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The Amino-Terminal Signal Peptide on the Porcine Transmissible Gastroenteritis Coronavirus Matrix Protein Is Not an Absolute Requirement for Membrane Translocation and Glycosylation

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cDNA clones mapping within the first 2601 bases of the 3' end of the porcine transmissible gastroenteritis coronavirus (TGEV) genome were sequenced by the method of Maxam and Gilbert and an open reading frame yielding a protein having properties of the matrix (M or E1) protein was identified. It is positioned at the 5' side of the nucleocapsid (N) gene from which it is separated by an intergenic stretch of 12 bases. The deduced M protein comprises 262 amino acids, has a molecular weight of 29,544, is moderately hydrophobic, and has a net charge of +7 at neutral pH. Thirty-four percent of its amino acid sequence is homologous with the M protein of the bovine coronavirus (BCV), 32% with that of the mouse hepatitis coronavirus (MHV), and 19% with that of the avian infectious bronchitis coronavirus (IBV). Judging from alignment with the BCV, MHV, and IBV M proteins, the amino terminus of the TGEV M protein extends 54 amino acids from the virion envelope which compares with only 28 for BCV, 26 for MHV, and 21 for IBV. Eleven of the sixteen amino-terminal amino acids are hydrophobic and the positions of charged amino acids around this sequence suggest that the first 16 amino acids comprise a potentially cleavable signal peptide for membrane insertion. A similar sequence is not found in the M proteins of BCV, MHV, or IBV. When mRNA from infected cells, or RNA prepared by in vitro transcription of the reconstructed M gene, was translated in vitro in the presence of microsomes, the M protein became translocated and glycosylated. When a protein without the amino-terminal signal peptide was made by translating a truncated version of the M gene transcript, some translocation and glycosylation also occurred suggesting that the amino-terminal signal peptide on the TGEV M protein is not an absolute requirement for membrane translocation. Interestingly, the amino-terminal peptide did not appear to be cleaved during in vitro translation in the presence of microsomes suggesting that a step in virion assembly may be required for proper exposure of the cleavage site to the signal peptidase.

INTRODUCTION

The porcine transmissible gastroenteritis coronavirus (TGEV) comprises three major structural proteins: an internal nucleocapsid phosphoprotein (N) of 43 kDa and two glycosylated envelope proteins, one of 29 kDa (a matrix-like protein, M or E1) and one of 200 kDa (the peplomeric, P, or E2 protein) (Brian et al., 1983; Garwes and Pocock, 1975; Kapke and Brian, 1986; Wesley and Woods, 1986). While the 200-kDa P glycoprotein is demonstrably important in stimulating neutralizing antibody (Garwes et al., 1978), the 29-kDa M glycoprotein may also be important, especially if complement is part of the virus–antibody reaction (Woods et al., 1987).

To investigate the role of individual viral proteins in virus replication and in induction of immunity, we have prepared cDNA clones beginning from the polyadenylated 3' end of the TGEV genome and examined the sequences of potential genes (Kapke and Brian, 1986). Within the first 3' 2000 bases, we deduced, from an examination of open reading frames, a noncoding region of 276 bases, and genes for a 9101 mol wt hypothetical hydrophobic polypeptide, a 43,426 mol wt nucleocapsid protein, and part of a matrix protein, arranged in that order from the 3' end of the genome. Assuming that a conserved intergenic sequence would be found in TGEV as has been found in the mouse hepatitis coronavirus (MHV) (Budzilowicz et al., 1985), and the avian infectious bronchitis coronavirus (IBV) (Brown and Boursnell, 1984), we prepared a synthetic oligodeoxynucleotide that is complementary to the TGEV intergenic sequence and used it as a primer for first-strand DNA synthesis in the preparation of additional genomic cDNA clones. Several cDNA clones were thus prepared and seven that mapped within the first 3' 2601 bases were sequenced in part and another clone was sequenced completely to derive a potential gene sequence for the M protein. The nucleotide sequence predicted an M protein that shared many features with the M proteins of the mouse hepatitis virus, the bovine coronavirus, and the avian infec-
tious bronchitis virus, but also predicted an unexpected, potentially cleavable amino-terminal signal peptide that makes the TGEV M protein strikingly different. In this study, we have confirmed our preliminary report of the M gene sequence (Kapke et al., 1987), and have examined the behavior of the amino-terminal peptide during synthesis of the M protein.

