EXPRESSION OF THE PORCINE TRANSMISSIBLE
GASTROENTERITIS CORONAVIRUS M PROTEIN

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ABSTRACT

Cloned cDNA encoding the M protein of the porcine transmissible gastroenteritis coronavirus (TGEV) was introduced into a vaccinia virus to examine the function of the amino-terminal signal peptide. The M protein expressed by the recombinant virus was targeted to the Golgi region of infected cells, as is the M protein in cells infected with TGEV. The protein appeared not to undergo processing other than glycosylation. However, the vaccinia-expressed M protein was slightly larger than the protein found in TGEV-infected cells, suggesting that a difference in modification exists between the proteins.

INTRODUCTION

The TGEV M protein has been cloned and shown to be different from the M proteins of the mouse hepatitis virus (MHV), the bovine coronavirus (BCV), and the avian infectious bronchitis virus (IBV)^{1,2}. Unlike the others, the TGEV M protein has a sixteen-amino-acid amino-terminal domain, which has the properties of a cleavable signal sequence. Otherwise, the TGEV M is like the others, in that it has three internal hydrophobic domains that presumably result in the protein being anchored in the lipid bilayer by three successive transmembrane helices³. Initial experiments suggested that there may be two forms of TGEV M, one that is virion-associated and has the signal peptide removed² and another intracellular uncleaved form¹. It was hypothesized that interaction with other viral components is required for cleavage and that the additional hydrophobic sequence results in the intracellular transport of TGEV M being different from that of the other coronaviruses¹.

This paper reports the initiation of studies on the intracellular biogenesis of the M protein in the absence of other TGEV proteins. We have expressed the M protein using a vaccinia virus recombinant which includes a cDNA copy of the gene for the protein. The synthesis and processing of the protein has been studied and compared with the protein synthesized in TGEV-infected cells. The results indicate that the protein is localized to the Golgi. The vaccinia-expressed protein is slightly larger than the M from TGEV-infected cells, a result consistent with noncleavage of the signal. Other recent data suggest, however, that signal cleavage does occur, even in the absence of other viral components.

MATERIALS AND METHODS

Generation of a Vaccinia Recombinant Expressing the TGEV M Protein

A cDNA copy of the TGEV M protein gene was removed from the pGEM-M-1 construct¹ with Bam HI and Eco RI and was filled in with the Klenow fragment of DNA polymerase I. The gene was then subcloned into the pSC11 vector⁴ in the unique Sma I site under the vaccinia promoter P7.5K. The plasmid containing the M gene in the correct orientation was transfected into CV-1 cells by calcium phosphate coprecipitation, after infection with wild-type vaccinia (WR strain) had been performed at a multiplicity of 0.05. Recombinant viruses were isolated by plaque assay on TK⁻ cells in the presence of 5-bromodeoxyuridine and by including 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal) in the plaquing overlay as previously described⁴. Recombinant viruses encoding the M protein (VVM) were plaque-purified three times on TK⁻ cells prior to preparation of large stocks in HeLa cells.

Protein Expression Analyses

The Purdue strain of TGEV was grown on swine testicle cells (ST) as previously described⁵. Cells were infected at a multiplicity of infection (moi) of 5. At 8-9 hours postinfection (hpi) cells were labeled with 100 μ Ci of [³⁵S]methionine or 50 μ Ci each of [³⁵S]methionine and [³⁵S]cysteine. After labeling, cells were lysed on ice in 0.5 ml of buffer containing 50 mM pH 8.0 Tris, 1% TX-100, 0.5% deoxycholate, 150 mM NaCl, 20 mM EDTA, and 100 kallikrein units/ml Aprotinin. The M protein was immunoprecipitated using 1-2 μ l of a monoclonal against the TGEV M (1A6)⁶. Antigen-antibody complexes were isolated with protein A sepharose CL-4B (Pharmacia) and washed in the buffer described above, to which was added 0.1% SDS. In some cases, cells were pretreated with 10 μ g/ml tunicamycin for 2 h and then labeled in the presence of the same concentration of the drug. Endo H digestions were carried out as previously described⁷, after elution of immunoprecipitated M from protein A sepharose by boiling in the presence of 1.0% SDS. Proteins were detected by immunofluorescence after cells were fixed with acetone:methanol (1:1) for 10 minutes at -20°C. M was detected by incubation with the 1A6 monoclonal (dilution 1:100) followed by incubation with affinity-purified fluorescein-conjugated goat anti-mouse IgG. Cells were visualized with a Nikon Optiphot microscope equipped with fluorescence epiillumination and a Nikon 40X objective. For vaccinia expression of the M protein, CV-1 or ST cells were infected with a moi of 1. Infected cells were harvested at 16 hpi. All procedures were carried out as described above.

RESULTS

Cellular Localization of Vaccinia Expressed M

To determine the cellular localization of M synthesized in the absence of other TGEV proteins, cells infected with the vaccinia recombinant were monitored by indirect immunofluorescence. Using monoclonal 1A6, specific M fluorescence was observed near the nuclei in the Golgi region of infected cells (Fig. 1, lower left). The fluorescence pattern was similar to that observed for the internal expression of M in TGEV-infected cells (Fig. 1, upper left).

Biosynthesis of Vaccinia Expressed M

The biosynthesis of M was followed by immunoprecipitation of infected cell lysates. Using monoclonal 1A6, a protein with a molecular weight of

Fig. 1. Internal immunofluorescence of M in ST cells infected with TGEV and VVM: Upper left, TGEV-infected cells; lower left, VVM-infected. Controls were either uninfected cells (upper right) or cells infected with a vaccinia recombinant expressing the influenza neuraminidase (lower right).

