Intracellular Processing and Transport of NH$_2$-Terminally Truncated Forms of a Hemagglutinin–Neuraminidase Type II Glycoprotein

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Abstract. Six amino-terminal deletion mutants of the NH$_2$-terminally anchored (type II orientation) hemagglutinin–neuraminidase (HN) protein of parainfluenza virus type 3 were expressed in tissue culture by recombinant SV-40 viruses. The mutations consisted of progressive deletions of the cytoplasmic domain and, in some cases, of the hydrophobic signal/anchor. Three activities were dissociated for the signal/anchor: membrane insertion, translocation, and anchoring/transport. HN protein lacking the entire cytoplasmic tail was inserted efficiently into the membrane of the endoplasmic reticulum but was translocated inefficiently into the lumen. However, the small amounts that were successfully translocated appeared to be processed subsequently in a manner indistinguishable from that of parental HN. Thus, the cytoplasmic domain was not required for maturation of this type II glycoprotein. Progressive deletions into the membrane anchor restored efficient translocation, indicating that the NH$_2$-terminal 44 amino acids were fully dispensable for membrane insertion and translocation and that a 10-amino acid hydrophobic signal sequence was sufficient for both activities. These latter HN molecules appeared to be folded authentically as assayed by hemagglutination activity, reactivity with a conformation-specific antiserum, correct formation of intramolecular disulfide bonds, and homooligomerization. However, most (85–90%) of these molecules accumulated in the ER. This showed that folding and oligomerization into a biologically active form, which presumably represents a virion spike, occurs essentially to completion within that compartment but is not sufficient for efficient transport through the exocytotic pathway. Protein transport also appeared to depend on the structure of the membrane anchor. These latter mutants were not stably integrated in the membrane, and the small proportion (10–15%) that was processed through the exocytotic pathway was secreted. The maturation steps and some of the effects of mutations described here for a type II glycoprotein resemble previous observations for prototypic type I glycoproteins and are indicative of close similarities in these processes for proteins of both membrane orientations.

THE majority of integral membrane glycoproteins span the membrane once and are oriented with either their carboxy-terminal (type I) or amino-terminal (type II) amino acids exposed to the cytoplasm. Type I glycoproteins typically have a cleavable signal sequence at the amino terminus, a carboxy-proximal hydrophobic membrane anchor and a carboxy-terminal hydrophilic cytoplasmic tail. Type II glycoproteins are characterized by an amino-terminal cytoplasmic tail, followed by a 20–30-amino acid hydrophobic transmembrane domain, with the remainder of the protein being oriented externally, comprising the ectodomain.

The intracellular processing and transport of membrane glycoproteins has been the object of extensive study. Eluci-

dation of the function of signal sequences and their role in signal recognition particle-mediated translocation of nascent polypeptides across the membrane of the RER has defined major steps in membrane insertion and translocation (1, 22, 28, 29, 32, 39, 40, 43, 44). With regard to type II glycoproteins, site-specific mutagenesis experiments with the influenza neuraminadase protein and the transferrin receptor have established that the amino-proximal hydrophobic region functions as a noncleaved signal/membrane anchor sequence (3, 21, 44).

Major advances have also been made in characterizing the steps in polypeptide maturation that can occur following translocation across the ER, including intramolecular folding, disulfide bond formation, and oligomerization (6, 10, 13, 17, 23, 24, 30, 38, 41, 42). However, most of these studies have involved type I glycoproteins such as the influenza virus hemagglutinin and the VSV attachment G glycoprotein.

The hemagglutinin-neuraminidase (HN)[1] protein of human parainfluenza type 3 virus (PIV3) is a type II surface glycoprotein that contains a 31-amino acid cytoplasmic domain, a 22-amino acid putative signal/anchor sequence, and a 516-amino acid ectodomain (11). There are four potential acceptor sites for N-linked glycosylation within the predicted ectodomain. The HN protein is responsible for virus attachment to sialic acid-containing cellular receptors, and also has a neuraminidase (or sialidase) activity (5, 33).

Using oligonucleotide directed mutagenesis directly on a single-stranded copy of an SV-40 expression vector containing the HN gene, we generated a series of six amino-terminal deletion mutants of the HN protein and expressed them from recombinant SV-40 viruses. The resulting HN proteins were analyzed for intracellular expression, biological activity, glycosylation pattern, reactivity with convalescent (conformation-specific) versus antipeptide serum, formation of intracellular disulfide bonds and homooligomers, transport to the cell surface and secretion. The purpose of these experiments was to (a) analyze more precisely the role of the cytoplasmic tail and anchor regions of the HN protein in membrane insertion and intracellular transport, (b) identify possible intermediates in the folding and intracellular transport pathway, and (c) identify possible secreted forms of the HN protein which, when expressed from a high-expression mammalian or insect system (5, 33) could be used to produce purified protein for vaccine and structural studies.

Materials and Methods

SV-40-HN Recombinant Viruses

A cDNA containing the coding sequence of the HN gene flanked by Bam HI restriction sites within three to six nucleotides of the translational initiation and termination sites (33) was inserted into the unique Bam HI site of the SV-40-pBR322 shuttle vector pSV2330 (21, 26). The SV40 moiety of pSV2330 contains deletions at bp 335–521 and 832–2538; the latter deletion is replaced by a unique Bam HI site, and foreign DNAs inserted in the Bam HI site are under the control of the SV-40 late promotor. To construct a new shuttle vector to facilitate mutagenesis, this recombinant vector was cut with Xba I to separate SV-40 and plasmid sequences, and the SV-40 moiety bearing the HN cDNA was inserted into the Xba I site of pTZ18R (Pharmacia Fine Chemicals, Piscataway, NJ), a plasmid vector that contains the origin of replication of bacteriophage M13. A single-strand template (positive sense with regard to the HN cDNA) was prepared according to suppliers' protocols. Mutagenesis was performed according to Zoller and Smith (45). Appropriate nucleotides were deleted from the beginning of the HN open reading frame while preserving the Bam HI site and the initiating methionine codon. The negative sense oligonucleotides used were as follows, with the Bam HI site and initiating methionine codon underlined:

dl10, 5′ ATTACCAGCATCTTTCATCTCGGATCCGGC;
dl20, 5′ GTTGCCATGAGTAGCCATCTCGGATCCGGC;
dl31, 5′ CCATAAATATATATGTTATCATCTCGGATCCGGC;
dl40, 5′ TATTGATAATAACACCAGCATCTCGGATCCGGC;
dl44, 5′ CACTATGATGAAGACTATTGACATCTCGGATCC;
dl173, 5′ TTCTGTAACTTCCATGGTGGATCCGTTTACGTCTTGTAG.

Mutant cDNAs were identified by conventional methods of colony hybridization using graded wash temperatures, and the fidelity of the mutagenesis was confirmed by restriction site analysis and dideoxynucleotide sequencing. Mutant cDNAs were purified and recloned by transformation to insure segregation from the parental plasmid. 5–10 µg of each recombinant vector was cut with Xba I to separate plasmid and SV-40 sequences, ethanol

precipitated and circularized by ligation in an 800-µl reaction. Similarly, the SV-40-pBR322 shuttle vector pSVrINS7 (18) was digested with Eco RI to separate the plasmid and SV-40 sequences; the SV-40 moiety of pSVrINS7 contains a defective early region and was used here as a helper DNA. Equal amounts of recombinant and helper SV-40 DNAs were mixed and used to cotransfect 10[5] secondary African Green Monkey (AGMK) cells (Microbiological Associates Bioproducts, Walkersville, MD) using DEAE-dextran (8). Recombinant SV-40 virus was harvested from the media 10–14 d transfection and diluted 1:5 for use in subsequent infections.

Antisera

Convalescent PIV3-specific hyperimmune antiserum (a generous gift of Dr. Gregory Prince, Johns Hopkins University, Baltimore, MD) was generated by three rounds (2-wk intervals) of intranasal infection of cotton rats (Sigmodon hispidus) with 10[6] plaque-forming units PIV3 per animal. Antiserum against denatured PIV3 was prepared as follows: gradient-purified PIV3 was heated at 95°C for 5 min in the presence of 0.5% SDS and 0.5% 2-mercaptoethanol, diluted fivefold, and was used to immunize cotton rats by intramuscular injection in the presence of CFA; followed by a second immunization in incomplete adjuvant 2 wk later. Because cotton rat immunoglobulin is not efficiently bound by the Staphylococcus aureus A protein, a second antibody prepared by sequential immunization of rabbits with purified cotton rat IgG was added before collecting the antibody-antigen complexes with Sepharose beads containing protein A (Pharmacia Fine Chemicals) (26, 33).

Antipeptide serum (a generous gift from Dr. John Rice (Battelle Inc., Columbus, OH) was raised in rabbits to a 24-amino acid peptide derived from the sequence of the bovine parainfluenza virus type 3 HN protein. The peptide, LVPDLNPRVTHTFNIDDNRKSCSL corresponds to a closely related region in human PIV3 at amino acids 235–258, differing at positions 243 (V to I), 244 (T to S), and 250 (D to N). In two experiments (Figs. 4 and 7), an additional rabbit antiserum was used that had been raised against a synthetic peptide representing the NH_2-terminal 12 amino acids of the parental HN protein followed by a cysteine residue for attachment to keyhole limpet hemocyanin. The two antipeptide sera were essentially indistinguishable with regard to their ability to react with both native and denatured forms of the HN protein.

