Integration of a Small Integral Membrane Protein, M2, of Influenza Virus into the Endoplasmic Reticulum: Analysis of the Internal Signal-Anchor Domain of a Protein with an Ectoplasmic NH₂ Terminus

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Abstract. The M₂ protein of influenza A virus is a small integral membrane protein of 97 residues that is expressed on the surface of virus-infected cells. M₂ has an unusual structure as it lacks a cleavable signal sequence yet contains an ectoplasmic amino-terminal domain of 23 residues, a 19 residue hydrophobic transmembrane spanning segment, and a cytoplasmic carboxyl-terminal domain of 55 residues. Oligonucleotide-mediated deletion mutagenesis was used to construct a series of M₂ mutants lacking portions of the hydrophobic segment. Membrane integration of the M₂ protein was examined by in vitro translation of synthetic mRNA transcripts prepared using bacteriophage T₇ RNA polymerase. After membrane integration, M₂ was resistant to alkaline extraction and was converted to a Mr ≈ 7,000 membrane-protected fragment after digestion with trypsin. In vitro integration of M₂ requires the cotranslational presence of the signal recognition particle. Deletion of as few as two residues from the hydrophobic segment of M₂ markedly decreases the efficiency of membrane integration, whereas deletion of six residues completely eliminates integration. M₂ proteins containing deletions that eliminate stable membrane anchoring are apparently not recognized by signal recognition particles, as these polypeptides remain sensitive to protease digestion, indicating that in addition they do not have a functional signal sequence. These data thus indicate that the signal sequence that initiates membrane integration of M₂ resides within the transmembrane spanning segment of the polypeptide.

The insertion of most eukaryotic integral membrane proteins into the rough endoplasmic reticulum (ER) is initiated by recognition of an ER-specific signal sequence by the signal recognition particle (SRP). Substantial evidence has been obtained to indicate that SRP exists as a soluble cytoplasmic complex that can bind weakly to free ribosomes (49, 52) and bind with high affinity to the signal sequence of a nascent secretory polypeptide as it emerges from the ribosome (23, 49, 55). High affinity binding of SRP to the ribosome in many but not all cases induces an arrest of elongation (30, 31, 50), which extends the time window available for the SRP-ribosome complex to interact with the SRP receptor or docking protein upon the surface of the ER membrane (15, 16) (for a mathematical analysis of SRP function, see reference 37). However, data obtained using experimentally reconstituted subparticles of SRP have demonstrated that an elongation arrest of translation is not an obligatory event in the translocation process (44). Interaction of SRP with the SRP receptor causes SRP displacement from the ribosome with the concomitant release of the elongation arrest (13), thereby allowing the initiation of nascent chain transport across the membrane bilayer. In addition to the mechanism discussed above, a limited number of membrane proteins including cytochrome b₅ and M₁₃ preprocoat protein have been shown to posttranslationally insert into microsomal membranes in an SRP and SRP-receptor independent manner (2, 53).

Integral membrane proteins can be classified into several categories based on their membrane topology (5, 12, 40, 54). Membrane proteins that span the bilayer a single time (bipart) can be subdivided into two classes based on the orientation of the polypeptide in the membrane. The most common type (class I) contains a cleavable signal sequence that initiates translocation of the NH₂-terminus of the protein into the lumen of the ER. A separate stop-transfer sequence eventually interrupts transport of the nascent chain to integrate the polypeptide in the bilayer with the COOH-terminal domain exposed to the cytoplasm. Well characterized examples of class I proteins (12) include the influenza virus hemagglutinin (HA), vesicular-stomatitis virus G protein,
and the low density lipoprotein receptor (36, 38, 39). Class II integral membrane proteins (12), e.g., influenza virus neuraminidase (NA), asialoalyclyprotein receptor, paramyxovirus hemagglutinin–neuraminidase (HN), transferrin receptor (18, 20, 43), are integrated in the membrane in the opposite orientation, with an NH2-terminal cytoplasmic domain and a COOH-terminal ectodomain. The transmembrane spanning sequence of class II proteins is typically located near the amino terminus of the polypeptide and serves as both a signal sequence and a membrane anchor. Polypolyic integral membrane proteins (class III) contain multiple transmembrane spanning segments and are proposed to achieve their final topology in the membrane by expression of a series of signal and stop-transfer sequences (3, 22).

