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Hepatitis C Virus RNA Polymerase and NS5A Complex with a SNARE-like Protein

Hong Tu,* Lu Gao,* Stephanie T. Shi,* Deborah R. Taylor,* Tao Yang,†‡ Austin K. Mircheff,†‡§ Yumei Wen,¶ Alexander E. Gorbalenya,‖ Soon B. Hwang,** and Michael M. C. Lai*,††,[1]

††*Howard Hughes Medical Institute,* **Department of Molecular Microbiology and Immunology,* †*Department of Physiology,* ‡*Department of Biophysics, and* §*Department of Ophthalmology, University of Southern California School of Medicine, Los Angeles, California 90033;* ¶*Institute of Molecular Virology, Shanghai Medical University, Shanghai 200032, P.R. China;* ‖*Advanced Biomedical Computer Center, National Cancer Institute, Frederick, Maryland 21702-1201; and* ***Institute of Environmental and Life Science, Hallym University, 1 Ockcheon-dong, Chuncheon, 200-702, Korea*

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Hepatitis C virus (HCV) NS5A is a phosphoprotein that possesses a cryptic *trans*-activation activity. To investigate its potential role in viral replication, we searched for the cellular proteins interacting with NS5A protein by yeast two-hybrid screening of a human hepatocyte cDNA library. We identified a newly discovered soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor-like protein termed human vesicle-associated membrane protein-associated protein of 33 kDa (hVAP-33). *In vitro* binding assay and *in vivo* coimmunoprecipitation studies confirmed the interaction between hVAP-33 and NS5A. Interestingly, hVAP-33 was also shown to interact with NS5B, the viral RNA-dependent RNA polymerase. NS5A and NS5B bind to different domains of hVAP-33: NS5A binds to the C-terminus, whereas NS5B binds to the N-terminus of hVAP-33. Immunofluorescent staining showed a significant colocalization of hVAP-33 with both NS5A and NS5B proteins. hVAP-33 contains a coiled-coil domain followed by a membrane-spanning domain at its C-terminus. Cell fractionation analysis revealed that hVAP-33 is predominantly associated with the ER, the Golgi complex, and the prelysosomal membrane, consistent with its potential role in intracellular membrane trafficking. These interactions provide a mechanism for membrane association of the HCV RNA replication complex and further suggest that NS5A is a part of the viral RNA replication complex.

INTRODUCTION

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is a positive-sense, single-stranded RNA virus, which has been shown to be the major causative agent of non-A, non-B hepatitis (Kuo *et al.,* 1989; Aach *et al.,* 1991; Choo *et al.,* 1991). The 9.5-kb viral genome encodes a single polyprotein of about 3010 amino acids, which is proteolytically processed by a combination of host- and virus-encoded proteases into at least 10 distinct products in the order of NH_2-C-E1-E2-p7-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Grakoui *et al.,* 1993; Lohmann *et al.,* 1996). Among the nonstructural proteins (NS2–5B), NS3 is a serine protease and also exhibits NTPase (Suzich *et al.,* 1993) and RNA helicase activities (Tai *et al.,* 1996), suggesting its potential role in viral RNA synthesis. NS5B contains a "GDD" sequence motif, which is highly conserved among all RNA-dependent RNA polymerases (RdRps) (Poch *et al.,* 1989). RdRp activities of recombinant NS5B proteins purified from transfected insect cells (Behrens *et al.,* 1996) and a bacterial expression system (Yamashita *et al.,* 1998) have been reported. Based on their enzymatic activities, NS3 and NS5B are presumed to be involved in viral RNA replication. The function of NS5A is not yet clear; it is a phosphoprotein (Tanji *et al.,* 1995) and contains a putative nuclear localization signal that can target a heterologous reporter gene product to the nucleus, although the native NS5A is retained in the cytoplasm (Ide *et al.,* 1996). The N-terminus-truncated, but not the full-length, NS5A is a potent transcriptional activator (Kato *et al.,* 1997). Whether this *trans*-activation activity is related to HCV RNA synthesis is not clear. NS5A contains an interferon-sensitivity-determining region (ISDR) in the central part of the protein (Enomoto *et al.,* 1996; Toshiko *et al.,* 1998). It can interact with the interferon-inducible double-stranded-RNA-dependent protein kinase (PKR) and function as a repressor of PKR (Gale *et al.,* 1997). This is believed to be one of the mechanisms of HCV-mediated interferon (IFN) resistance. Most of the viral nonstructural proteins have been shown to be associated with the cellular membrane structures (Hijikata *et al.,* 1993), probably forming a complex to perform critical functions during the viral replication cycle. A number of these proteins, i.e.,

[1] To whom correspondence and reprint requests should be addressed at Howard Hughes Medical Institute, Department of Molecular Microbiology and Immunology, University of Southern California School of Medicine, 2011 Zonal Avenue, HMR-401, Los Angeles, CA 90033-1054.

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p7, NS4A, NS4B, and NS5B, contain conserved hydrophobic stretches that may anchor this complex to membranes. The membrane retention ability of these hydrophobic sequences has been confirmed only for NS5B (Yamashita *et al.,* 1998), and the mechanism for membrane association of the replication complex is still not clear.

RNA replication of most RNA viruses involves certain intracellular membrane structures, including the ER (Restrepo-Hartwig and Ahlquist, 1996; Schaad *et al.,* 1997; van der Meer *et al.,* 1998), Golgi (Shi *et al.,* 1999), endosomes and lysosomes (Froshauer *et al.,* 1988), or a membrane structure derived from several membrane compartments (Bienz *et al.,* 1992). RNA synthesis of other flaviviruses, such as Dengue virus (Cauchi *et al.,* 1991) and West Nile virus (Wengler *et al.,* 1990), has also been shown to occur principally on the membrane of the ER. Similarly, some of the presumed HCV replicase components, NS3 and NS5B, sediment with membrane fractions isolated from transfected cells (Hijikata *et al.,* 1993; Hwang *et al.,* 1997). Thus, it appears that membrane association of the viral replication complex is essential for viral RNA replication. However, the mechanism by which the viral replication complex is associated with selected membrane components is largely unknown.

