indicating intracellular oligomer formation. The cross-linking pattern observed (species 1, 2, and 3) was found to be consistent with that observed previously when cross-linking studies were performed using SV5 virus-infected cells (Russell et al., 1994). Thus, all the F protein mutants could form oligomers similar to that of the wt F

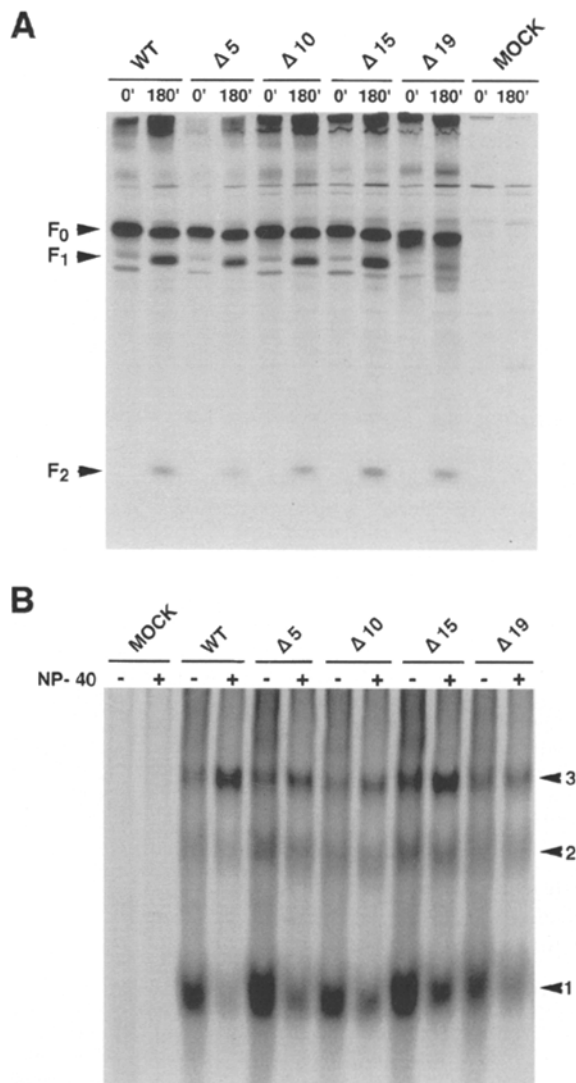


Figure 2. Expression and oligomeric form of the F protein COOH-terminal truncation mutants. (A) To express the altered F proteins, HeLa-T4 cells were infected with vaccinia virus vTF7-3 at 10 pfu per cell for 45 min at 37°C and then transfected with plasmids encoding wt and mutant F proteins as described in Materials and Methods. Transfected cells were metabolically labeled with Tran- ^{35}S -label (100 $\mu\text{Ci/ml}$) at 5 h posttransfection for 15 min and incubated in chase medium for 0 or 3 h. Polypeptides were immunoprecipitated with F_2 -specific antiserum and analyzed by SDS-PAGE on 15% gels under reducing conditions. Mock represents polypeptides immunoprecipitated from vTF7-3 infected but untransfected cells. Radioactivity was detected by fluorography. (B) HeLa-T4 cells expressing the F protein COOH-terminal truncation mutants were labeled with Tran- ^{35}S -label (100 $\mu\text{Ci/ml}$) at 5 h posttransfection for 30 min and incubated in chase medium for 2 h at 37°C. Cross-linking reactions were performed on cell suspensions in the absence (-) or presence (+) of 0.5% NP-40 as described in Materials and Methods. F protein species were immunoprecipitated and polypeptides analyzed on a 3.5% gel under nonreducing conditions. The three predominant species are indicated by arrows.

protein. However, the caveat has to be added that we cannot determine whether the mutant F proteins oligomers are as stable as the wt F protein oligomers.

Analysis of the Rate of Intracellular Transport of the F Protein COOH-Terminal Truncation Mutants

To determine the effect of the COOH-terminal region deletion mutations on transport of F proteins to the medial-Golgi apparatus, the rate of acquisition of carbohydrate chains resistant to endo H digestion was analyzed. Acquisition of endo H resistance is indicative of the conversion of carbohydrate chains from the simple to the complex form in the medial-Golgi compartment. We have shown previously that the two carbohydrate chains on the F₂ subunit remain in the high mannose form and do not acquire resistance to endo H digestion (Bagai and Lamb, 1995a;

Horvath and Lamb, 1992). However, most of the F₁ subunit carbohydrate chains acquire endo H resistance.

When the rate of transport to the medial-Golgi apparatus of the wt F protein expressed using the vaccinia virus-T7 system was determined, it was found to acquire endo H resistance with a $t_{1/2} \sim 70-75$ min. By 180 min, >90% of the F molecules were transported to the medial-Golgi apparatus (Fig. 3 A). Mutants $\Delta 5$, $\Delta 10$, $\Delta 15$, and $\Delta 19$ all showed rates of loss of carbohydrate chains sensitive to endo H digestion that were very similar to that for wt F protein (Fig. 3 A): quantification was done relative to the loss of the sensitive form, as the resistant form of $\Delta 19$ was heterogeneous in migration and difficult to quantify. Hence, the deletion in COOH-terminal domain of the F protein did not have a major effect on the rate of transport of the proteins to the medial-Golgi apparatus.