Radiolabeling, Endo H Digestion, and the Na_2CO_3 Fractionation of HN Protein

At the onset of cytopathology, infected AGMK cells were starved for 15 min in MEM lacking methionine and labeled with 100 µCi per ml [35S]methionine for indicated times. Lysates were prepared and HN protein was immunoprecipitated as described (26, 33). For endo-H digestion, immunoprecipitated proteins were eluted from protein-A Sepharose beads in 10 mM Tris pH 7.4, 10 mM DTT, 1 mM EDTA by boiling 3 min. An equal volume of 100 mM citrate buffer pH 5.3 and 2–4 mU endo H (Boehringer Mannheim Biochemicals, Indianapolis, IN) were added to each 40-µl reaction. Incubation was overnight at 37°C.

Sonicated radiolabeled cell lysates were separated into microsomal and cytoplasmic fractions by centrifugation at 200,000 g for 15 min. The microsomal pellet was treated with 100 mM Na_2CO_3 pH 11 according to Fujiki et al. (12). Proteins from the cytosol, after pH 11 treatment microsomal membrane and after pH 11 treatment supernatant fractions were immunoprecipitated as described above, boiled in sample buffer and analyzed by electrophoresis on 10% polyacrylamide gels in the presence of SDS-PAGE. The gel sample buffer contained 5% 2-mercaptoethanol except where noted in Figs. 6 and 7.

Hemadsorption and Hemagglutination

Infected cell monolayers were tested for their ability to hemadsorb by incubation with 0.1% (vol/vol) guinea pig erythrocytes for 30 min at 4°C. Hemagglutination titers were determined by performing serial twofold dilutions of tissue culture supernatants or sonicated cell lysates (corresponding to 10[4] cells) in 96-well microtiter dishes. Equal volumes of 0.5% guinea pig erythrocytes were added and samples were read after 1-h incubation at room temperature. To compare hemagglutination titers with levels of expressed intracellular protein, parallel cultures of infected cells were radiolabeled, immunoprecipitated with a mixture of convalescent cotton rat and antipeptide serum under conditions of antibody excess, and analyzed by SDS-PAGE. HN protein was excised from the gel and quantitated by liquid scintillation counting.

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1. Abbreviations used in this paper: AGMK, African green monkey cells; DTBP, dimethyl 3,3′ dithiobispropionimidate; HN, hemagglutinin-neuraminidase.

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                          10        20        30        40        50
                       -  +     +-  -  -       -     +
Parental   MEYWKHTNHGKDAGNELETSMATHGNKITNKITYILWTIILVLLSIVFIIVLINSIK...

d110                   MKDAGNELETSMATHGNKITNKITYILWTIILVLLSIVFIIVLINSIK...

d120                            MATHGNKITNKITYILWTIILVLLSIVFIIVLINSIK...

d131                                  MITYILWTIILVLLSIVFIIVLINSIK..

d140                                       MLVLLSIVFIIVLINSIK...

d144                                          MSIVFIIVLINSIK...

d173                                                  (74-MEVTE...)
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Figure 1. Amino acid sequences at the NH$_2$ terminus of expressed parent and mutant forms of the HN protein. Oligonucleotide mutagenesis was used to construct progressive NH$_2$-terminal deletions of the HN protein. The mutant designations (dl10, etc.) describe the number of NH$_2$-terminal amino acids deleted in each mutant relative to the parental sequence; in most cases an exogenous methionine start codon was inserted as a translational start site, and this residue is not counted. The hydrophobic signal/anchor region is underlined. Charged residues in the cytoplasmic tail are indicated.

In Vitro Translation and Transmembrane Insertion

The cDNAs for the parental HN, dl31, and dl73 proteins were cloned into the Bam HI site of the plasmid pGem3Zf+ (Promega Biotec, Madison, WI), and recombinants in the clockwise orientation were identified using conventional procedures. Purified plasmids were linearized by digestion with Xba I and used as template for the in vitro synthesis of mRNA by T7 phage polymerase (Promega Biotec). The transcripts were capped by GMP using vaccinia virus guanylyltransferase (Bethesda Research Laboratories, Gaithersburg, MD). Capped mRNAs that were prepared by the alternate procedure of transcription in the presence of m^{7}G(5')ppp(5')G (Pharmacia Fine Chemicals) yielded essentially identical results (not shown). In vitro translation and transmembrane insertion were performed in rabbit reticulocyte lysates using dog pancreatic membranes (Promega Biotec) in the presence of [^{35}S]methionine. All procedures followed the suggested protocols of the suppliers. For trypsin digestions, aliquots of the translation mixes were added to equal volumes of 0.1 M NaCl containing 1 mM MgCl, 10 mM Tris hydrochloride, pH 7.4 and twice the desired final concentration of TPCK-treated trypsin (Worthington Biomedicals). Incubation was at room temperature for 30 min and was terminated by the additions of aprotinin to 0.2 mg/ml and phenylmethyl fluoride to 10 mM. Samples were adjusted to the composition of standard SDS-PAGE sample buffer, immediately boiled for 5 min, and analyzed by SDS-PAGE.

Results

Expression of Parental and Mutant Forms of the HN Proteins

Site-directed mutagenesis was used to modify the parental HN cDNA to encode proteins with progressive NH$_2$-terminal deletions (Fig. 1). Previous work with both type II and type I glycoproteins suggested that positive charges in the cytoplasmic domain facilitate association of this region with the cytosol and might assist in positioning the anchored protein in the membrane (1, 7, 19, 32, 35). Parental HN protein possesses four positively charged (Lys) and four negatively charged (Asp, Glu) amino acids within the NH$_2$-terminal 31 residues that comprise the cytoplasmic tail. Three deletion mutations were constructed to examine the requirements for length and charge in this region. Mutant dl10 lacks the first 10 NH$_2$-terminal amino acids, leaving a truncated cytoplas-

mic domain that contains three positive and three negative charges; dl20 lacks the first 20 amino acids, leaving two positive charges in the remaining tail; and dl31 lacks all 31 amino acids of the cytoplasmic domain (Fig. 1).

The HN protein has 22 uncharged hydrophobic amino acids in its transmembrane domain (residues 31–53). We constructed a mutant, dl40, which lacks the first 40 NH$_2$-terminal amino acids leaving a truncated transmembrane domain of 14 amino acids. Another mutant, dl44, was constructed that lacked the first 44 amino acids and contained only ten remaining residues to act as a signal/anchor sequence. A sixth mutant, dl73, lacked all of the cytoplasmic tail, transmembrane domain and the first 20 amino acids of the ectodomain (Fig. 1).

Infectious recombinant SV-40 viruses bearing the parental or truncated HN gene were prepared and used to infect AGMK cell monolayers, and [^{35}S]methionine-labeled HN proteins were immunoprecipitated using convalescent PIV3 antiserum and analyzed by SDS-PAGE (Fig. 2). The HN proteins expressed by the parental recombinant, dl10, dl20, dl40, and dl44, reacted strongly with the convalescent antisera and had apparent molecular weights which were in good agreement with the values predicted for glycosylated, truncated forms of the HN protein (Fig. 2 A). When this experiment was repeated using [^{3}H]glucosamine-labeled infected cell lysates, the pattern of immunoprecipitated proteins (not shown) was essentially identical to that shown in Fig. 2 A.