MATERIALS AND METHODS

Cells and virus

The Purdue strain of TGEV was grown on swine testicle (ST) cells as previously described (Kapke and Brian, 1986).

cDNA cloning of TGEV genomic RNA

Virus genomic RNA was prepared as previously described (Kapke and Brian, 1986). cDNA cloning was accomplished by the method of Gubler and Hoffman (1983) essentially as described (Kapke and Brian, 1986) except that the synthetic oligodeoxynucleotide 5'TTAGAAGTTAGTTA3' was used as primer for first-strand cDNA synthesis. The primer was synthesized by the phosphoramidite method and was purified by polyacrylamide gel electrophoresis. Clones were selected by colony hybridization to random-primed cDNA prepared from size-selected genomic RNA (Kapke and Brian, 1986). Clones were initially physically mapped to obtain their approximate position on the genome by using a matrix cross-hybridization method in which plasmid DNA from individual clones was probed with purified inserts or segments of inserts that had been radiolabeled with 32P by nick-translation.

DNA sequencing and sequence analyses

DNA sequencing was done by the chemical method of Maxam and Gilbert (1980) and sequence analyses were done with the aid of the computer programs developed by Queen and Korn (1984) and marketed as part of the Beckman Microgenie system, October 1986 version (Beckman Instruments, Inc.). For protein homology determinations among the coronavirus M proteins, the "protein alignment" program of the Beckman Microgenie was used with MAXDIST set at 1000. For protein homology searches among signal peptides, the "IFIND" program of the Intelligenetics system (Intelligenetics, Mountain View, CA) was used to search the National Biomedical Research Foundation Protein database.

Reconstruction of the M gene and synthesis of transcripts in vitro

To reconstruct the full-length M gene, clones FT36 and C4 were digested with NcoI and the small fragment of FT36 (which contained the first 782 bases of the sequence shown in Fig. 2) and the large fragment of C4 (which contained bases 783 through 934, an oligo(dC) tail of 13 bases, and the rest of the pUC9 sequence) were ligated and used to transform Escherichia coli strain 294. The insert was removed from this plasmid using PstI, digested with Bsp1286 to remove the 5' 114 bases, and blunt-ended with mung bean nuclease. The fragment now contained 13 bases upstream (5'-ward) from the CTAAAC presumed intergenic sequence (or 22 bases upstream from the presumed M gene start codon) and extended to the end of the 13-base C-tail which begins 9 bases downstream from the M gene stop codon (bases 922–924 in Fig. 2). The blunt-ended fragment was ligated into the Smal site of the pGEM3 vector (Promega Biotec) and the orientation yielding a sense-strand RNA by transcription with SP6 polymerase was chosen. The construct was designated pGEM-M-1. Transcripts were prepared from EcoRI-cut plasmid using the SP6 transcription system marketed by Promega Biotec.

To reconstruct the truncated version of the M gene (i.e., the M gene with no N-terminal signal peptide), clone pGEM-M-1 was cut with SphI, which cuts within the multiple cloning region of the pGEM vector and between bases 174 and 175 in Fig. 2, and the resulting large fragment was isolated, religated, and designated pGEM-M-2. Translation of pGEM-M-2 transcripts allows initiation at the second AUG downstream from the CTAAAC intergenic sequence (beginning at base 200 in Fig. 2), i.e., at the fifth amino acid downstream from the potential peptidase cleavage site between glycine and lysine (von Heijne, 1986).

The sequence at the 5' junction of the insert (virus sense) and the vector were confirmed by sequencing for both the pGEM-M-1 and pGEM-M-2 constructs.

