Transposition of Domains between the M_2 and HN Viral Membrane Proteins Results in Polypeptides Which Can Adopt More Than One Membrane Orientation

Griffith D. Parks, J. David Hull, and Robert A. Lamb

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

Abstract. The influenza A virus M_2 polypeptide is a small integral membrane protein that does not contain a cleaved signal sequence, but is unusual in that it assumes the membrane orientation of a class I integral membrane protein with an NH_2-terminal ectodomain and a COOH-terminal cytoplasmic tail. To determine the domains of M_2 involved in specifying membrane orientation, hybrid genes were constructed and expressed in which regions of the M_2 protein were linked to portions of the paramyxovirus HN and SH proteins, two class II integral membrane proteins that adopt the opposite orientation in membranes from M_2. A hybrid protein (MgMH) consisting of the M_2 NH_2-terminal and membrane-spanning domains linked precisely to the HN COOH-terminal ectodomain was found in cells in two forms: integrated into membranes in the M_2 topology or completely translocated across the endoplasmic reticulum membrane and ultimately secreted from the cell. The finding of a soluble form suggested that in this hybrid protein the anchor function of the M_2 signal/anchor domain can be overridden. A second hybrid which contained the M_2 NH_2 terminus linked to the HN signal anchor and ectodomain (MgHH) was found in both the M_2 and the HN orientation, suggesting that the M_2 NH_2 terminus was capable of reversing the topology of a class II membrane protein. The exchange of the M_2 signal/anchor domain with that of SH resulted in a hybrid protein which assumed only the M_2 topology. Thus, all these data suggest that the NH_2-terminal 24 residues of M_2 are important for directing the unusual membrane topology of the M_2 protein. These data are discussed in relationship to the loop model for insertion of proteins into membranes and the role of charged residues as a factor in determining orientation.

INTEGRAL membrane proteins that span the lipid bilayer once can be divided into two major groups based on their topology with respect to the membrane (3, 37). The most common are the class I proteins, which are orientated with an NH_2-terminal ectodomain and a COOH-terminal cytoplasmic tail, e.g., the low density lipoprotein receptor (26), vesicular stomatitis virus (VSV) G surface glycoprotein (25), and the influenza virus hemagglutinin (37). Class I proteins contain an NH_2-terminal signal sequence which initiates the interaction of the growing polypeptide with the ER through binding with signal recognition particle (reviewed in reference 36), and this signal is ultimately cleaved in the lumen of the ER by signal peptidase. Translocation of class I proteins across the ER membrane is halted by a COOH-terminal stop-transfer sequence which anchors the protein in the membrane (27, 37).

Class II membrane proteins are orientated with the NH_2-terminal domain in the cytoplasm and a COOH-terminal ectodomain (37). By definition, class II membrane proteins do not contain a cleavable NH_2-terminal signal peptide, but instead contain a stretch of hydrophobic residues near the NH_2 terminus which acts as both an internal signal sequence directing integration into the membrane and as a domain which anchors the polypeptide (signal/anchor domain; 27, 37). Examples of class II proteins include the transferrin receptor (31), asialoglycoprotein receptor (35), influenza virus neuraminidase (4), and the paramyxovirus SV5 hemagglutinin-neuraminidase (HN) and SH proteins (9, 11).

The influenza A virus M_2 polypeptide is a 97-amino acid integral membrane protein which is abundantly expressed at the surface of infected cells, where it has been suggested that M_2 is involved in virus assembly and budding (17, 39). The membrane orientation of M_2 is unusual in that it cannot be classified as either a class I or II integral membrane protein. Although M_2 contains a single 19-amino acid internal uncleaved signal/anchor typical of class II proteins, the polypeptide is orientated in the membrane in a class I topology, with a 24-amino acid NH_2-terminal ectodomain and a 55-residue COOH-terminal cytoplasmic tail (17). The topology is uncommon, as only a few other examples of proteins containing a single membrane spanning domain with this topology are known, including the influenza B virus NB glycoprotein (38), gp74 V-erb B of avian erythroblastosis virus (30), and cytochrome P450 (20, 28).

The signals for proper membrane integration and transport of M_2 to the cell surface are contained within the M_2 polypeptide itself, as shown by analysis of the protein expressed from a cDNA clone (40). To investigate the regions of the M_2 protein involved in establishing its unusual topology, we have constructed cDNA clones which express chimeric proteins containing precise exchanges of the membrane spanning and COOH-terminal domains from M_2 with those of two class II membrane proteins: the paramyxovirus SV5 HN and SH polypeptides (9, 11). Analysis of the properties of these chimeric proteins indicate that some of the polypeptides can assume more than one membrane orientation, whereas others are completely translocated across the ER membrane and are ultimately secreted from the cell. The data suggest that the NH_2-terminal ectodomain of M_2 is important in establishing the M_2 membrane topology and that this region is capable of reversing the orientation of a class II integral membrane protein.

Materials and Methods

Plasmid Construction

cDNA clones which express wild-type SV5 HN (pSVHNm) (23), SH (pSH) (10), and influenza A virus M_2 proteins (pSV40-M_2) (40) were used as starting materials for the construction of plasmids. Oligonucleotides were synthesized by the Northwestern University Biotechnology Facility on a DNA synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA).

Bacteriophage M13 containing M_2 (M13M_2) or SH (M13SH) DNA were constructed by inserting the entire M_2 cDNA (40) or the 270 bp SH gene (10) into the Bam HI site of the replicative form of M13. M13HN was constructed by subcloning the 290 bp Xho I to Pst I fragment from pSVHNm (encoding HN 5′ sequences and amino acid residues 1–81) into the corresponding sites of the replicative form of M13mp19X (22) such that single stranded phage DNA contained (−) sense template.

The M_2 DNA was altered such that it encoded a consensus site for N-linked glycosylation (Asn-Leu-Thr) at M_2 residues 3–5 (17). Site-specific substitution (42) of nucleotides CTT (encoding leucine, residue 3) with AAC (asparagine) on M13M_2 was done using a 27-mer mutagenic oligonucleotide primer. The resulting cDNA (designated M13M_2g) was excised from the replicative intermediate by digestion with Bam HI and subcloned into the Bam HI site of pGEM-1 (Promega Biotec, Madison, WI) to generate pGM_2g. The orientation of the insert was such that M_2 mRNA-sense transcripts could be generated using the T_7 promoter and T_7 RNA polymerase (12). To construct pSVM_2g, the cDNA fragment from M13M_2g was excised by digestion with Bam HI and inserted into the SV-40 expression vector pSV73E/K (40).

Chimeric proteins are named by three capital letters (M, H, or S) to describe the three domains of the protein: NH_2-terminal, membrane spanning, and COOH-terminal (see Fig. 1). A lower case "g" denotes the addition of an N-linked glycosylation site to the M_2 NH_2-terminal region. Thus, a protein containing an M_2 NH_2 terminus and membrane spanning region linked to the COOH-terminal domain of HN is referred to as MMH. MgSM consists of the M_2g NH_2 terminus, SH membrane spanning domain, and M_2 COOH-terminus.

To construct the MgSM gene, the M_2 hydrophobic domain (residues 25–43) was substituted with that of SH (residues 17–39) in two stages using site-directed mutagenesis of M13M_2g. The first step used a 60-mer oligonucleotide to replace the region coding for residues 25–34 of M_2 with that coding for residues 17–28 of SH. The resulting molecule M13MgSM′ was then used in turn as template for mutagenesis with a 57-mer oligonucleotide which was used to replace the region encoding M_2 residues 35–43 with that coding for SH residues 29–39 to produce M13M_gSM. The entire nucleotide sequence was confirmed before subcloning (designated pGMgSM) as described above.