Cleavage of F₀ to F₁ and F₂ occurs during its transit from

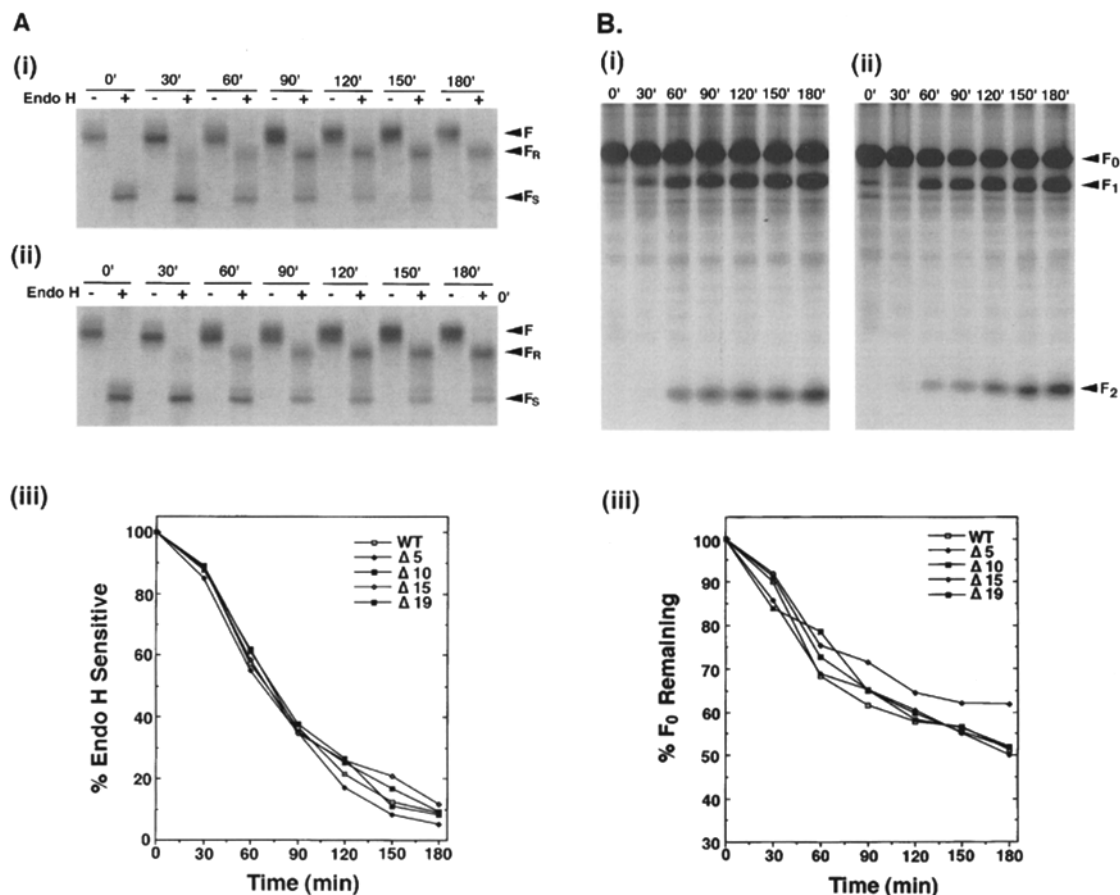


Figure 3. Kinetics of intracellular transport of F protein COOH-terminal truncation mutants to the medial-Golgi apparatus and the cleavage compartment. (A) HeLa-T4 cells expressing the F protein COOH-terminal truncation mutants were labeled with Tran-³⁵S-label (100 μ Ci/ml) at 5 h posttransfection for 15 min and incubated in chase medium for the times indicated. Polypeptides were immunoprecipitated with F₂-specific antiserum and incubated with (+) or without (-) endo H and analyzed by SDS-PAGE on a 10% gel under nonreducing conditions. (i) wt; (ii) mutant $\Delta 15$; F_s indicates mobility of both disulfide linked heterodimer of F₁+F₂ and F₀; F_s, endo H sensitive form of F; F_R, endo H-resistant form of F. (iii) Quantification by densitometric analysis of autoradiograms. Due to the presence of multiple endo H-resistant forms in mutant $\Delta 19$, it was difficult to quantify the endo H-resistant species, hence for all the mutants, data are plotted as the percentage of remaining endo H-sensitive species. (B) HeLa-T4 cells expressing the F protein COOH-terminal truncation mutants were labeled with Tran-³⁵S-label (100 μ Ci/ml) for 15 min at 5 h posttransfection and incubated in the chase medium for the times indicated. Polypeptides were immunoprecipitated with F₂-specific antiserum and analyzed by SDS-PAGE on a 15% gel under reducing conditions. (i) wt; (ii) mutant $\Delta 15$; (iii) rates of cleavage of F₀ to F₁ and F₂. Autoradiograms were quantified by densitometric analysis. Data are plotted as the percent of total F-specific species remaining as F₀.

the *trans*-Golgi apparatus (Klenk and Garten, 1994; Morrison et al., 1985). To determine the rate of transport of the mutant F proteins to the cleavage compartment, HeLa-T4 cells expressing the mutant F proteins at 5 h posttransfection were metabolically labeled with Tran³⁵S-label for 15 min, the cultures incubated in the chase medium for various time periods and the F polypeptides immunoprecipitated and the F₀, F₁ and F₂ polypeptides were analyzed by SDS-PAGE. The amount of F₀, as a percentage of total F polypeptide species was determined by densitometric scanning of autoradiographs (Fig. 3 B). The rate of cleavage for all the mutants was found to be similar to that of wt F protein; however, the final extent of cleavage was somewhat lower for mutant $\Delta 15$ as compared to that of wt F protein. For both wt F protein and the mutants, cleavage did not reach completion even after 180 min of chase period and only 50% of the molecules were found to be cleaved in 3 h. This finding is consistent with our earlier studies using the vaccinia virus transient expression system (Bagai and Lamb, 1995a), whereas in SV5 virus-infected cells cleavage of F goes to completion in 3 h (Horvath and Lamb, 1992). It is thought that the high level expression of F protein using the vac-T7 expression system saturates the amount of available cleavage enzyme, furin, present in the TGN (Klenk and Garten, 1994). Nonetheless, the rate and extent of cleavage was very similar for all the mutants and, as will be shown below, sufficient active F proteins are expressed to readily measure fusion activity.

Surface Expression of the F Protein COOH-Terminal Truncation Mutants

The cell surface expression of the altered F proteins was quantified by flow cytometry. Cells were stained with F-specific mAb, F1a. As shown in Table I, all the mutants showed both a comparable percentage of positive cells as compared to wt F protein and exhibited a similar mean fluorescent intensity (MFI) except for mutant $\Delta 15$ which had a somewhat reduced MFI, a finding which correlates with its somewhat reduced rate of cleavage (Fig. 3 B).

Induction of Cell Fusion by the F Protein COOH-Terminal Truncation Mutants

Cell fusion involves both the mixing of the outer leaflet membrane lipids and the mixing of inner leaflet membrane lipids with concomitant mixing of aqueous contents of donor and recipient cells (Kemble et al., 1994). We therefore determined the ability of CV-1 cells expressing

Table I. Surface Expression of F Proteins

Protein	Positive cells*	Mean fluorescent intensity [‡]
	%	
wt F	22.9	1.0
F $\Delta 5$	24.4	0.75
F $\Delta 10$	22.6	0.73
F $\Delta 15$	18.4	0.58
F $\Delta 19$	28.2	0.88

* Determined by flow cytometry.

[‡] Mean fluorescent intensity (MFI) was derived from flow cytometry data and is expressed as the amount compared to the value for wt F protein.

the wt F or COOH-terminal truncation mutants to fuse with target red blood cells (RBCs) prelabeled with fluorescent lipid and fluorescent soluble content probes either singly or in combination.