30 kDa was immunoprecipitated from cell lysates infected with VVM (Fig. 2A, lanes 8-12, and 2B, lanes 3-5). When electrophoresed in parallel with the M protein immunoprecipitated from TGEV-infected cells (Fig. 2A, lanes 2-5 and 7, and 2B, lane 2), the VVM expressed protein was observed to migrate slower. A molecular weight difference between the two protein species of approximately 2 kDa was estimated. The molecular weight of TGEV M has been estimated to be 28-30 kDa^{8,9,10}.

To follow posttranslational processing of the proteins, infected cells were pulsed for 5 minutes and chased. During a 3 hour chase, M synthesized by VVM did not appear to undergo processing, as evidenced by no change in molecular weight (Fig. 2A, lanes 8-12). The protein appeared to remain stable during this time. The TGEV synthesized M appeared to undergo some type of processing, since a heterogeneous smear began to appear above the major protein species after only a 10 minute chase (Fig. 2A, lane 3) and appeared to increase with time (Fig. 2A, lanes 4, 5, 7).

Infected cells were maintained in the presence of tunicamycin, to determine if the difference in mobility between VVM and TGEV M is due to an N-linked glycosylation difference. Glycosylation of the TGEV M has been shown to be of the N-linked type¹¹. In the presence of tunicamycin, the M synthesized by VVM (Fig. 2B, lanes 7 and 8) continued to run with a mobility less than the M from TGEV-infected cells (Fig. 2B, lane 7), indicating that the difference in molecular weight is not due to N-linked glycosylation. To assess the extent of processing of the N-linked sugar chains, proteins immunoprecipitated from both recombinant and TGEV-infected cells were

Fig. 2. In vivo expression of M in TGEV- and VVM-infected cells. (A) Pulse-chase results: Lanes 2 through 5 and 7 are immunoprecipitates from TGEV-infected cells harvested at 0, 10, 20, 30, and 40 minutes, respectively, after a 5 minute pulse with [35 S]methionine. Lanes 8-12 are immunoprecipitates from VVM-infected cells chased for 0, 15, 30, 45 minutes, and 3 hours, respectively. Lane 6 is uninfected cells after a 40-minute chase. Lanes 13 and 14 are wild-type vaccinia-infected cells chased for 0 and 3 hours, respectively. Lane 1 contains molecular weight markers. (B) Tunicamycin-treatment and endo H digestion results: Infected cells were pulsed for 30 minutes with [35 S]methionine and [35 S]cysteine then chased for 1.5 hours with excess cold amino acids. Lanes 6-8 are immunoprecipitations from cells maintained in the presence of tunicamycin. Lanes 9 and 10 are immunoprecipitations digested with endo H. Lanes 2-5 are untreated immunoprecipitates. Lane 1 contains molecular weight markers.

digested with endo H. Both species were sensitive to endo H (Fig. 2B, lanes 9 and 10), indicating that neither protein was transported as far as the medial Golgi compartment, where endo H resistance is acquired¹².

Results identical to those described above were obtained using a transient vaccinia expression system. A recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase¹³ was used in conjunction with a transfected cDNA copy of the M gene under a T7 promoter (data not shown).

DISCUSSION

We describe here the results of *in vivo* expression of the TGEV M protein in the absence of other TGEV proteins. We have generated a vaccinia recombinant virus which includes a cDNA copy of the M gene. Even though we did not quantify expression by the recombinant, it was high; therefore, this expression system is a reasonable one to be used for transport and function studies of M. The VVM-expressed protein was specifically recognized by a monoclonal against the TGEV M; however, as judged by electrophoretic mobility, the VVM expressed protein was larger than the protein precipitated from TGEV-infected cells.

The results of previous work¹ suggested that M signal cleavage may depend on interaction with another viral protein or viral RNA. The results described in this paper are consistent with this hypothesis. However, we now have sequenced the amino terminus of the VVM M protein and find, surprisingly, that it is cleaved (Hogue and Nayak, unpublished data). In addition, the M gene which lacks the amino-terminal signal¹ has been expressed *in vivo* and found to comigrate with the VVM expressed M. (Hogue and Nayak, unpublished data). Taken together, these findings suggest that some other modification is responsible for the molecular weight difference. We are presently developing new hypotheses and performing further analyses to account for the observed molecular weight difference between the M proteins synthesized in TGEV- and VVM-infected cells.

The localization of M in the Golgi region of permeabilized cells is consistent with that observed by others for the expression of the M proteins of MHV¹⁴ and IBV¹⁵. This site of localization also correlates with the site of intracellular budding for coronaviruses, consistent with the idea that M determines the budding site^{16,17}.

We did not detect definitive surface fluorescence of the VVM-expressed protein on nonpermeabilized cells (data not shown). No surface expression was seen in studies of MHV¹⁴ and IBV¹⁵, but we and others¹⁸ have detected apparent surface fluorescence of M on TGEV-infected cells. The failure to observe surface fluorescence from VVM may be due to the extensive cytopathic effect (cpe) on cells infected with vaccinia. However, using a transient vaccinia expression system¹³ which results in less cpe of infected cells, we are currently reexamining the surface fluorescence of cells expressing the M gene. We should be able to determine if the TGEV M protein is targeted to the cell surface in the absence of the other viral proteins or if the apparent surface expression on TGEV-infected cells represents transport of the protein to the surface by Golgi derived vesicles or mature virions remaining attached to the surface after release from the cell.

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