The relative levels of accumulation of the immunoprecipitated parental and truncated HN proteins were compared by liquid scintillation counting of gel bands from three separate experiments such as that shown in Fig. 2 A under conditions of antibody excess. The infections were performed under conditions where the number of SV-40-infected cells were similar. The levels of accumulation of mutants dl10, dl20, dl40, and dl44 were 66% or more of that of parental HN protein expressed in parallel. Thus, removal of up to 20 residues of the cytoplasmic tail (lanes *dl10, dl20*) or removal
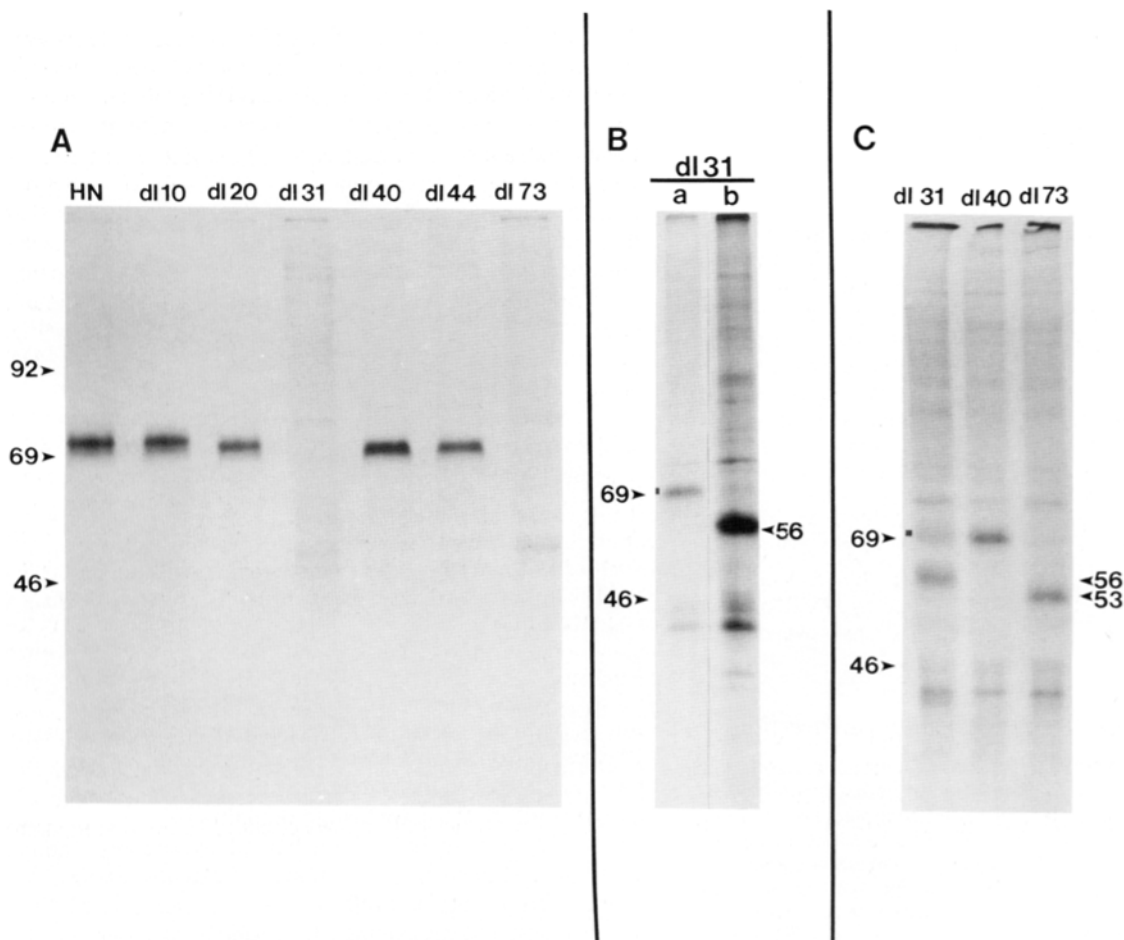
Figure 2. Intracellular expression of parental and truncated forms of the HN protein. Recombinant SV-40 virus-infected cells were labeled for 1 h with 100 μCi/ml [³⁵S]methionine. HN protein was immunoprecipitated from cell lysates using convalescent antiserum (*A* and *B*, lane *a*) or an anti-HN peptide serum (*B*, lane *b*) or both antisera (*C*). As described in the text, the convalescent antiserum was specific for conformationally authentic HN protein, whereas antipeptide serum reacted most efficiently with denatured HN protein. Molecular weight markers (in kilodaltons) are indicated on the left of each panel. In *B* and *C*, the truncated HN proteins are indicated on the right with arrows and estimated molecular weights in kilodaltons.

of 40 or 44 NH₂-terminal amino acids (lanes *dl40, dl44*), leaving 14 or 10 residues in the transmembrane domain, respectively, did not greatly reduce the association of nascent polypeptide with the membrane of the ER, or the subsequent translocation, glycosylation, and folding into a conformation recognized by convalescent antiserum.

In contrast, the immunoprecipitation of either dl31- or dl73-infected cell extracts with convalescent serum did not result in the detection of a major [³⁵S]methionine-labeled or [³H]glucosamine-labeled species (Fig. 2 *A*). The HN protein encoded by dl73 completely lacks the NH₂-proximal hydrophobic domain that is thought to be the signal for protein translocation. The lack of convalescent antibody recognition with the polypeptide product of dl73 might have been due to decreased synthesis, decreased stability and/or altered immunoreactivity of a cytoplasmic form of HN (see below).

In the case of the dl31-expressed protein, longer film exposures of fluorograms such as that shown in Fig. 2 *A* revealed a faint 69-kD species that could be labeled with [³⁵S]methionine (Fig. 2 *B*, lane *a*) or [³H]glucosamine (not shown). As described below, this minor band appeared to be

a form of dl31-encoded HN protein. This low level of immunoprecipitable dl31 protein was surprising because dl31 retains the complete transmembrane domain and might have been expected to be synthesized and translocated at least as efficiently as the mutant proteins with more truncated signal/anchor domains (dl40, dl44). To insure that this reduced accumulation was not the result of spurious damage in the SV-40 expression vector during mutagenesis, the dl31 cDNA sequence was confirmed by dideoxynucleotide sequencing and recloned into a preparation of pSV2330 that had not been used in site-directed mutagenesis. The resulting recombinant virus gave identical results to those obtained with the original dl31 mutant.

The convalescent serum had been generated in response to virus replication in the host respiratory tract and therefore might react preferentially with native HN protein. This was confirmed in pulse–chase radiolabeling experiments: the convalescent serum did not react detectably with newly synthesized HN protein after a 5-min labeling period, but immunoreactivity was acquired during a subsequent 10–20-min chase (unpublished data). HN-specific antipeptide serum
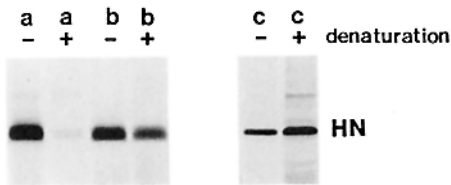
Figure 3. Differential reactivities of antisera with native and denatured HN protein. Cells infected with SV-40 recombinant virus expressing parental HN protein were labeled with [³⁵S]methionine and lysed in immunoprecipitation buffer. To prepare denatured HN protein, an aliquot was heated at 95°C for 5 min in the presence of 2% SDS and 0.5% 2-mercaptoethanol. The denatured HN protein sample was diluted 20-fold, adjusted to the composition of immunoprecipitation buffer, and analyzed by immunoprecipitation. A duplicate aliquot of cell lysate that had not been exposed to high concentrations of denaturants or heat was diluted and analyzed in parallel as native HN. The antisera were: (a) convalescent (post PIV3 infection) cotton rat serum; (b) serum from cotton rats immunized with PIV3 that had been denatured with heat in the presence of SDS and 2-mercaptoethanol; and (c) rabbit antipeptide serum. The immunoprecipitates were analyzed by SDS-PAGE, and fluorograms of portions of two different gels are shown.

(see Materials and Methods) reacted with both the early and chased forms (not shown). Similar observations regarding the maturation of immunoreactivity have been described for the HN proteins of Sendai and mumps viruses (24, 38, 41).

To determine whether dl31 and dl73 encoded proteins were present intracellularly in a form that did not react efficiently with convalescent serum, [³⁵S]methionine-labeled infected cell lysates were analyzed for reactivity with the above-mentioned antipeptide antiserum. This antiserum reacted with HN protein from parent-, dl10-, dl20-, dl40-, and dl44-infected extracts, and no forms of HN protein other than those previously detected using convalescent antiserum were observed in these infected cells (Fig. 2 *C*; data not shown). In addition, the antipeptide serum reacted with a previously undetected 56-kD form of HN in dl31-infected extracts (Fig. 2, *B* and *C*) and a previously undetected 53-kD form of HN in dl73-infected extracts (Fig. 2 *C*).

The observation that these two proteins reacted only with antipeptide serum, and not with antibodies specific to fully processed and biologically active HN protein, suggested that they were unfolded or incorrectly folded. Also, neither polypeptide was detectable in dl31- or dl73-infected extracts which had been labeled with [³H]glucosamine (not shown), indicating they were nonglycosylated, and therefore had not been translocated across the membrane of the ER. The cytoplasmic location of these species was confirmed in in vitro experiments described below. Thus, the HN protein encoded by dl31 accumulated intracellularly in two forms: a less abundant 69-kD glycosylated species that was reactive with conformation-specific antiserum, and a more abundant 56-kD unglycosylated species that was reactive only with antipeptide serum, and therefore appeared to be analogous to
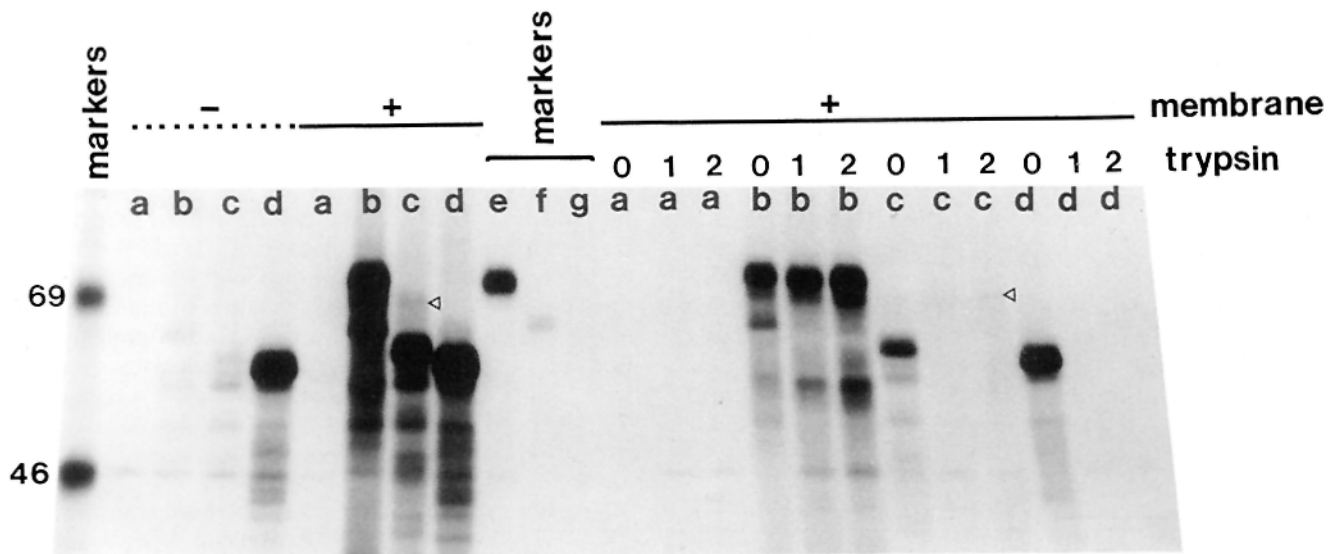