Isolation of viral mRNA

Cells were grown to confluency in 850-cm² roller bottles (Falcon) and infected with a multiplicity of infection of approximately 10. At 6 hr p.i., cells were rinsed twice with Earl's balanced salt solution (EBSS), scraped from the bottle, transferred to a 50-ml conical tip polypropylene tube, and pelleted. Cells from five roller bottles yielded a pellet of approximately 5 ml and constituted one batch for RNA extraction. Cells by lysing the addition of 5 vol (25 ml to a 5-ml cell pellet) of lysis buffer containing 10 mM Tris-HCl (pH 7.0), 10 mM NaCl, 5 mM MgCl₂, 1% NP-40(w/v) at room temperature followed by vigorous vortexing for 10 sec. Nuclei were removed immediately by centrifugation at 4K x g, 5 min, and to the supernatant was added 0.2 vol of 10% SDS(w/v) in water and 4 mg proteinase K crystals. The solution was incubated 30 min at 37° and
extracted with an equal volume of phenol–chloroform–isoamyl alcohol (24:24:1), and RNA was precipitated with 2.2 vol of ethanol after adding 0.1 vol 3 M Na acetate. RNA from a 5-ml cell pellet was dissolved in water and polyadenylated RNA was selected by oligo(dT)-cellulose chromatography using a binding buffer of 0.5 M NaCl, 0.1 M Tris–HCl (pH 7.5), 0.2% SDS (w/v), and an elution buffer of 0.1 M Tris–HCl (pH 7.5), 0.2% SDS (w/v). Polyadenylated RNA was ethanol precipitated with Na acetate, dissolved in 100 μl water, ethanol precipitated without salt, dissolved in 100 μl water, and 5 μl of this solution was used in a 50-μl translation reaction.

In vitro translation

In vitro translation was done using a wheat germ system (Amersham) in a 50 μl reaction volume that contained 25 μl wheat germ extract, 1 μl 1 mM amino acid solution deficient in methionine (Promega Biotec), 3 μl 1 M NaAc (to make a final K+ concentration of 1.17 mM), 2 μl Rnasin (Promega Biotec), 4 μl microsomes (Amersham, Promega Biotec), or as a gift from Dr. Peter Walter, University of California School of Medicine, San Francisco) or 4 μl microsome blank solution when microsomes were left out, 5 μl 300 μM octanoyl–asparagine–leucine–threonine or 5 μl water when the tripeptide was left out, 5 μl [35S]-methionine (>800 Ci/mmol, New England Nuclear), 5 μl RNA. Octanoyl–asparagine–leucine–threonine, a competitive inhibitor of N-linked glycosylation (Lau et al., 1983), was a kind gift from Dr. Fred Naider, City University of New York, and was prepared as a 300 μM stock in an aqueous solution containing 25% dimethyl sulfoxide. Translations were done for 1 hr at 25°C. Sodium carbonate treatment of microsomes followed the procedure of Fujiki et al. (1982). Deglycosylation of translation products was done with N-glycanase (Genzyme Corp.) or with endoglycosidase H (ICN) using methods recommended by the manufacturers. Immunoprecipitates were prepared as described by Anderson and Blobel (1983) except that iodoacetamide was not used to block SH groups prior to electrophoresis. Five microliters of porcine hyperimmune TGEV-specific serum was included in the medium used for refeeding. At 6 hr p.i., cells were rinsed with EBSS, scraped into a 15-ml conical tip polycarbonate tube and pelleted, and lysate was prepared by adding 0.5 ml phosphate-buffered saline, 1% NP-40, 10 units Aprotinin (Sigma)/ml, and incubating the mixture at 25°C for 30 min with frequent vortexing. Nuclei and cell debris were removed by centrifugation at 13,000 g for 5 min and 50 μl cell lysate supernatant was used in an immunoprecipitation reaction as described above for

In vivo labeling

For labeling intracellular M protein, ST cells in 60-mm plastic petri dishes were infected with a multiplicity of infection of approximately 10, incubated 1 hr, and refed, after rinsing, with 10 ml per dish of minimum essential medium containing 5% normal methionine concentration, 10% fetal calf serum (Sterile Systems), and 200 μCi [35S]methionine (Translabel, ICN). Where indicated, tunicamycin (Sigma) at a final concentration of 2 μg/ml was included in the medium used for refeeding. At 6 hr p.i., cells were rinsed with EBSS, scraped into a 15-ml conical tip polycarbonate tube and pelleted, and lysate was prepared by adding 0.5 ml phosphate-buffered saline, 1% NP-40, 10 units Aprotinin (Sigma)/ml, and incubating the mixture at 25°C for 30 min with frequent vortexing. Nuclei and cell debris were removed by centrifugation at 13,000 g for 5 min and 50 μl cell lysate supernatant was used in an immunoprecipitation reaction as described above for
400 430 460 490
SDFCESCFNGGDLIIIWLANWNFSWSIILIVFITVLQYGRPQ

