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

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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 \(M_2\) NH2 terminus linked to the HN signal anchor and ectodomain (MgHH) was found in both the \(M_2\) and the HN orientation, suggesting that the \(M_2\) NH2 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 oriented 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 oriented 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 oriented in the membrane in a class I topology, with a 24-amino acid NH2-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 M2 to the cell surface are contained within the M2 polypeptide itself, as shown by analysis of the protein expressed from a cDNA clone (40). To investigate the regions of the M2 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 M2 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 NH2-terminal ectodomain of M2 is important in establishing the M2 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 virus M2 proteins (pSV4O-M2) (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 M2 (M13M2) or SH (M13SH) DNA were constructed by inserting the entire M2 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 M2 DNA was altered such that it encoded a consensus site for N-linked glycosylation (Ann-Leu-Thr) at M2 residues 3-5 (17). Site-specific substitution (42) of nucleotides CTT (encoding leucine, residue 3) with AAG (asparagine) on M13M2 was done using a 27-mer mutagenic oligonucleotide primer. The resulting cDNA (designated M13M2g) was excised from the replicative intermediate by digestion with Bam HI and subcloned into the Bam HI site of pGEM-1 (Promega Biotech, Madison, WI) to generate pGM2g. The orientation of the insert was such that M2 mRNA-sense transcription could be generated using the T7 promoter and T7 RNA polymerase (12). To construct pSVM2g, the cDNA fragment from M13M2g 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: NH2-terminal, membrane spanning, and COOH-terminal (see Fig. 1). A lower case "g" denotes the addition of an N-linked glycosylation site to the M2 NH2-terminal region. Thus, a protein containing an M2 NH2-terminal and membrane spanning region linked to the COOH-terminal domain of HN is referred to as MMH. M2g consists of the M2 NH2-terminal, SH membrane spanning domain, and the M2 COOH-terminus.

To construct the M2gSM gene, the M2 hydrophobic domain (residues 25-43) was substituted with that of SH (residues 17-39) in two stages using site-directed mutagenesis of M13M2g. The first step used a 60-mer oligonucleotide to replace the region coding for residues 25-34 of M2 with that coding for residues 17-28 of SH. The resulting molecule M13M2gSM was then used in turn as template for mutagenesis with a 57-mer oligonucleotide which was used to replace the region encoding M2 residues 35-43 with that coding for SH residues 29-39 to produce M13M2gSM. The entire nucleotide sequence was confirmed before subcloning (designated pGM2gSM) as described above.

Hybrid M2/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 M13M2g and M13M2gt at M2 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 byideoxynucleotide 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 (CTTGGAG), which encodes the junction of the M2 membrane spanning and HN COOH-terminal domains (Leu-Glu), was used to join the HN and M2 cDNA fragments in a pGem-4 vector such that the resulting hybrid constructions encoded the M2 NH2-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 Mg2 and HN cDNA clones. Blunt end ligation of the Eco RV-cut Mg2 DNA fragment to the Sca I-cut HN segment produced a junction (GAT/ACT) encoding an Asp/Thr pair and resulted in a chimeric Mg2/HN cDNA which precisely linked the M2 NH2-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 dyeoxy 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 M2/HN genes or Mg2 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-3SS-label (ICN Radiochemicals Inc., Irvine, CA) in cysteine-methionine-free DME or (200 μCi/ml) [35S]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 M2 NH2-terminal domain (39), or monospecific polyclonal rabbit antiserum 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). Mg2 and MgSM were expressed from transfected pGM2g and pGM2gSM 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 T7 RNA polymerase (7). Cells were then transfected with 20 μg of pGM2g or pGM2gSM by calcium phosphate precipitation. At 3 h posttransfection, cells were labeled for 1 h with 100 μCi/ml [35S]cysteine in cysteine-free DME, lysed in RIPA buffer, immunoprecipitated with M2-specific mAb, and analyzed on 15% 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-mm-diam plates) 1. Abbreviation used in this paper: Endo H, endoglycosidase H. 