Hybrid M_2/HN genes were constructed by linking specific DNA fragments through a common restriction site or by a blunt end ligation such that no extraneous codons were generated. New restriction enzyme sites were introduced into M13M_2 and M13M_2g at M_2 nucleotides 153–158 (Xho I) or 96–101 (Eco RV) by oligonucleotide-directed mutagenesis using uracil-enriched single-stranded template as described (21). Similarly, new Xho I or Sca I sites were introduced into M13HN at HN nucleotides 169–177 and 115–120, respectively. Resulting mutants were screened by dideoxynucleotide sequence analysis (29). Mutant fragments were excised from the replicative form of M13 and subcloned into pGem-4 plasmids in place of the corresponding wild-type fragment.

To generate MMH and MgMH a new Xho I site (CTCGAG), which encodes the junction of the M_2 membrane spanning and HN COOH-terminal domains (Leu-Glu), was used to join the HN and M_2 cDNA fragments in a pGem-4 vector such that the resulting hybrid constructions encoded the M_2 NH_2-terminal and signal/anchor domains (residues 1–42) linked precisely to the HN ectodomain (residues 37–89). The resulting hybrid DNA fragment was then joined with a segment encoding the remaining HN residues (90–565) in the SV-40-based vector pSV103 (23).

Similarly, MgHH was constructed through the use of new Eco RV (GAT/ATC) and Sca I (AGT/ACT) sites added to the M_2g and HN cDNA clones, respectively. Blunt end ligation of the Eco RV-cut M_2g DNA fragment to the Sca I-cut HN segment produced a junction (GAT/ACT) encoding an Asp/Thr pair and resulted in a chimeric M_2g/HN cDNA which precisely linked the M_2 NH_2 terminus (residues 1–24) to the HN signal/anchor and ectodomains (residues 18–89). The hybrid gene fragment was then joined to the segment encoding the remaining HN residues as described above. Sequences were confirmed by dideoxy chain terminating analysis (29).

Cells

Monolayer cultures of the TC7 clone of CV-1 cells were grown in DME containing 10% FCS. Cells were passaged and infected as described (13).

Preparation and Expression of SV-40 Recombinant Virus

cDNA fragments encoding the hybrid M_2/HN genes or M_2g were inserted into the SV-40 late region replacement vectors pSV103 or pSV73E/K, respectively (23, 40). Recombinant SV-40 virus stocks were prepared by cotransfection of CV-1 cells with the recombinant DNA molecules and DNA from an SV-40 early region deletion mutant (24) using DEAE-dextran treatment as described (13).

Isotopic Labeling of Polypeptides in Infected Cells, Immunoprecipitation, and PAGE

Cells infected with recombinant SV-40 (13) were labeled between 40 and 48 h postinfection with 100–200 μCi/ml Tran-^{35}S-label (ICN Radiochemicals Inc., Irvine, CA) in cysteine-methionine-free DME or (200 μCi/ml) [^{35}S]cysteine in cysteine-free DME. Immunoprecipitation of proteins from cell extracts solubilized in RIPA buffer was done as described (16) using mAb 14C2 which is specific for the M_2 NH_2-terminal domain (39), or monospecific polyclonal rabbit antisera to SDS-denatured HN. Immunoprecipitation of SDS-solubilized cell extracts was done as described (2). Briefly, cells were lysed by the addition of 1% SDS, boiled for 5 min, and diluted into 4 vol of buffer (1.25% Triton X-100, 190 mM NaCl, 60 mM Tris-HCl, pH 7.4, 6 mM EDTA) before incubation with sera. Samples were analyzed by SDS-PAGE on 10% gels followed by fluorography (14, 15).

M_2g and MgSM were expressed from transfected pGM_2g and pGMgSM in CV-1 cells by a modification of previous procedures (7). Cells were infected (multiplicity of infection of 20) with recombinant vaccinia virus vTF7-3, which expresses the bacteriophage T_7 RNA polymerase (7). Cells were then transfected with 20 μg of pGM_2g or pGMgSM by calcium phosphate precipitation. At 3 h posttransfection, cells were labeled for 1 h with 100 μCi/ml [^{35}S]cysteine in cysteine-free DME, lysed in RIPA buffer, immunoprecipitated with M_2-specific mAb, and analyzed on 17.5% polyacrylamide gels containing 4 M urea (16, 40).

Endoglycosidase Analysis and Tunicamycin Treatment of Cells

Treatment of samples with β-endoglycosidase H (endo H) (Miles Laboratories, Inc. Elkhart, IN) was as described previously (38). For tunicamycin treatment, the drug (1 μg/ml) (Calbiochem-Behring Corp., La Jolla, CA) was added to cells 2 h before and maintained during the labeling period.

Trypsinization of Microsomal Membranes

Monolayers of recombinant SV-40–infected CV-1 cells (10-cm-diam plates)

1. *Abbreviation used in this paper*: Endo H, endoglycosidase H.

were labeled for 30 min with (200 μCi/ml) Tran-^{35}S-label, and intracellular microsomal membranes were prepared as described (1). Each sample was divided into three equal parts and incubated with either buffer, trypsin (11 μg/ml; Sigma Co., St. Louis, MO), or trypsin plus detergent (1% NP-40) at 37°C for 45 min. After addition of trypsin inhibitor (aprotinin, 5 TI units; Sigma Chemical Co.), membranes were isolated by centrifugation (35,000 rpm, 3.5 h, 4°C) in a SW41 rotor (Beckman Instruments Inc., Palo Alto, CA) through a 10-ml sucrose cushion (10% [wt/vol] 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Pellets were boiled in 1% SDS, diluted with buffer as described above, immunoprecipitated with HN-specific antisera, and analyzed by SDS-PAGE.

Alkali Treatment of Microsomes

6-cm-diam plates of CV-1 cells infected with recombinant SV-40 virus were labeled with (150 μCi/ml) Tran-^{35}S-label for 1 h. For MgSM and M_2g, vaccinia virus infected cells which had been transfected with pGMgSM or pGM_2g were labeled at 3 h posttransfection with [^{35}S]cysteine for 1 h. After preparation of microsomal membranes (1), 0.9 ml of lysate was clarified (3,000 *g*), mixed with 0.1 ml of 1 M Na_2CO_3 (pH 11), and incubated at 0°C for 30 min (33). The samples were centrifuged for 45 min at 40,000 rpm (130,000 *g*) at 4°C in a table top ultracentrifuge (model TL100) using a rotor (model TLS-55; both from Beckman Instruments). Equal portions of the pellet and supernatant were neutralized with acetic acid, solubilized in RIPA buffer, immunoprecipitated, and analyzed by SDS-PAGE.

Results

Expression of Chimeric Proteins in CV-1 Cells

To analyze the protein domains responsible for the membrane orientation of M_2, recombinant cDNA molecules were constructed with precise domain exchanges such that they encoded the NH_2-terminal ectodomain of M_2, a hydrophobic membrane spanning region from either M_2, HN, or SH and the ectodomain of HN or the cytoplasmic tail of M_2

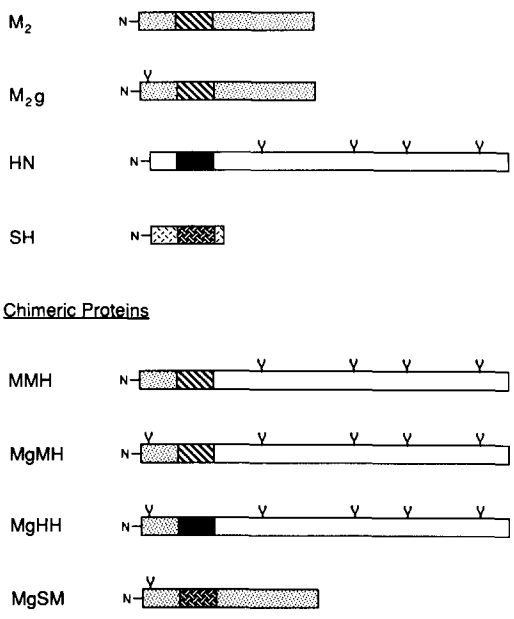


Figure 1. Schematic diagram of the domains of the wild-type integral membrane proteins and those of the chimeric proteins MMH, MgMH, MgHH, and MgSM. Membrane proteins are depicted schematically as horizontal bars consisting of three domains: amino terminal (*left*), membrane spanning (*middle*), and carboxy terminal (*right*). Chimeric proteins are named as described in the text. *N*, amino terminus. (Y), N-linked glycosylation sites. Proteins are not drawn to scale.