510 540 570 600
GFSIAAGAIVTFVLWIMYFVR51QLYRRRTKSWWSWNSEPTEK

610 640 670 700
ILCVSALGRSYFLPLEGVPTGVTTLINGNLIVARPFKIA

710 740 770 800
GNNIDNLPKYVMVALPSRTIVYTLVGGKKLKASSATGWAYY

810 840 870 900
VSKRACGDYSTEARTDNLSEQEKLLLMVMANOGQKRV

910 940 970 1000
CAUGTGAGAGAAGATCCTGCCCAACCCGACTGCCTTCC

Fig. 2. Nucleotide sequence of the TGEV M gene and deduced amino acid sequence for the protein. The nucleotide sequence comes from the part of the virus genome illustrated in Fig. 1. A continuous open reading frame beginning at nucleotide position 55 and continuing through nucleotide 922 is identified. The CTAAAC intergenic sequences are underlined. The proposed amino terminus for the M protein is identified by an underlined methionine residue near base position 137.

RESULTS

Deduced amino acid sequence of the matrix protein

Seven clones, C4, E2, F5, FT35, FT36, FT43, and FT44, mapping in the positions illustrated in Fig. 1, were sequenced in part to extend the TGEV genomic sequence that was known from clones FG5 and J21 (Kapke and Brian, 1986). Clone FG5 maps at the extreme 3' end of the genome and contains the sequence for the hypothetical hydrophobic protein gene, the N gene, and part of the M gene. Identification of the third open reading frame as the M gene sequence was based on regions of extensive amino acid homology with the M proteins of MHV (Armstrong et al., 1984) and IBV (Boursnell et al., 1984). The sequencing strategy we used is described in Fig. 1.

Polyacrylamide gel electrophoresis

In vitro translation reaction products or immunoprecipitates on protein A-Sepharose CL-4B beads were diluted with an equal volume of 2X Laemmli sample treatment buffer [1X sample treatment buffer is 0.0025 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol] that contained 5 M urea, heated 2 min at 100°, and electrophoresed using the method of Laemmli (1970).

products of a 50 µl in vitro translation reaction. The M protein was immunoprecipitated with 10 µl M-specific monoclonal antiserum (identified as 1A6; Woods et al., 1987).

For labeling virion M protein, cells grown in 150-cm² plastic flasks were infected as described above, and incubated with 500 µCi [³⁵S]methionine (Translabel, ICN) per flask. At 18 hr p.i., virus was purified from clarified supernatant fluids by isopycnic sedimentation in sucrose gradients as previously described (Brian et al., 1980). Virion proteins were solubilized in 4% SDS, and M protein was immunoprecipitated with M-specific monoclonal antibody and deglycosylated with N-glycanase.
and Brian, 1986) yielded a protein of 289 amino acids having a molecular weight of greater than 32,000 (Fig 2). Since the M proteins of the mouse hepatitis virus (Armstrong et al., 1984; Tooze et al., 1984) and bovine coronavirus (Lapps et al., 1987) migrate in sodium do- decyl sulfate-containing polyacrylamide gels with elec-

![Diagram](image)

