VIROLOGY 186, 676–683 (1992)

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

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Received September 16, 1991; accepted October 31, 1991

The open reading frame potentially encoding a 78 amino acid, 9101 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.

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 9101 Da and preceded nine nucleotides upstream by the CYAAAC 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), but a gene for a 101 amino acid homologous protein is found in a similar position near the 3′ end of the feline infectious peritonitis virus genome (De Groot *et al.*, 1988). For IBV, MHV, and BCV, however, other open reading frames for small (7 to 15 kDa) hydrophobic proteins are found, but they map farther upstream and on the 5′ side of the nucleocapsid or matrix protein genes (Abraham *et al.*, 1990; Boursnell and Brown, 1984; Kapke *et al.*, 1988; Skinner and Siddell, 1985; Skinner *et al.*, 1985). None of these for IBV, MHV, or BCV show sequence similarity to the TGEV 9101 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 *Pst*I fragment of clone FG5 which represents the 3′ end of the TGEV

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0042-6822/92 $3.00

genome (Kapke and Brian, 1986; Genbank accession number M14878) was digested with SfaNI that cuts at a position 6 bases upstream from the initiator codon of the HP gene, and the resulting 550 nt fragment was blunt-ended with mung bean nuclease, cloned into the *Sma*I site of pUC18 (Pharmacia) using *E. coli* JM83 as host, and named pHP. Although HP was cloned in-frame behind amino acid 12 of the lacZ gene, at no time did we observe synthesis of HP as a fusion protein in *E. coli* cells after induction with isopropyl-thiogalactoside in preliminary experiments. To construct a plasmid for the *in vitro* generation of HP transcripts, the insert from pHP was removed with *Bam*HI and *Eco*RI (which cut within the multiple cloning region of the vector), blunt-ended by 3′ fill-in with Klenow fragment, ligated to *Bam*HI linkers (5-d[pCGGATCCG]-3′), and cloned into the *Bam*HI site of 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′GGGAGACAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCGCGGATCCGCGGATCCGAATTCGAGCTCGGTACCCGAG3′ 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 pHP-1 was made by removing the HP-containing insert with *Kpn*I, blunt-ending it with mung bean nuclease, and ligating it into the *Sph*I site of pGEM-4-Z (Promega) that was likewise blunt-ended. The resulting clone, pHP-1A (Fig. 1A), had the 19 nt sequence 5′GGGAGACAAGCTTGCCGAG3′ between the T7 promoter and the HP initiator codon and was used in some of the *in vitro* translation studies as indicated. The pHP-1 and pHP-1A plasmids were linearized with *Sma*I and transcripts were prepared by the Promega protocol for generating 5′ capped RNA. Transcripts ($\approx$1 μg/50 μl reaction) were translated in the presence or absence of canine pancreatic microsomes (Promega) in wheat germ extract (Promega) that contained 1 mCi of [^{35}S]-cysteine (>800 Ci/mmol; ICN Pharmaceuticals) per ml, and no added potassium acetate above the endogenous level. Trypsin digestion for analysis of membrane translocation was done as described by Scheele (1983), and carbonate extraction at pH 11 for analysis of membrane anchorage was done as described by Fujiki *et al.* (1982), except that microsomes or membrane sheets were pelleted for 0.5 hr at 13,000 *g* in a microfuge at 4°. Microsomal washes in EDTA were done as described by Walter and Blobel (1983), except that the final EDTA concentration was 25 m*M*. Products of translation were analyzed by electrophoresis as previously described (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. ^{14}C-labeled proteins ovalbumin (MW 46,000), 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 (NH-RL-QLLERLLLDHSFNLKTVNDFNILYRSLAE-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-D-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 (Kemeny, 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 (10^8 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 [^{35}S]methionine and [^{35}S]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° 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 (Fig. 1A; 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