Figure 2. Expression and endoglycosidase treatment of M_2 and M_2g. CV-1 cells infected with recombinant SV-40 expressing M_2 or M_2g were labeled for 1 h with [^{35}S]cysteine and lysed in RIPA buffer. Samples were immunoprecipitated with an M_2-specific monoclonal antibody, and digested with (+) or without (−) β-Endo H before analysis by SDS-PAGE. Lane *C* shows a wild-type M_2 lysate immunoprecipitated with ascites fluids from control SP2/O cells.

(see Fig. 1). The analysis of the membrane orientation of the chimeric proteins was facilitated by the construction of an M_2 cDNA clone which encoded an asparagine-linked glycosylation site in the M_2 NH_2-terminal ectodomain (M_2g). The recombinant hybrid molecules (MMH, MgMH, MgHH, and MgSM) are named after the origin of the precise domains which were exchanged for their construction (Fig. 1). Thus, MMH contains the NH_2-terminal ectodomain and signal-anchor of M_2 (residues 1–42) linked precisely to the ectodomain of HN (residues 37–565). The chimeric genes were inserted into an SV-40 late-region replacement vector and lytic stocks of recombinant virus were produced.

To examine if the newly introduced NH_2-terminal glycosylation site in M_2 was used, CV1 cells infected with SV-40M_2 or SV-40M_2g recombinant viruses (the latter reconstructed and kindly made available by Hooshmand Sheshberadaran) were labeled for 1 h with [^{35}S]cysteine and immunoprecipitated with M_2-specific monoclonal antibody 14C2. As shown in Fig. 2, M_2g had a slower electrophoretic mobility as compared to M_2 (− lanes), compatible with the addition of a single N-linked carbohydrate chain to M_2g. Further evidence for glycosylation was provided by digesting M_2g with Endo H, and finding an increased electrophoretic mobility of M_2g (Fig. 2, + lane). The difference in mobility between wild-type M_2 and endo H-treated M_2g is due to both the amino acid change in M_2g and the *N*-acetylglucosamine residue remaining after Endo H treatment. Single amino acid changes in M_2 have been shown previously to alter its electrophoretic mobility (17). However, when the 24-residue ectodomain of M_2g is fused to the 548-residue transmembrane and COOH-terminal domains of HN, the amino acid substitution in M_2 does not detectably alter its electrophoretic mobility (see below). It will be shown elsewhere that M_2g is abundantly expressed at the cell surface and that the carbohydrate chain remains sensitive to Endo H digestion at all

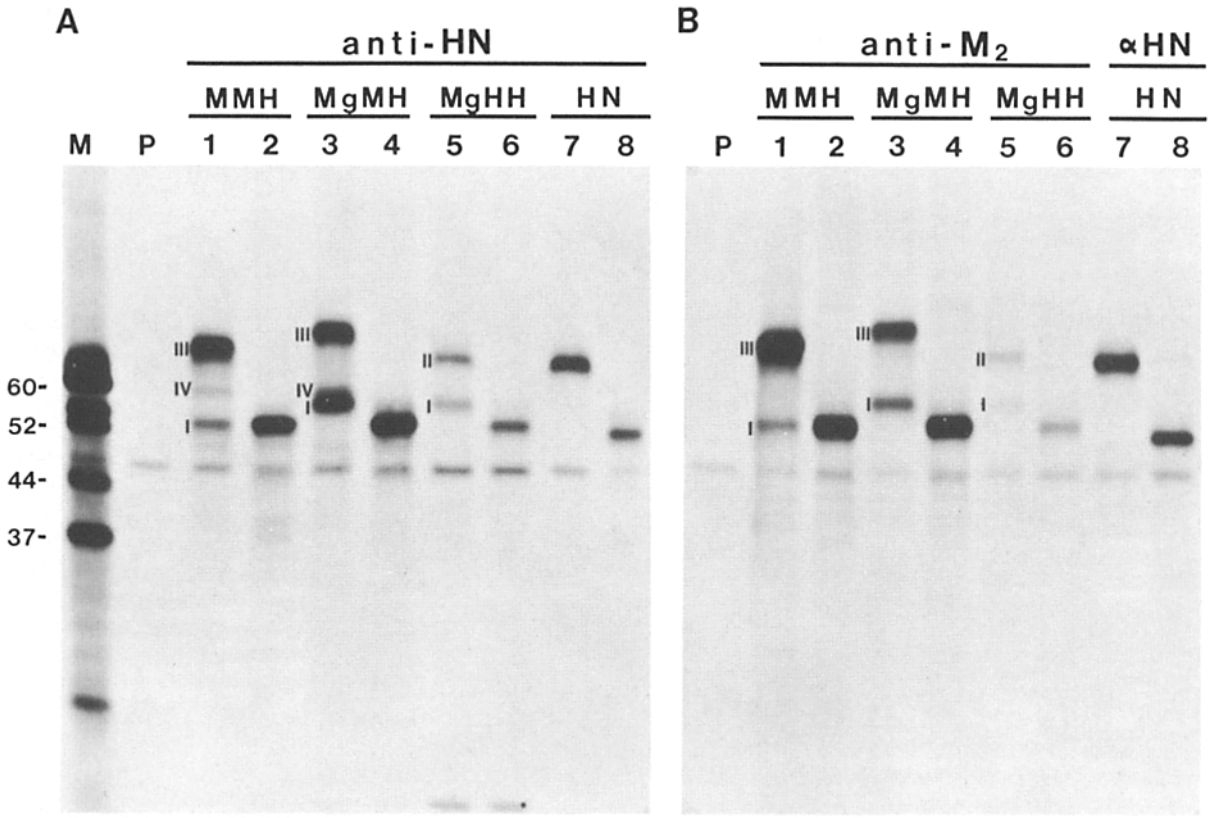


Figure 3. Expression of chimeric M_2/HN proteins in CV-1 cells. Cells infected with recombinant SV-40 MMH, MgMH, and MgHH were labeled for 1 h with Tran-^{35}S-label in the absence (lanes *1*, *3*, *5*, and *7*) or presence (lanes *2*, *4*, *6*, and *8*) of tunicamycin. Cells were lysed in RIPA buffer and immunoprecipitated with polyclonal antisera to HN (*A*) or an M_2-specific monoclonal antibody (*B*, except lanes *7* and *8*), and the resulting proteins were analyzed by SDS-PAGE and fluorography. The designation of the chimeric proteins is shown above the lanes. Lanes *7* and *8* are marker lanes of wild-type HN expressed from recombinant virus SVHNm (23) and immunoprecipitated with anti-HN serum. Lanes *P*, SV-40 recombinant virus MMH-infected cell lysate immunoprecipitated using preimmune sera. Lane *M*, marker lane of SV5-infected cell lysate; molecular masses (in kilodaltons) are indicated on the left. *I*, *II*, *III*, and *IV* denote forms of the chimeric proteins described in the text. αHN denotes anti-HN.

times (Sheshberadaran, H., unpublished observations). In the hybrid molecules described below, glycosylation of this NH_2-terminal region of M_2 is used as a marker for translocation of this domain into the ER lumen.

