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The 9-kDa Hydrophobic Protein Encoded at the 3' End of the Porcine Transmissible Gastroenteritis Coronavirus Genome Is Membrane-Associated

FRANK Y. T. TUNG,1 SUSHMA ABRAHAM,2 MANJIRI SETHNA, SHAN-LING HUNG,3 PHIROZE SETHNA, BRENDA G. HOGUE,4 AND DAVID A. BRIAN5

Department of Microbiology, the University of Tennessee, Knoxville, Tennessee 37996-0845

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The open reading frame potentially encoding a 78 amino acid, 9,101 Da hydrophobic protein (HP) and, mapping at the 3' end of the porcine transmissible gastroenteritis coronavirus (TGEV) genome, was shown to be expressed during virus replication. The cloned HP gene was placed in a plasmid under control of the T7 RNA polymerase promoter and in vitro translation of transcripts generated in vitro yielded a 9.1-kDa protein that was immunoprecipitable with porcine hyperimmune anti-TGEV serum. Antiserum raised in rabbits against a 31 amino acid synthetic polypeptide that represented the central hydrophilic region of HP specifically immunoprecipitated HP from TGEV-infected cells. HP was further shown to become associated with microsomal membranes during synthesis in vitro and was found to be closely associated with the endoplasmic reticulum and cell surface membranes in infected cells. The intracellular location of HP suggests that it may play a role in the membrane association of replication complexes or in virion assembly. © 1992 Academic Press, Inc.

INTRODUCTION

From sequence analysis of the porcine transmissible gastroenteritis coronavirus (TGEV) genome, an open reading frame potentially encoding a 78 amino acid hydrophobic protein (HP) of 9,101 Da and preceded nine nucleotides upstream by the CYAAAAC consensus intergenic sequence, was found to reside on the immediate 3' side of the nucleocapsid protein gene (Britton et al., 1988; Kapke and Brian, 1986; Rasschaert et al., 1987). This is the most 3' open reading frame in the genome, and a gene encoding a protein having these properties is not found in a similar position in the genomes of the avian infectious bronchitis virus (IBV; Boursnell et al., 1985), the mouse hepatitis virus (MHV; Armstrong et al., 1983; Skinner and Siddell, 1983), the bovine enteric coronavirus (BCV; Lapps et al., 1987), or the human coronaviruses OC43 (Kamahora et al., 1989) and 229E (Schreiber et al., 1989). None of these for IBV, MHV, or BCV show sequence similarity to the TGEV 9,101 Da protein.

The functions of the small coronavirus proteins have not been determined, nor has it been rigorously shown whether they reside on the virion (Spaan et al., 1988). In this paper we report that the TGEV 9.1-kDa hydrophobic protein is expressed in infected cells, and that it becomes associated with the membranes of the endoplasmic reticulum and cell surface. We speculate that HP may function in the formation of membrane-bound replication complexes or in the assembly of TGEV.

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).

Construction of plasmid, generation of HP transcripts, and in vitro translation analyses