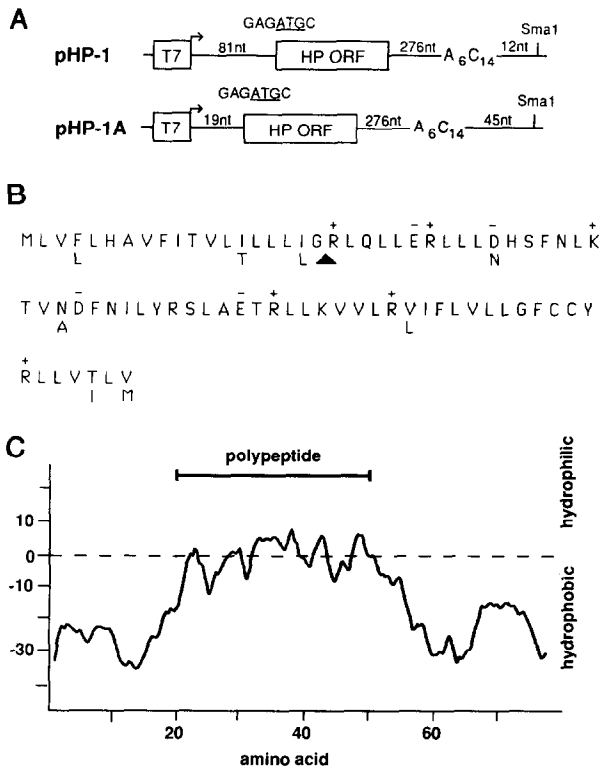

FIG. 1. Structural features of HP. (A) Plasmids used to generate transcripts for *in vitro* translation of HP. A_6 and C_{14} represent a portion of the TGEV 3′ poly(A) tail, and the poly(C) tract generated during the original cloning procedure (Kapke and Brian, 1986), respectively. The arrow represents the start site of transcription. (B) Deduced amino acid sequence of HP for the Purdue strain of TGEV. Amino acids that differ in the FS772/70 strain of TGEV (Garwes *et al.*, 1989) are shown below. Charged amino acids are indicated. A potential signal peptidase cleavage site is indicated by an arrowhead. (C) Hydrophobicity profile of HP as determined by the method of Kyte and Doolittle (1982). The region which represents the synthetic polypeptide is indicated.

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

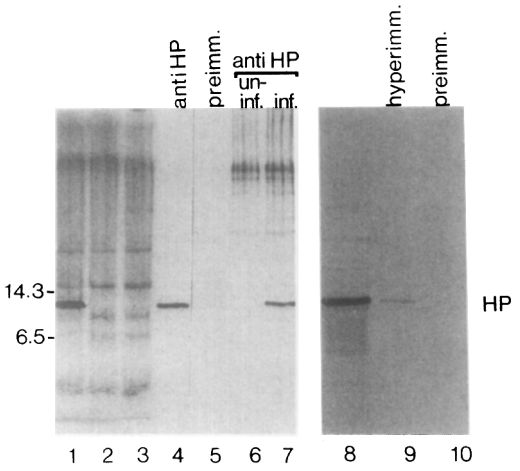

FIG. 2. Evidence that HP is made during virus replication. HP was analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions. Lanes 1 and 8, direct analysis of HP synthesized *in vitro* from transcripts of pHP-1. Lanes 2 and 3, direct analysis of products from the respective translation reactions in which vector transcripts or no RNA were added. Lanes 4 and 5, analysis of radiolabeled HP synthesized *in vitro* and immunoprecipitated with rabbit antipolypeptide serum or rabbit preimmune serum. Lanes 6 and 7, analysis of radiolabeled proteins from uninfected and TGEV-infected cells immunoprecipitated with rabbit antipolypeptide serum. Lanes 9 and 10, analysis of radiolabeled HP synthesized *in vitro* and immunoprecipitated with porcine hyperimmune antiTGEV serum or nonimmune (preimmune) serum.

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 m*M* 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 18-kDa dimer of HP (Fig. 3B, lanes 2 and 6) that was reducible to monomers 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 [^{35}S]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

