Defective Assembly and Intracellular Transport of Mutant Paramyxovirus Hemagglutinin-Neuraminidase Proteins Containing Altered Cytoplasmic Domains

GRIFFITH D. PARKS AND ROBERT A. LAMB*

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208-3500

Received 17 January 1990/Accepted 19 April 1990

The hemagglutinin-neuraminidase (HN) integral membrane protein of paramyxoviruses is expressed at the cell surface as a tetramer consisting of a pair of disulfide-linked dimers. HN has a large C-terminal ectodomain, a 19-residue uncleaved signal-anchor domain, and a 17-residue N-terminal cytoplasmic tail. Various mutant HN genes were constructed to examine the role of residues flanking the signal-anchor domain, including the cytoplasmic tail, on assembly and intracellular transport of the HN glycoprotein. Expression of the altered genes showed that by 90 min after synthesis the majority of the mutant HN proteins were in a conformationally mature form as assayed by their reactivity with conformation-specific monoclonal antibodies. However, the mutant proteins showed varied endoplasmic reticulum-to-Golgi apparatus transport rates, ranging from that of wild-type HN ($t_{1/2} \approx 90$ min) to slowly transported molecules ($t_{1/2} \approx 5$ h) and to molecules in which transport was not detected. Pulse-chase experiments indicated that the altered HN molecules had a specific and transient interaction with the resident endoplasmic reticulum protein GRP78-BiP, and thus the altered HN molecules were not retained in the endoplasmic reticulum by a prolonged interaction with GRP78-BiP. Sucrose density gradient sedimentation analysis of the mutant HN molecules indicated that they all had an oligomeric form that differed from that of wild-type HN; most of the molecules were found as disulfide-linked dimers rather than as tetramers. These data suggest that the HN cytoplasmic tail may function in the assembly of the final transport-competent oligomeric form of HN and that mutant HN molecules with seemingly properly folded ectodomains are retained in the endoplasmic reticulum by an as yet unidentified mechanism. The possible role of the HN cytoplasmic tail as a signal for intracellular transport is discussed.

Several models for the intracellular sorting of proteins have been proposed (reviewed in references 24, 42, and 46). Initially it seemed likely that transport would be mediated through receptors that would recognize positive-acting signals. However, despite an intensive effort, no direct evidence in favor of this hypothesis has been forthcoming. As an alternative mechanism, it has been suggested that molecules are transported in the exocytic pathway by bulk flow and that intracellular sorting is achieved by selective retention of proteins in the endoplasmic reticulum (ER) or Golgi apparatus (42). Evidence for retention signals in soluble ER proteins, ER integral membrane proteins, and Golgi apparatus integral membrane proteins has been obtained (27, 31, 35). In addition, a further layer of complexity in the control of intracellular transport is added by the findings that folding toward a native structure and oligomerization may be sequential and obligatory events for exit from the ER (46).

There has been considerable interest in the possible functions of the cytoplasmic tails of integral membrane proteins, because they are exposed at the cytoplasmic face of the ER membrane and thus may interact with cellular proteins that promote or delay the intracellular transport process. Previous work has focused predominantly on the cytoplasmic tails of type I proteins (molecules with an N-terminal luminal domain and a C-terminal cytoplasmic tail: $N_{out}C_{in}$ topology [3]), particularly the influenza virus hemagglutinin (HA) or vesicular stomatitis virus (VSV) G protein (46). It has been shown that mutations in the C-terminal cytoplasmic tail of the VSV G protein have differing effects ranging from no change in phenotype to the impairment of transport and in

some cases a complete block in transport from the ER (8, 44). In some cases it is thought that these phenotypes are a direct consequence of a change in the cytoplasmic tail, because when altered G tails are transferred to other membrane-bound proteins a similar phenotype is observed (16). However, complete removal of the cytoplasmic region of the influenza virus HA, Rous sarcoma virus envelope glycoprotein (*env*), or Semliki Forest virus E2 protein does not affect transport to the cell surface (10, 14, 41), and transfer of the *env* cytoplasmic tail to influenza virus HA does not transfer the slow transport properties of *env* to HA (9). Taken together, these data suggest that roles for the C-terminal cytoplasmic tail of type I proteins in providing information required for transport from the ER may depend on the protein in question (46).

In comparison to the type I proteins, the roles of the N-terminal cytoplasmic tail in the transport of the type II membrane proteins (those with an N-terminal cytoplasmic region and a C-terminal ectodomain: $N_{in}C_{out}$ topology [3]) have not been extensively studied. The HA-neuraminidase (HN) protein of the paramyxoviruses is a type II integral membrane protein that is expressed at the surface of infected cells. The paramyxovirus simian virus 5 (SV5) HN protein has an uncleaved signal-anchor (S/A) domain consisting of 19 hydrophobic residues and is oriented in the membrane such that it has a 529-residue ectodomain and a 17-residue cytoplasmic tail (17, 18). Studies on the intracellular transport of SV5 HN have indicated that native folding of the large C-terminal ectodomain and the formation of the homo-oligomer occur relatively slowly ($t_{1/2} \approx 25$ to 30 min) and precede transport of HN from the ER (33). Furthermore, mutations in the HN ectodomain that disrupt these assembly

* Corresponding author.

processes result in proteins that are defective in transport (32). These data suggest that native folding and assembly of the HN C-terminal ectodomain may be important for transport from the ER, but they do not address the role of the N-terminal domains of HN in this process.

To analyze the role of the N-terminal region of a type II protein in intracellular transport, a series of altered HN genes was constructed; these genes contained mutations in the residues immediately flanking the uncleaved S/A domain. Expression and analysis of the mutant proteins showed that alterations in the HN N-terminal cytoplasmic tail can significantly affect intracellular processing and can lead to proteins that are transported slowly or retained in the ER. The data indicate that mutations in the HN cytoplasmic tail do not disrupt proper folding of the ectodomain, and we suggest that they prevent transport from the ER by disrupting the assembly of the final oligomeric structure.

MATERIALS AND METHODS

Plasmid construction and preparation of recombinant simian virus 40 (SV40). DNA from pSV103HNm, which encodes a biologically active form of the SV5 HN protein (38), was used as a source of starting materials for the construction of mutant HN genes. Bacteriophage M13HN, which contains the HN N-terminal 5' bases (nucleotides 1 through 306) and encodes residues 1 through 81 as described previously (37), was used as a source of template DNA for oligonucleotide-directed mutagenesis (36). Oligonucleotides were synthesized by the Northwestern University Biotechnology Facility on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, Calif.).

The G1 gene was constructed by oligonucleotide-directed deletion of 42 nucleotides (nucleotides 73 through 114) from M13HN DNA, resulting in a cDNA segment encoding 3 amino acids (MVR; see Table 1) in place of the 17-residue HN N-terminal tail. The construction of HN mutant G3Y, which encodes a deletion (nucleotides 172 through 174) of the last residue (tyrosine, HN residue 36) from the C-terminal end of the membrane spanning domain, has been described (37). The HN mutant G13K gene combined both the G1 14-residue N-terminal deletion and the G3Y tyrosine deletion, but it also contained a Glu (HN residue 37) (GAG)-to-Lys (AAG) substitution.