The current model for vesicle docking and fusion at all locations, including synapses, involves SNARE proteins (Sollner *et al.,* 1993; Goda, 1997; Woodman, 1997). This universal vesicle transport machinery consists of an *N*-ethylmaleimide-sensitive fusion protein (NSF), soluble NSF attachment proteins (SNAPs), and two types of SNAP receptors (SNAREs), namely v-SNAREs and t-SNAREs. Vesicle docking is achieved by the interaction between v-SNAREs on the vesicle membrane and the t-SNAREs on the target membrane. Vesicle-associated membrane protein (VAMP), which is more often known as synaptobrevin, is one of the v-SNAREs (Trimble *et al.,* 1988; Baumert *et al.,* 1989; Schiavo *et al.,* 1995). The SNARE proteins have been found in many species and are distributed in a broad range of tissues and membrane compartments of the cell (Aalto *et al.,* 1993; Banfield *et al.,* 1994; Becherer *et al.,* 1996; Nichols *et al.,* 1997; Advani *et al.,* 1998), suggesting that they may play a fundamental role in vesicle biogenesis, protein sorting, and membrane fusion.

In this study, we screened an interferon-induced human hepatocyte cDNA library using HCV NS5A as bait and isolated a SNARE protein named human VAMP-associated protein of 33 kDa (hVAP-33) (Weir *et al.,* 1998). hVAP-33 can also bind to the NS5B protein, suggesting that hVAP-33 may be responsible for the association of the HCV replication complexes with the membrane.

RESULTS

Identification of a human cellular protein interacting with HCV NS5A protein

Since the role of NS5A in the HCV life cycle is not known, we sought to understand its potential function by studying its cellular partners. Therefore, we performed a yeast two-hybrid screening using the full-length NS5A of HCV genotype 1a as bait to identify cellular proteins that can potentially interact with HCV NS5A protein. We screened a cDNA library derived from HepG2 cells that had been treated with interferon, since previous studies showed that NS5A may potentially confer interferon resistance (Enomoto *et al.,* 1996; Gale *et al.,* 1997; Toshiko *et al.,* 1998). Of 2.8×10^6 transformants screened, seven positive clones, which contain cDNAs encoding NS5A-interacting proteins, were obtained. DNA sequence analysis revealed that two of the seven positive clones, 42 and 74, contained identical sequences within the open reading frames, although they differed in the length of their noncoding sequences. The other five cDNAs isolated during this screening will be described elsewhere.

A BLAST search against the GenBank database showed that this NS5A-binding protein has a sequence identical to that of hVAP-33, which is the human homolog of the *Aplysia californica* VAP-33 (Weir *et al.,* 1998). VAP-33 has been shown to be one of the SNAREs required for *A. californica* neurotransmitter release (Skehel *et al.,* 1995). The hVAP-33 protein contains 242 amino acids (aa) and shares 49% aa identity with *A. californica* VAP-33. Based on the predictions by computer analysis, the N-terminal 130 aa residues of the protein form a conserved domain that belongs to the IgG-like superfamily (Bullock *et al.,* 1996) (Fig. 1a). The region from aa 162 to 198 is predicted to be a leucine zipper able to form a parallel double-stranded coiled-coil structure, which is considered to be important for protein–protein interactions among the SNAREs (Canaves and Montal, 1998). It is noteworthy that another NS5A-binding protein identified during the same screening also harbors a leucine zipper motif within the binding domain (Tu and Lai, unpublished observations). The region from aa 221 to 239 has a highly hydrophobic sequence and is predicted to span the cellular membrane (Fig. 1a). The homology between the N-terminal regions (65%) of the *A. californica* VAP-33 and hVAP-33 is much higher than that between their C-terminal regions (35%). The hydrophobicity analysis (Fig. 1b) revealed that the hydrophobic and hydrophilic regions are conserved between the two species. hVAP-33 has a predicted molecular mass of 27.3 kDa. The apparent molecular mass was determined to be approximately 30 kDa when translated *in vitro* (data not shown) and by Western blotting of the endogenous hVAP-33 in HepG2 cells with a rat polyclonal antibody made against a recombinant hVAP-33 from *Escherichia coli.*

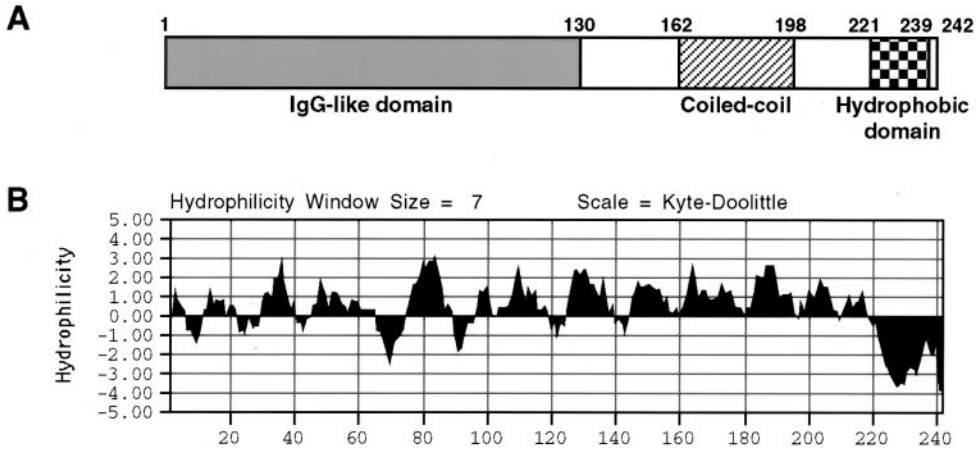

FIG. 1. Characteristics of hVAP-33 protein. (A) Structural domains of the hVAP-33 protein based on computer analysis. N-terminal 130 aa residues of the protein form a conserved domain that belongs to the IgG-like superfamily. The region of aa 162–198 is predicted to form a coiled-coil structure. The region of aa 221–239 has a highly hydrophobic sequence to form a transmembrane domain. (B) Hydrophilicity plots of hVAP-33 obtained by the MacVector program.

hVAP-33 interacts with both NS5A and NS5B *in vitro*

To confirm that hVAP-33 can interact with HCV NS5A directly *in vitro,* we first used a glutathione *S*-transferase (GST) pull-down assay. *E. coli*-expressed GST–hVAP-33 fusion protein was purified with glutathione–Sepharose 4B beads. *In vitro* translated ^{35}S-labeled NS5A was incubated with GST–hVAP-33 fusion protein to assay for binding. The result showed that NS5A was able to bind GST–hVAP-33 (Fig. 2A, lane 3) under the *in vitro* binding conditions. We also tested other HCV proteins, including core and NS4B. None of these proteins bound to GST–hVAP-33 (data not shown). Surprisingly, however, NS5B interacted with GST–hVAP-33 with at least the same binding intensity as did NS5A (Fig. 2A, lane 4). In contrast, neither NS5A nor NS5B bound GST protein under the same conditions (lane 6). These data indicate that NS5A and NS5B specifically interact with hVAP-33. Furthermore, the binding of NS5B did not interfere with the binding of NS5A or vice versa (lane 5). These results suggest that NS5A and NS5B may interact with two different domains of hVAP-33.