The influenza A virus M2 protein is an integral membrane protein (26, 29, 58) that cannot be classified in any of the above categories. The M2 protein has been shown to be integrated in the membrane with an extracellular domain of approximately 23 amino acid residues, a single hydrophobic domain of 19 residues, and a COOH-terminal domain of 55 residues (29, 58). To date, several other polypeptides have been identified with a similar structure, including influenza B virus NB protein (57) and gp 74 V-erb B (17). Therefore, it was of interest to determine whether small integral membrane proteins with this topology use the SRP-mediated mechanism of membrane integration, or alternatively partition directly into the lipid bilayer in a manner analogous to cytochrome b5 or MI3 preprocoat protein.

**Materials and Methods**

**Materials**

Canine pancreas rough microsomes (RM), salt-washed microsomes (K-RM), and SRP were extracted and purified as described previously (33, 49). The unit definition of these reagents are those defined previously (49). The production and specificity of the M2 NH2-terminal monoclonal antibody (14C2) will be described elsewhere (Zebedee, S. L., and R. A. Lamb, manuscript in preparation) and was kindly made available by S. L. Zebedee. The 14C2 M2 NH2 monoclonal antibody mix was kindly provided by Kathy Cole-

**Viruses, Cells, Radioisotopic Labeling Immunoprecipitation, and SDS-PAGE**

Influenza viruses (A/WSN/33 and A/Udorn/72) were grown as described previously (25). CVI cells were maintained and infected as described previously (24). Influenza virus–infected CVI cells were labeled with [35S]methionine as described above, and infected cell microsomes were isolated and subjected to digestion with 200 μg/ml TPCK-treated trypsin as described previously (58).

**In Vitro RNA Synthesis**

Plasmid DNAs were linearized at a site beyond the T7 promoter and the M2 cDNA insert with Xba I. Approximately 2 μg of linearized plasmid DNA was used as a template for RNA synthesis in a 100 μl reaction containing 10 μCi [3H]GTP, 40 mM Tris-HCl pH 7.5, 6 mM MgCl2, 1 mM spermidine, 10 mM NaCl, 0.5 mM each of ATP, CTP, and UTP, 150 μM GTP, 150 μM [32P]GTP, 5 μM dTTP, 40 μg RNasin (Promega Biotec), and 100 U T7 RNA polymerase. Samples were incubated for 60 min at 37°C, a further 50 U T7 RNA polymerase were added, and incubation was continued at 37°C for a further 60 min. The reaction was stopped with 10 μl 100 μM EDTA and the DNA template digested with 2 U RNase-free DNase (RQ DNase; Promega Biotec) at 37°C for 10 min. This reaction was stopped by the addition of 4 μl 0.25 M EDTA at 4°C and the RNA extracted with a 1:1 mixture of phenol and chloroform. The RNA was desalted on G50 Sephadex, ethanol precipitated, dissolved in H2O at 1 μl/μg, and stored at −20°C. The RNA yield was determined by measuring the incorporation of [3H]GTP into RNA, and the size of the RNA products was examined by electrophoresis on denaturing polyacrylamide gels.

**In Vitro Translation, Additon of Membranes, and Protease Digestions**

In all in vitro translations were done using wheat germ extract essentially as described (27). For translation of infected cell poly(A+) RNA, the amount used was equivalent to the RNA extracted from one 10-cm dish tissue culture plate per 100 μl reaction, and for translation of T7 RNA transcripts, 1 μg RNA per 100 μl reaction was used. When necessary, dog pancreas RM and K-RM were added to one equivalent per 15 μl translation and SRP to 30 μl per 25 μl. When these additions were made, the salt concentrations in the translation reactions were adjusted such that the final concentration remained at 30 mM Mg2+ KCl, 5 mM (octylthiolethylene-

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Alkali Extraction of Microsomes