The HNG5 gene was constructed by oligonucleotide-directed substitution of nucleotides 109 through 111 (Leu to Glu) and 115 through 117 (Arg to Ser), which resulted in the introduction of a new *ScaI* site in the DNA encoding the junction of the N-terminal and membrane-spanning domains. This recombinant DNA construction permitted the exchange of the *XhoI*-*ScaI* HN fragment with the *XhoI*-*ScaI* fragment from the lactate dehydrogenase (LDH) gene (30), to produce the L/HN gene. The resulting DNA segment encoded a hybrid protein composed of the first 72 NH₂-terminal residues of LDH linked precisely to the membrane-spanning domain and ectodomain of HN (residues 18 through 81). The hybrid MgHH gene was constructed from HN mutant G5 by blunt end ligation of an M₂g DNA segment (bases 1 through 98, residues 1 through 24) to the *ScaI* fragment from HN mutant G5 (base 118, residue 18) as described in detail previously (37). Altered HN genes were constructed in pGEM vectors as subclones, and their composition was confirmed by dideoxynucleotide sequencing (47).

To reconstruct the 5' mutations into the full-length HN gene, altered Asp-718 *PstI* segments were excised from their respective pGEM subclones and ligated with the *PstI*-*XhoI*

cDNA segment encoding the remaining HN ectodomain (residues 82 through 565) in the SV40 late region replacement vector pSV103 (38). Recombinant viruses were introduced into CV-1 cells by DEAE-dextran-mediated transfection (26) together with DNA from an SV40 early region mutant (*dl1055*) to act as a helper virus (43).

Cells. Monolayer cultures of CV-1 cells were grown and passaged in Dulbecco modified Eagle medium (DME) containing 10% fetal calf serum.

Metabolic labeling of polypeptides, immunoprecipitation, and polyacrylamide gel electrophoresis. CV-1 cells were infected with recombinant SV40 viruses and radiolabeled with Tran[³⁵S]-label (ICN Pharmaceuticals Inc., Irvine, Calif.) at 40 to 48 h postinfection (22). Cells were lysed in cold RIPA buffer and immunoprecipitated as described previously (21). Alternatively, cells were lysed in 1% sodium dodecyl sulfate (SDS), boiled, diluted with buffer, and immunoprecipitated with polyclonal rabbit antiserum to SDS-denatured HN as described previously (12, 32). Immunoprecipitation of cell extracts lysed in RIPA buffer with conformation-specific SV5 HN monoclonal antibody (MAb) HN-4b was done as described previously (33, 45). The immunoglobulin G fraction of rabbit sera raised to affinity-purified HN was that used previously (29). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels (21).

Co-immunoprecipitation of HN/GRP78-BiP complexes was done by using a rat MAb specific for cellular heavy-chain binding protein (BiP) (kindly provided by Linda Hendershot, St. Jude Children's Research Hospital, Memphis, Tenn., and John F. Kearney, University of Alabama, Birmingham) as described previously (33). CV-1 cells were infected with recombinant SV40 viruses and at 44 to 48 h postinfection were radiolabeled with Tran[³⁵S]-label for 15 min (pulse) and incubated for 90 min (chase) in DME containing 2 mM nonradioactive methionine (chase medium). Cell lysates were prepared in cold TNT buffer (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 1% Triton X-100) supplemented with 10 mM glucose and 2 IU of hexokinase (Calbiochem, La Jolla, Calif.) per ml to deplete ATP before immunoprecipitation with the anti-BiP MAb.

Alkali extraction of microsomal membranes. Monolayers of recombinant SV40-infected CV-1 cells were labeled with Tran[³⁵S]-label, and microsomal membranes were prepared (1). Microsomes were treated with 0.1 M Na₂HCO₃ (pH 11), incubated at 4°C for 30 min, and fractionated by centrifugation as described previously (37). Equal portions of the resulting pellet and supernatant were neutralized with acetic acid and immunoprecipitated.

Endoglycosidase digestion and tunicamycin treatment of cells. Cells were treated with 1 µg of tunicamycin (Calbiochem) per ml 2 h before and during the radiolabeling period. For endoglycosidase treatment, recombinant SV40-infected cells were labeled with Tran[³⁵S]-label and incubated in chase medium. Cell lysates were prepared in 1% SDS, boiled, and immunoprecipitated with polyclonal HN antiserum. The resulting antibody-antigen complexes were dissociated by boiling in 10 mM Tris hydrochloride (pH 7.4) containing 0.2% SDS and then diluted with an equal volume of 100 mM sodium citrate (pH 5.4). Samples were digested with 1 mU of endo-β-N-acetylglucosaminidase H (endo H; ICN) at 37°C overnight and analyzed by SDS-PAGE.

Sucrose gradient sedimentation. The oligomeric forms of the HN proteins were analyzed by sucrose gradient sedimentation as described previously (33). Briefly, recombinant SV40-infected CV-1 cells were radiolabeled with Tran[³⁵S]-

label for 30 min and incubated in DME for 2 h. Cells were lysed in 1 ml of lysis buffer (20 mM morpholineethanesulfonic acid, 30 mM Tris hydrochloride [pH 5.2], 100 mM NaCl, 1.0% Triton X-100, 1.0% aprotinin), clarified by centrifugation ($14,000 \times g$ for 5 min), and layered on a 11-ml linear 7.5 to 22.5% sucrose gradient prepared in lysis buffer containing 0.1% Triton X-100. Gradients were centrifuged at 38,000 rpm at 20°C for 22 h in an SW41 rotor (Beckman Instruments Inc., Palo Alto, Calif.). Sixteen 0.65-ml fractions were collected from the bottom of the tube and neutralized with 10 μ l of 1 M Tris hydrochloride (pH 9.5), and polypeptides were immunoprecipitated with polyclonal anti-HN serum and analyzed by SDS-PAGE.

Indirect immunofluorescence. Cover slips of recombinant SV40-infected CV-1 cells were fixed at 44 to 48 h postinfection for 10 min at room temperature in phosphate-buffered saline containing 0.2% freshly prepared paraformaldehyde (Sigma Chemical Co., St. Louis, Mo.). For internal staining, fixed cells were permeabilized in 100% acetone for 3 min at -20°C. Binding of MABs specific for HN (1:1,000 dilution of ascites fluids in phosphate-buffered saline-1% bovine serum albumin), followed by staining with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Organon Technica, West Chester, Pa.), was done as described previously (11). Quantitation of cell surface fluorescence was done by using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.).