To map the HCV NS5A- and NS5B-interacting domains of hVAP-33, three truncated constructs of hVAP-33 (Fig. 2B), expressed as GST-fusion proteins, were incubated with *in vitro* translated ^{35}S-labeled NS5A or NS5B. The results indicate that NS5A bound primarily to the C-terminal region (aa 156–242), which contains the coiled-coil sequence (Fig. 2B, lane 5). The N-terminal 75 or 155 aa of hVAP-33 bound NS5A only very weakly (lanes 3 and 4). In contrast, NS5B primarily bound the N-terminal 75 or 155 aa of hVAP-33 (lanes 9 and 10). The C-terminal fragment (aa 156–242) bound NS5B only very weakly (lane 11). These results indicate that the C-terminal one-third of hVAP-33 contains the critical residues for binding NS5A, whereas its N-terminal 75 aa are essential for NS5B interaction. These data established that NS5A and NS5B bound to different regions of hVAP-33, suggesting that both NS5A and NS5B could simultaneously and independently interact with hVAP-33.

hVAP-33 interacts with NS5A and NS5B *in vivo*

To demonstrate the potential interaction of hVAP-33 with both NS5A and NS5B in the cells, we performed a coimmunoprecipitation experiment from the cells expressing both NS5A and NS5B. For this purpose, Flag-tagged hVAP-33 was coexpressed with NS5A or NS5B in COS7 cells using T7 expression plasmids coupled with recombinant vaccinia virus (vTF7-3) infection expressing T7 polymerase. Cell lysates were immunoprecipitated with anti-Flag antibody-crosslinked Sepharose 4B beads. The immunoprecipitates were analyzed by SDS–PAGE followed by immunoblotting with a rabbit polyclonal anti-NS5A and HCV patient serum to detect NS5A and NS5B, respectively. The results showed that NS5A was co-immunoprecipitated with hVAP-33 (Fig. 3, lane 3), whereas NS5A alone could not be precipitated by the anti-Flag antibody (lane 2). Similarly, NS5B was specifically coprecipitated with hVAP-33 (lanes 4 to 6). Furthermore, when both NS5A and NS5B were coexpressed with N-Flag hVAP-33, they were coprecipitated almost equally efficiently (lanes 7 to 9). These results indicate that NS5A and NS5B bound specifically to hVAP-33 in the cells and that these two proteins bound independently to hVAP-33 at equimolar ratio.

Colocalization of NS5A and NS5B with the endogenous hVAP-33

The results shown above indicate that NS5A and NS5B can interact with the overexpressed hVAP-33 *in vitro* and *in vivo.* To determine the biological significance of these interactions, we further studied whether these

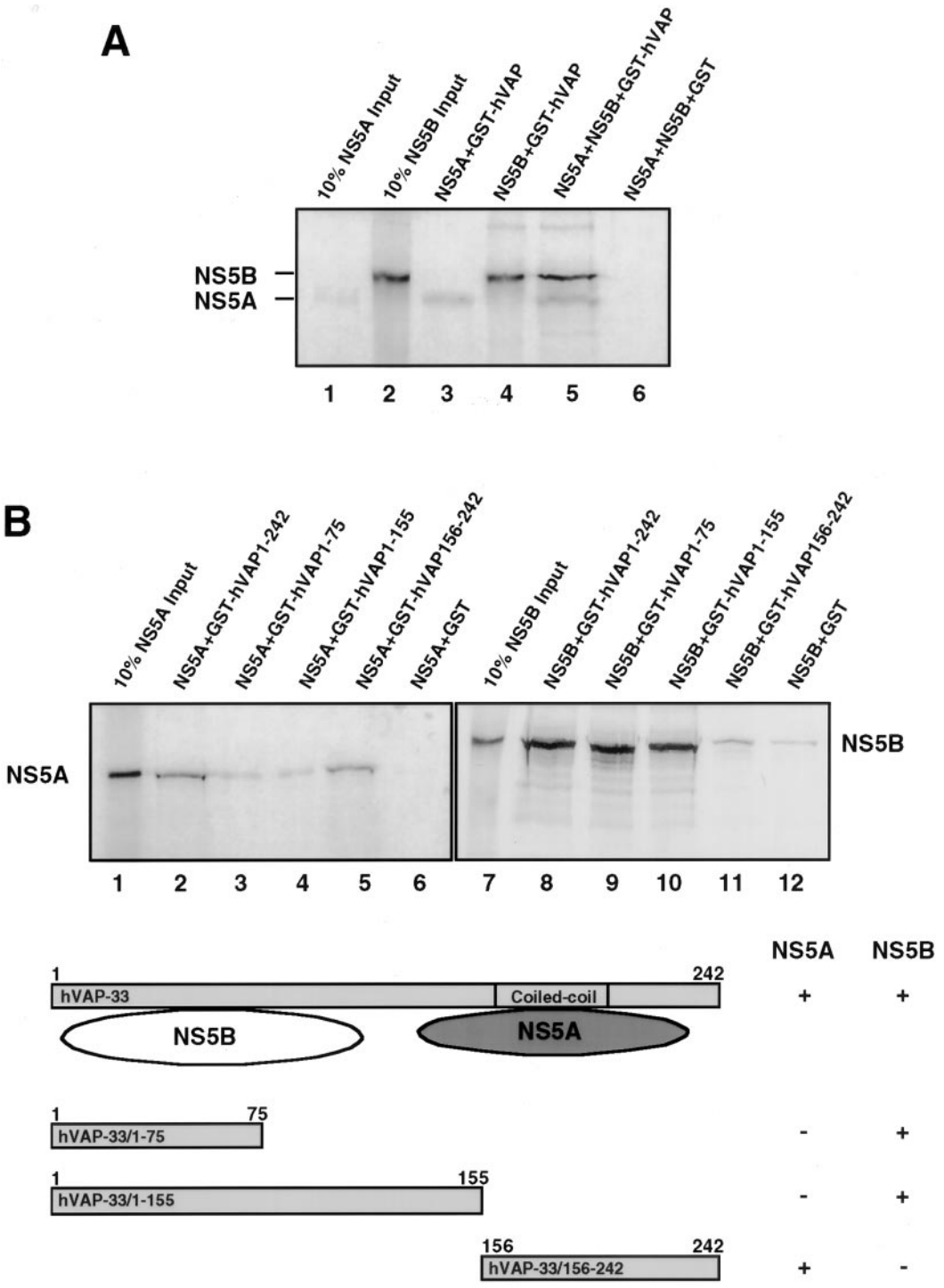

FIG. 2. *In vitro* interaction of hVAP-33 with NS5A and NS5B. (A) GST-hVAP-33 was incubated with *in vitro* translated ^{35}S-labeled NS5A and NS5B and precipitated by glutathione beads. GST protein alone was used as a negative control (lane 6). Lanes 1 and 2 represent 10% of input proteins used in lanes 3 to 6. (B) Mapping the NS5A- and NS5B-binding domains on hVAP-33. Various truncated hVAP-33 constructs were expressed as GST-fusion proteins and incubated with *in vitro* translated ^{35}S-labeled NS5A and NS5B. The binding sites on hVAP-33 for NS5A and NS5B are indicated by a schematic drawing.