Alkali fractionation of microsomes was done basically as described previously (35). Briefly, in vitro translation reactions were adjusted to a final volume of 300 μl with 50 mM triethanolamine (pH 7.5), 11 μl 1 N NaOH added, and the samples incubated at 4°C for 10 min. The samples were fractionated by layering on top of a 680 μl alkaline sucrose cushion (0.2 M sucrose, 30 mM Hepes, pH II, 150 mM KOAc, 2.5 mM Mg(OAc)₂) followed by centrifugation at 45,000 rpm at 4°C for 20 min in a TL-100 table-top ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) using a TLS-55 swinging-bucket rotor. The supernatants were removed, an equal volume of 20% TCA added, and the samples incubated at 4°C for 30 min. The pellets were resuspended in 100 μl of 1% SDS and precipitated with 100 μl 20% TCA at 4°C for 30 min. Precipitated proteins from both the supernatant and pellet were recovered by centrifugation at 4°C for 15 min in an Eppendorf microfuge. The pellets were washed with ethanol, vacuum dried, and solubilized at 37°C for 60 min in 10 μl of 0.1 M Tris-HCl pH 8.9, 1% SDS. The solubilized products were then immunoprecipitated before analysis by SDS-PAGE.

Results

In Vitro Synthesis of M₂ and Deletion Mutants of M₂

To facilitate experiments a cDNA to the M₂ mRNA (58) was subcloned into pGEM-1, a plasmid containing the bacteriophage T7 promoter (8), such that M₂ RNA transcripts could be synthesized using T7 RNA polymerase (see Materials and Methods). In addition, a series of M₂ mutants were constructed to examine the roles of the hydrophobic domain of M₂ (see Fig. 1). The deletion mutants lack between 2 and 10 amino acids from the COOH-terminal region of the M₂ hydrophobic domain and were constructed by oligonucleotide-directed mutagenesis of the M₂ cDNA in MI3mp8 followed by reconstruction into pGEM-1. M₂-specific recombinant pGEM plasmids were digested with Xba I to linearize the DNA molecules and synthetic ⁴⁰⁺GpppG-capped M₂ RNA was synthesized in vitro using T7 RNA polymerase. The RNAs were translated in wheat germ extracts and the products were immunoprecipitated with a monoclonal antibody specific for the NH₂-terminal region of M₂ and analyzed by gel electrophoresis. As shown in Fig. 2, the mobility of the mutants (ΔM₂) correlates well with the number of amino acids deleted from the M₂ protein (M₂ ≈ 15,000). Because there were no radioactive bands migrating above M₂ in the polyacrylamide gel (Fig. 2), subsequent figures show only that portion of the gel between the dye front (M₂ ≈ 4,000) and M₂ (M₂ ≈ 15,000).

Integration of M₂ into the ER Is SRP-dependent

To examine the insertion of M₂ into membranes in vitro, it was necessary to use a protease protection assay because M₂ lacks both a cleavable signal sequence and a consensus site that is used for asparagine-linked glycosylation (58), and therefore no mobility shift could be expected. When microsomal vesicles containing M₂ (isolated from influenza virus–infected cells) are treated with trypsin, the luminal NH₂-terminal region (M₂ ≈ 7000) is protected and the cytoplasmic COOH-terminal region is protease sensitive (58). The protease-protected fragment (M₂ ≈ 7000) from infected cell microsomes can be immunoprecipitated by the NH₂-terminal ectodomain specific sera (Fig. 3, lane 2, asterisk).

The detection of this fragment constitutes the major assay used in this study to demonstrate the in vitro insertion of M₂ protein into microsomal membranes. Control experiments used the integral membrane protein influenza virus HA where a gel mobility shift due to addition of N-linked carbohydrate as well as protease protection were used as the assay for proper membrane integration.

The M₂ mRNAs were translated in vitro in the presence and absence of RM or K-RM (i.e., depleted of SRP) with or without exogenous SRP. Each in vitro translation reaction was divided into three parts: one part was left untreated, and the third was treated with trypsin, and the third was treated with trypsin in the presence of 1% Triton X-100. The samples were immunoprecipitated with the M₂ NH₂-terminal–specific serum, and the results of such an experiment are shown in Fig. 3. The primary M₂-specific translation product (lane 3) comigrates with M₂ found in infected cell microsomes (lane 1). In the presence of added trypsin (lane 4) or trypsin plus detergent (lane 5), the vast majority of M₂ was digested. A small amount of a polypeptide that migrates slightly faster than the NH₂-terminal protease-protected fragment from infected cell microsomes (lane 2) was also synthesized (lane 3). This peptide, of unknown origin, is difficult to completely digest with trypsin even in the presence of detergent. However, the presence of this polypeptide does not adversely affect interpretation of the results described here. In the presence of added RM, no mobility shift in M₂ was observed as predicted based on the absence of a cleavable signal sequence or added carbohydrate (lane 6). Trypsin treatment of in vitro integrated M₂ yielded a protected fragment (lane 7, asterisk) of identical mobility to the marker (lane 2) that is sensitive to digestion by trypsin in the presence of detergent (lane 8). The addition of K-RM does not alter the mobility of M₂ (lane 9), and no trypsin-protected fragment was observed (lane 10). Addition of K-RM and SRP (lane 12) yielded a NH₂-terminal specific fragment of M₂ after trypsin digestion (lane 13) that has an identical mobility to the marker (lane 2), and was sensitive to trypsin digestion in the presence of detergent (lane 14).