Lipid mixing activity of F protein COOH-terminal truncation mutants: Kinetics. To obtain information about the rates and extents of wt F or mutant F protein induced fusion, we measured the kinetics of fluorescence dequenching of the lipophilic probe R18. The lipid probe R18 was incorporated into RBCs which were then mixed with the acceptor cells expressing the wt F or the COOH-terminal truncation mutants. As this assay is done in suspension, the F protein expressed alone yields no fluorescence dequenching on mixing with R18 labeled erythrocytes (Bagai and Lamb, 1995b) because the binding of one cell to another requires either the paramyxovirus sialic acid binding glycoprotein HN or the influenza virus sialic acid-binding protein HA. As shown in Fig. 4, wt F protein and the COOH-terminal truncation mutant F proteins coexpressed with HN mediated rapid and efficient lipid mixing. The kinetics of fluorescence dequenching was comparable for wt F protein and all the truncation mutants. The F protein-mediated fusion was inhibited by preincubation of R18 labeled RBC-acceptor cell suspensions with a monoclonal antibody specific to SV5 F protein, strongly suggesting that the fluorescence dequenching observed on coexpression of F and HN is due to the specific fusion activity of the F protein and is not an artifact as a result of nonspe-

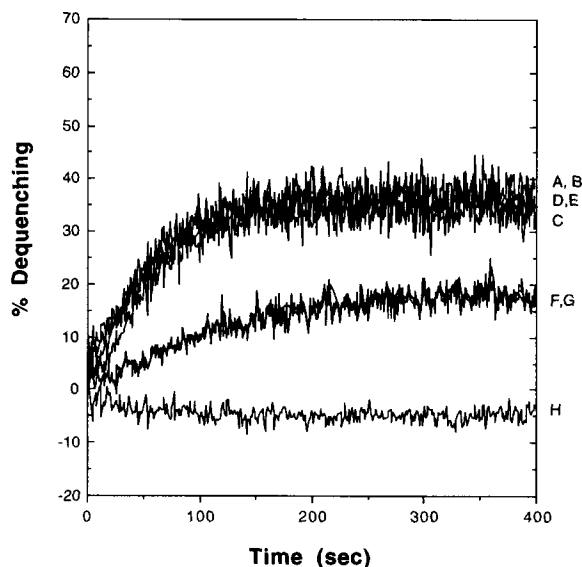


Figure 4. Kinetics of fusion of R18 labeled RBCs with cells expressing wt F or COOH-terminal truncation mutants. R18 labeled RBCs were bound to CV-1 cells expressing wt F and the F protein COOH-terminal truncation mutants and the fluorescence measurements were performed as described in Materials and Methods. Trace A, B, C, D, and E represents fusion of cells coexpressing SV5 HN protein and wt F, $\Delta 5$, $\Delta 10$, $\Delta 15$, and $\Delta 19$, respectively, with labeled RBCs. Trace F and G represents fusion of labeled RBCs with cells coexpressing influenza virus HA protein and wt F and $\Delta 19$, respectively. All other mutants also exhibited similar kinetics on coexpression with HA (data not shown). Trace H represents fusion of cells expressing F and HN proteins in the presence of the F-specific monoclonal antibody F1a.

cific dye transfer. Lipid mixing activity was also determined when the F proteins were coexpressed with HA. Significant fluorescence dequenching was observed and again the kinetics of fusion was similar for all the F protein COOH-terminal truncation mutants and the wt F protein when the F proteins were coexpressed with influenza HA. However, the rates as well as the extents of lipid mixing were found to be lower with the heterotypic-binding pro-

tein, confirming our earlier data (Bagai and Lamb, 1995b). Hence, the lipid mixing activity of the F protein COOH-terminal truncation mutants appears in all aspects to be the same as wt F protein.

Content mixing activity of the F protein COOH-terminal truncation mutants. We employed the reporter gene (β -galactosidase) activation assay (see Materials and Methods) to measure the content mixing potential of the F protein

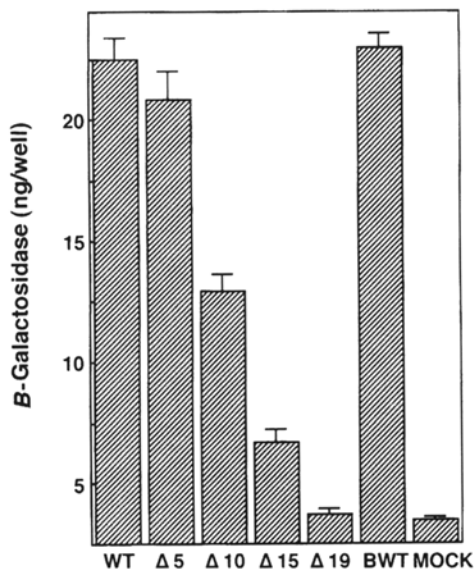
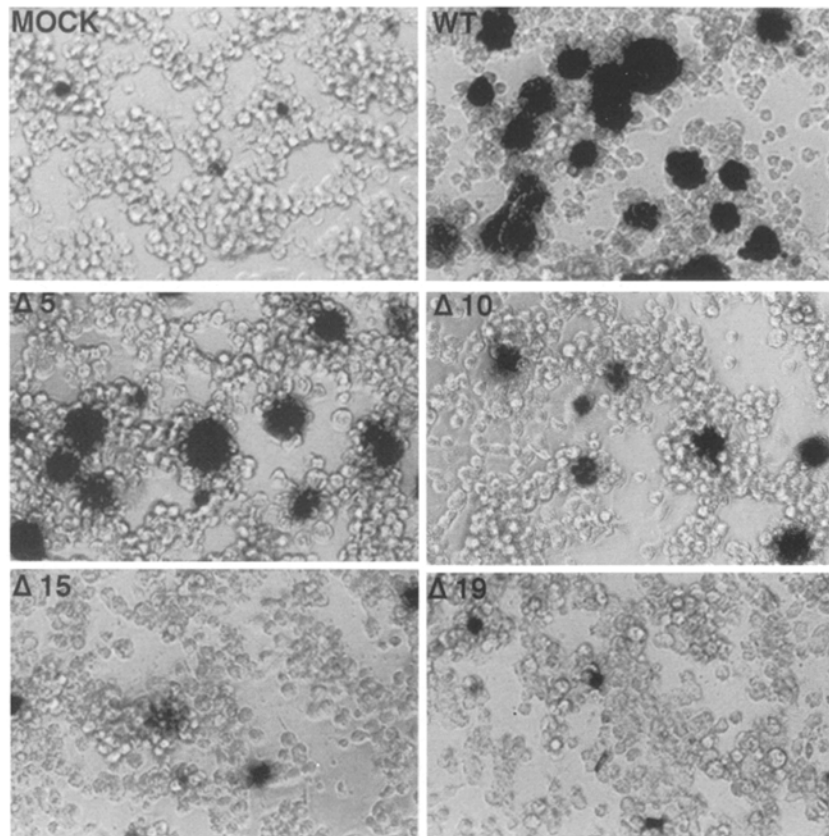


Figure 5. Reporter gene activation assay (content mixing) for the F protein COOH-terminal truncation mutants. One culture of CV-1 cells was infected with recombinant vaccinia virus vacTF7-3, which encodes T7 RNA polymerase, and cotransfected with plasmid DNA encoding the cDNAs for the SV5 HN and wt F or the F protein COOH-terminal truncation mutant proteins. Cells were then treated with bacterial neuraminidase as described in Materials and Methods. A second culture of CV-1 cells was infected with wt vaccinia virus (strain WR) and transfected with plasmid pGINT7 β -gal which encodes β -galactosidase. The cell populations were washed and suspended in OPTI-MEM containing CaCl₂ (2.5 mM) at 10⁶ per ml. Mixtures of the two populations of cells (0.1 ml each) were plated in triplicate in 96-well plates and incubated at 37°C. (Top) After incubation at 37°C for 4 h, samples were analyzed by using in situ X-gal staining (Materials and Methods). Cells were photographed using a Kodak Digital 420 camera at 10 \times magnification on a Nikon inverted phase contrast microscope. (Bottom) Samples were analyzed by the colorimetric lysate assay as described in Materials and Methods. Each point represents an average of three experiments \pm SD. BWT represents mutant Δ 19 that was reverted back to wt as described in Materials and Methods.