Figure 4. Synthesis of the HN, dl31 and dl73 proteins in vitro, and characterization of membrane insertion. mRNAs for (*b*) HN, (*c*) dl31, and (*d*) dl73 were synthesized in vitro from cDNA using phage polymerase and were translated in rabbit reticulocytes containing [³⁵S]methionine in the absence or presence (as indicated) of dog pancreatic membranes. Control reactions contained (*a*) no added mRNA. After incubation, portions of the reaction mixes were incubated in the presence of (*1*) 20 or (*2*) 100 µg/ml of trypsin or were (*0*) mock-treated in parallel. Trypsin-treated and untreated samples were analyzed by SDS-PAGE on a 10% gel. Samples that were trypsin-treated (*1* and *2*) contained 4.5-fold more reaction mix than did the mock-treated controls (*0*). In additional control reactions, the products in lanes *1* and *2* that were resistant to trypsin became fully sensitive upon the addition of 1% Triton X-100 (not shown). The sizes in kilodaltons of M_r markers electrophoresed in the left-hand lane are indicated. Other lanes contained markers consisting of (*e* and *f*) HN proteins immunoprecipitated with antipeptide serum from lysates of cells which had been infected with the SV-40 recombinant virus expressing parental HN protein and which had been radiolabeled by incubation for 3 h with 20 µCi [³⁵S]methionine in the (*e*) absence or (*f*) presence of 2 µg per ml tunicamycin, or (*g*) a negative control consisting of protein immunoprecipitated with the same HN-specific antiserum from cells which had been infected with an SV-40 recombinant virus expressing the respiratory syncytial virus G glycoprotein and labeled and processed in parallel.

the 53-kD unglycosylated cytoplasmic protein encoded by dl73.

The hypothesis that the convalescent serum reacted preferentially with native HN, whereas the antipeptide serum reacted with both forms, was also tested by comparing their reactivities with native HN proteins versus HN protein that had been denatured by heating in the presence of SDS and 2-mercaptoethanol. As shown in Fig. 3, the convalescent serum reacted poorly with the denatured HN protein in comparison with the native protein. In contrast, the antipeptide serum reacted with both the native and denatured forms, with reactivity being somewhat greater with the denatured form. This reactivity pattern was similar to that observed with antiserum that had been raised against PIV3 that had been denatured with heat in the presence of SDS and 2-mercaptoethanol (Fig. 3).

Association of dl31 with Membranes In Vitro

The cDNAs encoding the parental HN, dl31, and dl73 proteins were placed under the control of the T7 phage promoter, and synthetic mRNAs were made in vitro and analyzed by translation in rabbit reticulocyte lysates in the absence or presence of dog pancreatic membranes competent for transmembrane insertion and glycosylation (Fig. 4). The synthesis of the parental HN and dl31 proteins was greatly stimulated by the addition of membranes, suggesting that the translation of their mRNAs was subject to inhibition by signal recognition particle and that this inhibition was alleviated by association with membranes (40, 43). Consistent with this interpretation, the synthesis of dl73, lacking the signal sequence, was efficient in the absence of membranes and was not stimulated by their addition. These results suggested that the signal sequence of dl31, like that of parental HN protein, was fully competent for membrane insertion.

Parental HN protein synthesized in vitro in the absence of membranes was similar in relative molecular mass to the unglycosylated 61–62-kD intracellular HN protein synthesized in the presence of tunicamycin, whereas HN protein synthesized in vitro in the presence of membranes was similar in relative molecular mass to the glycosylated 70-kD intracellular HN protein (Fig. 4). Thus, parental HN protein appeared to be glycosylated and translocated across microsomal membranes in vitro. In contrast, the relative molecular mass of all of the dl73 protein and of most of the dl31

protein was unaffected by the addition of membranes, and the estimated sizes for the major in vitro forms of dl73 and dl31, 53 and 56 kD, respectively, were the same as their unglycosylated intracellular forms shown in Fig. 2. However, the synthesis of dl31 in vitro in the presence of membranes also resulted in the appearance of an additional, minor, 69-kD species (Fig. 4, *open triangle*) that was of the same size as the minor glycosylated intracellular form of dl31 (Fig. 2).

Thus, both in vitro and intracellularly, the dl31 protein appeared to be inserted into membranes, but only a small fraction was translocated and glycosylated. The majority of the dl31 protein remained unglycosylated and therefore presumably remained at the cytoplasmic membrane face. This was investigated directly by testing the sensitivity to trypsin of the proteins synthesized in vitro in the presence of membranes (Fig. 4). Upon the addition of trypsin, a significant fraction of the parental HN was protected from digestion, consistent with sequestration within closed vesicles as a result of membrane translocation. In contrast, the 53-kD dl73 protein and the 56-kD form of the dl31 protein were completely sensitive. This confirmed the cytoplasmic orientation of these species. On the other hand, the 69-kD form of dl31 synthesized in the presence of membranes was protected from digestion to the same extent as parental HN protein. This supported the interpretation that a small fraction of dl31 protein was translocated across the membrane and glycosylated.

Maturation of the Oligosaccharides in the HN Protein

N-linked oligosaccharides are added initially as high mannose side chains to nascent polypeptides in the lumen of the ER. These chains can be further processed by the trimming of glucose and mannose residues and by the subsequent addition of other sugars such as galactose or N-acetyl glucosamine (16). High-mannose side chains are sensitive to digestion by endo-H, a glycanase which cleaves between the first two N-acetylglucosamine residues, while processed or complex oligosaccharides containing fewer than four mannose residues are resistant (36). Resistance to endo H is acquired in the medial compartment of the Golgi complex. To determine the endo H sensitivities of the carbohydrate side chains of the parental and mutant forms of the HN protein, duplicate infected cell monolayers were labeled by incubation with [35S]methionine for 30 min. One plate was harvested
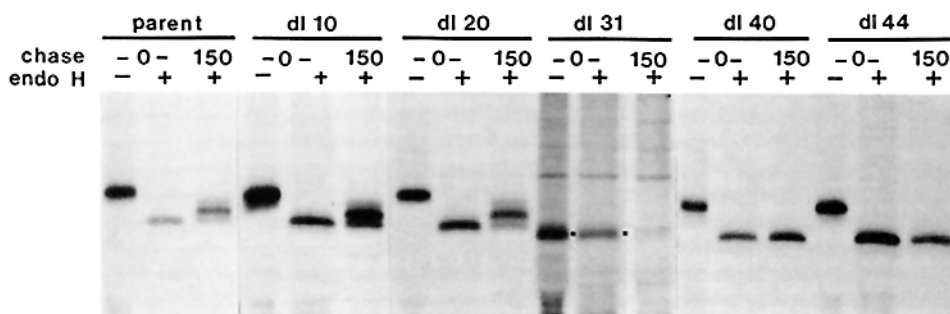

Figure 5. Endo H sensitivities of intracellular parental and truncated forms of expressed HN protein. Recombinant SV-40 virus-infected cells were labeled with 100 μCi/ml [35S]methionine for 30 min and then harvested immediately (0 chase) or chased in the presence of unlabeled medium for 150 min (150 chase). Harvested cells were lysed and immunoprecipitated using convalescent antiserum or, in the case of dl31-infected cells, antipeptide serum. Immunoprecipitated proteins were eluted from the protein A-Sepharose beads and then digested (+) or mock digested (−) with 2 mU endo-H. Digested samples were then analyzed on a 10% SDS polyacrylamide gel. The position of the more abundant species of HN protein from dl31-infected cells is indicated by a small dot.

immediately, and the other was harvested after a 150-min chase period. HN proteins were immunoprecipitated from the cell lysates with convalescent (parental HN, d110, d120, d140, and d144) or antipeptide (d173) serum, or with a mixture of the two (d131), and digested with endo H (Fig. 5). Parental HN protein harvested immediately after the labeling period was reduced in M_r from ~70,000 to 63,000 by treatment with endo H. This endo H-treated species was similar in size to HN protein synthesized in the presence of tunicamycin (not shown), confirming that the oligosaccharide side chains of newly-synthesized HN protein were fully sensitive to cleavage. Endo H treatment of HN protein isolated after the 150 min chase produced a major species of 65,000 D and two minor species of M_r 63,000 and 67,000. The minor 63,000-D species probably represents a subpopulation of molecules that remained fully sensitive because they had not yet passed through the medial Golgi compartment. A relatively slow transit time through the ER and Golgi complex has been described previously for the HN proteins of two other paramyxoviruses, Sendai virus and Newcastle disease virus (23, 24). The intermediate size of the 65,000 and 67,000 dalton major species showed that the HN protein acquired partial endo H resistance during the chase. One possibility is that the mature HN protein contains both high-mannose and complex oligosaccharides. The presence of both the 65,000- and 67,000-D species might represent incomplete endo H digestion, might mean that the 150-min chase was insufficient to allow complete processing, or might mean that one site on the mature HN protein can contain either a high-mannose or a complex side chain, as has been described for other proteins (15, 31). Nonetheless, these conditions were sufficient to document passage of HN through the Golgi complex.