**Fig. 3.** Translocation and glycosylation of the M protein with and without its amino-terminal peptide. (A) Comparison of M protein from in vitro and in vivo synthesis and from the virion. Lanes 1 through 12, mRNAs isolated from uninfected (U) or infected (I) cells, and in vitro transcripts prepared from the reconstructed full-length M gene (pGEM-M-1 or 1st AUG) or the truncated M gene (pGEM-M-2 or 2nd AUG), were translated in vitro and treated as noted. For immunoprecipitation, porcine hyperimmune antiserum was used. Lanes 13 and 14, M protein was immunoprecipitated from cell lysates with monoclonal antibody. Cell lysates were prepared as noted. Lanes 15 and 16, M protein was immunoprecipitated from virion proteins with monoclonal antibody. Immunoprecipitates were treated as noted. Lane 17, proteins from purified TGEV were electrophoresed and the position of the 28-kDa protein is identified. Lanes 16 and 17 are a longer exposure of part of lanes 1 and 2 used in (E). Lanes 1 through 4, 13, and 14 were overexposed to identify species in relatively lower abundance. (B) Endoglycosidase H treatment of the in vitro translation products of the reconstructed full-length M gene (1st AUG) and of the truncated M gene (2nd AUG). (C) Sodium carbonate treatment of microsomes containing the translation products of the reconstructed full-length M gene (1st AUG) and of the truncated M gene (2nd AUG). (D) Controls used for detecting signal peptidase activity in the microsomes employed. Positions of the uncleaved and cleaved forms of preprolactatin and β-lactamase are shown. (E) Immunoprecipitation showing the specificity of the monoclonal antibody 1A6. All gels were 10% polyacrylamide. 125I-Radiolabeled protein molecular weight markers were obtained from New England Nuclear.
The TGEV M protein of 262 amino acids (top row), BCV (Mebus strain) M protein of 230 amino acids (second row), MHV (A59 strain) M protein of 228 amino acids (third row), and IBV (Beaudette strain) M protein of 225 amino acids (fourth row) were aligned for maximum homology. For this, the 8 amino acid sequence SWWSFNPE was important in making the initial alignment. Identical amino acids among all four proteins are boxed with a solid line. The 21 amino acid hydrophobic transmembrane regions of the MHV and IBV proteins are boxed with a hatched line (Rottier et al., 1986) with the TGEV and BCV sequences drawn in parallel with MHV. Potential N-glycosylation sites (for TGEV and IBV) and O-glycosylation sites (for BCV and MHV) are indicated with a solid circle above the amino acid. The signal peptide-like properties of the amino-terminal 16 amino acids is depicted. Within this region the 11 hydrophobic amino acids are underlined. The basic amino acids at positions 2 and 18 and the acidic amino acid at position 17 are indicated. The potential site for peptidase cleavage, according to the rules of Von Heinje (1986) is indicated by an open triangle. The numbers 1 and 2 above the first and second methionine residues in the TGEV M sequence indicate initiation sites for translation of the in vitro transcripts derived from pGEM-M-1 and pGEM-M-2, respectively.

Also part of the intergenic sequence found in the MHV genome (Budzilowicz et al., 1985). If CTAAAC functions as part of an intergenic sequence that directs leader-primed synthesis and thereby defines the start of the M transcript for TGEV, then the M protein coding sequence could start with the first available methionine 3'-ward of the CTAAAC sequence, a codon that begins at base 137 in Fig. 2. It could also start with the second, downstream, in-frame AUG codon beginning at base 200, but this is surrounded by a much less favorable Kozak consensus sequence (Kozak, 1983).

To test our hypothesis, we prepared transcripts identical to the postulated functional mRNA structure (transcripts from the reconstructed full-length M gene, construct pGEM-M-1, that would initiate translation at the first AUG downstream from the CTAAAC sequence) and compared sizes of the resulting translation products with those of mRNA isolated from infected cells. Figure 3A, lanes 2 and 5, illustrates that...
M protein translated in vitro from cell-derived, poly(A)-
selected mRNA and immunoprecipitated with TGEV-
specific antibody had an electrophoretically deter-
mined molecular weight of 25k and comigrated with
protein translated from the reconstructed full-length M
gene. Protein translated from the reconstructed full-
length M gene immunoprecipitated with the same anti-
tbody thus confirming its authenticity (Fig. 3A, lane 8).
Furthermore, truncated M protein (generated from
construct pGEM-M-2), although also antigenically au-
thentic, migrated distinctly faster than full-length M but
with a migration rate much less than expected for a
2-kDa molecular weight difference (Fig. 3A, lanes 9
and 12). The small difference in electrophoretic mobi-
licity between the two forms of M again is an ostensible
function of the hydrophobic nature of the protein. The
electrophoretically determined molecular weight of the
truncated M polypeptide is 24.5k. Judging from the
size of the various translation products, it is unlikely
that initiation of translation in vivo starts at any place
other than at the first AUG downstream from the
CTAAC intergenic sequence.

When the first methionine codon downstream from
the CTAAAC sequence is used as the initiation site for
translation, the deduced M protein comprises 262
amino acids and has a molecular weight of 29,544. It
is moderately hydrophobic with 44% of its amino acids
being hydrophobic, and is basic since it carries a net
charge of +7 at neutral pH.