RESULTS

Expression and transmembrane orientation of mutant HN proteins. We previously showed that amino acid residues directly flanking the HN S/A domain are involved in establishing the membrane orientation of HN (37). The possible role of these residues in HN assembly and its intracellular transport was further examined by mutagenesis of the HN cDNA clone (Fig. 1A and Table 1). To maximize the possibility of introducing a new phenotype, a variety of mutations were introduced into the DNA such that they would cause changes ranging from single-amino-acid residue deletions and substitutions to large deletions and complete replacement of domains with foreign sequences. In mutant G5, 2 of the 17 cytoplasmic tail region residues were changed: Leu residue 15 was converted to a Glu residue, and Arg residue 17 was changed to a Ser. With mutants G1 and G13K, a deletion of 14 of the 17 N-terminal cytoplasmic residues was made. In addition, mutant G13K contained a deletion of tyrosine residue 36 (ΔY) and a substitution of the Glu residue 37 flanking the C-terminal side of the S/A with a Lys residue (E $\rightarrow$ K). Mutant G3Y contained only a deletion of tyrosine residue 36 (ΔY) from the membrane spanning domain. Last, MgHH and L/HN contained a precise substitution of the 17-amino-acid residue HN cytoplasmic tail with the 24-residue N-terminal ectodomain of a modified form of the influenza A virus M_2 protein, M_2g (37), or the first 72 N-terminal residues of human LDH (30), respectively. Mutant genes were inserted into an SV40 late region replacement vector, and recombinant virus stocks were produced by transfection of CV-1 cells.

To examine the synthesis of the wild-type and mutant HN molecules, CV-1 cells infected with the various recombinant SV40 viruses were radiolabeled for 1 h with Tran[35 S]-label in the presence or absence of tunicamycin, an inhibitor of N-linked glycosylation. Cell lysates were then prepared and immunoprecipitated with polyclonal HN antisera. HN was expressed as a polypeptide of $M_r \approx 68,000$ (Fig. 1B, lane 1)

FIG. 1. Construction and expression of mutant HN proteins. (A) Schematic diagram of mutant HN proteins. The N-terminal region of HN and mutant HN proteins is depicted as a box with the light shaded area representing the hydrophobic S/A domain. Solid and hatched boxes represent LDH and M_2g sequences, respectively. All mutants are described in detail in the text with N-terminal amino acid sequences listed in Table 1. Letters denote amino acid changes with the single-letter code. Δ , Deletion of a tyrosine residue; N, amino terminus; C, carboxy terminus. (B) Expression of mutant HN genes. CV-1 cells infected with recombinant SV40 virus expressing HN and mutant HN molecules were labeled for 1 h with Tran[35 S]-label in the absence (lanes 1, 3, 5, 7, 9, 11, and 13) or presence (lanes 2, 4, 6, 8, 10, 12, and 14) of tunicamycin (1 μ g/ml). Cells were lysed in 1% SDS and immunoprecipitated with HN-specific antiserum. Mutants are listed above their respective lanes. Lanes P and C, Recombinant SV40-infected cell lysate immunoprecipitated with preimmune sera and a control cell lysate immunoprecipitated with the anti-HN serum, respectively. The arrow indicates the position of wild-type glycosylated HN. Dots in lane 3 highlight the position of unglycosylated G5 protein. I and II denote forms of the MgHH protein as described in the text. M, SV5-infected cell polypeptides with the molecular weights indicated in thousands.

that was not precipitated by preimmune sera (lane P), and HN synthesized in the presence of tunicamycin was found to be a polypeptide species of $M_r \approx 52,000$ (lane 2), as reported previously (38). A faster-migrating polypeptide that is thought to be SV40 related was observed in all samples including a nonspecific control lysate (lane C) and an HN lysate incubated with preimmune serum (lane P). Mutant G3Y synthesized a polypeptide (lanes 11 and 12) that was indistinguishable in electrophoretic mobility from HN. Mu-

TABLE 1. Predicted amino acid sequences surrounding the membrane-spanning domain of wild-type and mutant HN proteins^a

Protein	Amino	Membrane	Carboxy ^b
HN	M V A E D A P V R A T C R V L F R	-----	E S L I T Q
G3Y	M V A E D A P V R A T C R V L F R	-----*	E S L I T Q
G5	M V A E D A P V R A T C R V E F S	-----	E S L I T Q
G1		M V R	E S L I T Q
G13K		M V R	E S L I T Q
MgHH	M S N L T E V E T P I R N E W G C R C N D S S D	-----	E S L I T Q
L/HN		72 amino acids of LDH	E S L I T Q

^a Amino acid sequence (one letter code) flanking the HN N-terminal membrane-spanning domain (-----). Amino, N-terminal side; Carboxy, C-terminal side.

^{*} Deletion of a tyrosine residue from the membrane-spanning domain.

^b Note that only the first six residues of the C-terminal HN ectodomain are listed.

tants G1 and G13K, which contained a truncated N-terminal domain, synthesized polypeptides with an electrophoretic mobility slightly faster than that of HN (lanes 5 through 8).

Mutants G5, L/HN, and MgHH synthesized polypeptides with electrophoretic mobility patterns significantly different from that of the HN protein. Mutant G5 synthesized a major polypeptide species of $M_r \approx 68,000$ (Fig. 1B, lane 3). In addition, with mutant G5 small amounts of a second polypeptide species were detected (lane 3). This minor G5 species had a gel mobility similar to that observed when mutant G5 was synthesized in the presence of tunicamycin (lane 4), and thus the minor species probably represented an unglycosylated form of G5. Mutant L/HN synthesized a polypeptide that had a similar electrophoretic mobility when synthesized in the absence or presence of tunicamycin (lanes 11 and 12), suggesting that the C-terminal ectodomain of the L/HN protein, which contains the consensus sites for N-linked glycosylation, had not been translocated across the ER membrane. Mutant MgHH synthesized two protein species, designated forms I and II (lane 13), but only a single polypeptide was synthesized in the presence of tunicamycin (lane 14). The nature of these two MgHH polypeptides (I and II) was determined previously (37). Mutant MgHH synthesizes a single polypeptide species that adopts two opposing transmembrane orientations: form I has an $N_{out}C_{in}$ topology, and form II is like HN with an $N_{in}C_{out}$ orientation (37).

Although glycosylation of the mutant HN polypeptides indicated that the polypeptide chain had been translocated into the ER lumen (with the exception of mutant L/HN), it was important to determine whether the polypeptides were stably anchored in the ER membrane or whether they had been completely translocated across the membrane and were soluble in the ER lumen. To distinguish between these possibilities, alkaline fractionation of microsomal membranes was performed. Recombinant SV40-infected CV-1 cells were labeled with Tran[³⁵S]-label, and microsomal vesicles were prepared and fractionated at alkaline pH into a pellet and supernatant. Polypeptides were immunoprecipitated with HN antiserum and analyzed by SDS-PAGE.