The proteins produced from the hybrid M_2/HN genes were examined by infecting CV-1 cells with the various recombinant SV-40 viruses and labeling for 1 h with Tran-^{35}S-label in the presence or absence of tunicamycin, an inhibitor of asparagine-linked glycosylation. Cell lysates were then immunoprecipitated with antisera specific for either HN or the NH_2-terminal domain of M_2 and analyzed by SDS-PAGE. In CV1 cells infected with recombinant virus SV-HNm, which has been shown previously to express biologically active wild-type HN (23), a single HN-specific glycosylated polypeptide species of ~68,000 M_r was synthesized (Fig. 3, lane *7*), and in the presence of tunicamycin a single band of ~52,000 M_r was observed (lane *8*). In contrast, SV-40–MMH synthesized two major polypeptides, designated form I (~53,000 M_r) and III (~70,000 M_r), which were reactive with antisera to HN (Fig. 3 *A*, lane *1*), but not with preimmune sera (lane *P*). SV-40–MgMH, which encodes the MgMH polypeptide with the additional NH_2-terminal glycosylation site, synthesized in infected cells two major polypeptides precipitated by HN antisera (forms I and III, lane *3*) which have a slower electrophoretic mobility compared to MMH form I and III (lane *1*). The increased size of the MgMH forms I and III (~58,000 and 75,000 M_r) was consistent with the addition of a carbohydrate chain to the NH_2 terminus of these two polypeptides. In the presence of tunicamycin, a single MMH-specific polypeptide of ~53,000 M_r was synthesized in both SV-40–MMH and SV-40–MgMH infected cells (Fig. 3 *A*, lanes *2* and *4*), suggesting that forms I and III are a single polypeptide species which differ by the extent of carbohydrate addition.

SV-40–MgHH synthesized two MgHH polypeptide species in infected cells (Fig. 3 *A*, lane *5*), one with an electrophoretic mobility matching that of MgMH form I (~58,000 M_r) and a second species (form II) with a mobility similar to wild-type HN (~68,000 M_r). In the presence of tunicamycin, the MgHH polypeptides migrated as a single species of ~53,000 M_r (lane *6*), suggesting that form I and II differed by carbohydrate addition. Further, the difference in mobility of ~3,000 M_r between MgMH form III and MgHH form II suggests that the four HN-specific glycosylation sites are used equivalently.

To examine the integrity of the M_2 NH_2-terminus in these hybrid molecules, cell lysates were immunoprecipitated with a site-specific antibody (M_2 monoclonal 14C2) (39). This

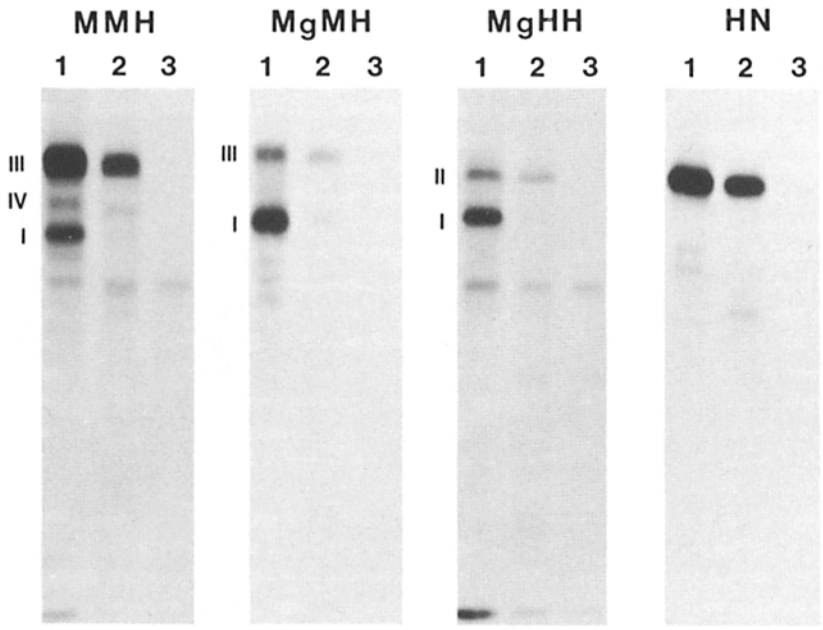


Figure 4. Protease treatment of microsomal membranes containing M_2/HN chimeric proteins. Recombinant SV-40–infected cells expressing MMH, MgMH, MgHH, or HN were labeled for 30 min with Tran-^{35}S-label. Microsomal membranes were prepared (1) and treated with buffer (lane *1*), trypsin (lane *2*), or trypsin plus detergent (lane *3*). After centrifugation through a sucrose cushion, pellets were boiled in 1% SDS, diluted, and immunoprecipitated with HN-specific antisera before analysis by SDS-PAGE.

antibody recognized two forms of the hybrid polypeptides which matched those identified by using HN-specific sera (Fig. 3 *B*, lanes *1–6*). The reactivity of these species with this M_2 NH_2-terminal–specific mAb indicates that the NH_2-terminal region of forms I, II, and III was present and had not been cleaved by signal peptidase during translocation across the ER membrane.

When a comparison of the hybrid polypeptides immunoprecipitated by the two antisera (Fig. 3, *A* and *B*) was made, another MMH- and MgMH-specific species (form IV, *A*) could be identified with the HN antisera that was not recognized by the M_2 antibody. The electrophoretic mobility of form IV was not altered in the case of MgMH by the presence of the M_2 NH_2-terminal glycosylation site (compare Fig. 3, lanes *1* and *3*). The precise nature of polypeptide IV has not been determined, but it is thought to lack the M_2 NH_2-terminal region and may represent a cleavage product of the MMH or MgMH polypeptides by a signal peptidase-like activity.

Taken together, the above data indicate that expression of MMH, MgMH, and MgHH chimeric genes in CV-1 cells results in the synthesis of three major protein species (forms I, II, and III) which differ in their glycosylation patterns. In addition, a polypeptide (form IV) was produced from the chimeras MMH and MgMH, and this product is thought to result from cleavage of its NH_2-terminal region.

Chimeric M_2/HN Proteins Exist in Multiple Orientations in the Membrane

Hybrid proteins I and III synthesized by MMH or MgMH and form I and II synthesized by MgHH differed in their extent of glycosylation, suggesting that they may be oriented differently in the ER membrane. To determine biochemically the membrane topology of the various hybrid species, microsomal membranes isolated from recombinant SV-40–infected cells were treated with trypsin and the protected fragments analyzed. Wild-type HN was resistant to protease digestion, unless the membrane had been disrupted by the addition of detergent (Fig. 4, *HN*, lanes *1–3*). Microsomes from recombinant SV-40–infected cells expressing MMH, MgMH, or MgHH were treated similarly (Fig. 4) and it was found that forms II and III were largely protected from digestion with added protease, while the majority of form I was accessible to proteolysis. Protein species IV is protected from digestion (Fig. 4, MMH, lanes *1* and *2*), but showed a slight alteration

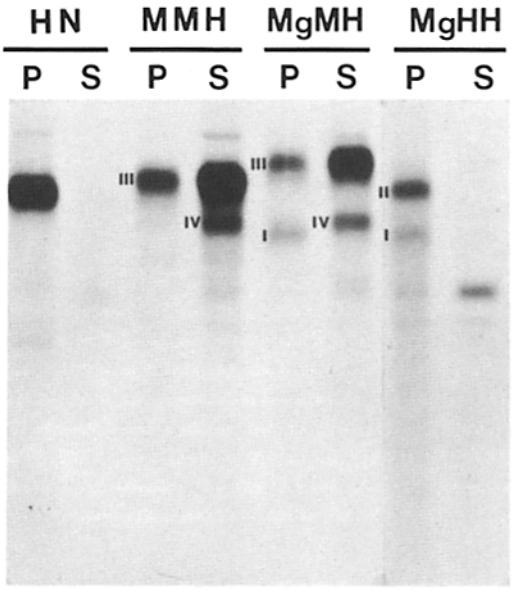


Figure 5. Alkali fractionation of chimeric M_2/HN proteins. CV-1 cells infected with recombinant SV-40 expressing HN, MMH, MgMH, or MgHH were radiolabeled with Tran-^{35}S-label for 1 h and microsomal membranes were prepared. Samples were adjusted to pH 11 by the addition of Na_2CO_3 and incubated at 4°C for 30 min. Membranes were then separated into pellet (*P*) and supernatant (*S*) fractions by centrifugation as described in Materials and Methods. Equal portions of the two fractions were neutralized, solubilized in RIPA buffer, immunoprecipitated with anti-HN sera, and analyzed by SDS-PAGE.

in electrophoretic mobility. This does not reflect proteolytic trimming of the polypeptide but is an artifact due to sample preparation before electrophoresis; all bands in lanes *2* have slightly altered mobilities.

