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Hepatitis C Virus Core Protein Interacts with the Cytoplasmic Tail of Lymphotoxin-β Receptor

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Hepatitis C virus (HCV) core protein is a multifunctional protein. We examined whether it can interact with cellular proteins, thus contributing to viral pathogenesis. Using the HCV core protein as a bait to screen a human liver cDNA library in a yeast two-hybrid screening system, we have isolated several positive clones encoding cellular proteins that interact with the HCV core protein. Interestingly, more than half of these clones encode the cytoplasmic domain of lymphotoxin-β receptor ($LT_\beta R$), which is a member of the tumor necrosis factor receptor family. Their binding was confirmed by in vitro glutathione *S*-transferase fusion protein binding assay and protein-protein blotting assay to be direct and specific. The binding sites were mapped within a 58-amino-acid region of the cytoplasmic tail of $LT_\beta R$. The binding site in the HCV core protein was localized within amino acid residues 36 to 91 from the N terminus, corresponding to the hydrophilic region of the protein. In mammalian cells, the core protein was found to be associated with the membrane-bound $LT_\beta R$. Since the $LT_\beta R$ is involved in germinal center formation and developmental regulation of peripheral lymphoid organs, lymph node development, and apoptotic signaling, the binding of HCV core protein to $LT_\beta R$ suggests the possibility that this viral protein has an immunomodulating function and may explain the mechanism of viral persistence and pathogenesis of HCV.

Hepatitis C virus (HCV) is the major cause of posttransfusion and community-acquired non-A, non-B hepatitis (1, 4, 61). It is characterized by a very high frequency (over 70%) of chronic, persistent infection (1, 59), leading to liver cirrhosis, hepatocellular carcinoma (16, 33, 48), and some autoimmune diseases (2, 3, 23, 29, 60). Virus infection persists despite the presence of circulating antibodies (11) and virus-specific, cytotoxic T cells (32, 34, 56). The mechanisms of HCV escape from host immunosurveillance and of its pathogenesis are still unclear.

HCV is a member of the *Flaviviridae* family. It contains a positive-strand RNA of 9.5 kb and encodes a large polyprotein, which is cleaved by both cellular and viral proteases into multiple proteins (12, 30). These proteins include three structural proteins (core, E1, and E2) and six nonstructural proteins. The core protein (191 amino acids [aa]) is cleaved from the remaining polyproteins by a host signal peptidase of the endoplasmic reticulum (22, 26). The mature core protein is associated with the cytoplasmic side of the endoplasmic reticulum (53) and is also found in the nucleus (36, 38). The variable localizations of the core protein in the cells suggest that it may have multiple roles in the viral life cycle. It can multimerize (41) and bind to viral RNA (28, 53), and is presumed to form HCV nucleocapsid. Recent studies have also demonstrated that core proteins have regulatory functions for viral and cellular genes, including the suppression of hepatitis B virus gene expression (54, 55), activation or suppression of promoters of some cellular or viral genes (47), and oncogenic transformation of rat embryo fibroblasts (46).

Many viruses escape immune defenses by producing viral proteins that modulate components of the host's defense mechanisms. Frequently targeted for interference by viral proteins are members of the tumor necrosis factor (TNF) superfamily of ligands and receptors (TNFR). Diverse types of viruses such as adenovirus, poxvirus, and herpesvirus all produce proteins that interact with ligands or receptors of this superfamily (21, 43). For example, the Shope fibroma poxvirus T2 open reading frame encodes a soluble version of the 80-kDa TNFR that functions as a TNF antagonist (43). Viral targets extend to the signaling proteins for this receptor family, as exemplified by the binding of the Epstein-Barr virus LMP-1 protein to the TRAF3, a putative signaling protein for several receptors in this family (44). TNF superfamily members regulate many cellular responses, such as growth or differentiation, that control inflammatory and immune defenses (57). Developmental processes essential to the immune system are also controlled by members of this family. For example, the lymphotoxin heterotrimer ($LT_{\alpha 1\beta 2}$) and its specific receptor, lymphotoxin-β receptor ($LT_\beta R$) (62), control the development of peripheral lymphoid tissue (13, 15) and also participate in formation of germinal centers during immune responses (42).

We set out to examine whether the HCV core protein interacts with cellular proteins that may be involved in regulating immune responses. Several candidate clones in a human liver cDNA library have been identified by a yeast two-hybrid screening system (10). Interestingly, a majority of these clones represent the coding region for the cytoplasmic domain of the $LT_\beta R$. This result implicates HCV core protein as a potential

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modulator of the host immune system, suggesting a mechanism for viral evasion of host defenses, perhaps allowing for virus persistence.