To construct a plasmid for the in vivo expression of HP in Escherichia coli cells, the 650 nt T7 promoter fragment of clone FG5 which represents the 3' end of the TGEV
cysteine (>800 Ci/mmol; ICN Pharmaceuticals) per ml, was likewise blunt-ended. The resulting clone, pHP-IA the HP initiator codon and was used in some of the in vitro translation of the HP gene, a subclone of pHP-1 (Fig. 1A), had the 19 nt sequence 5'GGGAGA-GATCCGCGGATCCGAATTCGAGCTCGGTACCC-
CATGCCTGCGAATTCGAGCTCGGTACC-GAG3' located between the T7 promoter and the start codon of HP was obtained, named pHP-1 (Fig. 1A), and used in some of the in vitro translation studies as indicated. Because a short (six amino acid) open reading frame was present within the 81 nt sequence (beginning at position 16) that might have interfered with optimal translation of the HP gene, a subclone of pHPl was made by removing the HP-containing insert with KpnI, blunt-ending it with mung bean nuclease, and ligating it into the Sphl site of pGEM-4-Z (Promega) that was likewise blunt-ended. The resulting clone, pHPl-1 (Fig. 1A), had the 19 nt sequence 5'GGGAGA-
CATGCCTGCAGGTCGACTCTAGAGGATCCGCGGATCC-
CAAGCTGAGAG3' located between the T7 promoter and the start codon of HP, and the resulting 550 nt fragment was cloned into the pGEM-4-Z vector (Promega) using E. coli JM105 as host. A clone having HP under control of the T7 polymerase promoter and having the 81 nt sequence 5'GGGAGACAAGCTG-
GATCCGCGGATCCGAATTCGAGCTCGGTACC-
CATGCCTGCGAATTCGAGCTCGGTACCC-GAG3' located between the T7 promoter and the start codon of HP was obtained, named pHP (Kapke et al., 1988) except that SDS gels of 15% polyacrylamide (Guilian et al., 1985) were used. In all experiments where various membrane treatments were examined, microsome-containing translation reaction mixes were divided equally before analysis, and proteins in the supernatant of pelleted microsomal membranes were recovered in full by precipitation with 10 vol of ice-cold acetone. Equivalent portions of each fraction were analyzed in parallel by electrophoresis. 3H-labeled proteins ovalbumin (MW 48,000), carbonic anhydrase (MW 30,000), soybean trypsin inhibitor (MW 21,500), lysozyme (MW 14,300) and aprotinin (MW 6,500) (Amersham) were used in all gels as molecular weight markers. Restriction endonuclease enzymes were purchased from New England Biolabs.

Preparation of HP-specific antiserum

A synthetic 31 amino acid polypeptide (NH2-QLLELDDHFSNLTVNDFNILYRSLE-COOH) representing the hydrophilic central region of HP, amino acid positions 20 through 50 (Fig. 1C) was prepared and used directly to immunize a rabbit. One milligram of peptide purified by HPLC was suspended in 0.5 ml water, emulsified in 0.5 ml Freund’s complete adjuvant containing 0.05 mg N-acetylmuramyl-L-alanyl-n-isoglutamine (Calbiochem-Behring), and injected subcutaneously at eight sites over the neck, back, and sides. The rabbit was boosted 2 weeks later with 1 mg peptide emulsified in Freund’s incomplete adjuvant, and serum was harvested 4 weeks after this.

Immunoprecipitation and immunofluorescence analyses

For immunoprecipitation analysis of HP synthesized in vitro, radiolabeled product from one 50-μl translation reaction was immunoprecipitated by the method of Anderson and Blobel (1983) as described (Kapke et al., 1988) using 5 μl porcine hyperimmune anti-TGEV serum (Kemony, 1976) or 10 μl rabbit anti HP polypeptide serum. Nonimmune porcine serum was obtained from a neonatal piglet produced on a TGEV-free herd. For immunoprecipitation and for all immunoreactive studies described below, sera were adsorbed against uninfected ST cells (10⁶ cells/ml) before use.

For immunoprecipitation analysis of HP synthesized in vivo, ST cells in a 60-mm dish were infected with an m.o.i. of 10 PFU per cell and incubated 17 hr in methionine-free DMEM containing 1% fetal calf serum and 400 μCi of [35S]methionine and [35S]cysteine (Transla-
bel, >1000 Ci/mmol, ICN Pharmaceuticals). Cell lysates were prepared as described by Sambrook et al. (1989) except that the lysis buffer was 1% NP-40 phosphate-buffered saline. Radioactivity quantitation in dried polyacrylamide gels was done with the Ambis Radioanalytic Imaging System (San Diego, CA).