Protection of forms II and III from proteolytic digestion of microsomes would indicate that the polypeptides had assumed either a membrane-anchored class II orientation or that they had been completely translocated across the membrane into the ER lumen. To distinguish between these possibilities microsomal membranes were prepared from recombinant SV-40–infected cells and fractionated at alkaline pH by centrifugation. By this criteria, integral membrane proteins are found in the pellet fraction and membrane peripherally associated and soluble proteins are found in the supernatant (5, 12, 33). Wild-type HN was found in the pellet fraction (Fig. 5, *HN*, lane *P*) as would be expected for an integral membrane protein. MgHH forms I and II were found in the pellet fraction (Fig. 5, *MgHH*, lane *P*) suggesting that they are integral membrane proteins. The majority of forms III and IV of MMH and MgMH were extracted with alkali (compare lanes *P* and *S*) suggesting that they are soluble proteins. Form I of MMH and MgMH was always difficult to detect in these experiments. However, when it could be observed, e.g., for MgMH, it was found in the pellet fraction suggesting that form I is associated with membranes. As will be described below, form I from MMH and MgMH is an unstable species in pulse–chase analysis and thus the relative loss of this protein during alkali fractionation is not unexpected.

The above data suggest that MMH and MgMH polypeptide form III was completely translocated across the membrane as both the NH_2-terminal M_2 and COOH-terminal HN domains were glycosylated, the molecule is resistant to digestion with protease and it is found to be soluble after alkali fractionation. As form I of MMH, MgMH, and MgHH was glycosylated only at its NH_2 terminus, can be digested with protease, and is strongly associated with membranes after alkali fractionation, it can be deduced that form I has its NH_2-terminal M_2 region in the lumen of vesicles and that the molecules are orientated like natural M_2. MgHH form II was not glycosylated at the M_2 site (electrophoretic mobility matching wt HN) but was glycosylated in the HN domain, was resistant to protease digestion, and was not extracted from membranes using the alkali fractionation procedure. These data are consistent with MgHH form II having assumed an HN-like class II integral membrane protein orientation. Thus, most importantly for the studies described here, MMH and MgMH always assumed orientations with their NH_2 terminus translocated across the ER membrane, while MgHH is expressed as two forms that assume opposite orientations in membranes.

Secretion and Stability of the Hybrid Molecules

To provide further evidence for the nature of the chimeric M_2/HN polypeptides, the stability of the different forms of the hybrids and their possible interconversion was examined. CV1 cells were infected with the recombinant SV-40 viruses and pulse-labeled with Tran-^{35}S-label for 30 min followed by incubation for varying periods (chase) with DME. At various times after the pulse-label, cells and medium were harvested, immunoprecipitated with anti-HN sera, and analyzed by SDS-PAGE.

After the 30-min labeling period MMH, MgMH, or MHH forms I, II, III, and IV could be immunoprecipitated with HN-specific sera (Fig. 6, lane *P*) but not control sera (lane *C*). During the chase period, form I of all three hybrid molecules was degraded with a half-time of <1 h. This was not entirely unexpected because the HN ectodomain when held in the cytoplasm would be unlikely to fold into its native form, and thus would be a candidate substrate for proteolysis. In the case of MgMH (*B*), forms I and IV were often difficult to separate electrophoretically (see also Fig. 3 *A*) but the result of many experiments indicated that form I and IV are distinct species and that it is form I that is degraded. Form III was lost from cells over a prolonged chase period (*A* and *B*) and as only a small amount of the molecule is secreted (see below), this loss of label probably reflects turnover of the polypeptide. In contrast, MgHH form II was relatively stable, up to 12 h after pulse-labeling. It can be seen that forms II, III, and IV exhibit a progressively faster mobility during the hours of the chase period, which probably reflects carbohydrate modifications during intracellular transport.

To determine if the lumen soluble polypeptide forms III and IV were secreted, the extracellular medium was immunoprecipitated. As shown in Fig. 6, MMH and MgMH polypeptides were found in the medium beginning 1 h after the pulse period and reaching a plateau by 9 h. The immunoprecipitated polypeptides shown in Fig. 6 were from equal volume aliquots of cell lysate or medium, and thus the unequal amount of proteins in the cells versus the medium, along with nearly complete loss from the cell by 6–9 h, suggests that species III and IV are turned over during the prolonged incubation. As expected for a polypeptide resistant to alkali extraction from membranes, MgHH form II was not secreted into the extracellular medium (Fig. 6 *C*).

The degree of native folding of the hybrid molecules, their oligomerization and rate of transport from the endoplasmic reticulum to the Golgi apparatus are beyond the scope of the experiments reported here. However, MgHH, can be detected at the cell surface by indirect immunofluorescence using HN but not M_2 ectodomain-specific monoclonal antibodies (data not shown) which provides further confirmation of the membrane orientation of the stable MgHH form II protein.

Substitution of a Class II Signal/Anchor into M_2 Does Not Alter Its Membrane Orientation

As described above, the MgHH hybrid molecule assumes two approximately equal but opposite membrane orientations. Since this molecule contains the M_2 NH_2 terminus linked to the HN signal/anchor and COOH-terminal domains, the relative importance of each region in bringing about the inversion in orientation of the HN ectodomain could not be assessed. To investigate further the role of the M_2 hydrophobic domain on membrane orientation, we wished to replace only the M_2 signal/anchor domain with that of the HN signal/anchor domain. This was not possible for technical reasons, and therefore we used the signal/anchor region from another class II integral membrane protein, the paramyxovirus SV5 SH polypeptide (11). A cDNA molecule (MgSM) was constructed by site-specific mutagenesis of M_2g, such that its 19–amino acid signal/anchor was replaced precisely with the 23-residue uncleaved signal/anchor domain of the SH polypeptide. Plasmids were constructed so that the