MATERIALS AND METHODS

Plasmid constructions. To construct the plasmids used in the yeast two-hybrid screening, two plasmid vectors, pGBT9, which encodes the GAL4 DNA-binding domain, and pGAD10, which encodes the GAL4 activation domain, were employed (Clontech). The HCV cDNA fragments representing various portions of the core protein-coding region (aa 1 to 191) of the HCV-T strain (9) were generated by PCR using two appropriate primers containing a *Bam*HI site and were cloned into the unique *Bam*HI site of the yeast plasmid pGBT9. In these constructs, the core protein-coding sequence was fused in frame with the GAL4 DNA-binding domain of pGBT9 vector. The human liver cDNA library, which was fused with the GAL4-activation domain in pGAD10 vector, was obtained from Clontech.

The plasmid used to express the core protein-glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* was constructed by inserting the PCR-generated HCV cDNA fragment as described above into the *Bam*HI site of pGEX-4T-1 vector. The plasmid used for in vitro transcription of HCV core protein was constructed by inserting the PCR-generated fragment into the *Bam*HI site of plasmid pCDNA3 (Invitrogen, San Diego, Calif.), in which the core protein-coding sequence was under the control of the T7 phage promoter.

To construct the mammalian expression vector for expressing HCV core protein in COS 7 cells (20), a derivative of pCDNA3 (Invitrogen) was used. For this purpose, an *Nru*I-*Hin*dIII fragment was removed from pCDNA3 and replaced with the *Eco*RI-*Not*I fragment of pCMV-β vector (Clontech), resulting in the removal of the T7 promoter and insertion of the simian virus 40 splicing donor and acceptor sequences into the region between the cytomegalovirus (CMV) promoter and the multiple cloning sites (this vector was designated pCMV). The core protein-coding region obtained by PCR was cloned into the *Eco*RV site of the pCMV vector. The resulting HCV core protein sequence was under the control of the CMV immediate-early promoter. The $LT_\beta R$-expressing vector was constructed with pCDNA3; thus, the $LT_\beta R$ sequence was under the control of both the CMV immediate-early promoter and the T7 promoter.

Yeast two-hybrid library screening. The screening procedure used was a modification of the previously published procedure (10). Briefly, *Saccharomyces cerevisiae* HF7C or SFY526 (Clontech) was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic minimal medium (0.67% yeast nitrogen base, 2% dextrose with appropriate auxotrophic supplements). Yeast strain HF7C, carrying two reporter genes, *HIS3* and *lacZ*, under separate promoters, was used as a host for cDNA library screening. Yeast was transformed with pGBT9/core(1-115) and pGAD10/cDNA pool by the lithium-acetate method (19) and selected for histidine, leucine, and tryptophan prototrophy. β-Galactosidase (β-Gal) activity was assayed on nitrocellulose filter replicas of yeast transformants. Filters were placed in liquid nitrogen for 30 s and incubated for 8 h in buffer containing 4 mM 6-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). The positive interaction was determined by the appearance of blue colonies. Blue colonies were isolated, replated, and retested for β-Gal activity. Yeast clones containing only pGAD10/cDNA plasmids were isolated from the positive clones as a result of the spontaneous loss of pGBT9/core plasmids in the absence of trytophan selection, and the isolated plasmids were retested for β-Gal activity in yeast strain SFY526, which carries only the *lacZ* reporter. For this assay, the isolated pGAD10/cDNA plasmid was transformed into yeast together with either pGBT9/core(1-115) or parent plasmid pGBT9. The selected plasmids that gave positive reactions only when cotransformed with the former were further transformed into yeast strain HF7C and retested for histidine prototrophy and β-Gal activity.

Sequence analysis of pGAD10/cDNA. cDNA sequences were obtained by the dideoxynucleotide chain termination sequencing method (52) using oligonucleotide primers that anneal to GAL4 activation domain sequences at one end and multiple cloning sites of the vector at the other. Resulting sequences were compared against the database of the National Center for Biotechnology Information by the BLAST program.

GST fusion protein binding assay. GST or GST-$LT_\beta R$ expression plasmids were grown in *E. coli* BL21(DE3) (Novagen) and induced with isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial lysates were incubated with glutathione Sepharose 4B beads (Pharmacia) and used for the GST fusion binding assay. Approximately equal amounts, as judged by Coomassie blue staining, of various GST-HCV core fusion proteins on glutathione-Sepharose beads were incubated with recombinant HCV core proteins obtained from *E. coli* (38) in incubation buffer (40 mM HEPES [pH 7.5], 100 mM KCl, 0.1% Nonidet P-40, 20 mM 2-mercaptoethanol) for 2 h at 4°C and then rinsed four times in the same buffer. The beads were boiled in Laemmli sample buffer (35), and the supernatants were analyzed by electrophoresis on a 15% polyacrylamide gel containing sodium dodecyl sulfate (SDS). Afterwards, the bound HCV core protein was detected by immunoblotting using a rabbit polyclonal antibody against HCV core protein or an HCV patient's serum. Alternatively, in vitro-translated, [^{35}S]methionine-labeled HCV core protein was incubated with GST-core fusion proteins. The bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and directly visualized by autoradiography.