COOH-terminal truncation mutants. The β -galactosidase activity was analyzed by *in situ* X-Gal staining assay (Fig. 5, *top*) and by a quantitative colorimetric assay for β -galactosidase activity (Fig. 5, *bottom*). In contrast to the data obtained for lipid mixing, the content mixing activity of the F protein COOH-terminal truncation mutants was found to decrease in parallel with the extent of the deletion of the F protein cytoplasmic tail (Fig. 5). The reduced content mixing activity observed with mutant $\Delta 15$ could be due to its lower surface expression level (Table I) but we do not believe this is the case as the lower expression level of $\Delta 15$ did not affect the rate or extent of lipid mixing activity. The most striking effect of the COOH-terminal deletions on content mixing was observed with mutant $\Delta 19$ where the level of β -galactosidase activity detected was similar to background levels observed when mock-infected cells were used in the assay.

To confirm that the complete loss in the content mixing activity in mutant $\Delta 19$ was a result of deletion of the F protein COOH-terminal and not due to another mutation that might have been inadvertently introduced in the F gene during mutagenesis, the back mutant (BWT) was made in which the translational stop codon was converted to leucine. As shown in Fig. 5, the content mixing activity was found to be completely restored in BWT confirming that the loss of the content mixing activity in mutant $\Delta 19$ is due to the lack of the F protein COOH-terminal domain. To determine whether the presence of the heterogeneous carbohydrate chains on the F₁ subunit of mutant $\Delta 19$ contributed to loss of content mixing activity, cells were treated with DMJ during expression of wt F protein and mutant $\Delta 19$ and during the content mixing assay. It was found that the content mixing activity of wt F protein was unaffected by DMJ treatment, and that treatment with DMJ did not restore detectable content mixing activity to mutant $\Delta 19$. Thus, taken together these findings indicate that, in contrast to the lipid mixing results, the removal of the COOH-terminal domain completely abolishes the content mixing activity.

Microscopy: Lipid dye but not aqueous dye spreads between RBCs and mutant $\Delta 19$ expressing cells under fusogenic conditions. To eliminate the possibility that the lipid mixing data and the content mixing data were reflecting properties of two subpopulations of F expressing cells, rather than being a property of the same cell, we used fluorescence microscopy. The F proteins with the most varied fusion promoting activity, wt F protein and mutant $\Delta 19$, were used for these experiments. First, we wished to show that the lipophilic probe R18, on fusion of the R18-labeled RBCs to F protein expressing cells was restricted to the lipid bilayer. As shown in Fig. 6, cells expressing wt F and mutant $\Delta 19$ proteins showed intense fluorescent staining of the lipid bilayer. For double-labeling of RBCs the dye NBD-*taurine* was trapped into the aqueous interiors and the lipid dye R18 was incorporated into the cell membranes. The colabeled RBCs were bound to wt F and mutant $\Delta 19$ expressing cells at 4°C. After incubation at 37°C, the cells were observed by fluorescence microscopy. The colabeled RBCs transferred R18 to both wt F and $\Delta 19$ expressing cells under fusogenic conditions (Fig. 7). However, only the wt F protein expressing cells took up the aqueous dye NBD-*taurine*. Mutant $\Delta 19$ expressing cells

bound to RBCs became labeled with the lipid probe R18 but no cells became labeled with the aqueous content probe. Control cells maintained at 4°C did not take up any of the dyes. Hence, even though mutant $\Delta 19$ can promote efficient transfer of outer leaflet lipids, it failed to promote transfer of cytoplasmic contents.

It has been shown recently that ability to detect small and/or transient fusion pores between RBCs and cells can be improved by loading RBCs with 1 mM ethidium bromide (EtBr) (mol wt 394) (Melikyan et al., 1995). Human RBCs do not contain a nucleus and are not stained strongly by EtBr. Upon fusion with cells containing a nucleus, EtBr intercalates into target cell DNA, leading to both a major increase in fluorescence quantum yield and irreversible accumulation in the cell nucleus. Thus, in an attempt to detect if small pore formation occurs with mutant $\Delta 19$ that could not be detected using NBD-*taurine*, we loaded RBCs with EtBr. Under fusogenic conditions, none of the cells expressing mutant $\Delta 19$ were stained with EtBr (Fig. 7). However, many of the wt F expressing cells were found to be stained with EtBr (Fig. 7). Very similar data were obtained using the DNA staining dye HOECHST 33258 (data not shown). Thus, the wt F protein expressing cells exhibited aqueous continuity with RBCs under fusogenic conditions whereas mutant $\Delta 19$ expressing cells did not, indicating that the presence of the F protein COOH-terminal domain is necessary for content mixing to occur.

Discussion

The data presented here indicate that progressive deletions in the COOH-terminal region of the SV5 F protein, causes an increasingly deleterious effect on the ability of the altered F proteins to mediate the final step in fusion, content mixing, whereas there is no discernable effect on the presumptive early step in fusion, hemifusion. Operationally, the transmembrane domain of a type I integral membrane protein is defined as a COOH terminally located hydrophobic region of ~20–30 residues that is usually bounded by two charged residues. In the case of the SV5 F protein, a computer-assisted analysis of the hydrophobicity of the COOH-terminal region suggests that the transmembrane domain is residues 485–510, which would yield a 20-residue cytoplasmic tail. However, the residues assigned to the transmembrane domain form a working hypothesis, especially as on the ectodomain side of the transmembrane domain there is not a charged residue until residue 471. Furthermore, some transmembrane domains are known to tolerate a charged residue and thus lysine residue 510 and the residues up to arginine residue 519 could form part of the transmembrane domain and if this is the case the F protein would only have a cytoplasmic tail of 11 residues.

The effect of the F protein truncation mutations on cytoplasmic content mixing was not secondary to a major defect in protein oligomerization, rate of intracellular transport, or cell surface expression level. Mutant $\Delta 19$ showed more heterogeneous carbohydrate modifications than wt F protein and this carbohydrate modification could be prevented by synthesis of $\Delta 19$ F protein in the presence of deoxymannojirimycin (DMJ), an inhibitor of α -mannosidase I. When $\Delta 19$ was synthesized in the presence of DMJ