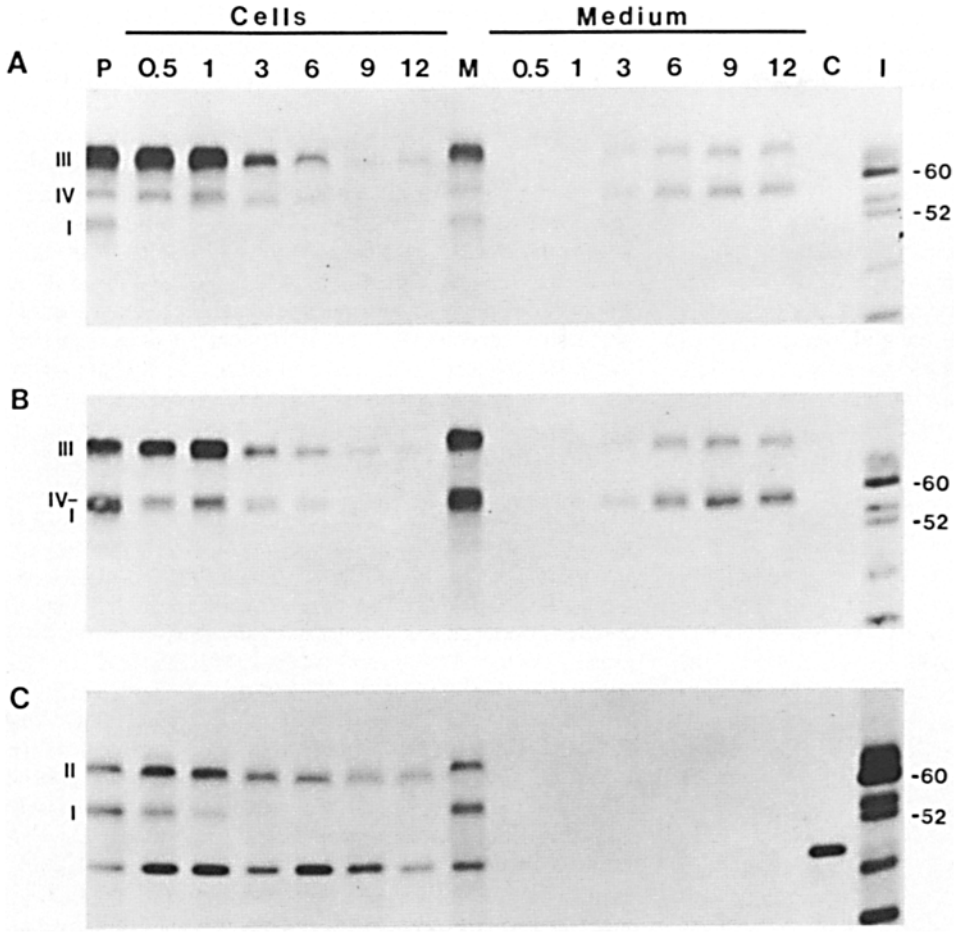


Figure 6. Time course of processing and secretion of chimeric M_2 HN proteins. CV-1 cells infected with recombinant SV-40 expressing MMH (*A*), MgMH (*B*), or MgHH (*C*) were pulse-labeled for 30 min with Tran-^{35}S-label (lane *P*), and incubated in DME containing excess nonradioactive methionine. At each timepoint (in hours), the medium was removed, clarified by centrifugation (10,000 *g* for 2 min), adjusted to 1% SDS and boiled. Cells were lysed in 1% SDS and boiled. Aliquots representing equal portions of the cells and medium were diluted, immunoprecipitated with HN-specific antisera, and analyzed by SDS-PAGE on 10% polyacrylamide gels. Lane *M*, marker of pulse-labeled proteins; lane *C*, control sample of cell lysate immunoprecipitated with preimmune sera; lane *I*, SV5-infected cell lysate with molecular masses (in kilodaltons) indicated. Note in *C* that the fastest migrating species is also found in lysates incubated with preimmune sera.

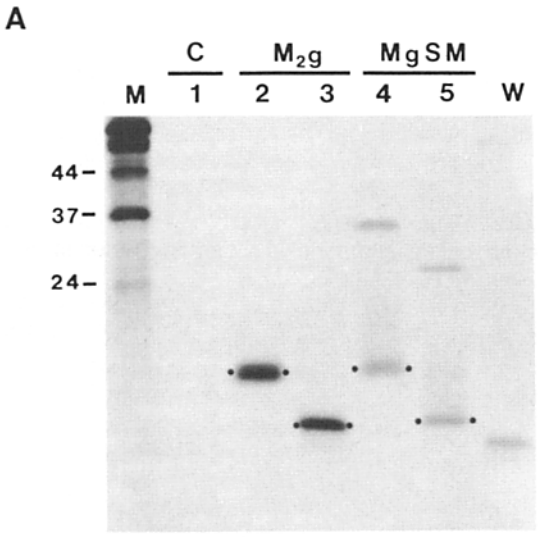
M_2g and MgSM DNA molecules were adjacent to the bacteriophage T_7 RNA polymerase promoter (pGM_2g and pGMgSM). To investigate the membrane orientation of MgSM, CV1 cells were infected with a recombinant vaccinia virus that expresses the T_7 RNA polymerase (7) and then transfected with either pGM_2g or pGMgSM. The cells were labeled with [^{35}S]cysteine and the M_2-specific polypeptides immunoprecipitated using the M_2 monoclonal antibody. As shown in Fig. 7 *A*, the MgSM polypeptide has an electrophoretic mobility similar to M_2g (~20,000 M_r). Treatment of M_2g and MgSM with Endo H yielded polypeptide species with ~15,000 M_r, consistent with the loss of one high mannose N-linked carbohydrate chain (lanes *3* and *5*). Glycosylation of MgSM indicates that its NH_2 terminus has been translocated across the ER membrane. The slightly slower electrophoretic migration of Endo H–treated M_2g compared to wild-type M_2 is in part due to the single *N*-acetylglucosamine residue which remains after Endo H treatment and to the amino acid change in M_2g. The protein of slower electrophoretic mobility (~35,000 M_r) observed in lane *4* which changes mobility with Endo H treatment (lane *5*) is of unknown origin, and may represent nonspecific precipitation of a vaccinia virus protein.

To investigate if MgSM had been translocated completely across the membrane and was a soluble protein, or whether the hydrophobic domain stably anchored MgSM in membranes, the resistance of MgSM and M_2g (as a control) to extraction by alkali was examined. As shown in Fig. 7 *B*, both M_2g and MgSM were found in the pellet fractions and none could be detected in the supernatant fraction. These data indicate that the observed M_2g and MgSM polypeptides are glycosylated and are stably integrated in membranes in the same orientation, i.e., glycosylated lumenal NH_2 termini. Thus, in MgSM the M_2 NH_2 terminus is correctly orientated, even though the SH hydrophobic signal/anchor has been inverted with respect to its normal orientation in the membrane.

Discussion

The M_2 protein of influenza virus is one of a small number of characterized integral membrane proteins with uncleaved signal/anchors that span the lipid bilayer once and are orientated with extracellular NH_2 termini (17, 20, 30, 38). To attempt to understand the role of the protein domains which are responsible for M_2 assuming an uncommon topology, we have constructed hybrid molecules consisting of exchanges of domains of the M_2 polypeptide with domains from HN and SH, two class II integral membrane proteins encoded by the paramyxovirus SV5. The hybrid molecules MMH, MgMH, MgHH, and MgSM were examined for their membrane topology and a summary of the deduced orientations is shown schematically in Fig. 8.

The MMH and MgMH hybrid polypeptides, in which the M_2 cytoplasmic tail (55 residues) was replaced with the much larger ectodomain of HN (529 residues), was found in



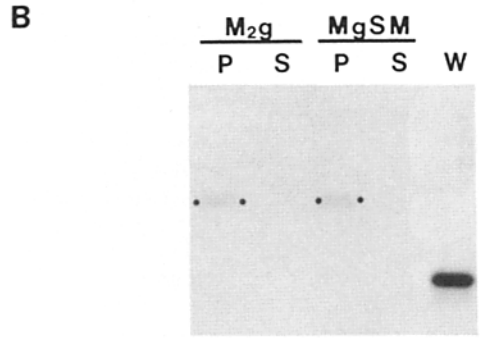


Figure 7. Expression and analysis of M_2g and MgSM. (*A*) Expression and β-Endo H treatment. CV-1 cells were infected with recombinant vaccinia virus vTF-3 which expresses the bacteriophage T7 RNA polymerase (7) and were then transfected with pGM_2g (lanes *2* and *3*) or pGMgSM (lanes *4* and *5*), and labeled with [^{35}S]cysteine for 1 h. Cells were lysed in RIPA buffer, immunoprecipitated with M_2-specific monoclonal antibody, and appropriate samples digested with Endo H. Lanes *2* and *4*, mock digested; lanes *3* and *5*, digested with Endo H. Lane *1* shows an immunoprecipitation of CV1 cells transfected with a nonspecific control plasmid (pGem1). Lane *W*, marker lane of wild-type M_2 expressed from a recombinant SV-40 (40). (*B*) Alkali extraction of M_2g and MgSM. Hybrid proteins were transiently expressed from pGM_2g or pGMgSM and alkaline extraction was performed as described in Materials and Methods. After centrifugation, equal portions of the pellet (*P*) and supernatant (*S*) were immunoprecipitated with anti-M_2 and analyzed by SDS-PAGE.