Thus, a comparison of the data in lanes 9 and 10 with those in lanes 12 and 13 strongly indicates that insertion of M₂ into membranes is mediated by a mechanism dependent on SRP.

The amount of trypsin-protected fragment found after translation with RM (lane 7) was less than that with K-RM and SRP (lane 13) even though the same amount of RNA and microsomal membranes was used in each case. Translocation of secretory proteins has also been shown to occur more efficiently with SRP-supplemented K-RM than with RM when using a wheat germ translation system (51), whereas SRP supplementation of either RM or K-RM using the reticulocyte lysate system does not alter the translocation efficiency (30). For experiments concerning SRP-dependent mechanisms, the wheat germ translation system must be used because reticulocyte lysate contains endogenous SRP (30). M₂ protein contains three cysteine residues, one of which located within the cytoplasmically exposed domain (29). Protease digestion of integrated M₂ should result in a one-third decrease in radioactivity recovered in the protease-protected domain. One interpretation of the low molar amount of the protected M₂ fragment relative to the total molar amount of M₂ synthesized (Fig. 3) would be that there is a
low efficiency of insertion of M2 into membranes. However, as discussed below, the most likely explanation for this finding is that the M2 NH2-terminal specific monoclonal antibody has a lower affinity for the protected fragment than for intact M2.

A control experiment was conducted to determine whether the RM, and K-RM with added SRP, were competent for insertion of integral membrane proteins and to show that the procedure for trypsin digestions did not rupture the vesicles. The mRNA encoding influenza virus HA (a class I integral membrane glycoprotein) was translated in vitro and the glycosylation and trypsin protection monitored. HA as a control is especially useful because although it is very well characterized in terms of synthesis and structure (56), it has not been formally shown that HA insertion into the ER requires SRP.

Influenza A virus-infected-cell poly(A+)—containing mRNA was translated in a wheat germ extract with the appropriate addition of pancreatic microsomal membranes (RM or K-RM) and SRP. Samples were treated with trypsin or trypsin and detergent and then immunoprecipitated using a mixture of HA monoclonal antibodies. The results of this experiment are shown in Fig. 3 B. In the absence of added microsomal membranes, only unglycosylated HA (HA0) was synthesized (lane 3). When RM (lane 4) or K-RM with SRP (lane 6) were added, a significant proportion of HA was glycosylated as demonstrated by a comigration of a more slowly migrating polypeptide (HA) and authentic glycosylated HA (lane 1). The proportion of HA0 converted to HA cannot be accurately quantitated from this experiment because the mixture of monoclonal antibodies precipitates HA much better than HA0. However, other experiments using either a polyclonal antiserum or direct-analysis of TCA-precipitated proteins indicate that 50–60% of HA0 becomes glycosylated (data not shown). When K-RM were added to the translation in the absence of added SRP (lane 5), a small amount of glycosylated HA could be observed, which is probably due to incomplete removal of SRP from