The endo H sensitivity patterns for the HN proteins encoded by d110 and d120 were similar to that of parental HN protein (Fig. 5), showing that these two truncated forms were processed authentically in the Golgi stack. As would be expected, endo H treatment had no effect on the M_r of the major, unglycosylated 56-kD HN protein encoded by d131. This species appeared less stable during the 150-min chase than did parental HN protein. As described in the previous section, expression of d131 also resulted in a second, less abundant species of M_r 69,000 that was glycosylated and reactive with convalescent antibodies. However, analysis of the endo H sensitivity of this minor species was precluded by its low abundance. Finally, the truncated HN proteins encoded by d140 and d144 remained completely endo H sensitive (Fig. 5) after the chase period, indicating that these molecules were not detectably transported through the medial Golgi compartment and therefore accumulated primarily at an earlier step in the exocytotic pathway.

To further identify the intracellular site of accumulation of the HN proteins encoded by d140 and d144, infected cell monolayers were permeabilized with acetone and analyzed by indirect immunofluorescence (not shown). HN proteins were detected by staining with rhodamine-conjugated IgG, and the location of the Golgi stack was determined by co-staining with fluorescein-conjugated WGA. The WGA produced a pattern of localized fluorescence adjacent to the cell nucleus, as is characteristic of staining of the Golgi stack (20). In contrast, the rhodamine-conjugated IgG used to identify the d140- and d144-encoded HN proteins exhibited

a diffuse network of staining throughout the cytoplasm, consistent with localization of these HN proteins in the ER.

The d140 and d144 Proteins Form Authentic Intramolecular Disulfide Bonds and Homooligomers

Studies with the type I influenza virus hemagglutinin protein and vesicular stomatitis virus G protein showed that intramolecular folding and subsequent intermolecular assembly or oligomerization can be prerequisites for transport beyond the ER (6, 10, 13, 17, 30). The reactivity of the d140 and d144 proteins with the conformationally dependent convalescent antibodies (Fig. 2) suggested that both proteins have a native conformation. However, an alternate possibility was that the observed immunoreactivity was due to the folding of only a subset of epitopes. We therefore further characterized the d140 and d144 proteins by SDS-PAGE under nonreducing conditions to investigate disulfide bonding and by chemical cross-linking to investigate oligomerization.

When analyzed under nonreducing conditions, parental HN protein migrates as a discrete monomeric species that has a greater electrophoretic mobility than does the reduced form (Fig. 6 and reference 33), indicating the presence of intramolecular disulfide bonds that result in a more compact structure. A contrasting situation is represented by HN253, a variant of HN protein expressed from a cDNA modified by site-directed mutagenesis (unpublished data) to contain a single amino acid change (R253 to A253). The HN253 protein reacts poorly with convalescent antibodies and does not acquire endo H resistance (unpublished data), and by those criteria is an improperly folded protein that is not transported to the medial Golgi compartment. Under reducing conditions, HN253 protein that had been immunoprecipitated with antipeptide serum migrated as a discrete species of the same mobility as parental HN protein, as would be expected, but under nonreducing conditions HN253 migrated as a heterogeneous smear rather than as the discrete, more compact form that was characteristic of parental HN protein (Fig. 6). This showed that HN253 contains heterogeneous, aberrant disulfide bonds. A similar result was observed for unglycosylated parental HN protein synthesized in the presence of tunicamycin: unglycosylated HN protein reacted poorly with convalescent antibodies (not shown), and when immunoprecipitated with antipeptide serum it migrated as a discrete band under reducing conditions but as a heterogeneous smear under nonreducing conditions (Fig. 6). These latter observations are in agreement with previous experiments involving the HN protein of Sendai virus, in which the acquisition of immunoreactivity with conformation-dependent antibodies was dependent upon glycosylation and the formation of correct intramolecular disulfide bonds (38). Under nonreducing conditions, the d140 and d144 proteins migrated as discrete species of increased electrophoretic mobility (Fig. 6), supporting the interpretation that both proteins contained a homogeneous, correct pattern of intramolecular disulfide bonds.

In work to be presented elsewhere, SDS-PAGE of HN protein that had been reacted with chemical cross-linking agents identified species of the appropriate sizes to be homooligomers consisting of two, three and, in some cases, four HN molecules. Small amounts of multimeric forms also can
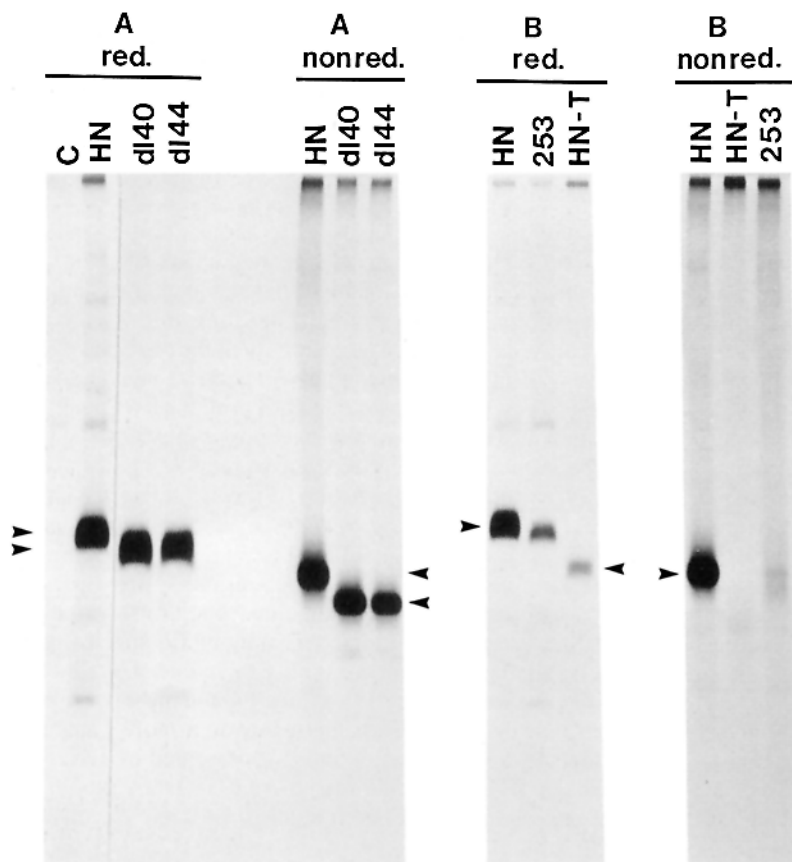
Figure 6. SDS-PAGE of parental HN, dl40, and dl44 proteins under reducing (*red.*) or nonreducing (*nonred.*) conditions. AGMK cells were infected with SV40 recombinant viruses that express the parental HN, dl40, dl44, or HN253 proteins (as indicated), the latter being a variant of parental HN protein that is defective in folding (see text) due to a single amino acid substitution (R253 to A253) effected by site-directed mutagenesis of the HN cDNA. As a control (*C*), additional cells were infected with an SV-40 recombinant virus that expresses the respiratory syncytial virus G glycoprotein. Additional cells were infected with the recombinant SV-40 virus expressing parental HN protein and were treated in parallel except that 2 μg tunicamycin per ml were present beginning 3 h before the radiolabeling period. At 40 h after infection, all cells were labeled for 1 h with [^{35}S]methionine, lysed in RIPA buffer and immunoprecipitated with (*A*) convalescent antibodies or (*B*) with antipeptide antibodies. Each immunoprecipitated sample was divided into two equal aliquots and analyzed by SDS-PAGE after denaturation at 100°C for 5 min in SDS-PAGE sample buffer in the presence (*red.*) or absence (*nonred.*) of 5% vol/vol 2-mercaptoethanol. The samples in parts *A* and *B* were analyzed on the same 10% gel, and the discrete electrophoretic bands of the various HN proteins are indicated with arrows.

sometimes be detected in the absence of cross-linking, but the HN protein of PIV3 differs from its counterparts in several other paramyxoviruses in that the multimers are not linked by disulfide bonds. In pulse–chase radiolabeling studies, homooligomerization was concurrent with or immediately followed folding and correct intramolecular disulfide bond formation as assayed by immunoreactivity and nonreducing SDS-PAGE, and oligomerization was not observed with unglycosylated HN protein (unpublished data). These observations support the interpretation that the multimeric species identified by cross-linking represent authentic homooligomers that form during the maturation of the HN protein. EM of purified Sendai virus HN protein identified dimeric and tetrameric forms (37), and it seems likely the mature cell-surface and virion-associated forms of the HN proteins of both Sendai virus and PIV3 are homotetramers. Incubation of parental PIV3 HN protein with the cross-linking agent dimethyl 3,3' dithiobispropionimidate (DTBP) (Fig. 7) and dimethyl suberimidate (not shown) resulted in the cross-linking of dimeric and trimeric forms. The relatively small fraction of material represented in the oligomers could be attributed to the characteristic inefficiency of cross-linking reactions. Treatment of dl40 and dl44 in parallel with parental HN with DTBP (Fig. 7) or dimethyl suberimidate (not shown) resulted in the formation of a similar profile of oligomeric forms. Although the dimeric forms of dl40 and dl44 appeared to be more diffuse relative to parental HN in the experiment shown in Fig. 7, this appeared to represent experimental variability between samples and in other experiments the dimeric forms were essentially indistinguishable. Thus, the two truncated forms formed homooligomers in a manner similar to parental HN protein.