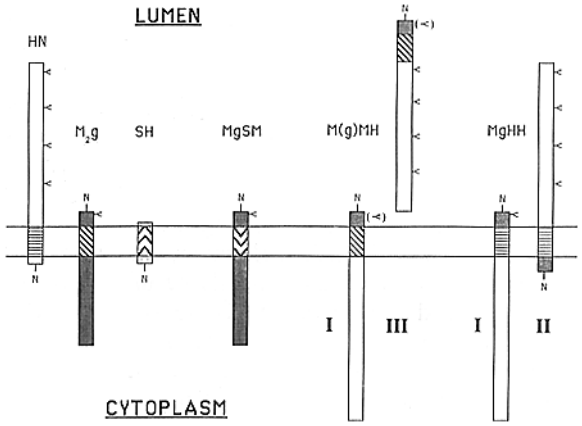


Figure 8. Summary of membrane orientation of HN, M_2g, M_2gSH, and the M_2/HN chimeric proteins. The ER membrane is depicted as two horizontal lines separating the lumenal and cytoplasmic compartments of the cell. Membrane proteins are drawn schematically as vertical boxes with cross-hatched regions representing signal/anchor domains. ≺ and *N* denote N-linked glycosylation sites and the NH_2 terminus of the proteins, respectively. *I*, *II*, and *III* are forms of the chimeric proteins as described in the text.

two major forms (I and III). MMH and MgMH form I (Fig. 8) is anchored in membranes and orientated with a lumenal NH_2 terminus like M_2. This conclusion is based on the observed shift in electrophoretic mobility of glycosylated MgMH as compared to MMH, the sensitivity of its cytoplasmic tail to protease digestion and the inability to extract MgMH on alkali treatment of microsomes. On the other hand, MMH (or MgMH) form III is a soluble protein that is completely translocated across the ER membrane and ultimately secreted from cells. As both MMH form I and III can be recognized by the site-specific M_2 NH_2-terminal antibody, these two species do not differ by cleavage at the NH_2 terminus. In contrast, the soluble and secreted form IV from MMH and MgMH was not recognized by the antisera specific for the M_2 NH_2 terminus and did not exhibit an altered electrophoretic mobility by addition of the M_2 NH_2-terminal glycosylation site. These properties of form IV are consistent with it being a modified version of form III which lacks its NH_2 terminus, possibly due to the action of a signal peptidase-like activity cleaving the hydrophobic domain, but the precise cleavage site remains to be determined. Cleavage of a membrane spanning domain has been reported previously for an altered form of the invariant chain of class II histocompatibility antigen (Iγ), a class II integral membrane protein (18).

The finding of soluble MMH and MgMH form III suggests that the membrane signal/anchor property of the M_2 hydrophobic domain sometimes can be overridden. Although the signal/anchor domain operated normally in form I, in form III it acted only as a signal sequence without anchoring ability. Previous attempts to dissect membrane signaling properties from membrane anchoring functions by deletion of residues in the hydrophobic domain of M_2 (12) and of class II integral membrane proteins (5, 19, 34) have been unsuccessful. In the case of MMH (and MgMH), the ability of the M_2 hydrophobic domain to act as an anchor when the molecule is being synthesized and cotranslationally inserted into the membrane may be borderline due to the driving force of polypeptide chain elongation. It has been suggested that for a region to function as a membrane anchor it must be above a certain critical threshold of hydrophobicity (6). In addition, the position of the hydrophobic domain within the protein is thought to play an important part in determining the value of the threshold of hydrophobicity: the threshold value is greater if the membrane spanning domain is in an internal position rather than an end position (6, 22). Thus, the alteration in the position of the hydrophobic domain due to the length of the cytoplasmic tail in MMH may also be a factor

that contributes to the relative inefficiency of anchoring by the M_2 signal/anchor domain. Although the MMH construction alone does not show which domain of M_2 is important for its topology in membranes, the orientation of MMH (or MgMH) form I does suggest that the M_2 COOH-terminal domain is not required for directing proper orientation.

It has usually been considered that integral membrane proteins assume only one orientation. However, with the hybrid molecule MgHH in which the M_2 NH_2-terminal ectodomain was substituted in place of the HN NH_2-terminal cytoplasmic domain, the polypeptide chain was inserted into the membrane in two opposing orientations: form I in the M_2 orientation and form II in the HN orientation (Fig. 8). The reversal of the usual topology of HN occurred about half the time as measured by accumulation, but this is difficult to quantitate because of the degradation of form I. Although the M_2 NH_2 terminus is strongly implicated in specifying the orientation of MgHH form I, other factors must be involved to explain the ability of the MgHH polypeptide to also adopt the opposite orientation which was found for form II. As MgMH (NH_2 terminus translocated in all species) and MgHH (NH_2 terminus translocated 50% of species) differ only by the origin of their transmembrane domain, it is possible that the nature of this domain influences membrane orientation in conjunction with the M_2 NH_2 terminus.

To directly test the role of the M_2 signal/anchor on membrane topology, we constructed a molecule (MgSM) in which the M_2 hydrophobic domain alone was exchanged with that of the signal/anchor domain from SH, a class II integral membrane protein (11). MgSM was found to have similar properties to M_2g, in that both proteins are stably integrated into membranes in the M_2 orientation. These data indicate that the SH hydrophobic domain can still act as a signal/anchor domain when inverted in membranes with respect to its original topology. In addition, they suggest that the membrane spanning domain of M_2 alone is not responsible for directing the topology of M_2, a result consistent with previous work which indicated that for class II proteins, foreign hydrophobic regions could function as a signal/anchor domain (41). In summary, it appears that the M_2 signal/anchor domain initiates the initial insertion of the nascent polypeptide into the ER membrane (12), and that in the large part signals outside of the M_2 hydrophobic domain direct the membrane orientation.

The initial interaction of the nascent polypeptide chain of both class I and II membrane proteins with the ER membrane may occur by a common mechanism (27, 32). It has been suggested that the signal sequence of a nascent polypeptide is inserted as a loop structure into the ER membrane and that after membrane insertion, the NH_2 terminus of the nascent protein remains on the cytoplasmic side of the ER. It has also been suggested that the final orientation of a protein is determined by either the presence (in class I proteins) or absence (in class II proteins) of both a signal peptidase cleavage site and a COOH-terminal anchor domain (32). However, this latter idea does not adequately explain the mechanism by which the orientation of M_2 is achieved. We have previously determined that the first step in the membrane integration of M_2 is mediated by the signal recognition particle (12), suggesting that the initial interaction of this protein with the ER membrane is by the same mechanism as for class II integral membrane proteins, yet the latter have lumenal COOH termini. If the signal/anchor domain of a nascent M_2 polypeptide is also inserted into the ER membrane as a loop, the flanking regions must contain signals different from those found in class II membrane proteins to direct the M_2 NH_2-terminal domain across the ER membrane and retain the COOH-terminal region in the cytoplasm. It is possible that the distribution of charged residues flanking uncleaved signal/anchor regions is a major determining factor in protein topology (8, 11, 12).

Recently, it has been proposed that negatively charged domains may be more favorable for translocation across the ER membrane than positively charged regions (8). For M_2, the net charge of the 24-residue NH_2-terminal domain is -3 and is $+2$ for the first 24 residues of the COOH-terminal domain (17). Thus, after presentation to the ER membrane as a loop structure, translocation of the negatively charged M_2 NH_2-terminal domain may be favored over the positively charged COOH-terminal flanking region. By contrast, the charge distribution surrounding the HN signal/anchor is somewhat different: a net $+1$ for the 17-residue NH_2-terminal tail and 0 for the first 17 residues in the COOH-terminal domain (9), and the positive charged NH_2 terminus is cytoplasmic. Any mechanism for directing membrane topology based on charge distribution alone however, would probably have difficulty accommodating the two observed opposite orientations of the MgHH polypeptide. Elucidation of the role of the charged residues in determining the orientation of M_2 will require extensive further site-specific alterations in M_2.