viral proteins colocalized with the endogenous hVAP-33. We first investigated the subcellular localization of the endogenous hVAP-33 by indirect immunofluorescent analysis in COS7 cells. The endogenous hVAP-33 was detected by rat polyclonal antibody generated against the bacteria-expressed hVAP-33. Immunofluorescent staining of the hVAP-33 protein showed that it was localized exclusively in the cytoplasm (Fig. 4). Double staining

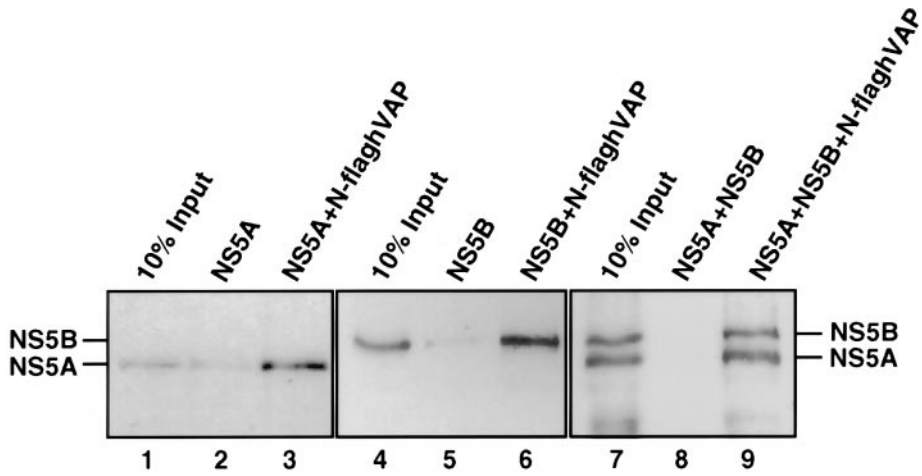

FIG. 3. *In vivo* coimmunoprecipitation of hVAP-33 with NS5A and NS5B. Flag-tagged hVAP-33 was cotransfected with NS5A or NS5B or both into COS7 cells. Cell lysates were immunoprecipitated with anti-Flag-crosslinked Sepharose 4B beads. Rabbit anti-NS5A and HCV patient serum were used to detect NS5A and NS5B, respectively. COS7 cells transfected with NS5A or NS5B alone (lanes 2, 5, and 8) were used as the negative controls. Lanes 1, 4, and 7 represent 10% of lysates prior to immunoprecipitation.

of hVAP-33 protein and a marker for the ER (GRP78) revealed partial colocalization of these proteins (data not shown), suggesting that at least some of the hVAP-33 protein is associated with the ER. There was no detectable staining when preimmune rat serum was used (data not shown). To compare the subcellular localization of hVAP-33 with NS5A and NS5B, we transfected COS7 cells with either pcDNA3/NS5A or pcDNA3.1/Flag-NS5B. Immunofluorescent staining was performed using rabbit polyclonal anti-NS5A and an anti-Flag antibody to detect NS5A and NS5B, respectively. Confocal microscopy showed a significant colocalization of the endogenous hVAP-33 with NS5A and NS5B (Fig. 4, top two rows). Furthermore, using a COS7 cell line stably expressing NS5A, we also found a similar colocalization of NS5A with hVAP-33 (data not shown). Finally, when cells were cotransfected with plasmids expressing both NS5A and NS5B, the two proteins also colocalized (Fig. 4, bottom row). These results indicate that NS5A and NS5B colocalized with the endogenous hVAP-33.

Subcellular localization of hVAP-33 by cell fractionation

The colocalization of NS5A and NS5B with hVAP-33 suggests that the HCV replication complex is associated with the membrane where hVAP-33 is localized. Therefore, it is of interest to fine-map the membrane localization of hVAP-33. For this purpose, we fractionated cellular membranes by a two-dimensional fractionation procedure with sorbital density gradient sedimentation on the first dimension, followed by phase partitioning at pH 7.0–6.6 shift on the second dimension (Gierow *et al.,* 1996; Mircheff, 1996; Hamm-Alvarez *et al.,* 1997). For technical reasons, we initially used rabbit lacrimal gland acinar cells, which have active secretory activities, for the subcellular localization of hVAP-33. The separation of cellular membranes was monitored by enzyme markers associated with the different membranes, while VAP-33 was detected by Western blotting with the rat polyclonal antibody. This procedure produced clear separation of most subcellular organelles, except that the ER and the Golgi overlapped considerably at pH 7.0–6.6 shift (Fig. 5, bottom). We also performed the phase partitioning at pH 7.6, which better separated the ER from the Golgi apparatus; however, the separation of other organelles was not as good as that at pH 7.0–6.6 shift (data not shown). A combination of the results generated under two different pH conditions allowed us to conclude that VAP-33 is present predominantly in the ER and Golgi apparatus, with some in the *trans*-Golgi network and prelysosomes (Fig. 5). Very little was detected on the plasma membrane, the lysosomes, or the endosomes (Fig. 5). Preliminary analysis also showed that hVAP-33 and NS5A have a similar subcellular distribution pattern in HepG2 cells (data not shown).