A potentially cleavable amino-terminal signal
peptide is not an absolute requirement for
membrane translocation and glycosylation

A comparison of the amino acid sequence for the M
proteins of TGEV, BCV, MHV, and IBV (Fig. 4) reveals
several features that are shared among all four viruses,
but also one feature for TGEV that is strikingly con-
trasting. Regions of high sequence homology are
found among the proteins. Most notable is a stretch of
8 amino acids beginning at position 132 on the TGEV
sequence that is identical for all four viruses. By com-
puter analysis using a protein alignment function, 34,
32, and 19% of the TGEV protein sequence is homolo-
gous with that of BCV, MHV, and IBV, respectively.
Furthermore, a hydrophobicity plot of the TGEV M
protein shows three internal hydrophobic domains
that align with similar domains in BCV, MHV, and IBV
(Fig. 4 and data not shown; Rottier et al., 1984; 1986),
BCV (Lapps et al., 1987), and IRV (Roursnell et al.,
1984). This suggests that the topology of the four pro-
teins is similar; that is, from its entrance into the virion
membrane and as it extends toward the carboxy termi-
nus, the protein spans the membrane three times and
has a relatively hydrophilic intravirion carboxy-terminal
region (Rottier et al., 1984, 1986).

The striking feature of the TGEV M protein is its
much longer amino terminus that includes a sequence
resembling a cleavable peptide for membrane inser-
tion. Assuming a parallel structure for the M proteins
of the four viruses and assuming the MHV M protein
enters the virion envelope at position 26 (Rottier et al.,
1986), then the external amino-terminal portion is 28
amino acids for BCV, 21 for IBV, and 54 for TGEV.
Within the first 54 amino acids there is one asparagine
at position 32 that has the appropriate surrounding
sequence required for N-linked glycosylation (Hubbard
and Ivatt, 1981), the kind of glycosylation shown for
the TGEV M protein (Jacobs et al., 1986). Unlike the
amino terminus of the MHV, BCV, and IBV proteins,
the TGEV M protein is hydrophobic for the first 16
amino acids. Eleven of the sixteen terminal amino
acids are hydrophobic, and amino acids at positions 2,
17, and 18 are charged (Fig. 4). By inspection, this
sequence has the properties of a cleavable amino-termi-
nal signal peptide and from the “−3, −1” rule (von
Heijne, 1986) peptidase cleavage would occur be-
tween amino acids 16 and 17.

Since an amino-terminal peptide is not required for
membrane translocation and glycosylation of the M
proteins of BCV, MHV, and IBV, we examined what
effect the peptide had on the translocation of the
TGEV M protein. Both forms of the reconstructed M
gene generate the asparagine glycosylation site when
translated, pGEM-M-1 at amino acid position number
32 and pGEM-M-2 at amino acid position number 11
together with the respective translation products (Fig. 4).
Both forms of transcripts were therefore translated in the
presence of microsomes known to have glycosylating
activity. Figures 3A, lanes 5, 6, 9, and 10, 3B, lanes 1,
2, 3, and 4, and 3C, lanes 1, 2, 3, and 4, illustrate that,
whereas the full-length reconstructed M gene (1st AUG) yielded a 25-kDa protein that was mostly glycosylated, 65–88% as determined from optical density tracings, the truncated M gene (2nd AUG) yielded a protein that was also glycosylated but to a far lesser extent, only 10–30%. The glycosylated products of both the 1st AUG and 2nd AUG transcripts were approximately 28 kDa (Fig. 3A, lanes 6 and 10), the same size as the glycosylated product from viral mRNA translation (Fig 3A, lane 3) and glycosylated virion M (Fig. 3A, lanes 16 and 17). Glycosylation was confirmed to have taken place since the products from in vitro translation migrated again with the un glycosylated polypeptides after they had been digested with N-glycanase (Fig. 3A, lanes 7 and 11) or endoglycosidase H (Fig. 3B, lanes 5 and 6). These results demonstrate that although the asparagine glycosylation site of both the full-length and truncated forms of M protein became translocated to the luminal side of the microsome, translocation with the amino-terminal peptide present was far more efficient. Furthermore, translocation of the amino terminus appeared to be specifically enhanced by the peptide since carbonate treatment showed both full-length and truncated forms of the protein as a whole to be equally membrane embedded (Fig. 3C, lanes 5, 6, 7, and 8), presumably as a result of internal translocation signals of the kind described for the MHV and IBV M proteins (Rottier et al., 1985; Machamer and Rose, 1987). Following carbonate treatment greater than 85% of both the full-length and truncated forms of the M protein remained membrane bound as determined by optical density tracing of the autoradiogram.