We thank Margaret Shaughnessy for excellent technical assistance; David Simpson for his advice with the transient T_7 expression system; and Hooshmand Sheshberadaran for reconstructing the M_2g mutant into the recombinant SV-40 and for helpful discussions concerning properties of M_2g. We thank Bernard Moss and Thomas Fuerst for supplying vaccinia virus vTF7-3.

G. D. Parks was supported by an American Cancer Society postdoctoral fellowship (PF-3177). This research was supported by research grants AI-20201 and AI-23173 from the National Institutes of Health.

Received for publication 19 May 1989 and in revised form 5 July 1989.

References

1. 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.
2. Anderson, D. J., and G. Blobel. 1983. Immunoprecipitation of proteins from cell-free translations. *Methods Enzymol.* 96:111–120.
3. Blobel, G. 1980. Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA.* 77:1496–1500.
4. Bos, T. J., A. R. Davis, and D. P. Nayak. 1984. NH_2-terminal hydrophobic region of influenza virus neuraminidase provides the signal function in translocation. *Proc. Natl. Acad. Sci. USA.* 81:2327–2331.
5. Brown, D. J., B. G. Hogue, and D. P. Nayak. 1988. Redundancy of signal and anchor function in the NH_2-terminal uncharged region of influenza virus neuraminidase, a class II membrane glycoprotein. *J. Virol.* 62:3824–3831.
6. Davis, N. G., and P. Model. 1985. An artificial anchor domain: hydrophobicity suffices to stop transfer. *Cell.* 41:607–614.
7. Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA.* 83:8122–8126.
8. Haeuptle, M.-T., N. Flint, N. M. Gough, and B. Dobberstein. 1989. A tripartite structure of the signals that determine protein insertion into the endoplasmic reticulum membrane. *J. Cell Biol.* 108:1227–1236.
9. 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.
10. Hiebert, S. W., R. G. Paterson, and R. A. Lamb. 1985. Identification and predicted sequence of a previously unrecognized small hydrophobic protein, SH, of the paramyxovirus simian virus 5. *J. Virol.* 55:744–751.
11. Hiebert, S. W., C. D. Richardson, and R. A. Lamb. 1988. Cell surface expression and orientation in membranes of the 44-amino acid SH protein of simian virus 5. *J. Virol.* 62:2347–2357.
12. Hull, J. D., R. Gilmore, and R. A. Lamb. 1988. Integration of a small integral membrane protein, M_2, of influenza virus into the endoplasmic reticulum: analysis of the internal signal-anchor domain of a protein with an ectoplasmic NH_2 terminus. *J. Cell Biol.* 106:1489–1498.
13. 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 (M_1). *Virology.* 123: 237–256.
14. Lamb, R. A., and P. W. Choppin. 1976. Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P polypeptides, from RNA produced by primary transcription. *Virology.* 74:504–519.
15. Lamb, R. A., and P. W. Choppin. 1977. The synthesis of Sendai virus polypeptides in infected cells. *Virology.* 81:382–397.
16. Lamb, R. A., P. R. Etkind, and P. W. Choppin. 1978. Evidence for a ninth influenza viral polypeptide. *Virology.* 91:60–78.
17. Lamb, R. A., S. L. Zebedee, and C. D. Richardson. 1985. Influenza virus M_2 protein is an integral membrane protein expressed on the infected-cell surface. *Cell.* 40:627–633.
18. Lipp, J., and B. Dobberstein. 1986. The membrane spanning segment of invariant chain (Iγ) contains a potentially cleavable signal sequence. *Cell.* 46:1103–1112.
19. Lipp, J., and B. Dobberstein. 1988. Signal and membrane anchor functions overlap in the type II membrane protein IγCAT. *J. Cell Biol.* 106:1813–1820.
20. Monier, S., P. Van Luc, G. Kreibich, D. D. Sabatini, and M. Adesnik. 1988. Signals for the incorporation and orientation of cytochrome P450 in the endoplasmic reticulum membrane. *J. Cell Biol.* 107:457–470.
21. 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.
22. Paterson, R. G., and R. A. Lamb. 1987. Ability of the hydrophobic fusion related external domain of a paramyxovirus F protein to act as a membrane anchor. *Cell.* 48:441–452.
23. 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.
24. 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 deletion in the T antigen gene. *Mol. Cell Biol.* 3:203–213.
25. Rose, J. K., W. J. Welch, B. M. Sefton, F. S. Esch, and N. C. Ling. 1980. Vesicular stomatitis virus glycoprotein is anchored in the viral membrane by a hydrophobic domain near the COOH terminus. *Proc. Natl. Acad. Sci. USA.* 77:3884–3888.
26. Russell, D. W., W. J. Schneider, T. Yamamoto, K. L. Luskey, M. S. Brown, and J. L. Goldstein. 1984. Domain map of the LDL receptor: sequence homology with the epidermal growth factor precursor. *Cell.* 37:577–585.
27. Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* 92:1–22.
28. Sakaguchi, M., K. Mihara, and R. Sato. 1984. Signal recognition particle is required for co-translational insertion of cytochrome P-450 into microsomal membranes. *Proc. Natl. Acad. Sci. USA.* 81:3361–3364.
29. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.
30. Schatzman, R. C., G. I. Evan, M. L. Privalsky, and J. M. Bishop. 1986. Orientation of the v-erb-B gene product in the plasma membrane. *Mol. Cell Biol.* 6:1329–1333.
31. Schneider, C., M. J. Owen, D. Banville, and J. G. Williams. 1984. Primary structure of human transferrin receptor deduced from the mRNA sequence. *Nature (Lond.).* 311:675–678.
32. 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.
33. Simon, K., V. R. Lingappa, and D. Ganem. 1988. Secreted hepatitis B surface antigen polypeptides are derived from a transmembrane precursor. *J. Cell Biol.* 107:2163–2168.
34. Spiess, M., and C. Handschin. 1987. Deletion analysis of the internal signal-anchor domain of the human asialoglycoprotein receptor HI. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2683–2691.
35. Spiess, M., and H. F. Lodish. 1986. An internal signal sequence: the asialoglycoprotein receptor membrane anchor. *Cell.* 44:177–185.
36. Walter, P., and V. Lingappa. 1986. Mechanism of protein translocation across the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 2:499–516.
37. Wickner, W. T., and H. F. Lodish. 1985. Multiple mechanisms of protein insertion into and across membranes. *Science (Wash. DC).* 230:400–407.
38. Williams, M. A., and R. A. Lamb. 1986. Determination of the orientation of an integral membrane protein and sites of glycosylation by oligonucleotide mutagenesis: influenza B virus NB glycoprotein lacks a cleavable signal sequence and has an extracellular NH_2-terminal region. *Mol. Cell. Biol.* 6:4317–4328.
39. Zebedee, S. L., and R. A. Lamb. 1988. Influenza A virus M_2 protein: monoclonal antibody restriction of virus growth and detection of M_2 in virions. *J. Virol.* 62:2762–2772.
40. Zebedee, S. L., C. D. Richardson, and R. A. Lamb. 1985. Characterization of the influenza virus M_2 integral membrane protein and expression at the infected-cell surface from cloned cDNA. *J. Virol.* 56:502–511.
41. Zerial, M., D. Heylebroeck, and H. Garoff. 1987. Foreign transmembrane peptides replacing the internal signal sequence of transferrin receptor allow its translocation and membrane binding. *Cell.* 48:147–155.
42. Zoller, M. J., and M. Smith. 1982. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13-derived vectors. *Methods Enzymol.* 100:468–500.