As expected for an integral membrane protein, HN remained associated with membranes after alkali treatment and, after centrifugation, was only detected in the pellet fraction (Fig. 2; HN, lane P, arrow). Likewise, the vast majority of the mutant HN proteins G5, G1, G13K, and G3Y fractionated with the membrane pellet, and only trace amounts of these polypeptides were found in the supernatant fraction (lanes G5, G1, G13K, and G3Y). Small amounts of the unglycosylated form of the G5 protein were also detected in the pellet fraction (G5 lane), suggesting that this species was tightly associated with the ER membrane. Trypsin treatment of microsomes from cells expressing the G5 protein resulted in the digestion of the unglycosylated species

but not the major $M_r \approx 68,000$ polypeptide (data not shown), which suggests that the membrane-associated minor G5 species may be an aborted translocation intermediate or that the species inserted into membranes in the opposite $N_{in}C_{out}$ orientation. We found previously that both forms I and II of MgHH fractionate with the membrane after alkali treatment (37). The L/HN protein was found in both the pellet and supernatant fractions, suggesting that it has a weak membrane association (lanes L/HN), and the low abundance of the L/HN protein relative to the other mutants reflects the unstable nature of this chimeric polypeptide ($t_{1/2}$, <10 min; data not shown). Thus, the altered polypeptide L/HN was not glycosylated and did not form a stable interaction with the ER membrane and was not examined in further assays. In contrast, N-terminal extensions to the VSV G protein signal sequence did not abrogate membrane insertion (49). The N-terminal 72-residue LDH extension may have prevented the initial interaction of the HN S/A domain with the ER membrane, because it had folded such that the S/A was not exposed, a phenomenon that was suggested previously (2).

These data concerning membrane orientation and integration indicate that, with the exception of the L/HN protein,

FIG. 2. Stability of the interaction of the altered HN polypeptides with the ER membrane. Recombinant SV40-infected CV-1 cells were radiolabeled for 1 h with Tran[³⁵S]-label, and microsomal membranes were prepared. Samples were treated with 100 mM Na₂HCO₃ buffer (pH 11) and separated into pellet (P) and supernatant (S) fractions by centrifugation. Equal portions of the two fractions were neutralized, immunoprecipitated with polyclonal HN serum, and analyzed by SDS-PAGE. Dots indicate the position of the unglycosylated G5 protein species, and stars denote the L/HN protein.

the mutant HN proteins are all stably integrated in membranes. This is in contrast to the situation reported for two other type II membrane proteins, the asialoglycoprotein receptor (48) and I γ CAT (23), in which alterations to the cytoplasmic tail caused complete translocation of the molecules across the ER membrane due to cleavage of the S/A domain.

Endoglycosidase analysis. The rate of acquisition of carbohydrate chains resistant to endo H digestion, which is indicative of a polypeptide having been transported to the medial Golgi complex, was determined. CV-1 cells were infected with the recombinant SV40 viruses that express the HN mutants, radiolabeled with Tran³⁵S-label for 15 min, and then incubated in chase medium for various periods. Cell lysates were prepared and immunoprecipitated with polyclonal HN antisera. After the antigen-antibody complexes were incubated with or without endo H, the polypeptides were analyzed by SDS-PAGE.

HN expressed from the SV40 recombinant virus acquired carbohydrate chains that were resistant to endo H digestion, with a $t_{1/2}$ of approximately 90 min (Fig. 3, panel HN), a rate very similar to that found for HN synthesized in an SV5 infection (33). However, it is difficult to measure a precise rate of transport of HN to the medial Golgi complex, because HN is extensively internalized and degraded on arrival at the cell surface (33). The mobility of the HN endo H-resistant form was not identical to that of the untreated molecule, because only a subset of the four HN carbohydrate chains is converted from the simple to the complex form after transport to the Golgi apparatus. The rate of acquisition of carbohydrate chains resistant to endo H digestion for the G3Y protein was very similar to that found for HN (Fig. 3, panel G3Y). In contrast, only a small amount of the G1 protein gained carbohydrate residues that were resistant to endo H digestion in 5 h (this is difficult to visualize in Fig. 3, panel G1). Mutant G5 protein containing carbohydrate chains that were resistant to endo H digestion was not detected, even on prolonged exposure of the autoradiographs (data not shown). Likewise, the carbohydrate chains on both form I and II proteins of HN mutant MgHH were completely sensitive to endo H digestion up to 5 h after their synthesis (Fig. 3, panel MgHH). An increase in electrophoretic mobility of MgHH form II was observed with time; this probably reflects trimming of the high-mannose carbohydrate chain.

The G13K protein, which migrated as a single species after the pulse-label, showed a more complex electrophoretic mobility pattern 1 to 5 h after the labeling period. A prominent $M_r \approx 68,000$ species could be observed, but in addition there was heterogeneously migrating HN-specific material ranging from $M_r \approx 70,000$ to 100,000 (Fig. 3, panel G13K). After endo H digestion, two populations with faster electrophoretic mobility were observed: the expected endo H-sensitive species and a heterogeneously migrating species. The heterogeneous mixture of G13K species detected by the pulse-chase analysis suggested that the carbohydrate chains were being modified by heterogeneous extensions such as the addition of polylactosamine. Therefore, the characteristics of the carbohydrate chains on G13K were examined by digestion with endoglycosidase β , which cleaves the $\beta 1 \rightarrow 4$ linkage between the Gal and *N*-acetylglucosamine of a lactosamine repeating unit (13). The heterogeneously migrating G13K material was sensitive to endoglycosidase β digestion, which suggests that the modification is the addition of polylactosaminoglycan (data not shown).

Internal and cell surface immunofluorescence of mutant HN

FIG. 3. Time course of acquisition of HN-specific carbohydrate chains resistant to endo H digestion. Cells infected with recombinant SV40 viruses expressing the altered HN molecules were radiolabeled with Tran³⁵S-label for 15 min and then incubated in chase medium for various periods. At each time point, cells were lysed in 1% SDS and immunoprecipitated with HN-specific serum. Immune complexes were divided into two portions and incubated with (+) or without (-) endo H, and polypeptides were analyzed by SDS-PAGE. The times of chase period are indicated at the top of the figure, and for each panel lanes 1 through 6 represent 0, 0.5, 1, 1.5, 2.5, and 5 h of chase, respectively. Note that digestion of G3Y at the zero time point was incomplete. R and S denote endo H-resistant and endo H-sensitive HN species, respectively. In the panel for mutant MgHH, I and II indicate the two MgHH species that have opposing membrane orientations as described in the text. The bar flanking the G13K panel denotes the heterogeneous population of G13K proteins, which contain polylactosaminoglycan extensions.

proteins. The cellular distribution of the altered HN proteins was examined by indirect immunofluorescence. CV-1 cells infected with the various recombinant viruses were fixed in paraformaldehyde and permeabilized in acetone for intracellular fluorescent staining or fixed in paraformaldehyde for cell surface staining. The cells were then incubated with HN conformation-specific MABs (33) and stained with fluorescein-conjugated secondary antibody. The staining pattern of HN in permeabilized cells (Fig. 4A) showed extensive perinuclear staining characteristic of the Golgi apparatus; such a pattern is typical for proteins in transport from the ER to the cell surface. In addition, as found previously, some localization to endosomes and lysosomes of internalized HN could be seen (33). Mutant G3Y showed an intracellular staining pattern very similar to that of HN (Fig. 4B). In comparison, cells expressing the G1, G5, and MgHH polypeptides showed predominantly a reticular pattern characteristic of the ER (Fig. 4C, D, and F) with very little Golgi apparatus

FIG. 4. Indirect intracellular immunofluorescence of HN mutants. CV-1 cells infected with recombinant SV40 virus expressing HN (A), G3Y (B), G5 (C), G1 (D), G13K (E), or MgHH (F) were fixed in paraformaldehyde and permeabilized with acetone at -20°C for 3 min. Cells were then stained with MAb HN-4b, followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin.

staining, a pattern consistent with the localization of the majority of the protein to the ER. However, the intracellular staining pattern of G13K was very different (Fig. 4E) and showed strong perinuclear staining, characteristic of the

Golgi apparatus, as well as a reticular pattern characteristic of the ER. This staining pattern is consistent with a delayed rate of transport through the medial Golgi apparatus, as was also suggested by the kinetics of conversion to complex

carbohydrate chains (Fig. 3), which was slower than that observed with HN.