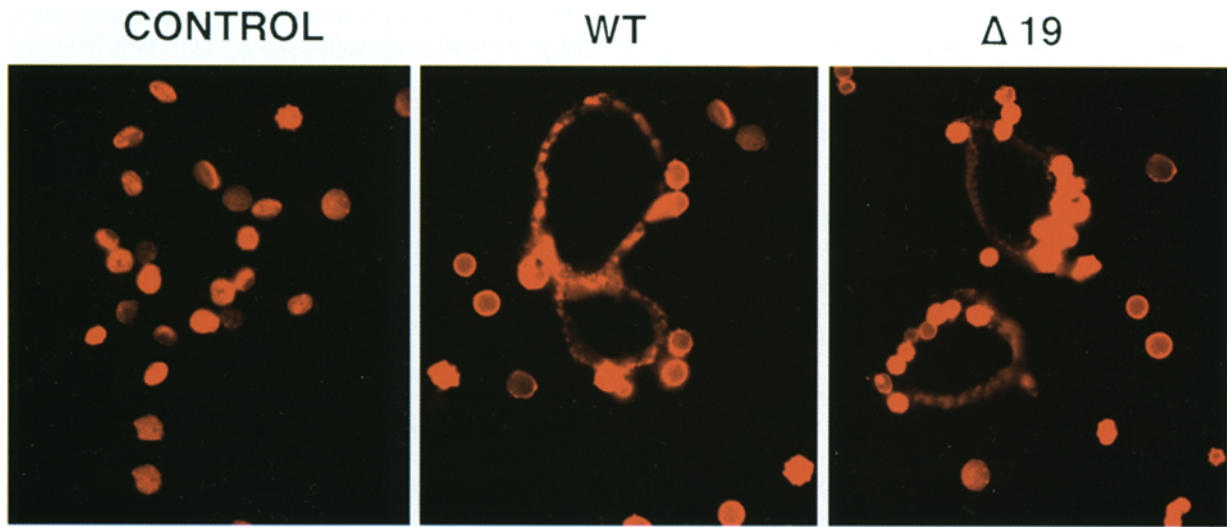


Figure 6. Lipid mixing with cells expressing wt F or mutant $\Delta 19$ F protein. RBCs labeled with only R18 were bound to CV-1 cells coexpressing SV5 HN and either wt F or mutant $\Delta 19$ F protein and fusion was triggered by shifting incubation to 37°C. After 10 min incubation at 37°C, the distribution of dye was monitored microscopically.

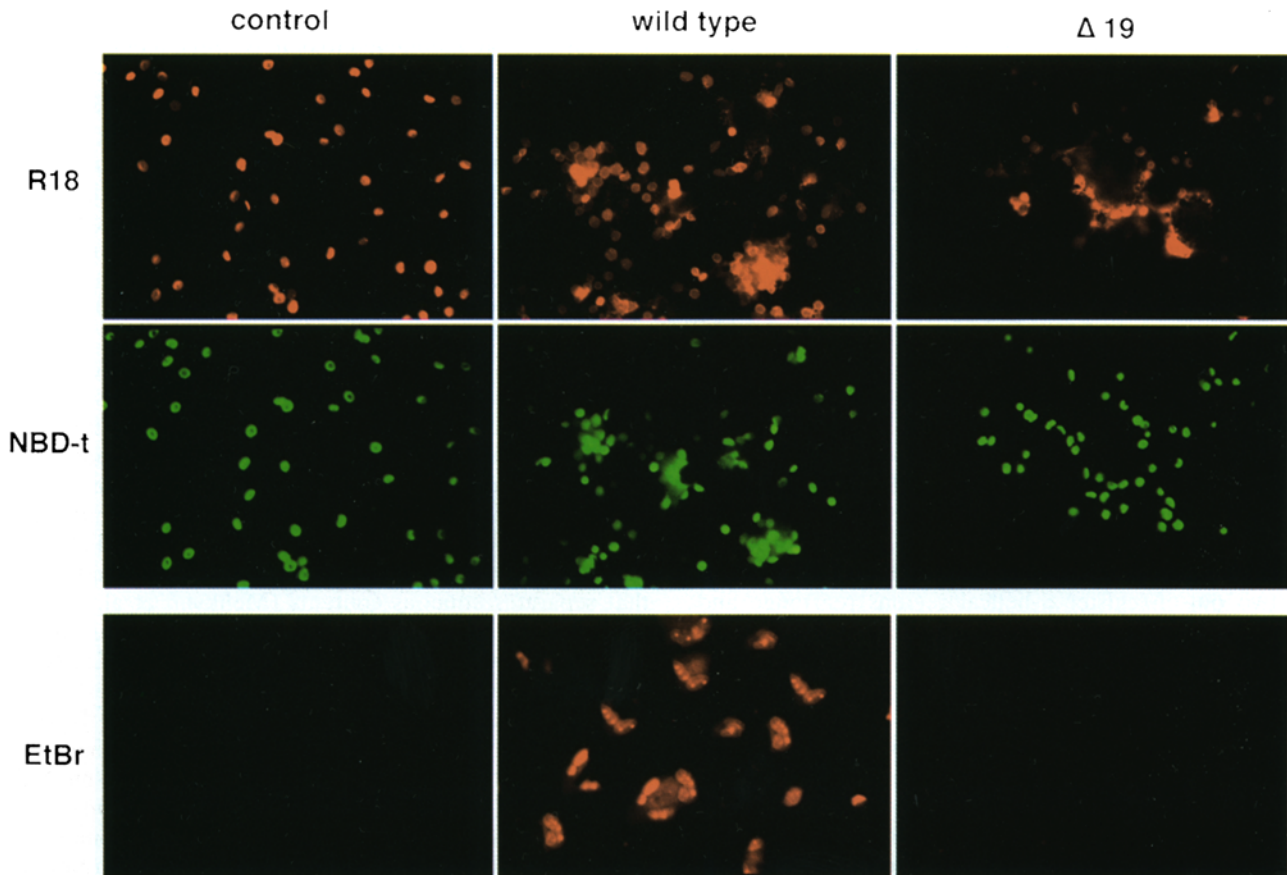


Figure 7. Lipid and aqueous content dye mixing with cells expressing wt F protein or truncation mutant $\Delta 19$ F protein. RBCs colabeled with R18 and NBD-*t*aurine or labeled singly with EtBr were bound to CV-1 cells coexpressing SV5 HN and either wt F protein or F $\Delta 19$ COOH-terminal truncation mutant protein and fusion was triggered by shifting incubation to 37°C. After 30 min incubation at 37°C, dye transfer from RBCs to cells was visualized in a fluorescent microscope using appropriate filters as described in Materials and Methods. Control panel shows the cells coexpressing SV5 HN and either wt F protein or the mutant $\Delta 19$ F protein bound to labeled RBCs and maintained at 4°C.

its fusion properties were unaffected, indicating that the carbohydrate modifications did not influence fusion activity. The reason that $\Delta 19$ F carbohydrate chains are modified differently from wt F protein is unknown but it is interesting to note that GPI-linked HA, unlike wt HA, is also modified by heterogeneous carbohydrate extensions (Kemble et al., 1993, 1994).

Alterations to the cytoplasmic tails of viral glycoproteins have varying effects on properties of the proteins. In some cases deletions or changes in the sequence of the cytoplasmic tail delay or inhibit intracellular transport of the proteins, e.g., paramyxovirus HN and VSV G protein (Parks and Lamb, 1990; Rose and Bergmann, 1983). In other cases deletion or changes in the sequence of the cytoplasmic tail have little effect on intracellular transport of the proteins, e.g., Semliki Forest virus E2 glycoprotein, influenza virus HA, Rous sarcoma virus env glycoprotein, Friend murine leukemia virus env glycoprotein (Garoff et al., 1983; Jin et al., 1996; Kilpatrick et al., 1987; Perez et al., 1987; Simpson and Lamb, 1992). For human immunodeficiency virus and simian immunodeficiency virus env glycoproteins deletion of the cytoplasmic tail does not affect intracellular transport but, by an unknown mechanism, it increases syncytia formation (Mulligan et al., 1992; Ritter et al., 1993). Even among paramyxovirus F protein, changes in the cytoplasmic tail have varying effects on intracellular transport (Sergel and Morrison, 1995; Yao and Compans, 1995). Deletion of the presumed cytoplasmic tail of the SV5 F protein has little effect on its intracellular transport, thus permitting determination of effects on fusion activity.