The Journal of Cell Biology, Volume 109, 1989
were labeled for 30 min with (200 μCi/ml) Tran-35S-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 (200 μCi/ml) Tran-35S-label for 1 h. For MgSM and MgMg, vaccinia virus infected cells which had been transfected with pGMgSM or pGMgMg were labeled at 3 h postransfection with 150 μCi/ml Tran-35S-label 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 Na2CO3 (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 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 M2, recombinant cDNA molecules were constructed with precise domain exchanges such that they encoded the NH2-terminal ectodomain of M2, a hydrophobic membrane spanning region from either M2, HN, or SH and the ectodomain of HN or the cytoplasmic tail of M2 (see Fig. 1). The analysis of the membrane orientation of the chimeric proteins was facilitated by the construction of an M2 cDNA clone which encoded an asparagine-linked glycosylation site in the M2 NH2-terminal ectodomain (M2g). 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 NH2-terminal ectodomain and signal-anchor of M2 (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 NH2-terminal glycosylation site in M2 was used, CV1 cells infected with SV-40M2 or SV-40Mg recombinant viruses (the latter reconstructed and kindly made available by Hooshmand Sheshberadaran) were labeled for 1 h with [35S]cysteine and immunoprecipitated with M2-specific monoclonal antibody 14C2. As shown in Fig. 2, MgMg had a slower electrophoretic mobility as compared to M2 (- lanes), compatible with the addition of a single N-linked carbohydrate chain to MgMg. Further evidence for glycosylation was provided by digesting M2g with Endo H, and finding an increased electrophoretic mobility of M2g (Fig. 2, + lane). The difference in mobility between wild-type M2 and endo H-treated M2g is due to both the amino acid change in M2g and the N-acetylglucosamine residue remaining after Endo H treatment. Single amino acid changes in M2 have been shown previously to alter its electrophoretic mobility (17). However, when the 24-residue ectodomain of M2g is fused to the 548-residue transmembrane and COOH-terminal domains of HN, the amino acid substitution in M2 does not detectably alter its electrophoretic mobility (see below). It will be shown elsewhere that M2g is abundantly expressed at the cell surface and that the carbohydrate chain remains sensitive to Endo H digestion at all times.
Figure 3. Expression of chimeric M2/HN proteins in CV-1 cells. Cells infected with recombinant SV-40 MMH, MgMH, and MgHH were labeled for 1 h with Tran-35S-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 M2-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 SVS-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.

The proteins produced from the hybrid M2/HN genes were examined by infecting CV-1 cells with the various recombinant SV-40 viruses and labeling for 1 h with Tran-35S-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 NH2-terminal domain of M2 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 Mq was synthesized (Fig. 3A, lane 7), and in the presence of tunicamycin a single band of ~52,000 Mq was observed (lane 8). In contrast, SV-40–MMH synthesized two major polypeptides, designated form I (~53,000 Mq) and III (~70,000 Mq), which were reactive with antisera to HN (Fig. 3A, lane 7), but not with preimmune sera (lane P). SV-40–MgMH, which encodes the MgMH polypeptide with the additional NH2-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 7). The increased size of the MgMH forms I and III (~58,000 and 75,000 Mq) was consistent with the addition of a carbohydrate chain to the NH2 terminus of these two polypeptides. In the presence of tunicamycin, a single MMH-specific polypeptide of ~53,000 Mq, was synthesized in both SV-40–MMH and SV-40–MgMH infected cells (Fig. 3A, 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. 3A, lane 5), one with an electrophoretic mobility matching that of MgMH form I (~58,000 Mq) and a second species (form II) with a mobility similar to wild-type HN (~68,000 Mq). In the presence of tunicamycin, the MgHH polypeptides migrated as a single species of ~53,000 Mq (lane 6), suggesting that form I and II differed by carbohydrate addition. Further, the difference in mobility of ~3,000 Mq 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 M2 NH2-terminus in these hybrid molecules, cell lysates were immunoprecipitated with a site-specific antibody (M2 monoclonal 14C2) (39). This
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 M2 NH2-terminal–specific mAb indicates that the NH2-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 M2 antibody. The electrophoretic mobility of form IV was not altered in the case of MgMH by the presence of the M2 NH2-terminal glycosylation site (compare Fig. 3, lanes 1 and 2). The precise nature of polypeptide IV has not been determined, but it is thought to lack the M2 NH2-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 NH2-terminal region.