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Figure 3. Insertion of M2 protein into membranes is dependent on SRP. (A) Synthetic 7mGpppG-capped RNAs were translated in wheat germ extracts in the presence (+) and absence (−) of microsomal membranes and SRP. The samples were divided into thirds, with one-third as an untreated control and the other two samples treated with trypsin in the presence and absence of detergent as indicated at the top of each lane. A + sign above a lane indicates the addition, as appropriate, of RM, K-RM, SRP, TRYP (TPCK-treated trypsin), and DET (Triton X-100). Reaction products were immunoprecipitated with M2 NH2-terminal-specific monoclonal antibody and analyzed by SDS-PAGE. Lane 1 (also marked C), control lane showing marker M2 protein immunoprecipitated from an influenza A virus (strain, A/Udorn/72) infected cell lysate. Lane 2 (marked M), marker lane showing the NH2-terminal fragment (Mr = 7,000) that is protected from trypsin digestion of microsomes isolated from influenza A virus (strain A/Udorn/72) infected cells. Fragments that comigrate with this marker are indicated by an asterisk in other lanes. (B) Poly(A+)-containing RNA molecules isolated from influenza A virus (strain A/WSN/33) infected cells were translated in wheat germ extracts under the conditions described in Fig. 3 A. The translation products were immunoprecipitated with a mixture of HA-specific monoclonal antibodies and analyzed on an 8% SDS-polyacrylamide gel containing 4 M urea and visualized by fluorography. Lane 1 (also marked C), glycosylated HA immunoprecipitated from influenza A/WSN/33 infected-cell lysates. Lane 2, control translation with no added mRNA. The unglycosylated form of HA synthesized in vitro is designated HA0 and its position indicated by an arrow. RM, K-RM, SRP, TRYP, and DET are as indicated in the legend to Fig. 3 A.

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the salt-washed membranes. Trypsin treatment of the translation products digested HA0 but not the ectodomain of HA (lanes 7–10), indicating that the microsomal vesicles are intact and not damaged by the procedure. (The extramicrosomal tail of HA is digested by trypsin, but the small shift in mobility cannot be detected on this gel system.) In the presence of trypsin and detergent, all HA-derived translation products were sensitive to proteolysis. Thus, these data indicate that the RM and K-RM + SRP are competent for translocation of proteins across membranes and that translocation of HA is dependent on an SRP-mediated mechanism.

Deletion of Amino Acids from the COOH-Terminal Region of the M2 Signal-Anchor Domain Inhibits Signal Sequence Function

The above data suggest that the hydrophobic membrane-spanning domain of M2 (residues 25–43) both targets M2 to the ER in a SRP-dependent manner and acts as a stop-transfer sequence to anchor M2 in the membranes. Transmembrane spanning segments of integral membrane proteins are typically both longer and more hydrophobic than are signal sequences (48). To determine whether signal sequence function and membrane anchor function could be ascribed to specific sections of the M2 transmembrane segment, we constructed a series of deletions ranging between 2 and 10 residues near the COOH-terminus of the M2 hydrophobic domain. These seven deletion mutants (M2Δ) are indicated diagrammatically in Fig. 1, and the translation products are shown in Fig. 2 as discussed above. Mutants M2Δ2 and M2Δ2' have deletions of two residues (40 + 41 and 42 + 43, respectively). M2Δ4 has a deletion of four residues (38–41, inclusive) and M2Δ6 a deletion of six residues (36–41, inclusive). Translation of M2Δ2, Δ2', and Δ4 mutant RNAs, in the presence of K-RM and SRP, yielded trypsin-protected NH2-terminal fragments (asterisk), whereas in the presence of K-RM alone, the ΔM2 proteins were all protease sensitive (compare Fig. 4 A, lanes 6 and 9, Fig 4 B, lanes 3 and 6, and Fig. 4 C, lanes 6 and 9). In each case, the protected fragment was slightly smaller than the control wt M2 fragment and correlated directly with the size of the deletion in the hydrophobic domain. Thus, these data suggest that the 15 uncharged or hydrophobic residues in M2Δ4 are suf-
Figure 4. Analysis of the membrane integration properties of ΔM₂ proteins. Synthetic \(^{7}m\)G-pppG-capped mRNAs of the M₂ deletion mutants were translated in wheat germ extracts in the presence and absence of microsomal membranes and SRP. Equal fractions were treated as a control or treated with trypsin in the presence or absence of detergent as indicated at the top of each lane. RM, K-RM, SRP, TRYP, and DET are as indicated in the legend for Fig. 3 A. In each panel of this figure, the synthetic mRNA of the deletion mutant that was translated in vitro is identified at the top of the gel. In each panel, lane 1 (also marked M) is a control containing the M₂ NH₂-terminal fragment (M₂ = 7000) that is protected from trypsin digestion of microsomes isolated from influenza A virus (strain A/Udorn/72) infected cells. Fragments that are specific for this marker are identified by an asterisk. A–D show the results obtained for M₂Δ2, Δ2', Δ4, and Δ6, respectively.
Figure 5. Alkaline extraction of wt $M_2$ and AM$_2$ proteins. Synthetic 7mGpppG-capped mRNAs of wt $M_2$ and M2 deletion mutants were translated in the presence (+) and absence (−) of K-RM + SRP. The translation products were alkaline extracted, separated into pellet (P) and supernatant (S) fractions, TCA precipitated, resolubilized, and immunoprecipitated with $M_2$ NH$_2$-terminal monoclonal antibody as described in Materials and Methods. The products were analyzed on a 17.5% SDS-polyacrylamide gel containing 4 M urea and visualized by fluorography.