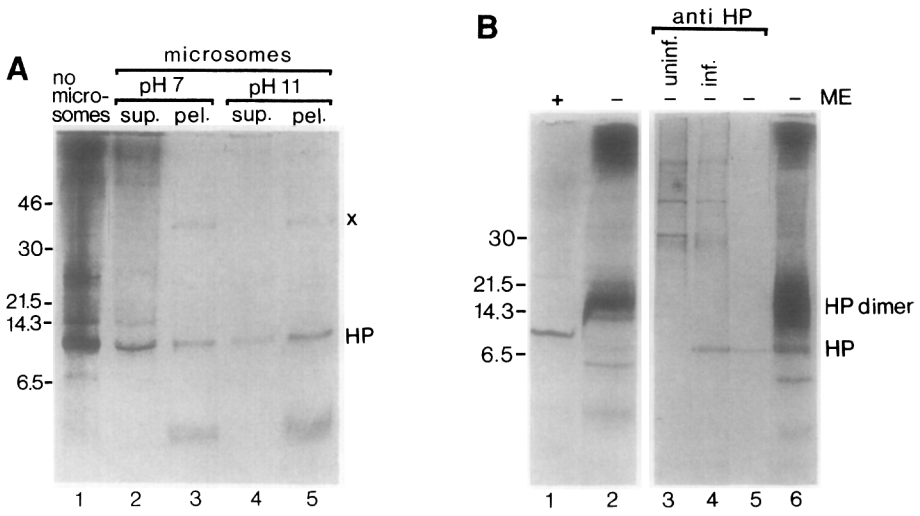

FIG. 3. Membrane association and multimeric forms of HP. (A) Association of HP with microsomal membranes. HP synthesized *in vitro* from transcripts of pHP-1 was analyzed by SDS–polyacrylamide gel electrophoresis under reducing conditions. Lane 1, direct analysis of HP synthesized in the absence of microsomes. Lanes 2 through 5, analysis of HP recovered from the supernatant or pellet of microsomes treated as indicated. For recovery from the supernatant, HP was precipitated with acetone. A protein species of unknown identity (x) is discussed in the text. (B) Disulfide-linked multimeric forms of HP. HP was analyzed by SDS–polyacrylamide gel electrophoresis. HP synthesized *in vitro* from transcripts of pHP-1A was analyzed in the presence (lane 1) or absence (lanes 2 and 6) of 2-mercaptoethanol. HP synthesized *in vitro* (lane 5) or in TGEV-infected cells (lane 4) was immunoprecipitated with rabbit antipolypeptide serum and electrophoresed in the absence of 2-mercaptoethanol. Radiolabeled proteins from uninfected cells (lane 3) were likewise treated and analyzed.

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 coronaviruses 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

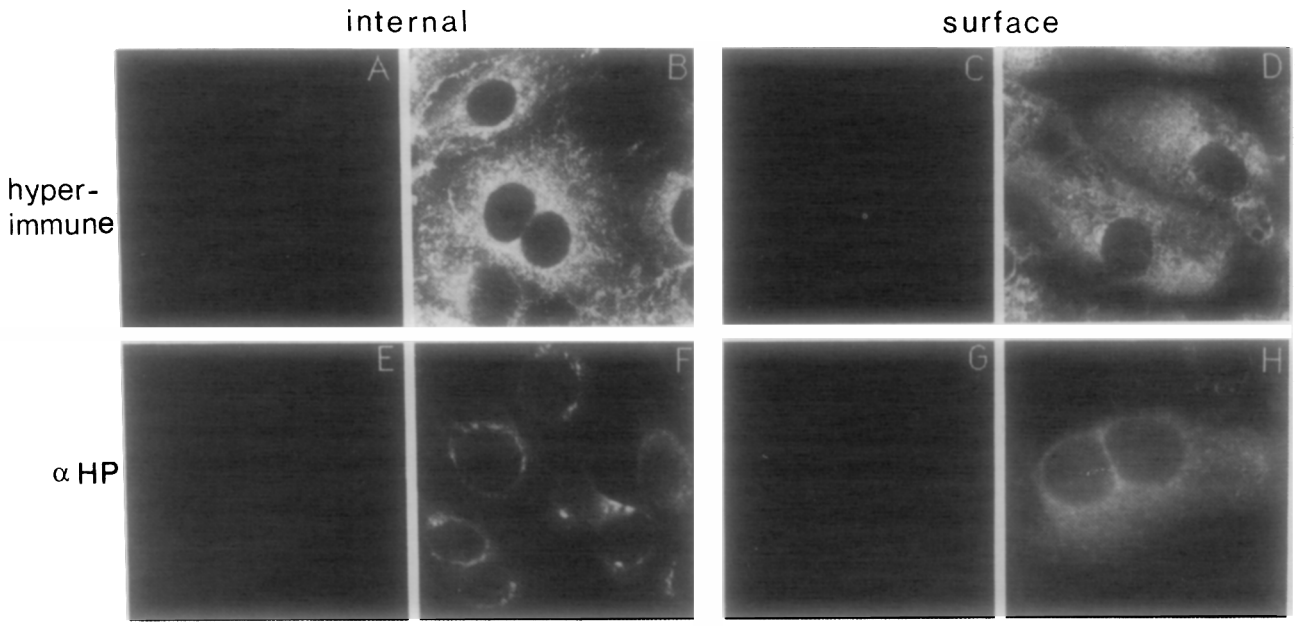

FIG. 4. Immunofluorescence analysis of HP. TGEV-infected ST cells were analyzed for internal (B) or surface (D) fluorescence with porcine hyperimmune antiTGEV serum, and for internal (F) or surface (H) fluorescence with rabbit antipolypeptide serum. (A, C, E, and G) Uninfected cells that underwent the corresponding treatments.

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 antiHPpolypeptide 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.

ACKNOWLEDGMENTS

This work was supported by Grants AI-14367 from the National Institute of Allergy and Infectious Diseases, and 82-CRSR-2-1090 from the U.S. Department of Agriculture, and in part by a grant from the Upjohn Company.

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