The cell surface fluorescence staining patterns were also examined for the altered HN molecules. The HN and mutant G3Y showed bright surface staining (Fig. 5A and B, respectively), a finding consistent with their intracellular staining pattern and their acquisition of complex carbohydrate chains. Mutant G13K also showed surface staining (Fig. 5E). Mutants G1 and MgHH, which showed a strong ER intracellular staining pattern and had only a very slow rate of conversion to endo H-resistant carbohydrate chains, showed an overall weak surface fluorescence pattern (data not shown), but a few brightly fluorescent cells could be found (Fig. 5D and F). Fluorescent flow cytometry indicated that the number of cells that were weakly fluorescent on mutant G1 infection was similar to the number of cells that were strongly fluorescent with HN (data not shown). Thus, these data suggest that mutants G1 and MgHH are inefficiently transported to the cell surface. Mutant G5 did not show any detectable cell surface fluorescence (Fig. 5C). Examination of the surfaces of cells expressing the MgHH polypeptide with MAbs to either the N-terminal M₂ ectodomain or the HN ectodomain demonstrated that the chimeric protein was transported to the plasma membrane in only the HN orientation (data not shown), indicating that the M₂ ectodomain can also function as a cytoplasmic tail.

After infection with recombinant SV40 viruses, the number of cells observed to express the G3Y and G1 molecules was similar to that in the case of HN ($\approx 70\%$), and the level of protein expression as determined by immunoprecipitation of radiolabeled polypeptides was equivalent (data not shown). Although it would be ideal to quantitate the surface fluorescent intensities of the altered HN molecules so that they could be compared with that of HN, it would be misleading. This is because HN is extensively internalized from the cell surface and degraded (33), and at the present time it is not known whether the mutant HN molecules accumulate stably at the cell surface or whether they are internalized at a rate similar to or different from that of wild-type HN.

Conformation-specific monoclonal reactivity. Conformation-specific MAbs have proven to be useful reagents for examining the folded status of membrane proteins (8, 15, 33). It has been shown that the native folding of nascent SV5 HN molecules is nearly complete by 60 min after synthesis and that only mature HN molecules exit the ER (33). Thus, the large variations in mutant transport rates ($t_{1/2} \approx 90$ min to >5 h) could reflect differences in the degree of native folding of the proteins.

CV-1 cells infected with recombinant SV40 viruses were labeled for 15 min with Tran[³⁵S]-label and incubated in DME for 90 min. Cell lysates were prepared and sequentially immunoprecipitated three times with MAb HN-4b, which recognizes a mature epitope that is formed only after oligomerization of the HN protein (33). Lysates depleted of MAb HN-4b-immunoreactive species were finally immunoprecipitated with a polyclonal antiserum specific for all forms of HN. The samples were analyzed on SDS-PAGE under nonreducing conditions, and the data for three mutants, G3Y, G1, and G5, which have greatly differing rates of transport to the medial Golgi apparatus, are shown in Fig. 6. With all of the mutants (data for mutants G13K and MgHH not shown), irrespective of their intracellular transport rate, nearly all of the molecules could be found as a disulfide-linked dimer (with only small amounts of monomers), which could be recognized by the conformation-specific MAb.

Only very small quantities of monomers were detected by the polyclonal antisera after the depletion of MAb HN-4b-reactive forms. Thus, these data indicate that mutants G3Y, G1, G5, G13K, and MgHH matured in 90 min to a form that was reactive with MAb HN-4b and that there was very little unfolded HN.

Oligomeric structure of mutant HN proteins. Oligomerization of integral membrane proteins is thought to be a prerequisite for transport from the ER (6, 15, 20). The paramyxovirus HN protein is thought to exist as disulfide-linked dimers that form either noncovalently linked tetramers or disulfide-linked tetramers (28, 33, 50). To determine whether the various transport rates of the HN mutants reflected different degrees of assembly, the oligomeric state of the altered HN proteins was analyzed by sucrose gradient sedimentation. CV-1 cells were infected with the various recombinant SV40 viruses, radiolabeled for 30 min with Tran[³⁵S]-label, and incubated for 2 h in chase medium. Cell lysates were analyzed by sucrose gradient sedimentation under conditions that stabilize the tetrameric form of the wild-type HN protein (33). HN sedimented as a single species found near the bottom of the gradient (Fig. 7A; fast sedimenting form, fractions 3 and 4). This is consistent with previous results, which showed that by 60 min after synthesis the vast majority of HN is in an oligomeric form (33). Mutant HN polypeptides G1, G13K, and G5 sedimented as a single protein species (Fig. 7C through E; slow-sedimenting form, fractions 6 and 7) that sedimented through the gradient at a rate slower than that of wild-type HN. However, the sedimentation pattern of the G3Y protein was different in that fast- and slow-sedimenting species were detected (Fig. 7B).

Analysis of the MgHH mutant showed that the two polypeptide forms sedimented in different regions of the gradient. Form II sedimented in approximately the same fractions as mutant HN proteins G1, G13K, and G5 (arrow S), whereas a small amount of form I was found near the top of the gradient (panel MgHH, fraction 12). The reduced levels of form I relative to form II species recovered from the gradient may be due to the relative stabilities of the proteins during the 2-h chase period (Fig. 3).

The slower sedimentation of the mutant HN proteins in the sucrose gradient (Fig. 7, arrow S) relative to HN (arrow F) suggests that these altered polypeptides exist as dimers rather than in the wild-type oligomeric form, a conclusion that is supported by the observation that on SDS-PAGE under nonreducing conditions the HN-specific proteins in peaks F and S were both composed of disulfide-linked polypeptides (data not shown). We cannot readily distinguish whether the mutants only matured as far as forming dimers during assembly of HN or whether the dimers resulted because the mutations caused dissociation of a weakly associated tetramer under the centrifugation conditions used.