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sufficient for both SRP-mediated insertion into the membrane and for anchoring of the protein. Analysis of $M_2$Δ6, Δ8, Δ8', and Δ10, which have deletions from 6 to 10 residues (see Fig. 1), gave a different result. A protease-protected fragment derived from $M_2$Δ6, Δ8, Δ8', and Δ10 was not observed after translation in the presence of K-RM and SRP. In addition, no $\Delta M_2$ protein was found that was completely trypsin resistant, as would be expected for a soluble $\Delta M_2$ molecule in the lumen of the microsomal vesicles. Thus, we could not obtain evidence indicative of a functional signal sequence and a nonfunctional anchor domain. Only the data for $M_2$Δ6 are shown in Fig. 4 D, as it is representative of all the data obtained for the other larger deletion mutants. It should be noted that the apparent efficiency of integration observed in the experiments shown in Fig. 4 cannot be compared with that in Fig. 3 as the experiments were performed at different times and with different batches of wheat germ extract.

To examine further the insertion of the $M_2$ deletion proteins into membranes, and to use an assay independent of the analysis of trypsin-protected fragments, alkaline extraction was used. This procedure is based on the criteria that upon alkaline treatment (pH 11) and centrifugation through an alkaline sucrose cushion, integral membrane proteins fractionate in the pellet while peripheral membrane proteins and soluble proteins are recovered in the supernatant (10, 14, 47). When translation of wt $M_2$ mRNA was conducted in the absence of K-RM + SRP, most of the $M_2$ protein fractionated in the supernatant (Fig. 5). When K-RM + SRP were present at the beginning of the translation, a significant amount of $M_2$ fractionated in the pellet (Fig. 5). Although the level of membrane integration of $M_2$ varied somewhat between experiments, the percent integration as determined by alkaline fractionation was apparently greater than that indicated using the protease-protection assay. Both assays rely upon immunoprecipitation of $M_2$ related polypeptides with a monoclonal antibody. After alkaline fractionation, the intact $M_2$ protein was immunoprecipitated from both the supernatant and pellet fractions. In contrast, in the trypsin-protection experiments, intact and protease-digested forms of the $M_2$ protein would need to be immunoprecipitated with equal efficiency for a direct comparison to be made. Additional data suggest that the monoclonal antibody cannot precipitate the $M_2$ fragment protected in microsomes as efficiently as intact $M_2$ (data not shown).

When $M_2$Δ2, Δ2', Δ4, and Δ6 proteins were analyzed by alkaline fractionation after translation in the presence of K-RM + SRP, a proportion of the mutant polypeptide was
found in the pellet for Δ2, Δ2', and Δ4, indicating insertion into membranes (Fig. 5). However, almost no M2Δ6 was found in the pellet fraction (Fig. 5). These data for M2Δ6, coupled with the lack of any protease-protected fragment, suggest that the I3 uncharged or hydrophobic residues of M2Δ6 are insufficient to interact with SRP and provide a functional signal to initiate membrane insertion. Hence, without the initial insertion, M2 cannot be anchored in the membrane. A reproducible lower efficiency of integration of all the M2 deletion proteins (M2Δ2, Δ2', and Δ4) as compared with wt M2 was observed (Fig. 5). This finding suggests that even small deletions within the 19 residue M2 membrane-spanning domain have a deleterious effect on SRP-dependent targeting of M2 to the ER membrane.

Discussion

The M2 protein of influenza A virus is an example of a low molecular weight integral membrane protein that lacks a cleavable signal sequence yet contains an ectoplasmic NH2-terminal domain and a COOH-terminal cytoplasmic domain. These structural features raised several intriguing questions concerning the mode of membrane integration of the M2 protein.