**Chimeric M2/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...
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 NTH-terminal M2 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 NH2 terminus, can be digested with protease, and is strongly associated with membranes after alkali fractionation, it can be deduced that form I has its NH2-terminal M2 region in the lumen of vesicles and that the molecules are oriented like natural M2. MgHH form II was not glycosylated at the M2 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 NH2 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 M2/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-35S-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 M2 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 M2 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 M2 NH2 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 M2 hydrophobic domain on membrane orientation, we wished to replace only the M2 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 M2g, 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
M2g and MgSM DNA molecules were adjacent to the bacteriophage T7 RNA polymerase promoter (pGM1 and pGMgSM). To investigate the membrane orientation of MgSM, CVI cells were infected with a recombinant vaccinia virus that expresses the T7 RNA polymerase (7) and then transfected with either pGM1 or pGMgSM. The cells were labeled with [35S]cysteine and the M2-specific polypeptides immunoprecipitated using the M2 monoclonal antibody. As shown in Fig. 7 A, the MgSM polypeptide has an electrophoretic mobility similar to M2g (~20,000 Mr). Treatment of M2g and MgSM with Endo H yielded polypeptide species with ~15,000 Mr, consistent with the loss of one high mannose N-linked carbohydrate chain (lanes 3 and 5). Glycosylation of MgSM indicates that its NH2 terminus has been translocated across the ER membrane. The slightly slower electrophoretic migration of Endo H–treated M2g compared to wild-type M2 is in part due to the single N-acetylglucosamine residue which remains after Endo H treatment and to the amino acid change in M2g. The protein of slower electrophoretic mobility (~35,000 Mr) 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 M2g (as a control) to extraction by alkali was examined. As shown in Fig. 7 B, both M2g and MgSM were found in the pellet fractions and none could be detected in the supernatant fraction. These data indicate that the observed M2g and MgSM polypeptides are glycosylated and are stably integrated in membranes in the same orientation, i.e., glycosylated lumenal NH2 termini. Thus, in MgSM the M2 NH2 terminus is correctly oriented, even though the SH hydrophobic signal/anchor has been inverted with respect to its normal orientation in the membrane.

Discussion
The M2 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 oriented with extracellular NH2 termini (17, 20, 30, 38). To attempt to understand the role of the protein domains which are responsible for M2 assuming an uncommon topology, we have constructed hybrid molecules consisting of exchanges of domains of the M2 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.
two major forms (I and III). MMH and MgMH form I (Fig. 8) is anchored in membranes and orientated with a luminal NH₂ terminus like M₂. 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 alkaline 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 M₂ NH₂-terminal antibody, these two species do not differ by cleavage at the NH₂ terminus. In contrast, the soluble and secreted form IV from MMH and MgMH was not recognized by the antisera specific for the M₂ NH₂ terminus and did not exhibit an altered electrophoretic mobility by addition of the M₂ NH₂-terminal glycosylation site. These properties of form IV are consistent with it being a modified version of form III which lacks its NH₂ 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 M₂ 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 M₂ (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 M₂ 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.

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**Figure 7.** Expression and analysis of M₂g and M₂gSM. (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 pGM₂g (lanes 2 and 3) or pGMgSM (lanes 4 and 5), and labeled with [35S]cysteine for 1 h. Cells were lysed in RIPA buffer, immunoprecipitated with M₂-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 M₂ expressed from a recombinant SV-40 (40). (B) Alkali extraction of M₂g and M₂gSM. Hybrid proteins were transiently expressed from pGM₂g 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-M₂ and analyzed by SDS-PAGE.
that contributes to the relative inefficiency of anchoring by the M₂ signal/anchor domain. Although the MMH construction alone does not show which domain of M₂ is important for its topology in membranes, the orientation of MMH (or MgMH) form I does suggest that the M₂ 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 M₂ NH₂-terminal ectodomain was substituted in place of the HN NH₂-terminal cytoplasmic domain, the polypeptide chain was inserted into the membrane in two opposing orientations: form I in the M₂ 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 M₂ NH₂ 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 (NH₂ terminus translocated in all species) and MgHH (NH₂ 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 M₂ NH₂ terminus.

To directly test the role of the M₂ signal/anchor on membrane topology, we constructed a molecule (MgSM) in which the M₂ 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 MgHg, in that both proteins are stably integrated into membranes in the M₂ 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 M₂ alone is not responsible for directing the topology of M₂, 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 M₂ 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 M₂ 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 NH₂ 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 M₂ is achieved. We have previously determined that the first step in the membrane integration of M₂ 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 M₂ 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 M₂ NH₂-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 M₂, the net charge of the 24-residue NH₂-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 M₂ NH₂-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 NH₂-terminal tail and 0 for the first 17 residues in the COOH-terminal domain (9), and the positive charged NH₂ 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 M₂ will require extensive further site-specific alterations in M₂.

We thank Margaret Shaughnessy for excellent technical assistance; David Simpson for his advice with the transient T₇ expression system; and Hooshmand Sheshberadaran for reconstructing the Mg mutant into the recombinant SV-40 and for helpful discussions concerning properties of MgHg. 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.

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