Association of altered HN molecules with the cellular protein GRP78-BiP. The ER resident protein GRP78-BiP has been suggested to play roles both in the normal folding and assembly of integral membrane proteins and in the retention of misfolded integral membrane proteins in the ER (4, 15, 19, 40). A specific and transient association of GRP78-BiP with SV5 HN during its normal folding pathway has been suggested (33), based on the observations that immature folded forms of the HN polypeptide are associated with GRP78-BiP but that just before HN oligomerization the GRP78-BiP/HN complex dissociated. In addition, misfolded forms of HN that do not acquire reactivity with the HN conformation-

FIG. 5. Indirect surface immunofluorescence of cells expressing the HN mutants. Recombinant SV40-infected cells expressing HN (A), G3Y (B), G5 (C), G1 (D), G13K (E), or MgHH (F) were fixed in paraformaldehyde and incubated with MAb HN-4b, followed by staining with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin. The exposure time for all panels was manually adjusted to be the same.

FIG. 6. Conformation-specific MAb reactivity of HN G3Y, G5, and G1 mutants. Monolayers of CV-1 cells were infected with recombinant SV40 that expressed G3Y, G5, or G1, labeled for 15 min with Tran³⁵S-label, and incubated for 90 min in chase medium. Cell lysates were prepared and sequentially immunoprecipitated three times with conformation-specific MAb HN-4b (lanes 1 through 3). Lysates depleted of MAb HN-4b-reactive species were then incubated with the polyclonal HN sera (lane P). Samples were analyzed by SDS-PAGE under nonreducing conditions. HN₁, Monomeric HN; HN₂, dimeric HN; G1HN₂, dimeric form of G1 mutant.

specific MAbs and do not oligomerize to the native form remain in a stable complex with GRP78-BiP for several hours (32). Although the HN N-terminal mutants were in a mature folded form as evaluated by their reactivity with the conformation-specific HN-4b MAb, it was of interest to determine whether the slow rate of transport of the altered HN proteins was due to a prolonged association with GRP78-BiP in the ER. CV-1 cells infected with the recombinant SV40 viruses expressing the G5, G3Y, G1, or G13K proteins were radiolabeled with Tran³⁵S-label for 15 min. Cell lysates were prepared immediately after labeling or after a 90-min chase period and immunoprecipitated with the anti-BiP MAb under ATP-depleting conditions (33). After the pulse-label, the anti-BiP MAb coimmunoprecipitated GRP78-BiP and the altered HN polypeptides (Fig. 8, lanes P). Many cellular proteins were also coimmunoprecipitated by this MAb, as would be expected (33). However, after a 90-min chase period, coimmunoprecipitation of the HN polypeptides with the anti-BiP MAb was greatly reduced (Fig. 8, lanes C). Since we showed previously that the majority of newly synthesized HN molecules interact with GRP78-BiP (33), these data suggest that by 90 min after synthesis the majority of the altered HN molecules were not retained in the ER due to a prolonged association with GRP78-BiP.

DISCUSSION

The tails of integral membrane proteins are exposed to the cytoplasm, and therefore they may have the opportunity to interact with cytoplasmic proteins to form a signal that could control the transport of proteins from the ER. However, analysis of the role of this cytoplasmic region of type I integral membrane proteins, including the viral proteins influenza virus HA, Rous sarcoma virus *env*, Semliki Forest virus E2 protein, and VSV G protein, has not led to a single unifying conclusion (46). To determine whether the N-terminal cytoplasmic domain of a type II integral membrane protein plays a role in its intracellular assembly and transport, cDNA clones encoding mutant HN proteins were

FIG. 7. Sucrose density gradient analysis of oligomeric forms of HN mutants. Cells were infected with recombinant SV40 expressing HN (A) or mutant protein G3Y (B), G1 (C), G13K (D), G5 (E), or MgHH (F). Infected cells were labeled for 30 min with Tran³⁵S-label, incubated for 2 h in chase medium, lysed by the addition of lysis buffer, and analyzed on sucrose density gradients as described in Materials and Methods. Gradients were fractionated from the bottom of the tube, and the first 13 fractions were immunoprecipitated with HN-specific polyclonal serum and analyzed by SDS-PAGE. Centrifugation was from right (top) to left (bottom). Large vertical arrows indicate the position of fast (F)- or slow (S)-migrating protein species, and small horizontal arrows indicate forms I and II of the MgHH protein.

FIG. 8. Association of mutant HN proteins with GRP78-BiP. CV-1 cells infected with recombinant SV40 expressing G5, G3Y, G1, and G13K were pulse-labeled with Tran³⁵S-label for 15 min (lanes P) and incubated in chase medium for 90 min (lanes C). Cell lysates were prepared under ATP-depleting conditions, immunoprecipitated with an MAb to GRP78-BiP, and analyzed by SDS-PAGE. Arrows indicate the position of GRP78-BiP (BiP) and HN proteins. Lanes U and I contained direct lysates from mock-treated and tunicamycin-treated uninfected cells, respectively, as a marker for GRP78-BiP. Stars indicate the position of the G5 protein. M contained SV5-infected cell polypeptides.

TABLE 2. Summary of phenotypes of N-terminally altered HN proteins

Protein	HN ectodomain in the ER ^a	MAb reactivity ^b	Transiently associated with BiP ^c	Oligomer form ^d	<i>t</i> _{1/2} of endo H resistance ^e	Immunofluorescence	
						Internal ^f	Surface ^g
HN	+	+	+	F	1.5	G	Bright
G3Y	+	+	+	F + S	1.5	G	Bright
G5	+	+	+	S	>6	ER	None
G1	+	+	+	S	>6	ER	Weak
G13K	+	+	+	S	~5	G	Bright
MgHH	±	+	N/D ^h	S	>6	ER	Weak
L/HN	—	N/A ⁱ	N/A	N/A	N/A	N/A	N/A

^a +, HN ectodomain glycosylated; ±, approximately half in the HN and half in the M₂ orientation; —, HN ectodomain not glycosylated and in isolated microsomes is susceptible to protease digestion. A small amount of the G5 mutant was membrane associated but not glycosylated and is thought to be in the inverse orientation.

^b +, Majority of protein recognized by native conformation-specific MAbs.

^c +, Coimmunoprecipitation of nascent but not mature HN with MAb to GRP78-BiP.

^d F and S, Fast- and slow-sedimenting disulfide-linked oligomers, respectively.

^e Time (hours) for half of the protein to gain carbohydrate residues resistant to endo H digestion.

^f Internal localization of protein by immunofluorescence with MAb HN-4b. G, Golgi staining; ER, ER staining.

^g Cell surface immunofluorescence staining with MAb HN-4b.

^h N/D, Not determined.

ⁱ N/A, Not applicable.

constructed and expressed. These molecules were examined for their membrane stability, native folding, oligomerization, intracellular transport, and cell surface expression (Table 2).