DISCUSSION

Considerable evidence suggests that most of the HCV nonstructural proteins, including NS3 and NS5B, which are presumably involved in viral RNA synthesis, are associated with host cell membranes (Hijikata *et al.,* 1993). Thus, HCV replication most likely occurs in close association with the cellular membrane (Hijikata *et al.,* 1993), which is a characteristic feature shared by most positive-sense RNA viruses (Grun and Brinton, 1988; Wengler *et al.,* 1990; Cauchi *et al.,* 1991; Bienz *et al.,* 1992; Schaad *et al.,* 1997; van der Meer *et al.,* 1998; Shi *et al.,* 1999). However, the RNA replication complexes of different viruses appear to be associated with different membrane compartments; the mechanism underlying the direction

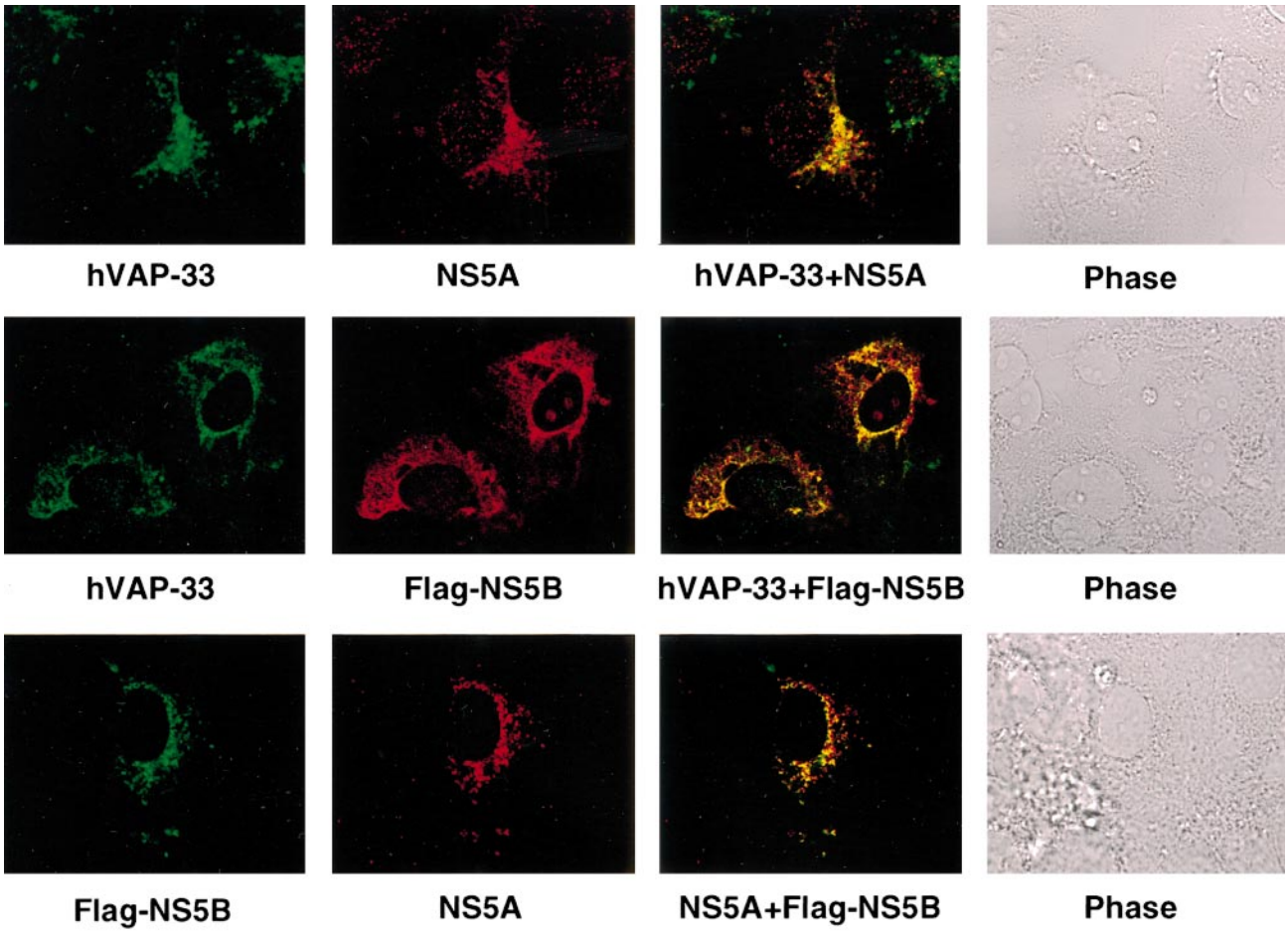

FIG. 4. Colocalization of NS5A, NS5B, and endogenous hVAP-33. NS5A or Flag-tagged NS5B or both were transfected into COS7 cells. Immunofluorescent staining was performed using a rat polyclonal antibody against hVAP-33, a rabbit polyclonal antibody against NS5A, and the anti-Flag antibody to detect hVAP-33, NS5A, and NS5B, respectively.

of viral replication complexes to different cellular membranes is largely unknown. In the case of a plant RNA virus, tobacco etch virus, it has been shown that a virus-encoded protein containing hydrophobic domains targets the replication complex to the membrane derived from the ER, forming a membrane-bound scaffold for the viral replication–transcription complexes (Schaad *et al.,* 1997). In this study, we report the isolation of a SNARE-like membrane protein, hVAP-33, by yeast two-hybrid screening in human hepatocytes using NS5A as bait. Our data showed that, in addition to NS5A, NS5B (RNA polymerase) is complexed with hVAP-33 both *in vitro* and *in vivo,* suggesting that hVAP-33 may serve as a membrane receptor for HCV replication complexes.

The C-terminal 21 aa residues of NS5B, including an anchoring domain and a C-terminal tail, has been shown to play a role in anchoring the protein to the membrane (Yamashita *et al.,* 1998). However, it is not known whether it targets the protein to any specific membrane site. Our findings here provide a mechanism for the association of the RNA replication complex with specific cellular membranes, that is, through binding to a specific cellular membrane protein, hVAP-33. However, this conclusion does not rule out the possibility that the viral proteins may also directly interact with membrane or other cellular membrane proteins. In fact, it is likely that HCV proteins engage in multiple protein–protein interactions. hVAP-33 is predicted to be a transmembrane protein with a membrane-spanning domain very close to the C-terminus of the protein. Although hVAP-33 has not been extensively characterized, it is likely that it has a membrane orientation similar to that of the SNARE proteins, inasmuch as the hVAP-33 preserves all of the hydrophobicity characteristics of the *A. californica* VAP-33, which has been shown to have the vesicle transport function of the SNARE proteins (Skehel *et al.,* 1995). Thus, the majority of the hVAP-33 protein is likely present on the cytoplasmic side of the intracellular membrane compartments. This extensive cytoplasmic "arm" of hVAP-33 provides an ideal membrane-docking site for the NS5A and NS5B proteins (Fig. 2B). Additional viral or cellular proteins may also be recruited by interacting with one or more of these proteins to form a functional HCV replication complex.