Hemagglutination by Expressed Intracellular HN Proteins

To further investigate the conformational authenticity of the dl40 and dl44 proteins, lysates of infected cells were serially diluted and tested for biological activity as assayed by the ability to agglutinate guinea pig erythrocytes. To determine whether the hemagglutination titers observed with infected cell lysates was proportional to the amount of expressed intracellular HN proteins, duplicate cultures of infected cells were radiolabeled in parallel, immunoprecipitated under conditions of antibody excess, and analyzed by SDS-PAGE and liquid scintillation counting of excised HN bands (see Materials and Methods). The ratios of hemagglutination titer to the radioactivity of immunoprecipitated protein was within 2.5-fold for all positive samples (Table I; data not shown). The hemagglutination titers shown in Table I were not corrected for differences in the level of expression of mutant proteins, and the slightly lower titers for several mutants appeared to be attributable in large part to differences in the level of expressed protein (Table I, footnote). The HN proteins encoded by dl110 and dl120 were similar to parental HN protein in their ability to agglutinate erythrocytes whereas dl131-infected extracts had no hemagglutination activity. This suggests that the minor glycosylated species of HN protein found in dl131-infected extracts was either unable to agglutinate erythrocytes or was expressed at levels below the sensitivity of this assay. Evidence for the latter possibility is presented below. Interestingly, HN proteins encoded by dl40 and dl44 were as active in hemagglutination as was the parental HN protein, even though they are arrested in their intracellular maturation in the ER.
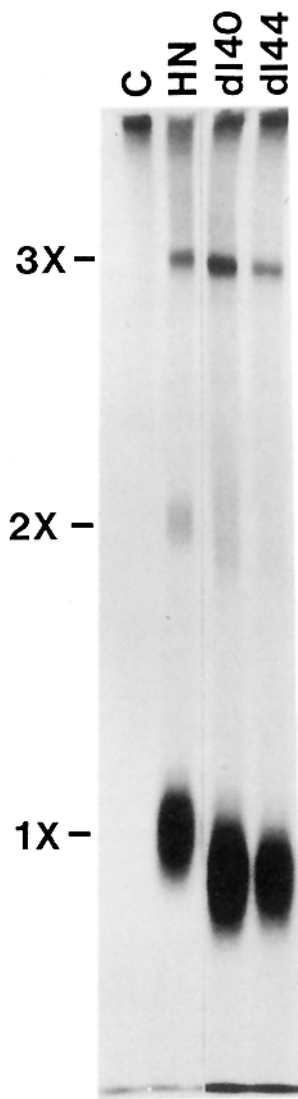
Figure 7. SDS-PAGE of multimers of HN, dl40, and dl44 proteins identified by cross-linking with the agent DTBP. AGMK cells were infected with SV-40 recombinant viruses expressing the HN, dl40, dl44 (as indicated), or (C) respiratory syncytial virus G protein, labeled at 40 h after infection by incubation for 1 h in the presence of [³⁵S]methionine, and lysed in 50 mM Tris hydrochloride, pH 7.0, 150 mM NaCl containing 1% vol/vol Triton X-100. The lysates were mixed with 4 vol of 100 mM triethanolamine, pH 8.0, containing 200 μg per ml DTBP, incubated at room temperature for 1 h, adjusted to contain 5 mM glycine and incubated for an additional 5 min to inactivate the DTBP, and adjusted to the composition of RIPA buffer. The HN proteins were immunoprecipitated with convalescent antibodies and analyzed by nonreducing SDS-PAGE on an 8% gel. The positions of (1×) monomeric, (2×) dimeric, and (3×) trimeric forms of the HN protein, identified in work to be presented elsewhere, are indicated.

Cell Surface Expression of Parent and Mutant HN Protein

Tissue culture monolayers that had been infected with the recombinant SV-40 viruses were tested for their ability to bind guinea pig erythrocytes, as described previously for the parental HN protein expressed by recombinant vaccinia virus and baculovirus (5, 33).

Table I. Hemagglutination and Hemadsorption by Parental and Truncated HN Proteins

SV-40-HN recombinant	Hemagglutination by cell lysates	Hemadsorption of infected cell monolayer	Hemagglutination by media
Parent	1:32*	+++	ND
dl10	1:16	+++	ND
dl20	1:32	+++	ND
dl31	ND	+/−	ND
dl40	1:16*	ND	1:4
dl44	1:8*	ND	1:4
dl73	ND	ND	ND

* The ratio of relative levels of intracellular accumulation of the parental, dl40 and dl44 proteins as determined by radiolabeling of duplicate cultures (see text) was 1 (parental): 0.6 (dl40): 0.5 (dl44).

The levels of hemadsorption observed for parental HN and for the truncated HN proteins encoded by dl10 and dl20 were essentially indistinguishable (not shown, summarized in Table I), indicating that these two truncated species were biologically active and were efficiently transported to the cell surface. Surface expression of these HN proteins was confirmed by indirect immunofluorescence (not shown). dl31-infected monolayers exhibited low levels of hemadsorption (<5% that of parental HN). A likely explanation was that this represented surface expression of the minor, glycosylated species of HN protein produced in these cells, and this was supported by the detection of low levels of specific surface immunofluorescence (not shown). In all cases where hemadsorption was observed, the addition of warm PBS and incubation at 37°C resulted in a rapid and nearly complete elution of erythrocytes. This suggested that the presence of hemadsorbing activity in the mutant HN proteins was paralleled by the presence of a functional neuraminidase. Cell monolayers infected with the recombinant viruses for dl40, dl44, and dl73 were completely negative for hemadsorption, and the absence of these HN proteins at the cell surface was confirmed by indirect immunofluorescence using both antipeptide and convalescent antisera (not shown).

Identification of Secreted Forms of the dl40 and dl44 Proteins

It was of interest to determine whether the parental or truncated HN proteins were released into the media of infected
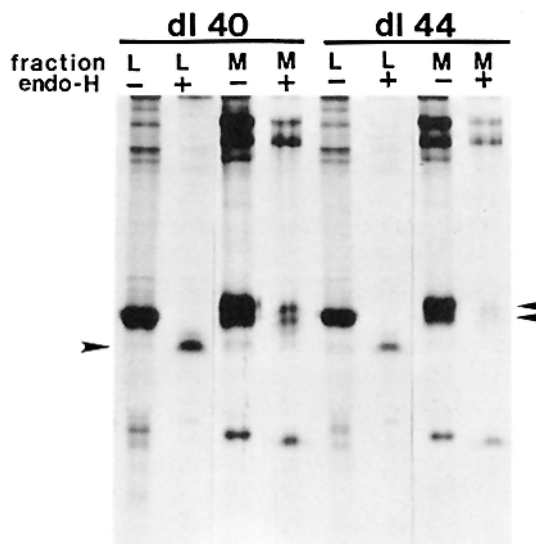

Figure 8. Endo H sensitivity of the secreted forms of dl40- and dl44-expressed HN protein. Cell monolayers were infected with dl40 or dl44 recombinant viruses, labeled with [³⁵S]methionine for 30 min, and harvested immediately. Intracellular dl40 and dl44 expressed HN proteins were immunoprecipitated with convalescent antiserum and digested (L+) or mock digested (L−) with endo-H. A duplicate set of dl40 and dl44 recombinant infected cultures was labeled with [³⁵S]methionine for 1 h followed by a nonradioactive chase for 4 h. The chase media were collected and clarified, and the radiolabeled secreted dl40 and dl44 proteins were immunoprecipitated and digested (M+) or mock digested (M−) with endo H. The arrow on the left indicates the position of intracellular, endo H sensitive HN protein; arrows on the right indicate the position of secreted, endo H-resistant HN proteins.

cells. For example, soluble forms of several viral surface glycoproteins have been described; in some cases the soluble forms were slightly shorter than the parental proteins and appeared to result from proteolytic release of the polypeptide chain from the membrane anchor (14 and references therein).