With the exception of the mutant L/HN, the altered HN molecules were all translocated across the membrane of the ER and formed a stable interaction with the membrane. In addition, the altered HN molecules assumed a folded conformation such that they gained reactivity with the conformation-specific MAb HN-4b, which recognizes an epitope formed at a late step in the folding pathway of HN (33), and very few of the altered molecules were in an unfolded form. The N-terminally altered molecules, like HN, formed intermolecular disulfide bonds and on SDS-PAGE under nonreducing conditions were found as disulfide-linked dimers. However, when the rate of intracellular transport of the altered molecules was examined, as determined by acquisition of carbohydrate chains resistant to endo H digestion, the HN molecules that contained altered cytoplasmic tails were defective in transport from the ER. The intracellular fluorescent staining pattern of the altered molecules is consistent with the observed rates of transport. Molecules for which a transport rate could not be determined were found predominantly in the ER, whereas molecules for which a transport rate could be determined showed Golgi apparatus staining. Cell surface staining patterns of the G3Y and G13K molecules were also consistent with the observed transport rates and the internal staining patterns. Interestingly, mutants G1 and MgHH, which did not have a measurable transport rate, did show some cell surface staining, which suggests that these altered HN molecules were inefficiently transported to the cell surface.

The transport defect of the altered HN molecules, as far as can be determined, was not attributable to a defect in the folding of the HN ectodomain; consistent with this was the finding that the retention of mutants G1, G5, and G13K in the ER was not due to a prolonged interaction with the resident ER protein GRP78-BiP. The mechanism of retention of the HN mutant proteins is not known at the present time but may result from an association with other as yet unidentified ER proteins.

When examined on sucrose density gradients under conditions that stabilize the tetrameric form of HN (33), the mutant molecules were detected only as disulfide-linked dimers. Thus, the feature common to the HN mutants that

can be assayed, and which might explain their transport defect, is that assembly into the final oligomeric HN structure may be blocked. The exception was mutant G3Y, which seemingly had a normal transport rate and formed both tetramers and dimers, but it is not known whether these oligomers exhibit differing transport rates. Although we favor the interpretation of the data as suggesting that the altered HN molecules failed to assemble into the tetrameric form, we cannot eliminate the possibility that the HN mutants formed a weakly associated tetramer that dissociated under the centrifugation conditions used. Irrespective of whether the mutations blocked tetramer formation or rendered the tetramer unstable, the data suggest that a property of the transmembrane and cytoplasmic domain is important for proper oligomerization and intracellular transport. HN tetramer assembly could require noncovalent interactions between the N-terminal tails of two disulfide-linked dimers, and a role for the transmembrane and cytoplasmic tail in the stability and oligomerization of molecules has been proposed previously for the chicken hepatic lectin receptor (25) and the low-density lipoprotein receptor (51).

HN mutants that showed defective assembly properties were not always completely blocked in exit from the ER, as exemplified by mutant G13K. Both G1 and G13K have the same three residue cytoplasmic tail (Met-Val-Arg) but differ by a tyrosine deletion (common to G3Y also) and a charge change at the G13K transmembrane domain-ectodomain junction. G1 is largely transport defective, whereas G13K is only slowed in transport. Thus, the charge alteration in G13K seemingly overcomes the effect of reducing the length of the cytoplasmic tail in mutant G1. One possible explanation of these phenomena is that, rather than there being a specific transport signal in the cytoplasmic tail of HN, efficient transport may require a proper spatial configuration between the HN transmembrane domains. In support of such a proposal, synthetic oligopeptides corresponding to the transmembrane domain of glycoporphin A can form a specific complex with the native molecule *in vitro*, and it has been suggested that this binding mimics the normal interaction between the transmembrane domains of glycoporphin A monomers (5).

The transmembrane domains inevitably play some role in the oligomerization process of integral membrane proteins, since the monomers have to migrate in the plane of the

membrane, but it is not known whether the role is active or passive. It seems possible that there could be cellular molecules that facilitate the bringing together of the hydrophobic transmembrane domains and that the interactions depend on the appropriate conformation of the transmembrane region (which in turn is altered by the structure of the tail), and perhaps this explains the sensitivity of the intracellular transport of HN to charge alterations around the transmembrane domain. This could also explain the data obtained with VSV G protein mutants, in which alteration to the cytoplasmic tail permitted normal ectodomain oligomerization but the molecules had slowed rates of transport (7, 8, 44).

Another possibility that may explain the slowed or undetectable transport of cytoplasmic tail mutants of HN and VSV G is that the critical event for transport is not just the formation of the native ectodomain but could also be the appropriate conformation of the cytoplasmic tails. This structure could itself be dependent on oligomerization, and it creates the transport signal. In this regard it is interesting to note that alterations in the C-terminal cytoplasmic tail sequences of the adenovirus E3/19K protein that normally resides in the ER of infected cells eliminate the ER retention of this protein (34, 35). It has been proposed that the normal retention of the E3/19K protein in the ER may result from an interaction of its cytoplasmic tail with cellular cytoplasmic factors (34). In addition, it has been suggested that cytosolic factors play a role in the compartmentalization of some membrane proteins, since the Kex 2P endopeptidase is not retained in the Golgi apparatus of *Saccharomyces cerevisiae* lacking clathrin (39). Thus, a proper conformation in the cytoplasmic tail of HN might allow interactions with cytoplasmic factors during assembly and transport.

ACKNOWLEDGMENTS

We thank Margaret Shaughnessy for excellent technical assistance. We thank Erwin Goldberg for LDH antisera and the cDNA clone to human LDH (C) and Linda Hendershot and John Kearney for providing the anti-BiP MAb. We thank Davis T. W. Ng for helpful discussions concerning the GRP78-BiP immunoprecipitations.

G.D.P. was supported by an American Cancer Society postdoctoral fellowship (PF-3177). This research was supported by Public Health Service research grant AI-23173 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