Immunofluorescence was done on infected ST cells at 10 h.p.i. For surface fluorescence, cells were fixed with 4% paraformaldehyde by the procedure of Kaariainen et al. (1983), or unfixed and treated by the procedure of Vennema et al. (1990). For internal fluorescence, cells were fixed for 10 min at 4 °C with absolute ethanol. Fluorescence isothiocyanate-conjugated rabbit anti-swine IgG and goat anti-rabbit IgG were obtained from Boehringer-Mannheim.

RESULTS

HP is made during virus replication

In earlier analyses of radiolabeled, oligo(dT)-selected, virus-specific RNA obtained from TGEV-infected cells, we identified a potential mRNA species of approximately 600 nt that, from the 3'-coterminal nested set pattern of coronavirus mRNAs, suggested a small gene mapping on the 3' side of the N gene was being expressed (Dennis and Brian, 1982). More recently, with knowledge of genome sequence which identified the gene at this position (Kapke and Brian, 1986), we have shown by Northern hybridization analyses with strand-specific oligodeoxynucleotide probes that a transcript of approximately 600 nt in length and having the appropriate structure to be translated into HP is made, and that it becomes the second-most abundant viral mRNA species in infected cells (Sethna et al., 1989). The nucleotides surrounding the HP initiator codon fit a consensus that is only moderately favorable for translation according to the rules of Kozak (1989).

To determine whether the hydrophobic protein is made during virus replication, two approaches were taken. In the first, transcripts of the cloned HP ORF made in vitro were translated, and attempts were made to immunoprecipitate the protein product with hyperimmune porcine anti-TGEV serum. Fig. 2, lanes 1 and 8, illustrate that the HP ORF was translated into a protein of approximately 9.1 kDa, when electrophoretically analyzed under reducing conditions, and lane 9 illustrates that HP was immunoprecipitated with TGEV hyperimmune serum. Nonimmune pig serum failed to precipitate the protein (Fig. 2, lane 10). These results indicate that HP is made during virus replication utilizing the moderately favorable sequence context for initiation of translation (i.e., a G at -4 and C at +3 relative to the initiation codon [Kozak, 1989]) and, furthermore, they show for the first time that HP induces a significant antibody response in the infected pig.

In the second approach, rabbit serum prepared against the 31 amino acid hydrophilic core of HP and capable of immunoprecipitating HP made in vitro (Fig. 2, lane 4) was used to determine if HP could be immunoprecipitated from TGEV-infected cells. Lane 7 in Fig. 2 illustrates that such a protein is specifically immunoprecipitated. This confirms that HP is made during virus replication and further establishes the authenticity of HP as a TGEV protein.

HP is membrane-associated

The deduced primary structure of HP shows it to be sufficiently hydrophobic at its termini that either end of
The molecule could theoretically function as a transmembrane domain (Fig. 1C). The N-terminal 19 amino acids, in addition, show many features of a transmembrane signal peptide with a potential signal peptidase cleavage site between amino acids 19 and 20 (Fig. 1B; von Heijne, 1986). To test for membrane association, the HP transcript was translated in the presence of pancreatic microsomes and its microsomal association was studied. Figure 3A, lane 3 illustrates that HP, apparently unchanged in its molecular weight, sediments with the microsomal pellet. A minor species with a molecular weight of around 40 kDa (identified as species x in Fig. 3A) also appeared after translation with microsomes and sedimented with the microsomal pellet. Although species x is a potential tetramer unaffected by reducing agent, its identity as an HP-specific protein could not be confirmed by immunoprecipitation with polypeptide-specific antiserum (data not shown). The mechanism by which monomeric HP becomes associated with microsomes was examined by three experimental approaches. To determine whether HP was bound to the external surface of microsomal membranes as a result of divalent cation bridging, microsomes used in the translation reaction were washed in a solution containing 25 mM EDTA before pelleting and electrophoretic analysis. After washing, there was no apparent decrease in the amount of bound HP when compared to unwashed microsomes, indicating that cationic bridging is not the mechanism for HP adherence (data not shown). To determine whether HP had undergone complete translocation into the microsomal lumen to become packaged as a secreted protein, microsomes used in the translation reaction were converted from closed vesicles to open membrane sheets by treatment with sodium carbonate buffer at pH 11, and the membranes were pelleted and analyzed for HP content. Nonanchored proteins would be released by this treatment and therefore not pelleted with the membrane fraction (Fujiki et al., 1982). Fig. 3A, lane 5 illustrates that after microsomal lysis, HP remained with the pelleted membranes indicating that it may be adhering to the membranes by virtue of its hydrophobic nature, or by anchorage through a fatty acid side chain, or perhaps by partial translocation and anchorage as an integral membrane protein. Experiments employing protease digestion to further resolve whether HP was completely external to the microsomal membranes or had undergone partial translocation were inconclusive because of an inherent resistance of free HP to protease digestion. Digestion of HP made in the absence of membranes for up to 2 hr at 20° with trypsin (100 μg/ml), chymotrypsin (100 μg/ml), or a combination of the two enzymes (each at 100 μg/ml), conditions which completely digested β-lactamase and yeast α-mating factor, was incomplete, and the HP remaining resistant to digestion was full size (data not shown). Conditions which did completely digest HP, such as higher enzyme concentrations, warmer incubation temperatures, longer incubation times, or the use of proteinase K (100 μg/ml) for 30 min at 20°, also destroyed microsomal integrity as determined by digestion of the internalized β-lactamase and yeast α-mating factor (data not shown).