Cleavage of the amino-terminal peptide did not appear to occur during in vitro translation

To test for amino-terminal peptide cleavage, viral mRNA from infected cells and transcripts of the cloned M gene were translated in the presence of microsomes known to contain signal peptidase activity (Fig. 3D). In initial experiments, a tripeptide serving as a competitive inhibitor of asparagine-linked glycosylation (Lau et al., 1983) was incubated with the microsomal translation mixture in order to inhibit concurrent glycosylation that would otherwise obscure the results of peptide cleavage. Although inhibition of glycosylation was never complete, at no time was there evidence of peptide cleavage (data not shown). To use a second approach, mRNA and transcripts were translated in the presence of microsomes and the products were deglycosylated with either N-glycanase or endoglycosidase H, and sizes were compared by electrophoresis (Figs. 3A, lanes 4, 7, and 11, and 3B, lanes 5 and 6). Interestingly, the sizes of the deglycosylated products, as described above, appeared to be no smaller than the polypeptides synthesized without microsomes. There appeared, therefore, to be no amino-terminal peptide removal during in vitro translation in the presence of microsomes.

Results of experiments to determine whether the N-terminal peptide is cleaved in vivo suggest that cleavage in vivo may be dependent upon the glycosylation of M or other glycoproteins. When infected cells were incubated in the presence of tunicamycin and radiolabeled M was immunoprecipitated from cell lysate using monoclonal antibody. only one major band was found and it migrated as an uncleaved protein of 25 kDa (Fig. 3A, lane 14). Similar results were obtained when polyclonal hyperimmune TGEV serum was used (data not shown). On the other hand. in the absence of tunicamycin, two forms of M were precipitated. These were a 28-kDa species, the size of fully glycosylated M, and a 24.5-kDa species, the size of M from which the N-terminal signal had been removed (Fig. 3A, lane 13). Glycosylation in vivo may have important consequences on the exposure of the peptidase cleavage site because of interactions between M and other viral components in vivo.

In light of a recent report demonstrating by aminoterminal amino acid sequencing that the virion form of TGEV M protein is indeed cleaved (Laude et al., 1987), we included deglycosylated, radiolabeled virion M in our electrophoretic analysis (Fig. 3A, lane 15). The deglycosylated virion M did not migrate as a cleaved polypeptide but rather migrated with an apparent molecular weight of approximately 26 kDa suggesting that it had perhaps undergone a second as yet undetermined modification after becoming virion-associated.

DISCUSSION

The M protein of MHV was the first coronavirus M protein to be sequenced (Armstrong et al., 1984) and its topography with regard to membrane orientation and insertion has been carefully documented (Rottier et al., 1984, 1986). It therefore serves as the prototypic coronavirus M protein to which others can be compared. The coronavirus M protein apparently functions to direct the budding of virus into the rough endoplasmic reticulum and the Golgi since it inserts into these membranes and is found at highest concentrations there (Holmes et al., 1984; Tooze et al., 1984). Presumably virus assembly is mediated through an interaction between the M protein (in the membrane) and the N protein (in the cytoplasm) or the RNA (in the cytoplasm), or both. The M protein of MHV does not have an amino-terminal cleavable signal peptide for membrane translocation, but rather utilizes
one or more of its three internal hydrophobic domains for membrane insertion (Rottier et al., 1985). The same is also true for the IBV M protein (Machamer and Rose, 1987), and is probably true for the BCV M protein (Lapps et al., 1987) which has a structure similar to that of the MHV M protein.