The function of NS5A is so far unknown. Our finding

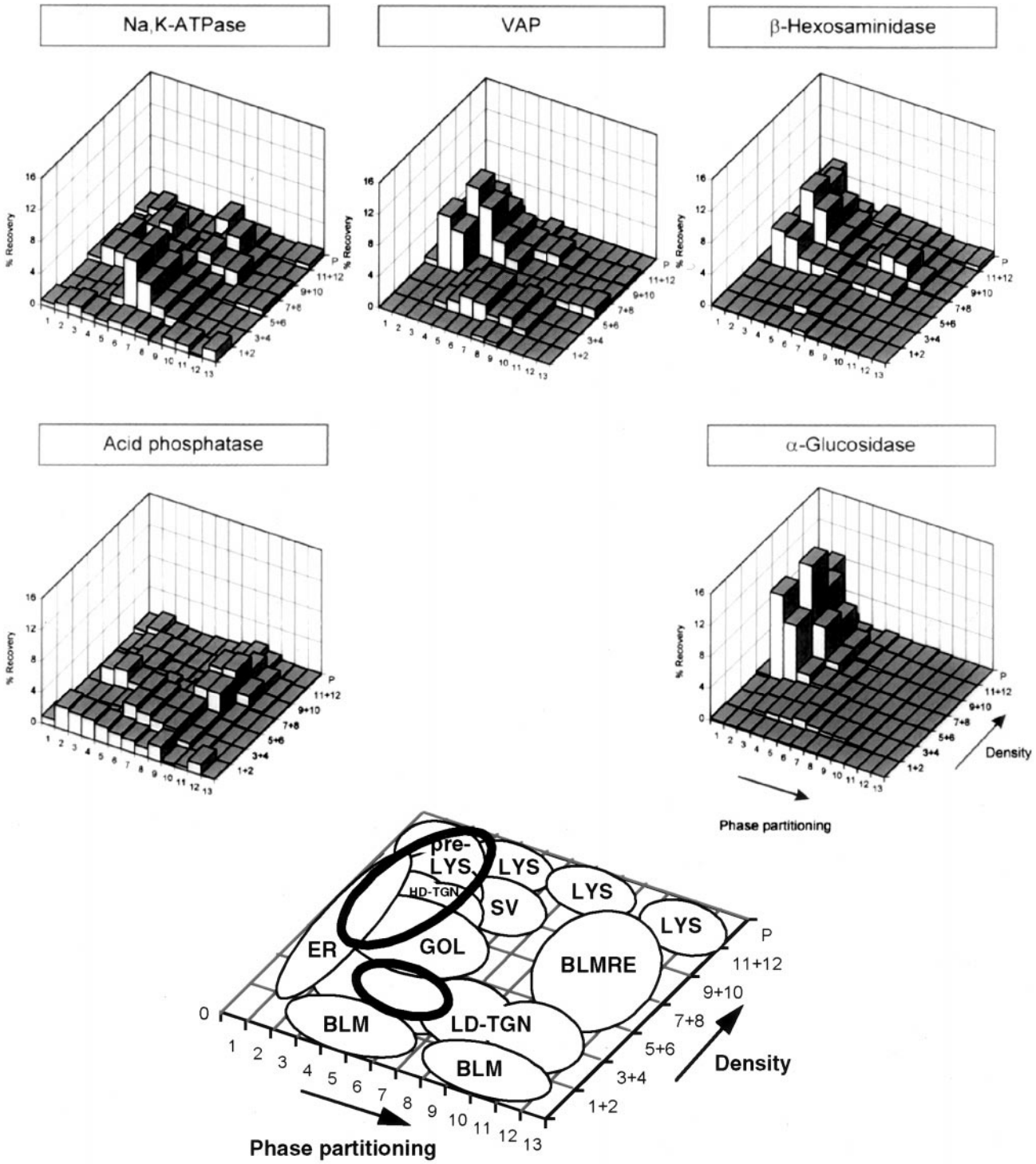

FIG. 5. Two-dimensional fractionation analysis of VAP-33. The detailed method was described previously (Gierow *et al.,* 1996; Mircheff, 1996; Hamm-Alvarez *et al.,* 1997). Female rabbit lacrimal gland acinar cell lysates were first separated into 13 fractions on a 26.5–80% sorbitol gradient. Fractions were pooled (1–2, 3–6, 7–8, 9–10, 11–12, 13) and then subjected to phase partitioning at pH 7.0–6.6 shift. Biochemical analysis of enzyme markers in each fraction was performed as described previously (Mircheff, 1989; Gierow *et al.,* 1996; Hamm-Alvarez *et al.,* 1997). VAP-33 was detected by Western blotting using a rat polyclonal antibody. ER, endoplasmic reticulum; GOL, Golgi; BLM, basal-lateral membrane; BLMRE, basal-lateral membrane-related endosome; LYS, lysosome; pre-LYS, prelysosome; HD-TGN, high-density *trans*-Golgi network; LD-TGN, low-density *trans*-Golgi network; SV, secretory vesicle; P, pellete. The circles outlined by thick lines indicate the approximate fractions that contain the VAP-33 protein.

that NS5A, together with NS5B, is associated with hVAP-33 suggests that NS5A is also involved in HCV RNA replication by direct or indirect interaction with NS5B. Interestingly, NS5A and NS5B bind to separate domains of hVAP-33; thus NS5A can be associated with the membrane independently of NS5B. NS5A is a phos-

phoprotein (Tanji *et al.,* 1995) and has a *trans*-activation activity (Kato *et al.,* 1997); these properties may enable NS5A to participate in viral RNA synthesis. This putative role of NS5A will need to be confirmed in an HCV RNA replication system in mammalian cells. Unfortunately, such a system is currently unavailable.