To determine whether disulfide-linked multimeric forms of HP occur, products synthesized in vitro or in vivo were analyzed by gel electrophoresis in the absence of 2-mercaptoethanol. Interestingly, at times the product of in vitro synthesis was an apparent 19-kDa dimer of HP (Fig. 3B, lanes 2 and 6) that was reducible to monomer of 9.1 kDa with 2-mercaptoethanol (Fig. 3B, lane 1). Although dimeric HP was often the predominant product, it could not be predictably reproduced, possibly because of varying amounts of reducing agent already present in the wheat germ translation mix or in the radiolabeled [35S]methionine mix, or as a result of the varying amounts of HP produced. Attempts to stabilize dimeric forms by adding varying amounts of oxidized glutathione, or microsomes, were...
not successful. To examine the status of disulfide-linked multimeric forms made in vivo, radiolabeled proteins from infected cells were immunoprecipitated with antipolypeptide serum and electrophoretically analyzed in the absence of 2-mercaptoethanol (Fig. 3B, lane 4). Only monomeric HP was recovered suggesting that only monomeric forms of HP are made in vivo. Interestingly, only monomeric HP was recovered from an in vitro translation reaction in which dimeric forms were present (Fig. 3B, lanes 5 and 6), raising the possibility that the antipolypeptide serum may not recognize the dimeric form of HP. This could happen if the hydrophilic central core of HP were inaccessible to the antibody. Experiments using monospecific antiserum to the whole of the HP molecule will be needed to resolve whether disulfide linked forms are found in vivo.

To determine the intracellular location of HP, rabbit antipolypeptide serum was used in immunofluorescence studies on TGEV-infected ST cells. Whereas porcine anti-TGEV serum produced the expected generalized intracytoplasmic immunofluorescence on infected cells fixed with ethanol, because of the presence of antibodies to the full range of viral soluble and membrane-bound proteins, (Fig. 4B), rabbit antipolypeptide serum produced fluorescence that was concentrated on perinuclear membranes in the region of the rough endoplasmic reticulum (Fig. 4F). With either unfixed cells (data not shown) or cells fixed with paraformaldehyde, both the porcine anti-TGEV serum (Fig. 4D) and the rabbit antipolypeptide serum (Fig. 4H) produced fluorescence on the cell surface. HP therefore appears to be closely associated with the intracellular membranes at the sites where coronavirus have been shown to bud (Tooze et al., 1984) and on the surface of infected cells.