The mechanism by which the paramyxovirus F protein mediates fusion is not understood. It seems unlikely that the F protein hydrophobic fusion peptides are freely exposed to an aqueous environment after cleavage-activation (either in the *trans*-Golgi network or at the cell surface) as aggregation of the F protein oligomers would most likely occur. Thus, by analogy to influenza virus HA, it has been hypothesized that the cleaved F protein undergoes a conformational change to expose the fusion peptide at the right time and in the right place (Lamb, 1993; Sergel et al., 1993). For influenza virus HA the metastable cleaved neutral pH form of HA undergoes a low pH-induced conformational change to expose the buried fusion peptide (Skehel et al., 1982). From the X-ray structure of a proteolytic fragment of the low pH-induced form of HA, it is known that two out-of-register α -helical regions rearrange and form a triple stranded coiled-coil 105 Å in length, with the fusion peptide at the extremity of the coiled-coil (Bullough et al., 1994). Several studies have suggested that the paramyxovirus F fusion peptide forms an α helix (Brasseur et al., 1988; Hsu et al., 1981) and this putative α -helical domain is followed in the F₁ protein sequence by an out-of-register 42 residue heptad repeat (Chambers et al., 1990; Lamb, 1993). Thus, by analogy to influenza virus HA, it has been suggested that paramyxovirus F proteins may undergo a conformational change to form a related coiled-coil structure (Carr and Kim, 1993; Lamb, 1993). However, unlike influenza virus where the biologically relevant trigger to the conformational change is low pH, the trigger for the proposed paramyxovirus conformational change has not been identified.

Hemifusion is a presumptive intermediate event in the fusion process, in which the outer but not the inner leaflets of two fusing membranes have merged but there is no mixing of their aqueous contents. Ultrastructural studies on the steps of fusion of biomembranes have indicated that an intermediate single bilayer occurs but such intermediates are very transitory structures: e.g., in exocytosis of vascular endothelial vesicles (Palade and Bruns, 1968; Palade, 1975), zoospore secretion in *Phytophthora palmivora* (Pinto da Silva and Nogueira, 1977), myoblast fusion (Kalderon and Gilula, 1979), and the fusion of carrot protoplasts by poly (ethylene glycol) (Kanchanapoom and Boss, 1986). Other work using fluorescent probes to study the fusion of phospholipid vesicles has also shown that under certain conditions, an intermixing of lipid probes can occur without mixing of the vesicular contents (Chernomordik et al., 1995; Ellens et al., 1985; Fischer and Parker, 1984; Helm et al., 1989, 1992; Horn, 1984; Leventis et al., 1986; Neher, 1974). Such hemifused bilayer intermediates have been observed as stable states during the fusion of planar phospholipid bilayers in model membrane systems. Hemifusion has also been detected during artificially induced fusion between biological membranes such as during electrically induced fusion of RBCs (Song et al., 1991; Stenger and Hui, 1986) and PEG induced fusion of human erythrocytes (Ahkong et al., 1987). However, the only evidence for hemifusion in a well-characterized protein-mediated fusion system obtained to date is with a modified form of the influenza HA protein in which the transmembrane domain was substituted for a GPI anchor (Kemble et al., 1994). Our studies with the SV5 F tail truncation mutant $\Delta 19$ have demonstrated hemifusion with another fusion protein. For HA, removal of the cytoplasmic tail does not affect formation of the fusion pore or content mixing (Jin et al., 1996). However, as discussed above, removal of the HA transmembrane domain and replacement with a GPI-anchor abolishes pore formation (Kemble et al., 1994; Melikyan et al., 1995).

For influenza HA, it has been suggested that separate domains of HA may control individual steps in fusion and an elastic coupling model has been proposed (Melikyan et al., 1995). A mechanical coupling between the ectodomain and the transmembrane domain of HA is hypothesized to be responsible for pore formation within the hemifusion diaphragm and it is suggested that the hydrophilic cytoplasmic tail may also help in destabilizing the diaphragm that separates aqueous contents (Melikyan et al., 1995). We assume that HA and SV5 F protein mediate fusion by similar mechanisms. However, because of the difficulty in defining precisely the residues that constitute the SV5 F protein cytoplasmic tail, it cannot be determined if the HA and F protein cytoplasmic tails have differing effects on the final fusion event. If the SV5 F protein cytoplasmic tail consists of 20 residues, it is possible that the cytoplasmic tail is needed to maintain the transmembrane domain in an appropriate conformation which facilitates formation of the fusion pore but this interaction between the cytoplasmic tail and transmembrane domain is not required for HA mediated fusion. It is also possible that the hypothesized role of the cytoplasmic tail in destabilizing the hemifusion diaphragm is more critical in SV5 F protein-induced fusion. However, if the SV5 F protein cytoplasmic

tail only consists of 11 residues then removal of a part of the transmembrane domain in mutant $\Delta 19$, may directly affect fusion pore formation.

We thank Bernard Moss and Edward Berger (National Institutes of Health) for providing vTF7.3 and pGINT7 β -gal and Rebecca Dutch for helpful discussions.

This research was supported by Public Health Service Research grant AI-23173 from the National Institute of Allergy and Infectious Diseases. S. Bagai is an Associate and R.A. Lamb is an Investigator of the Howard Hughes Medical Institute.

Received for publication 20 May 1996 and in revised form 12 July 1996.