Recombinant virus infected cell monolayers were labeled with [³⁵S]methionine for 1 h, chased in the presence of unlabeled medium for 4 h, and then the media were clarified and analyzed by immunoprecipitation and SDS-PAGE. Similar results were obtained with convalescent (Fig. 8) or antipeptide (not shown) serum. Secreted HN protein was not detected in the tissue culture supernatants for parental HN and for most of the truncated forms (not shown). However, secreted forms of HN were detected in the media of both dl40 and dl44 infected monolayers (Fig. 8). Under these conditions of radiolabeling and chase, the released forms accounted for ~10-15% of the labeled HN protein as assayed by immunoprecipitation, SDS-PAGE and densitometry of fluorograms (not shown). For both dl40 and dl44, the released protein appeared as a doublet by SDS-PAGE; one band of each doublet appeared to be identical in relative molecular mass to the intracellular form, and the second band appeared to be of slightly greater abundance and migrated slightly slower. Whereas the carbohydrate side chains of the intracellular forms of dl40 and dl44 were completely sensitive to endo H, those of the secreted forms were completely resistant, indicating that the secreted forms had been processed through the exocytotic pathway. Minor heterogeneity in the processing of the carbohydrate side chains might account for the greater relative molecular mass of one of the bands in each of the doublets of released dl40 and dl44 protein.

The secreted HN proteins of dl40 and dl44 also were biologically active as assayed by the ability of clarified tissue culture supernatants to agglutinate guinea pig erythrocytes (Table I).

Association of the dl40 and dl44 Proteins with Intracellular Membranes

As described above, most of the dl40 and dl44 protein expressed in infected cells accumulated in the ER, with a small fraction being processed further by a secretory pathway. The nature of the membrane association of the intracellular forms was characterized by cell fractionation by differential centrifugation and exposure to high pH (12). Infected cells were sonicated and separated into a cytosolic supernatant and a membrane pellet. The membrane pellet was then incubated in carbonate buffer at pH 11, a treatment that disrupts membranes without solubilizing them. Under these conditions, integral membrane proteins remain membrane associated and are pelleted by centrifugation whereas peripherally associated proteins (and soluble proteins that were trapped within vesicles) are released into the supernatant.

Fig. 9 shows SDS-PAGE patterns of radiolabeled proteins immunoprecipitated with convalescent antiserum from the cytosol (containing the soluble proteins), from the supernatant following carbonate treatment of the membrane fraction (soluble proteins and peripherally associated proteins), and from the pellet fraction after carbonate treatment of the membrane fraction (integral membrane proteins). Parental HN protein was detected only in the carbonate pellet, consis-

tent with its status as an integral membrane protein. In contrast, small amounts of dl40 and dl44 were released into the cytosolic fraction by sonication, suggesting that this material was soluble or loosely associated with the membrane of the ER. Of the dl40 and dl44 proteins that pelleted in the membrane fraction, approximately two-thirds were released by the carbonate treatment. These results indicated that most of the intracellular dl40 and dl44 proteins were present in soluble form or in loose association with the membrane of the ER.

In addition, the membrane association of the HN protein expressed by dl31 was analyzed by differential centrifugation of infected cell lysates (Fig. 9 B). As described above, most of the dl31 protein synthesized intracellularly was not translocated into the ER despite the presence of the complete hydrophobic signal/anchor domain. Instead, most of the dl31 protein appeared to accumulate in the cytoplasm in a less stable, unglycosylated form that, by these criteria, resembled the dl73 protein, which lacked the signal/anchor domain. However, whereas the dl73 protein was present only in the cytosolic fraction and thus was a soluble protein, essentially all of the dl31 protein was found in the membrane pellet (Fig. 9 B), consistent with the interpretation that it was inserted into the membrane. Attempts to further analyze the pellet fraction by carbonate treatment were unsuccessful, apparently due to protein instability or loss of immunoreactivity during the carbonate treatment. Nonetheless, the single-step fractionation was sufficient to establish that the dl31 protein was firmly associated with intracellular membranes despite the absence of translocation into the ER.

Discussion

Six mutants were constructed which contain progressive deletions of the NH₂-terminal amino acids that comprise the cytoplasmic tail and putative transmembrane domain of the HN protein. The expression, glycosylation, folding, oligomerization, intracellular transport, and biological activity of the parental and truncated HN proteins were examined.

The conformation-specific convalescent antibodies bound detectably only to biologically active HN proteins (parental HN, dl10, dl20, dl40, dl44, and the 69-kD form of dl31). The peptide-specific antiserum reacted with the biologically active forms and, in addition, bound to and thereby identified forms of the truncated HN proteins (dl31 and dl73) that were not bound detectably by convalescent antibodies, were not glycosylated, and were biologically inactive. These appeared to be unfolded or incorrectly folded species that had not been translocated into the ER. The use of conformation-specific antisera to monitor the folding of the HN proteins of other paramyxoviruses has been reported previously (24, 38, 41).

The dl10 and dl20 proteins, which each lacked part of the cytoplasmic domain, were essentially indistinguishable from parental HN protein with regard to their intracellular processing, cell surface expression and biological activity. Thus, removal of the NH₂-terminal 20 residues of the cytoplasmic tail had no detectable effect on the translocation and maturation of the HN protein, although relatively minor differences in the kinetics of transport might exist that were not detected in the experiment in Fig. 5.

A contrasting situation was represented by the dl73 protein, which completely lacked both the NH₂-terminal cytoplasmic tail and the hydrophobic domain. dl73 was not detect-
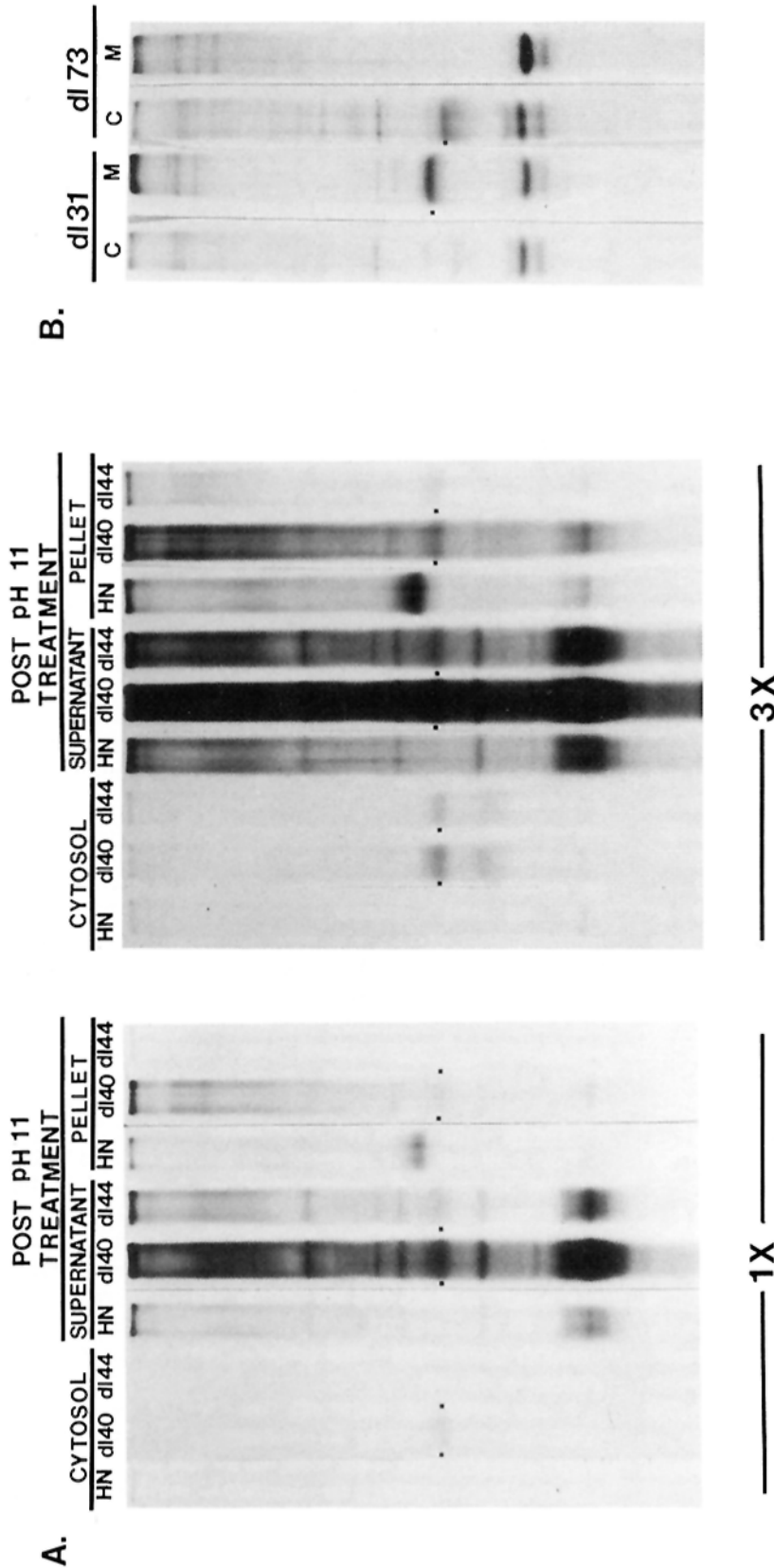
Figure 9. Characterization of the membrane associations of the truncated HN proteins by cell fractionation and pH 11 treatment. (*A*) AGMK cells were infected with recombinant SV-40 viruses expressing the HN, dl40, or dl44 proteins. The cells were labeled with [^{35}S]methionine for 1 h, harvested, sonicated, and separated by differentiated centrifugation into cytosolic (lanes marked *cytosol*) and membrane fractions. The membranes were then treated with Na_2CO_3 buffer, pH 11, and separated by centrifugation into a supernatant fraction (lanes marked *post pH 11 treatment, supernatant*), which would contain soluble and peripherally associated membrane proteins, and a pellet fraction (lanes marked *post pH 11 treatment, pellet*), which would contain integral membrane proteins. Radiolabeled HN proteins were immunoprecipitated with convalescent antiserum and analyzed by 10% PAGE. Two exposures (1×, 3×) of the fluorographed gel are shown and the position of the dl40 and dl44 HN proteins are marked with small dots. (*B*) AGMK cells were infected with recombinant SV-40 viruses expressing the dl31 and dl73 proteins. The cells were labeled with [^{35}S]methionine for 1 h, harvested, sonicated, and separated by centrifugation into cytosolic (*C*) and membrane (*M*) fractions. Radiolabeled HN proteins were immunoprecipitated with antipeptide serum and analyzed by 10% SDS-PAGE. The positions of dl31- and dl73-encoded HN proteins are indicated by small dots.