Our finding thus suggests that the HCV RNA replication complex is likely associated with cellular membranes, similar to many other viruses. RNA synthesis of many RNA viruses has been reported to involve the ER, Golgi, endosomes, or lysosomes. For example, the replication complexes of equine arteritis virus (van der Meer *et al.,* 1998), brome mosaic virus (Restrepo-Hartwig and Ahlquist, 1996), and tobacco etch virus (Schaad *et al.,* 1997) are associated with the ER. Viral RNA synthesis of murine hepatitis virus has been detected on the Golgi apparatus in human cells (Shi *et al.,* 1999). Alphavirus RNA replicase has been located to the cytoplasmic surface of endosomes and lysosomes (Froshauer *et al.,* 1988). More remarkably, the replication of poliovirus RNA has been shown to occur on a unique membrane compartment derived from membranes of the ER, Golgi, and other cellular membranes (Bienz *et al.,* 1992). Among flaviviruses, the functional replication complexes of various viruses have been obtained from membrane fractions (Grun and Brinton, 1988), and flaviviral RNA synthesis appears to occur principally on the membrane of the ER (Wengler *et al.,* 1990; Cauchi *et al.,* 1991). Furthermore, HCV proteins, including the presumed replicase components NS3 and NS5B, sediment with membrane fractions isolated from transfected cells (Hijikata *et al.,* 1993). Whether NS3 is associated with the membrane through hVAP-33 or indirectly through binding to NS5B will be an interesting question.

hVAP-33 is a membrane protein ubiquitously expressed in mammalian cells (Weir *et al.,* 1998), which shares 49% identity with the *A. californica* VAP-33 at the amino acid level. The *A. californica* VAP-33 is a plasma membrane protein of neuron cells (Schiavo *et al.,* 1995). By direct interaction with VAMP, which is an integral protein present in the membrane of vesicles, VAP-33 functions in the exocytosis of synaptic vesicles. hVAP-33 and the *A. californica* VAP-33 share a typical SNARE structure, i.e., a coiled-coil structure followed by a membrane-spanning domain at their C-terminal regions. However, VAP-33 mRNA was detected exclusively in the central nervous system of *A. californica* while hVAP-33 mRNA showed a wide range of tissue distribution, based on our Northern blotting results (data not shown) and the previous observation by Weir *et al.* (1998). The clear difference in the expression patterns of VAP-33 of different species may be related to the obvious difference in the complexity between human and *Aplysia.* Alternatively, if this protein family is multigenic, these two VAP-33 proteins may not be orthologous proteins playing an identical role in the two organisms. hVAP-33 has all of the attributes of a characteristic SNARE protein, although its precise functions have not been reported. Thus, it is likely that hVAP-33 may participate in diverse vesicle trafficking between membrane compartments of the cell, including endocytotic, exocytotic, ER–Golgi, and intra-Golgi transport pathways. Our finding that hVAP-33 is predominantly in the ER and the Golgi apparatus suggests that it may be involved in vesicle transport between the ER and the Golgi. hVAP-33 is also present in small amounts on the *trans*-Golgi network and prelysosomes. It may be speculated that the interaction of NS5A and NS5B with hVAP-33 may affect the vesicle transport functions of the host cells.

MATERIALS AND METHODS

Plasmid construction

To construct the plasmids used in the yeast two-hybrid screening, vector pGBT9, which encodes the GAL4 DNA-binding domain (GAL4 BD), and vector pGAD10, which encodes the GAL4 activation domain (GAL4 AD), were employed (Clontech, Palo Alto, CA). The HCV NS5A (genotype 1a) was amplified by PCR from a full-length HCV cDNA (Choo *et al.,* 1991) and cloned into GBT9 at *Eco*RI and *Sal*I sites to generate the pGBT9/NS5A construct, which can express the GAL4 BD–NS5A fusion protein in yeast. The cDNA library was custom-made by Clontech using mRNAs from HepG2 cells that had been treated with 1000 u/ml of interferon α (Sigma, St. Louis, MO) at 37°C for 20 h. The cDNA was cloned into the *Eco*RI site of pGAD10 vector to generate GAL4 AD–cDNA fusion proteins.

The plasmid used for expressing GST–hVAP-33 fusion protein in *E. coli* was constructed by inserting the hVAP-33 cDNA fragment into the *Bam*HI and *Eco*RI sites of the pGEX-4T-1 vector (Novagen, Madison, WI). The plasmid used to express HCV NS5A protein in COS7 cells was constructed by inserting the PCR-generated NS5A cDNA fragment into *Kpn*I and *Xba*II sites of pcDNA3 vector (Invitrogen, Carlsbad, CA). Similarly, NS5B sequence derived from the same HCV cDNA as that used for NS5A was cloned into the *Nhe*I and *Bam*HI sites of pcDNA3.1 (Invitrogen). These constructs, which contain a T7 promoter as well as a cytomegalovirus immediate early promoter, were also used for the *in vitro* translation of NS5A and NS5B proteins.

To construct the mammalian expression vector for hVAP-33 tagged with a Flag epitope, the PCR-generated hVAP-33 fragment containing Flag sequence (GAT TAC AAG GAT GAC GAC GAT AAG) at its 5′-end was first cloned into a TA cloning vector (Invitrogen) and then subcloned into the *Bam*HI and *Eco*RI sites of pcDNA3.1.

Yeast two-hybrid screening

Yeast two-hybrid screening was performed with *Saccharomyces cerevisiae* Y190 according to the manufac-

turer's instructions (Clontech). Yeast cells were first transformed with pGBT9/NS5A plasmid and then sequentially transformed with 100 μg of pGAD10/cDNA using the lithium acetate method. Double transformants were selected on Trp-/Leu-/His- glucose plates supplemented with 25 mM 3-amintriazole. Seven days after transformation, the surviving cells were tested for β-galactosidase (β-Gal) activity using a filter lift assay. After being frozen in liquid nitrogen and thawed at room temperature, filter replicas of yeast transformants were overlaid on Waterman 3 MM paper saturated with 6-bromo-4-chloro-3-indolyl-β-D-galacosidase solution (0.033% in Z buffer) and incubated at 30°C for 8 h. Blue colonies were isolated, plated, and tested again for β-Gal activity. Yeast clones containing only pGAD10/cDNA plasmid were isolated from the positive clones as a result of the spontaneous loss of pGBT9/NS5A plasmid in the Trp^- SD medium. The isolated pGAD10/cDNA plasmids were verified by cotransformation with either pGBT9/NS5A or the parental plasmid pGBT9. Only those pGAD10/cDNA clones that develop blue color when cotransformed with pGBT9/NS5A, but not with pGBT9 vector, were considered true positives.

Sequence analysis

The positive pGAD10/cDNA clones were sequenced using a Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham, Piscataway, NJ). The DNA sequences were translated and compared with nonredundant sequence database using a family of BLAST programs (Altschul *et al.,* 1997) through the National Center for Biotechnology Information network service. Statistically significant matches were analyzed with the CLUSTAL_X program (Thompson *et al.,* 1997), producing multiple sequence alignments, or the PCGENE program. The resulting alignments were sent as input for the PHD program to predict secondary structure (Rost, 1996) and transmembrane (Rost *et al.,* 1996) and coiled-coil (Lupas *et al.,* 1991) regions. Hydrophobicity profiles were obtained by the MacVector program.