References

- Ahkong, Q.F., J.-P. Desmazes, D. Georgescauld, and J.A. Lucy. 1987. Movement of fluorescent probes in the mechanism of cell fusion induced by poly(ethylene glycol). *J. Cell Sci.* 88:389-398.
- Bagai, S., and R.A. Lamb. 1995a. Individual roles of N-linked oligosaccharide chains in intracellular transport of the paramyxovirus SV5 fusion protein. *Virology*. 209:250-256.
- Bagai, S., and R.A. Lamb. 1995b. Quantitative measurement of paramyxovirus fusion: differences in requirements of glycoproteins between simian virus 5 and human parainfluenza virus 3 or Newcastle disease virus. *J. Virol.* 69:6712-6719.
- Bagai, S., A. Puri, R. Blumenthal, and D.P. Sarkar. 1993. Hemagglutinin-neuraminidase enhances F protein-mediated membrane fusion of reconstituted Sendai virus envelopes with cells. *J. Virol.* 67:3312-3318.
- Brasseur, R., P. Lorge, E. Goormaghtigh, J.-M. Ruyschaert, D. Espion, and A. Burny. 1988. The mode of insertion of the paramyxovirus F1 N-terminus into lipid matrix, and initial step in host cell/virus fusion. *Virus Genes*. 4:325-332.
- Bullough, P.A., F.M. Hughson, J.J. Skehel, and D.C. Wiley. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature (Lond.)*. 371:37-43.
- Carr, C.M., and P.S. Kim. 1993. A spring-loaded mechanism for the conformational change of influenza haemagglutinin. *Cell*. 73:823-832.
- Chambers, P., C.R. Pringle, and A.J. Easton. 1990. Heptad repeat sequences are located adjacent to hydrophobic regions in several types of virus fusion glycoproteins. *J. Gen. Virol.* 71:3075-3080.
- Chernomordik, L., A. Chanturia, J. Green, and J. Zimmerberg. 1995. The hemifusion event and its conversion to complete fusion: regulation by membrane composition. *Biophys. J.* 69:922-929.
- Deng, W.P., and J.A. Nickoloff. 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.* 200:81-88.
- Ebata, S.N., M.J. Cote, C.Y. Kang, and K. Dimock. 1991. The fusion and hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus 3 are both required for fusion. *Virology*. 183:437-441.
- Ellens, H., J. Bentz, and F.C. Szoka. 1985. H^+ and Ca^{2+} -induced fusion and destabilization of liposomes. *Biochemistry*. 24:3099-3106.
- Ellens, H., S. Doxsey, J.S. Glenn, and J.M. White. 1989. Delivery of macromolecules into cells expressing a viral membrane fusion protein. *Methods Cell Biol.* 31:155-176.
- Fischer, L.R., and N.S. Parker. 1984. Osmotic control of bilayer fusion. *Biophys. J.* 46:253-258.
- Fuerst, T.R., E.G. Niles, F.W. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA*. 83:8122-8126.
- Garoff, H., C. Kondor-Koch, R. Petterson, and B. Burke. 1983. Expression of Semliki Forest virus proteins from complementary DNA. II. The membrane-spanning glycoprotein E2 is transported to the cell surface without its normal cytoplasmic tail. *J. Cell Biol.* 97:652-658.
- Gething, M.-J., K. McCammon, and F. Sambrook. 1989. Protein folding and intracellular transport. Evaluation of conformational changes in nascent exocytotic proteins. *Methods Cell Biol.* 32:185-206.
- Helm, C.A., J.N. Israelachvili, and P.M. McGuigan. 1989. Molecular mechanisms and forces involved in the adhesion and fusion of amphiphilic bilayers. *Science (Wash. DC)*. 246:919-922.
- Helm, C.A., J.N. Israelachvili, and P.M. McGuigan. 1992. Role of hydrophobic forces in bilayer adhesion and fusion. *Biochemistry*. 31:1794-1805.
- Horn, R.G. 1984. Direct measurement of the force between two lipid bilayers and observation of their fusion. *Biochim. Biophys. Acta*. 778:224-228.
- Horvath, C.M., and R.A. Lamb. 1992. Studies on the fusion peptide of a paramyxovirus fusion glycoprotein: roles of conserved residues in cell fusion. *J. Virol.* 66:2443-2455.
- Horvath, C.M., R.G. Paterson, M.A. Shaughnessy, R. Wood, and R.A. Lamb. 1992. Biological activity of paramyxovirus fusion proteins: factors influencing formation of syncytia. *J. Virol.* 66:4564-4569.
- Hsu, M.-C., A. Scheid, and P.W. Choppin. 1981. Activation of the Sendai virus fusion protein (F) involved a conformational change with exposure of a new hydrophobic region. *J. Biol. Chem.* 156:3557-3563.
- Hu, X., R. Ray, and R.W. Compans. 1992. Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza viruses. *J. Virol.* 66:1528-1534.
- Jin, H., K. Subbarao, S. Bagai, G.P. Leser, B.R. Murphy, and R.A. Lamb. 1996. Palmitoylation of the influenza virus hemagglutinin (H3) is not essential for virus assembly or infectivity. *J. Virol.* 70:1406-1414.
- Kalderon, N., and N.B. Gilula. 1979. Membrane events involved in myoblast fusion. *J. Cell Biol.* 81:411-425.
- Kanchanapoom, K., and W.F. Boss. 1986. Osmoregulation of fusogenic protoplast fusion. *Biochim. Biophys. Acta*. 861:429-439.
- Kemble, G.W., T. Danieli, and J.M. White. 1994. Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell*. 76:383-391.
- Kemble, G.W., Y.I. Henis, and J.M. White. 1993. GPI- and transmembrane-anchored influenza hemagglutinin differ in structure and receptor binding activity. *J. Cell Biol.* 122:1253-1265.
- Kilpatrick, D.R., R.V. Srinivas, E.B. Stephens, and R.W. Compans. 1987. Effect of deletion of the cytoplasmic domain upon surface expression and membrane stability of a viral glycoprotein. *J. Biol. Chem.* 262:16116-16121.
- Klenk, H.-D., and W. Garten. 1994. Host cell proteases controlling virus pathogenicity. *Trends Microbiol.* 2:39-43.
- Lamb, R.A. 1993. Paramyxovirus fusion: a hypothesis for change. *Virology*. 197:1-11.
- Lamb, R.A., and P.W. Choppin. 1976. Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P polypeptides, from RNA produced by primary transcription. *Virology*. 74:504-519.
- Lamb, R.A., P.R. Etkind, and P.W. Choppin. 1978. Evidence for a ninth influenza viral polypeptide. *Virology*. 91:60-78.
- Leventis, R., J. Gagne, N. Fuller, R.R. Rand, and J.R. Silvius. 1986. Divalent cation-induced fusion and lipid lateral segregation in phosphatidylcholine-phosphatidic acid vesicles. *Biochemistry*. 25:6978-6987.
- Melikian, G.B., J.M. White, and F.S. Cohen. 1995. GPI-anchored influenza hemagglutinin induces hemifusion to both red blood cell and planar bilayer membranes. *J. Cell Biol.* 131:679-691.
- Morrison, T., C. McQuain, and L. McGinnes. 1991. Complementation between avirulent Newcastle disease virus and a fusion protein gene expressed from a retrovirus vector: requirements for membrane fusion. *J. Virol.* 65:813-822.
- Morrison, T.G., L.J. Ward, and A. Semerjian. 1985. Intracellular processing of the Newcastle disease virus fusion glycoprotein. *J. Virol.* 53:851-857.
- Mulligan, M.J., G.V. Yamshchikov, G.D. Ritter, J. Gao, F., M.J. Jin, C.D. Nail, C.P. Spies, B.H. Hahn, and R.W. Compans. 1992. Cytoplasmic domain truncation enhances fusion activity by the exterior glycoprotein complex of human immunodeficiency virus type 2 in selected cell types. *J. Virol.* 66:3971-3975.
- Neher, E. 1974. Asymmetric membranes resulting from the fusion of two black lipid membranes. *Biochim. Biophys. Acta*. 373:327-336.
- Ng, D.T., R.E. Randall, and R.A. Lamb. 1989. Intracellular maturation and transport of the SV5 type II glycoprotein hemagglutinin-neuraminidase: specific and transient association with GRP78-BiP in the endoplasmic reticulum and extensive internalization from the cell surface. *J. Cell Biol.* 109:3273-3289.
- Nussbaum, O., C.C. Broder, and E.A. Berger. 1994. Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. *J. Virol.* 68:5411-5422.
- Palade, G.E. 1975. Intracellular aspects of the process of protein synthesis. *Science (Wash. DC)*. 189:347-358.
- Palade, G.E., and R.R. Bruns. 1968. Structural modifications of plasmalemmal vesicles. *J. Cell Biol.* 37:633-649.
- Parks, G.D., and R.A. Lamb. 1990. Defective assembly and intracellular transport of mutant paramyxovirus hemagglutinin-neuraminidase proteins containing altered cytoplasmic domains. *J. Virol.* 64:3605-3616.
- Paterson, R.G., and R.A. Lamb. 1987. Ability of the hydrophobic fusion-related external domain of a paramyxovirus F protein to act as a membrane anchor. *Cell*. 48:441-452.
- Paterson, R.G., and R.A. Lamb. 1993. The molecular biology of influenza viruses and paramyxoviruses. In *Molecular Virology: A Practical Approach*. Davidson, A., and R.M. Elliott, editors. IRL Oxford University Press, Oxford. pp. 35-73.
- Paterson, R.G., T.J.R. Harris, and R.A. Lamb. 1984a. Analysis and gene assignment of mRNAs of a paramyxovirus, simian virus 5. *Virology*. 138:310-323.
- Paterson, R.G., T.J.R. Harris, and R.A. Lamb. 1984b. Fusion protein of the paramyxovirus simian virus 5: nucleotide sequence of mRNA predicts a highly hydrophobic glycoprotein. *Proc. Natl. Acad. Sci. USA*. 81:6706-6710.
- Paterson, R.G., S.W. Hiebert, and R.A. Lamb. 1985. Expression at the cell surface of biologically active fusion and hemagglutinin-neuraminidase proteins of the paramyxovirus simian virus 5 from cloned cDNA. *Proc. Natl. Acad. Sci. USA*. 82:7520-7524.
- Perez, L.G., G.L. Davis, and E. Hunter. 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. *J. Virol.* 61:2981-2988.
- Pinto da Silva, P., and M.L. Nogueira. 1977. Membrane fusion during secretion. A hypothesis based on electron microscope observation of *Phytophthora palmivora* zoospores during encystment. *J. Cell Biol.* 73:161-181.
- Randall, R.E., D.F. Young, K.K.A. Goswami, and W.C. Russell. 1987. Isolation