The initial question to be addressed was whether integration of M2 was an SRP-mediated event, or was instead promoted by partitioning of a hydrophobic insertion sequence into the membrane bilayer. The data presented here indicate that integration of the M2 protein of influenza A virus into the ER was strictly dependent upon the signal recognition particle. High affinity binding of SRP to signal sequences can induce a complete arrest (50) or substantial decrease in the protein synthesis elongation rate (21), and in so doing effectively increase the time window for interaction of the SRP-ribosome complex with the ER membrane (37). Neither an arrest of translation nor a pronounced inhibition of M2 synthesis was detected in a 1-h translation containing SRP (data not shown). A lack of detectable inhibition of membrane protein synthesis by SRP is not unprecedented (2). When in vitro translations of M2 protein were treated with puromycin before incubation with K-RM and SRP, no protease-protected fragments of the M2 protein could be detected (data not shown). However, a low level of posttranslational integration of this low molecular weight protein cannot be totally excluded due to the detection limits that are implicit in the protease protection assay used in this study. Nonetheless, the data strongly suggest that integration of the M2 protein proceeds by an SRP-mediated cotranslational mechanism, in contrast to the low molecular weight polypeptides discussed below.

The minimum length of a ribosome-bound polypeptide that could potentially be recognized by SRP is apparently equal to the length of the signal sequence plus the 40–50 residues of a nascent chain which are buried in a protease, and by inference, SRP-inaccessible groove in the large ribosomal subunit (4). Because M2 protein has 23 amino acid residues that precede the hydrophobic transmembrane spanning sequence (29, 58), the majority of the 97 residue M2 protein will be synthesized before emergence of the functional signal from the ribosome. Recently, translocation of several other low molecular weight polypeptides has been investigated to elucidate the mechanism of transport for polypeptides that fall near the minimal size range outlined above. Translocation of frog prepropeptide GLa, a 64 amino acid protein (19), has been shown to occur by a posttranslational SRP-independent mechanism (42). An initial report proposing an SRP-independent posttranslational mechanism for translocation of the 70 residue polypeptide prepromelinin (33) has been disputed (21). Clearly, further research will need to be done to determine the predominant mechanism of translocation and integration of low molecular weight polypeptides.

The M2 protein contains a single hydrophobic domain that performs the dual role of acting as an SRP-dependent signal sequence and as a stop-transfer sequence. Previous data have shown that a single hydrophobic domain in a class II integral membrane protein is both necessary and sufficient to provide a signal sequence and a membrane anchor function (6, 46, 59). Moreover, stop-transfer sequences from class I integral membrane proteins will provide a signal sequence function when placed near the amino terminus of a reporter protein lacking a cleaved signal sequence (32, 59). Such observations clearly reflect the common feature of hydrophobicity shared by signal sequences, stop-transfer sequences, and signal-anchor sequences. Important structural differences between these sequences clearly exist, as internally duplicated signal sequences do not function as stop-transfer sequences (11). The hydrophobic core of cleavable signal sequences is typically shorter than a transmembrane spanning segment of an integral membrane protein (48), suggesting that the signal sequence function of the M2 hydrophobic sequence could perhaps be experimentally located by deletion mutagenesis.

With this goal in mind, we constructed a series of deletion mutants to regions within the M2 hydrophobic domain that provide signal and stop-transfer function. The data obtained using a series of deletions constructed within the COOH-terminal region of the M2 hydrophobic domain demonstrate that membrane integration of the M2 protein requires a minimum of at least 15 residues of the 19 residue hydrophobic domain. Substantial decreases in membrane integration of M2 were detected after deletion of as little as two amino acid residues from this region. Approximately 20 amino acid residues are required to span a lipid bilayer in an a-helical conformation (9). Thus, deletion of two residues from the M2 hydrophobic segment will result in either a distortion of the a-helix or an insertion of two additional charged amino acid residues into the lipid bilayer. Deletion analysis of the stop-transfer sequence of the vesicular stomatitis virus G protein (1), and experiments using artificially constructed apolar sequences (7), have both indicated a minimum length of approximately 12 residues for hydrophobic sequences that can interrupt translocation of a protein across a membrane bilayer. Below this threshold length we would anticipate complete translocation of the M2 polypeptide provided that signal sequence function has been maintained. Translocation of hydrophobic sequences that do not meet such threshold values is a biologically relevant phenomenon for naturally occurring viral membrane fusion sequences (35). To date, our deletion analysis of the M2 hydrophobic segment has not yielded a mutant that retains signal sequence function without also retaining stop-transfer function. In all cases, an increase in sensitivity to alkali extraction was accompanied
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