**DISCUSSION**

In this report we show that a 9.1-kDa protein encoded by the most 3' ORF on the TGEV genome is made during virus replication, and that it is found concentrated on the membranes of the endoplasmic reticulum and to a lesser extent on the infected cell surface. In preliminary experiments with TGEV that had been harvested at 18 h.p.i. when cytopathic effect was minimal and purified by isopycnic sedimentation on a sucrose gradient, we found no evidence by immunoblotting that proteins smaller than 14 kDa were present on the virion (unpublished data). We therefore conclude at this time that monomeric HP, despite its abundance on membranes at the site of virus assembly, is probably not a virion structural protein. Consistent with this notion is our failure to find any virus neutralizing activity with the rabbit antipolypeptide serum in cell culture (data not shown), but rigorous conclusions on this point must await tests with antiserum prepared against
the entire HP molecule. Whether higher molecular weight multimeric forms of HP are found on the virion or in the cell will require additional studies.

The manner by which HP becomes associated with intracellular membranes and the process by which it becomes distributed on the cell surface remain to be resolved. The association of HP with microsomes after synthesis in vitro indicates that it has inherent properties for membrane association, either as a feature of its own hydrophobicity, by anchorage through an acylated side chain, or by anchorage after membrane translocation. If HP is associated or anchored only on the cytoplasmic side of the endoplasmic reticulum, then one possibility for migration to the cell surface might be through its release from cytoplasmic compartments during cell lysis and its readherence to the surface of adjacent cells. If, on the other hand, HP becomes partially translocated and anchored in the endoplasmic reticulum, then it could by the exocytic pathway become part of the cytoplasmic membrane during fusion of the exocytic vesicles at the time of virus release (a process reviewed in Dubois-Dalcq et al., 1984).

The function of HP is unknown at this time, but its membrane association at the endoplasmic reticulum, the probable site of RNA replication complex formation (Dennis and Brian, 1982) and the demonstrated site of virion assembly (Tooze et al., 1984), suggests that it may play a role in one or both of these processes by interacting with viral RNA or other viral proteins. In preliminary experiments to determine whether HP is an RNA binding protein, the RNA-protein (Northwestern) blotting procedure described by Stohlman et al. (1988) was used. In our study, RNA from TGEV-infected cells was electrophoresed, blotted onto nitrocellulose, incubated with lysates of infected cells, and separately probed with polyclonal rabbit antiHP polypeptide or rabbit antiN serum. Although N binding to RNA was readily observed (as reported by Stohlman et al., 1988, for mouse hepatitis virus), no HP binding to RNA was found (data not shown). We therefore conclude at this time that HP is probably not an RNA binding protein, but, again, a rigorous conclusion cannot be drawn until antiserum against the entire HP molecule is tested. We have no data on the interaction of HP with other viral proteins.

Our results on the behavior of HP differ dramatically from those reported by Garwes et al. (1989) despite the fact that our experimental approaches were similar. In the studies by Garwes et al., HP was found only as small foci in the nuclei of infected cells when studied by immunofluorescence, suggesting that the protein had been transported to the nucleus and had a nuclear function. At no time did we observe this pattern with the Purdue strain of TGEV, nor with the FS772/70 strain used by Garwes et al., when studied with our reagents on ST cells. Two factors differ between our studies, however, that may explain the discrepancies.
First, 8 of the 78 HP amino acids differ between the two virus strains (Fig. 1B) and this may lead to a dramatic difference in the behavior of the two proteins. Most notable is a potential glycosylation site at Asn 30 in the FS772/70 strain of TGEV used by Garwes et al., that is not present in the Purdue strain. Since the behavior of HP from both virus strains appeared identical in ST cells, this seems to be an unlikely explanation. Second, the cells used by Garwes et al. were the porcine LLC-PK1 cells in which HP may behave quite differently. The structural bases for the strikingly different cellular distribution patterns for HP will be important to determine since they may be exploitable in the long-term goal of determining the function of HP in virus replication.

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