GST pull-down assay

pGEX-4T-1 or pGEX-4T-1/hVAP-33 plasmids were transformed and expressed in *E. coli* BL21 (DE3) (Novagen). GST or GST-fusion proteins were purified with glutathione–Sepharose 4B beads. The amounts of purified proteins were estimated by Coomassie blue staining of SDS–polyacrylamide gels. ^{35}S-labeled NS5A protein was *in vitro* translated in the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) using pcDNA3/NS5A and pcDNA3.1/NS5B plasmid DNA as templates. Equal amounts of GST and GST-fusion proteins were incubated with 5 μl of *in vitro* translated NS5A or NS5B for 2 h at 4°C in binding buffer (40 mM HEPES, pH 7.5, 100 mM KCl, 0.1% Nonidet-P40, 20 mM 2-mercaptoethanol). After being washed four times in wash buffer (40 mM HEPES, pH 7.5, 100 mM KCl, 0.4% Nonidet-P40, 20 mM 2-mercaptoethanol), the beads were boiled in Laemmli's sample buffer for 3 min. The dissociated proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and detected by autoradiography.

Antibodies

For the production of anti-hVAP-33, rats were immunized with hVAP-33 purified as follows: hVAP-33 was expressed as a GST-fusion protein in *E. coli* BL21 strain and purified with glutathione–Sepharose 4B beads. hVAP-33 was then released from the GST moiety by digestion with thrombin (Novagen). Proteins were separated by SDS–PAGE, and the specific band was excised, electroeluted, and mixed with Freund's adjuvant before injecting into rats. To generate polyclonal antibody against NS5A, nucleotides 6255–7595 of HCV genotype 1b were expressed as a GST-fusion protein and purified as described above. Approximately 100 μg of the fusion protein was used to immunize New Zealand White rabbits. HCV patient serum was used to detect NS5B. FITC-conjugated antibody against rat immunoglobulin G (IgG) was purchased from American Qualex (La Mirada, CA). FITC- or TRITC-conjugated goat anti-rabbit IgG was obtained from Pierce (Rockford, IL).

Transfection, immunoprecipitation, and immunoblotting

COS7 cells were transfected using the calcium phosphate precipitation method in 60-mm-diameter plates. Two hours before transfection, cells were infected with recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase (Fuerst *et al.,* 1986). For coimmunoprecipitation study, 5 μg of each plasmid DNA was transfected into COS7 cells. After 12 h of incubation at 37°C under 5% CO_2, the cells were harvested and washed with cold phosphate-buffered saline. The samples were then collected in 150 μl of Buffer A (10 mM HEPES–KOH, pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.5 mM DTT). After the cells were passed through a 25-gauge needle five times, the cell lysates were centrifuged at maximum speed for 5 min at 4°C. The supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in 120 μl of ice-cold Buffer C (20 mM HEPES–KOH, pH 7.8, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT) and incubated on ice for 20 min. The nuclear debris was pelleted by centrifugation at 4°C for 5 min, and the supernatant was collected as the nuclear fraction. Coimmunoprecipitation was performed at 4°C in 1× TM[10] buffer (50 mM Tris–HCl, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). Cell lysates (50 μl) were incubated with 3 μl of Sepharose 4B

beads crosslinked with anti-Flag (Eastman Kodak Co., Rochester, NY) in a total volume of 200 μl of reaction buffer. After overnight incubation, beads were washed four times with 1× TM[10] buffer. The precipitates were then boiled for 5 min in Laemmli's sample buffer and run on a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Hybond-ECL) and analyzed by enhanced chemiluminescence detection method (Amersham).

Immunofluorescent staining

Cells were plated on 8-well chamber slides and cotransfected with pcDNA3- or pcDNA3.1-derived constructs. After being fixed in 4% formaldehyde for 20 min and permeabilization in cold acetone for 5 min, cells were incubated in 5% bovine serum albumin (BSA) for 20 min to inhibit nonspecific antibody binding. Primary antibodies were diluted in 5% BSA (1:100) and incubated with cells for 1 h at room temperature. After three washes in PBS, the cells were incubated with fluorochrome-conjugated secondary antibodies for 1 h at room temperature. Cells were then washed three times in PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Confocal microscopy was performed on a Zeiss 210 Laser Scanning Confocal Microscope equipped with an argon and a HeNe laser and appropriate filters. Image analysis was performed using the standard system operating software provided with the microscope. Fluorescence images were superimposed digitally to allow fine comparison. Colocalization of green (FITC) and red (TRITC) signals in a single pixel produces yellow while separated signals remain green or red.

Subcellular fractionation analysis

Female rabbit lacrimal gland acinar cells or HepG2 cells were subjected to two-dimensional fractionation analysis according to the method developed by Mircheff and co-workers (see Gierow *et al.,* 1996; Mircheff, 1996; Hamm-Alvarez *et al.,* 1997). In brief, cell lysates were prepared from primary cultures of rabbit lacrimal glands or HepG2 cell cultures and subjected to isopycnic centrifugation on 26.5–80% sorbitol density gradients on a SW-28 rotor (Beckman, Fullerton, CA) at 48,000 rpm for 5 h (Gierow *et al.,* 1996; Hamm-Alvarez *et al.,* 1997). Each gradient was collected into 13 membrane fractions, some of which were combined, yielding 6 samples (fractions 1–2, 3–6, 7–8, 9–10, 11–12, 13). Each sample was further separated into 13 fractions by phase partitioning in a thin-layer countercurrent distribution apparatus at pH 7.0–6.6 shift or pH 7.6 (Mircheff, 1996). Specific enzyme markers were used to determine the nature of intracellular membranes in each fraction (Gierow *et al.,* 1996; Hamm-Alvarez *et al.,* 1997). The ER marker, α-glucosidase, was determined with 4-methylumbelliferyl-α-D-glucoside as substrate (Peters *et al.,* 1972). β-Hexosaminidase, which is predominantly localized on the Golgi and prelysosomes, was determined with 4-methylumbelliferyl-α-D-glucosaminide as substrate (Barrett and Heath, 1977). The endosome and basal-lateral membrane markers, Na,K-ATPase and acid phosphatase, were assayed as described previously (Mircheff, 1989). Na,K-ATPase is primarily associated with the constitutive endosomal system and, to a lesser extent, with the basal-lateral membranes. The distribution of acid phosphatase is generally similar to that of Na,K-ATPase, but relatively more of the acid phosphatase appeared to be associated with the basal-lateral membranes and the stimulation-dependent endosomal compartments. The distribution of the VAP-33 protein was determined by Western blotting with the rat polyclonal anti-hVAP-33.

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