- and characterization of monoclonal antibodies to simian virus 5 and their use in revealing antigenic differences between human, canine and simian isolates. *J. Gen. Virol.* 68:2769-2780.
- Ritter, G.D., M.J. Mulligan, S. Lydy, and R.W. Compans. 1993. Cell fusion activity of the simian immunodeficiency virus envelope protein is modulated by the intracytoplasmic domain. *Virology*. 197:255-264.
- Rose, J.K., and J.E. Bergmann. 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. *Cell*. 34:513-524.
- Rose, J.K., L. Buonocore, and M.A. Whitt. 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *Biotechnology*. 10:520-525.
- Russell, R., R.G. Paterson, and R.A. Lamb. 1994. Studies with cross-linking reagents on the oligomeric form of the paramyxovirus fusion protein. *Virology*. 199:160-168.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 74:5463-5467.
- Sarkar, D.P., S.J. Morris, O. Eidelman, J. Zimmerberg, and R. Blumenthal. 1989. Initial stages of influenza hemagglutinin-induced cell fusion monitored simultaneously by two fluorescent events: cytoplasmic continuity and lipid mixing. *J. Cell Biol.* 109:113-122.
- Scheid, A., and P.W. Choppin. 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity of proteolytic cleavage of an inactive precursor protein of Sendai virus. *Virology*. 57:475-490.
- Scheid, A., L.A. Caliguri, R.W. Compans, and P.W. Choppin. 1972. Isolation of paramyxovirus glycoproteins. Association of both hemagglutinating and neuraminidase activities with the larger SV5 glycoprotein. *Virology*. 50:640-652.
- Sergel, T., and T.G. Morrison. 1995. Mutations in the cytoplasmic domain of the fusion glycoprotein of Newcastle disease virus depress syncytia formation. *Virology*. 210:264-272.
- Sergel, T., L.W. McGinnes, M.E. Peeples, and T.G. Morrison. 1993. The attachment function of the Newcastle disease virus hemagglutinin-neuraminidase protein can be separated from fusion promotion by mutation. *Virology*. 193:717-726.
- Sergel-Germano, T., C. McQuain, and T.G. Morrison. 1994. Mutations in the fusion peptide and heptad repeat regions of the Newcastle disease virus fusion peptide blocks fusion. *J. Virol.* 68:7654-7658.
- Simpson, D.A., and R.A. Lamb. 1992. Alterations to influenza virus hemagglutinin cytoplasmic tail modulate virus infectivity. *J. Virol.* 66:790-803.
- Skehel, J.J., P.M. Bayley, E.B. Brown, S.R. Martin, M.D. Waterfield, J.M. White, I.A. Wilson, and D.C. Wiley. 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc. Natl. Acad. Sci. USA*. 79:968-972.
- Song, L., Q.F. Ahkong, D. Georgescauld, and J.A. Lucy. 1991. Membrane fusion without cytoplasmic fusion (hemifusion) in erythrocytes that are subjected to electrical breakdown. *Biochim. Biophys. Acta*. 1065:54-62.
- Stegmann, T., R.W. Doms, and A. Helenius. 1989. Protein mediated membrane fusion. *Annu. Rev. Biophys. Biophys. Chem.* 18:187-211.
- Stenger, D.A., and S.W. Hui. 1986. Kinetics of ultrastructural changes during electrically induced fusion of human erythrocytes. *J. Membr. Biol.* 93:43-53.
- Tanabayashi, K., K. Takeuchi, K. Okazaki, M. Hishiyama, and A. Yamada. 1992. Expression of mumps virus glycoproteins in mammalian cells from cloned cDNAs: both F and HN proteins are required for cell fusion. *Virology*. 187:801-804.
- Wang, C., G. Raghu, T. Morrison, and M.E. Peeples. 1992. Intracellular processing of the paramyxovirus F protein: critical role of the predicted amphipathic α -helix adjacent to the fusion domain. *J. Virol.* 66:4161-4169.
- Ward, C.D., R.G. Paterson, and R.A. Lamb. 1995. Mutants of the paramyxovirus SV5 fusion protein: regulated and extensive syncytium formation. *Virology*. 209:242-249.
- White, J.M. 1990. Viral and cellular membrane fusion proteins. *Annu. Rev. Physiol.* 52:675-697.
- Wild, T.F., E. Malvoisin, and R. Buckland. 1991. Measles virus: both the haemagglutinin and fusion glycoproteins are required for fusion. *J. Gen. Virol.* 72:439-442.
- Yao, Q., and R.W. Compans. 1995. Differences in the role of the cytoplasmic domain of human parainfluenza virus fusion proteins. *J. Virol.* 69:7045-7053.