- Adams, G. A., and J. K. Rose. 1985. Incorporation of a charged amino acid into the membrane spanning domain blocks cell surface transport but not membrane anchoring of a viral glycoprotein. *Mol. Cell. Biol.* 5:1442-1448.
- Bernstein, H. D., T. A. Rapoport, and P. Walter. 1989. Cytosolic protein translocation factors. Is SRP still unique? *Cell* 58:1017-1019.
- Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA* 77:1496-1500.
- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.
- Bormann, B.-J., W. J. Knowles, and V. T. Marchesi. 1989. Synthetic peptides mimic the assembly of transmembrane glycoproteins. *J. Biol. Chem.* 264:4033-4037.
- Copeland, C. S., K.-P. Zimmer, K. R. Wagner, G. A. Healey, I. Mellman, and A. Helenius. 1988. Folding, trimerization, and transport are sequential events in the biogenesis of influenza virus hemagglutinin. *Cell* 53:197-209.
- Crise, B., A. Ruusala, P. Zagouras, A. Shaw, and J. K. Rose. 1989. Oligomerization of glycolipid and soluble forms of the vesicular stomatitis virus glycoprotein. *J. Virol.* 63:5328-5333.
- Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose. 1988. Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. *J. Cell Biol.* 107:89-99.
- Doyle, C., M. G. Roth, J. Sambrook, and M.-J. Gething. 1985. Mutations in the cytoplasmic domain of the influenza virus hemagglutinin affect different stages of intracellular transport. *J. Cell Biol.* 100:704-714.
- Doyle, C., J. Sambrook, and M.-J. Gething. 1986. Analysis of progressive deletions of the transmembrane and cytoplasmic domains of influenza hemagglutinin. *J. Cell Biol.* 103:1193-1204.
- Dreyfuss, G., Y. D. Choi, and S. A. Adam. 1984. Characterization of heterogeneous nuclear RNA-protein complexes in vivo with monoclonal antibodies. *Mol. Cell. Biol.* 4:1104-1114.
- Erickson, A. H., and G. Blobel. 1979. Early events in the biosynthesis of lysosomal enzyme cathepsin. *J. Biol. Chem.* 254:11771-11774.
- Fukuda, M. 1985. Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. *Biochim. Biophys. Acta* 780:119-150.
- Garoff, H., C. Kondor-Koch, R. Pettersson, and B. Burke. 1983. Expression of Semliki Forest virus proteins from cloned complementary DNA. II. The membrane spanning glycoprotein E2 is transported to the cell surface without its normal cytoplasmic domain. *J. Cell Biol.* 97:652-658.
- Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wildtype and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell* 46:939-950.
- Guan, J.-L., A. Ruusala, H. Cao, and J. K. Rose. 1988. Effects of altered cytoplasmic domains on transport of the vesicular stomatitis virus glycoprotein are transferable to other proteins. *Mol. Cell. Biol.* 8:2869-2874.
- Hiebert, S. W., and R. A. Lamb. 1988. Cell surface expression of glycosylated, nonglycosylated and truncated forms of a cytoplasmic protein pyruvate kinase. *J. Cell Biol.* 107:865-876.
- Hiebert, S. W., R. G. Paterson, and R. A. Lamb. 1985. Hemagglutinin-neuraminidase protein of the paramyxovirus simian virus 5: nucleotide sequence of the mRNA predicts an N-terminal membrane anchor. *J. Virol.* 54:1-6.
- Hurtley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and C. S. Copeland. 1989. Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). *J. Cell Biol.* 108:2117-2126.
- Kreis, T. E., and H. F. Lodish. 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. *Cell* 46:929-937.
- Lamb, R. A., P. R. Etkind, and P. W. Choppin. 1978. Evidence for a ninth influenza viral polypeptide. *Virology* 91:60-78.
- Lamb, R. A., and C.-J. Lai. 1982. Spliced and unspliced messenger RNAs synthesized from cloned influenza virus M DNA in an SV40 vector: expression of the influenza virus membrane protein (M₁). *Virology* 123:237-256.
- Lipp, J., and B. Dobberstein. 1986. The membrane spanning segment of invariant chain (I gamma) contains a potentially cleavable signal sequence. *Cell* 46:1103-1112.
- Lodish, H. F. 1988. Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi. *J. Biol. Chem.* 263:2107-2110.
- Loeb, J. A., and K. Drickamer. 1987. The chicken receptor for endocytosis of glycoproteins contains a cluster of N-acetylglucosamine-binding sites. *J. Biol. Chem.* 262:3022-3029.
- Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb. 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethylsulfoxide or glycerol shock treatment. *Nucleic Acids Res.* 12:5707-5717.
- Machamer, C. E., and J. K. Rose. 1987. A specific transmembrane domain of a coronavirus E1 glycoprotein is required for

- its retention in the Golgi region. *J. Cell Biol.* **105**:1205–1214.
28. Markwell, M.-A. K., and C. F. Fox. 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking. *J. Virol.* **33**:152–166.
 29. Merz, D. C., A. Scheid, and P. W. Choppin. 1981. Immunological studies of the functions of paramyxovirus glycoproteins. *Virology* **109**:94–105.
 30. Millan, J. L., C. E. Driscoll, K. M. LeVan, and E. Goldberg. 1987. Epitopes of human testis specific lactate dehydrogenase deduced from a cDNA sequence. *Proc. Natl. Acad. Sci. USA* **84**:5311–5315.
 31. Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**:899–907.
 32. Ng, D. T. W., S. W. Hiebert, and R. A. Lamb. 1990. Different roles of individual N-linked oligosaccharide chains in folding, assembly, and transport of simian virus 5 hemagglutinin-neuraminidase. *Mol. Cell. Biol.* **10**:1989–2001.
 33. Ng, D. T. W., R. E. Randall, and R. A. Lamb. 1989. Intracellular maturation and transport of the SV5 type II glycoprotein HN: specific and transient association with GRP78-BiP in the ER and extensive internalization from the cell surface. *J. Cell Biol.* **109**:3273–3289.
 34. Nilsson, T., M. Jackson, and P. A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* **58**:707–718.
 35. Paabo, S., B. M. Bhat, W. S. M. Wold, and P. A. Peterson. 1987. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell* **50**:311–317.
 36. Parks, G. D., J. C. Baker, and A. C. Palmenberg. 1989. Proteolytic cleavage of encephalomyocarditis virus capsid region substrates by precursors to the 3C enzyme. *J. Virol.* **63**:1054–1058.
 37. Parks, G. D., J. D. Hull, and R. A. Lamb. 1989. Transposition of domains between the M₂ and HN viral membrane proteins results in polypeptides which can adopt more than one membrane orientation. *J. Cell Biol.* **109**:2023–2032.
 38. 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 SV5 from cloned cDNA. *Proc. Natl. Acad. Sci. USA* **82**:7520–7524.
 39. Payne, G. S., and R. Schekman. 1989. Clathrin: a role in the intracellular retention of a Golgi membrane protein. *Science* **245**:1358–1365.
 40. Pelham, H. 1988. Heat shock proteins: coming in from the cold. *Nature (London)* **332**:776–777.
 41. 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.
 42. Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* **56**:829–852.
 43. Pipas, J. M., K. W. C. Peden, and D. Nathans. 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene. *Mol. Cell. Biol.* **3**:203–213.
 44. Puddington, L., C. E. Machamer, and J. K. Rose. 1986. Cytoplasmic domains of cellular and viral integral membrane proteins substitute for the cytoplasmic domain of the vesicular stomatitis virus glycoprotein in transport to the plasma membrane. *J. Cell Biol.* **102**:2147–2157.
 45. 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.
 46. Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **4**:257–288.
 47. Sanger, F., S. Nicklin, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 48. Schmid, S. R., and M. Spiess. 1988. Deletion of the amino-terminal domain of asialoglycoprotein receptor H1 allows cleavage of the internal signal sequence. *J. Biol. Chem.* **263**:16886–16891.
 49. Shaw, A. S., P. J. M. Rottier, and J. K. Rose. 1988. Evidence for the loop model of signal-sequence insertion into the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **85**:7592–7596.
 50. Thompson, S. D., W. G. Laver, K. G. Murti, and A. Portner. 1988. Isolation of a biologically active soluble form of the hemagglutinin-neuraminidase protein of Sendai virus. *J. Virol.* **62**:4653–4660.
 51. Van Driel, I. R., C. G. Davis, J. L. Goldstein, and M. S. Brown. 1987. Self-association of the low density lipoprotein receptor mediated by the cytoplasmic domain. *J. Biol. Chem.* **262**:16127–16134.