ably inserted or translocated across the ER and accumulated as a cytoplasmic, unfolded form. This provided direct evidence that the hydrophobic domain represented by amino acids 32–53 is the signal for membrane insertion and translocation of the HN protein.

As shown by the dl31 mutant, removal of the cytoplasmic tail, leaving the complete hydrophobic signal/anchor domain intact, resulted in the accumulation of two forms of HN protein. A small fraction (<5%) of the dl31-expressed protein was glycosylated, was reactive with convalescent antibodies, and could be detected at the cell surface by hemadsorption. These results showed that there was not an absolute requirement for any of the cytoplasmic tail for posttranslational processing and cell-surface expression of the HN protein. However, it is likely that the cytoplasmic domain is important for other activities such as association with the PIV3 M protein or with nucleocapsids during virion maturation. It would be interesting to determine whether these truncated proteins can be incorporated into PIV3 virions upon superinfection with PIV3.

In contrast to the efficient membrane translocation of dl10 and dl20, most of the expressed dl31 protein was not translocated across the ER and instead remained in the cytoplasm, based on its lack of glycosylation and lack of reactivity with conformation-specific antibodies. The cytoplasmic orientation was confirmed using an in vitro translation–transmembrane insertion system (Fig. 4). But unlike the unfolded dl73 protein, which accumulated in a soluble form in the cytoplasm, all of the unfolded dl31 protein was associated with the membrane. This was evidenced by its copurification with intracellular membrane vesicles (Fig. 9 B) and, in vitro, by the observation that the addition of membranes released a translational block of the dl31 mRNA that presumably was mediated by signal recognition particle (Fig. 4, references 40 and 43). Thus, essentially all of the dl31 protein appeared to be inserted in membrane but was translocated very inefficiently.

One possible explanation for the inefficient translocation of dl31 could be that the presence of charged residues immediately upstream of the signal/anchor is important for signal function; the presence of such residues is characteristic of both type I and type II glycoproteins (1, 7, 19, 32, 35). However, efficient translocation of dl31 was restored simply by shortening the length of the hydrophobic domain (as in dl40 and dl44), indicating that the HN signal functioned efficiently without upstream charged residues. Another possible explanation for the low efficiency of the translocation of dl31 is that residues of the cytoplasmic domain that precede the anchor serve to counteract its hydrophobic character and facilitate passage through membrane, and in the absence of these residues the hydrophobic domain acted as a stop–transfer signal (9). Consistent with this, reducing hydrophobicity by shortening the sequence (dl40 and dl44) restored translocation. Analogous situations of hydrophobic sequences acting to prematurely arrest translocation have been reported, for example, for a chimeric protein that consisted of the NH₂-terminal hydrophobic domain of the cytochrome P450IIC2 fused to the ectodomain of preparathyroid hormone (35), and for the fusion glycoprotein of simian virus 5, where an internal hydrophobic domain acted as a stop-transfer signal following the removal of upstream charged residues (27).

The ability of dl10, dl20, dl40, and dl44 to be inserted

and translocated with equal efficiencies despite the extensive differences in their NH₂-terminal structures showed that these processes do not have strict structural requirements and possibly can proceed by more than one mechanism. Specifically, translocation of parental HN, dl10 and dl20 might initiate by the insertion of a hydrophobic loop into the membrane with the polar, charged NH₂-terminus remaining cytoplasmic, whereas translocation of dl31, dl40, and dl44 might initiate by head-on insertion of the hydrophobic NH₂-terminus.

dl40 and dl44 were efficiently translocated across the membrane of the ER, but appeared to have peripheral rather than integral associations with the membrane. Thus, the signal and anchoring activities could be dissociated. Similarly, Brown et al. have reported that a mutant form of the influenza neuraminidase protein which was missing the first 15 amino acids, leaving a 20-amino acid signal–anchor domain, was efficiently translocated in vitro, but appeared to be unstably anchored in the membrane (4). It seems likely that the presence of charged residues upstream of the hydrophobic domain, whereas not required for signal function, are important for subsequently stabilizing the protein in the membrane.

The dl40-, and dl44-expressed proteins accumulated primarily in the ER, with a small fraction being processed further in the Golgi complex (as evidenced by the acquisition of endo H-resistance) and secreted into the medium. The secreted forms, like the intracellular forms, directed the agglutination of erythrocytes (Table I). This implied that the secreted proteins are present in oligomeric (multivalent) form. This was confirmed for the intracellular species (Fig. 7) but was not investigated for the secreted forms. Previous studies with soluble forms of the Sendai virus HN protein released by proteolysis (37) showed that the soluble protein was 250-fold less active in hemagglutination than was the membrane-bound form. This showed that HN protein present on large multivalent virions was much more efficient in agglutinating erythrocytes than were small divalent and tetravalent oligomers. In light of this, the secreted forms of dl40 and dl44 appeared to be surprisingly efficient in hemagglutination relative to intracellular parental HN in cell lysates. Unlike the protease-treated soluble Sendai virus HN protein, dl40 and dl44 retained part of the hydrophobic anchor, which might allow the formation of aggregates with increased hemagglutinin activity. Also, components of the cell lysate might have interfered with the activity of the intracellular forms, making their secreted counterparts appear artifactually more active by comparison.

The dl40 and dl44 proteins that accumulated in the ER reacted strongly with conformation-specific antiserum (Fig. 2), appeared to form authentic intramolecular disulfide bonds (Fig. 6) and oligomers (Fig. 7), and bound erythrocytes with an efficiency similar to that of parental HN protein (Table I). The results indicate that the folding and oligomerization of PIV3 HN into a mature, biologically active form (which presumably represents a virion spike) occurs essentially to completion in the ER.

It was somewhat surprising that most of the expressed dl40 and dl44 proteins accumulated in the ER. For some type I glycoproteins, it has been shown that correct polypeptide folding is important for transport to the cell surface and incorrectly folded proteins have been shown to accumulate in the ER or in the Golgi complex (6, 13, 17), although the

requirement for folding might not apply to all proteins (for example, see reference 26). The d140 and d144 proteins appeared to be conformationally indistinguishable from wild type in the assays described here, suggesting that defects in the structures of the ectodomains were not responsible for the transport block. This is offered with the caveat that we cannot eliminate the possibility that the folded ectodomains of d140 and d144 contain undetectable alterations in conformation resulting in ER retention. Alternatively, perhaps the less-stable association of d140 and d144 with the membrane resulted in the exposure of part of the hydrophobic anchor, which might identify d140 and d144 as "unfolded." At least part of the mechanism for retention of incorrectly folded proteins might involve binding by a 78-kD cellular protein, the GRP78 or BiP protein, which is a resident of the ER (2, 25). It would be interesting to determine whether d140 and d144 are associated with the GRP78/BiP protein, or whether that putative component of the folding or retention or degradative machinery associates only with grossly unfolded intracellular HN protein such as HN253 or with newly synthesized unfolded HN protein.

On the other hand, if the ectodomains of d140 and d144 are correctly folded, their retention in the ER indicates that the membrane anchor contributes to a structure that is critical for surface transport but is not directly related to the folding of the ectodomain. Similarly, Doms et al. (10) described mutants of the type I vesicular stomatitis virus G protein, expressed from cDNA, that contained modifications in the cytoplasmic region which did not appear to affect folding and oligomerization but which greatly reduced the efficiency of transport through the exocytotic pathway, and mutants that might be analogous have also been described for the influenza virus HA protein (34). These experiments demonstrate that correct folding of most of the ectodomain is not sufficient for transport of these proteins to the cell surface, and that the localized structure of the transmembrane and flanking regions also has an important role in protein transport.

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