It came as a surprise to us, therefore, that the deduced TGEV M protein has, in addition to the three internal hydrophobic domains, an amino-terminal sequence that possesses the properties of a cleavable signal peptide for membrane insertion (von Heijne, 1980). The potentially cleavable N-terminal peptide was also revealed by the work of Laude et al. (1987) in which a nearly identical M gene sequence was reported for the same strain of TGEV. In their sequence, bases in positions 375, 468, and 720 of Fig. 2 were T, C, and A, respectively, making the corresponding amino acids at these codon positions Val, Asn, and Asp. Furthermore, they obtained direct evidence that the N-terminal signal peptide was cleaved from the virion-associated M protein. At least two fundamental questions are therefore raised by the existence of an amino-terminal signal peptide in the TGEV M protein: (i) What is the evolutionary origin of such a sequence, and (ii) what role does the sequence play for the TGEV M protein?

With regard to the evolutionary origin of the hydrophobic amino terminus, two possibilities can be entertained, assuming the four coronaviruses, TGEV, BCV, MHV, and IBV have a common evolutionary origin. (i) There was an amino-terminal hydrophobic sequence in the primordial protein which was lost during the evolution of BCV, MHV, and IBV since it is superfluous for membrane insertion and virus assembly. Mechanistically, the loss of a genetic sequence could be explained by the dissociating-reassociating polymerase hypothesized by Lai et al. (Makino et al., 1986a). The nucleotide sequence encoding the amino-terminal hydrophobic sequence could have been eliminated by the polymerase as it copied the negative-strand template. (ii) There was no amino-terminal hydrophobic sequence in the primordial protein but the TGEV M protein acquired one during evolution. The polymerase during replication of the TGEV genome could have become dissociated and then reassociated with another minus-strand RNA template that carried the sequence for a hydrophobic signal. While negative-strand RNA of this kind is probably nonexistent or of low abundance in eucaryotic cells normally, it does exist in cells coinfected with another RNA virus. Conceivably the TGEV M protein could have acquired its amino-terminal sequence by copying the negative strand of another RNA virus. In this regard, it is interesting that 6 of the first 8 amino acids in the TGEV M sequence are identical to the VSV G protein signal sequence (Rose and Gallione, 1981).

With regard to the function of the amino-terminal hydrophobic sequence, we propose that it aids in the translocation of the amino terminus of the TGEV M protein through the endoplasmic reticulum as would other amino-terminal signal peptides, but it is not an absolute requirement. The TGEV M protein without its amino-terminal signal peptide, as approximated by the translation product of the pGEM-M-2 construct which is 4 amino acids shorter than the polypeptide identified by the peptidase cleavage site (Laude et al., 1987), can translocate apparently by the use of an internal signal(s) of the type described for the MHV and IBV M proteins (Rottier et al., 1985; Machamer and Rose, 1987). It is interesting to note that even after removal of the amino-terminal peptide, the TGEV M protein extends 12 amino acids farther from the envelope than does BCV, 11 amino acids farther than MHV, and 16 amino acids farther than IBV. Perhaps the amino-terminal signal peptide, while not being an absolute requirement for translocation of the TGEV M protein, aids enough in the translocation of the additional external amino-terminal sequence that it was evolutionarily selected.

Since the amino-terminal peptide is apparently not removed from the M protein during in vitro translation in the presence of microsomes, but is removed from the M protein in the assembled virion (Laude et al., 1987), we propose that its cleavage depends on the context in which it finds itself. Perhaps a step in virion assembly, for example, an interaction with another viral protein or viral RNA, may be required for cleavage to occur. Glycosylation of the M protein alone is apparently not a prerequisite for cleavage in vitro (Figs. 3A and B) but may be important in the in vivo context. Context is important for the cleavability of other signal peptides. For example, under certain constraints the potentially cleavable signal on the invariant (Iγ) chain of class II histocompatibility antigens (Lipp and Dobberstein, 1986) is not cleaved. We further propose that the M protein with an uncleaved signal peptide would have the orientation depicted in Fig. 5. With this orientation, the cleavage site is buried in the membrane and fewer than 37 amino acids are exposed as a loop on the lumenal side of the endoplasmic reticulum. Once the virion assembly begins, the cleavage site becomes exposed to the signal peptidase, cleavage occurs, and 37 amino acids, including a glycosylated asparagine, are left remaining on the virion surface. Because of the amino-terminal hydrophobic sequence, the TGEV M protein may behave differently than its MHV, BCV, or IBV counterparts with regard to intracellular trafficking.
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