Far Western protein-protein blotting. Recombinant HCV core protein expressed in *E. coli* (38) and hepatitis delta antigen (HDAg) (of hepatitis delta virus) expressed by recombinant baculovirus (27) were lysed by Laemmli sample buffer, separated by SDS-PAGE on a 15% polyacrylamide gel, and stained with Coomassie brilliant blue or electrotransferred to a nitrocellulose membrane. The membrane was washed with buffer A (10 mM HEPES-KOH [pH 7.5], 60 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) and incubated with 6 M guanidine HCl for 15 min at 4°C and then sequentially with 3, 1.5, 0.75, 0.38, 0.19, and 0.09 M guanidine HCl for 5 min each to renature the proteins. The membrane was subsequently blocked for 1 h at 4°C with 5% nonfat dry milk in buffer A containing 0.05% Nonidet P-40. The in vitro-translated, [^{35}S]methionine-labeled $LT_\beta R$ was incubated with the membrane in buffer A containing 3% nonfat dry milk and 0.05% Nonidet P-40 overnight at 4°C. Unbound proteins were removed by washing three times with buffer A containing 1% nonfat dry milk and 0.05% Nonidet P-40. Protein binding was detected by autoradiography.

Membrane flotation analysis. This method (51) was used for demonstration of protein-protein interactions in the cells. COS 7 cells (20) were transfected with various plasmids by the calcium phosphate precipitation method (8). Forty-eight hours posttransfection, the transfected cells were suspended in 0.5 ml of hypotonic lysis buffer (10 mM Tris HCl [pH 7.5], 10 mM KCl, 5 mM $MgCl_2$) and incubated on ice for 10 min before disruption of the cells by passage through a 26-gauge hypodermic needle 15 times. Unbroken cells and nuclei were removed by centrifugation at 1,000 × *g* for 5 min, and the resulting supernatant was subjected to fractionation by the membrane flotation method as described previously (51). Briefly, 0.5-ml aliquots of lysates were dispersed into 2 ml of 72% (wt/wt) sucrose in low-salt buffer (LSB) (50 mM Tris HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$) and overlaid with 2.5 ml of 55% (wt/wt) sucrose in LSB and 0.6 ml of 10% (wt/wt) sucrose in LSB. Sucrose gradients were then centrifuged in a Beckman SW55Ti rotor at 4°C for 12 h at 38,000 rpm. After centrifugation, 0.8-ml fractions were collected successively from the top of the gradient. Any material pelleted by centrifugation was resuspended in 0.8 ml of LSB and designated as the final fraction of the gradient. All fractions were diluted with 4 ml of LSB and recentrifuged in a Beckman SW55Ti rotor at 46,000 rpm for 90 min at 4°C, and the resulting pellets were dissolved in Laemmli sample buffer. Proteins were boiled for 3 min and separated by SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membrane.

Immunoblot detection of HCV core protein and $LT_\beta R$. The proteins blotted onto nitrocellulose membrane were first treated with 5% nonfat milk in phosphate-buffered saline for 60 min and then incubated with rabbit polyclonal antibody against HCV core protein (diluted 1:500) or with goat anti-$LT_\beta R$ antibody (diluted 1:500) for 2 h at room temperature. After three washes in phosphate-buffered saline, the blots were incubated with ^{125}I-labeled protein A for 2 h at room temperature or incubated with rabbit anti-goat immunoglobulin G (American Qualex) (diluted 1:500) for 2 h at room temperature and then incubated with ^{125}I-labeled protein A. The membrane was washed, and bound antibody was detected by autoradiography.

Sequences and comparative sequence analysis of HCV core proteins and $LT_\beta R$. The RNA and amino acid sequences of different HCV core proteins and $LT_\beta R$ and its related proteins were obtained from the EMBL/GenBank and Swissprot databases, respectively. Database searches were performed with the Blitz program (58) and a family of the Blast programs (5) through the EMBL and National Center for Biotechnology Information network servers, respectively. Multiple sequence alignments were produced with the CLUSTAL V program (25) utilizing the PAM250 (14) or different Blossum (24) scoring tables. Pairwise sequence comparisons were performed in dot plot fashion, by using the high-resolution DotHelix program (37) in conjunction with the PAM250 or Blossum 62 tables. Secondary structure predictions were produced with the help of the PHD program (49) through the EMBL network server.

RESULTS

A yeast two-hybrid screen of cellular proteins interacting with HCV core protein. To identify cellular proteins that can interact with HCV core protein, the hydrophilic portion (aa 1 to 115) of the HCV core protein (41) was fused to the GAL4 DNA-binding domain (pGBT9) to serve as a bait in the yeast two-hybrid screen (10) for cDNAs that encode interactive proteins. The GAL4 activation domain (pGAD10) was fused to a cDNA library of human adult liver. The two plasmids were cotransformed into yeast strain HF7c. Of 4.7×10^6 transformants used for screening, 47 grew in the absence of tryptophan, leucine, and histidine and expressed β-Gal activity. Thirty-one pGAD10/cDNA plasmids were successfully isolated and retested for specificity of β-Gal expression. Of these, 20 clones expressed β-Gal activity only when cotransformed with pGBT9/core and were considered to be true positives. DNA

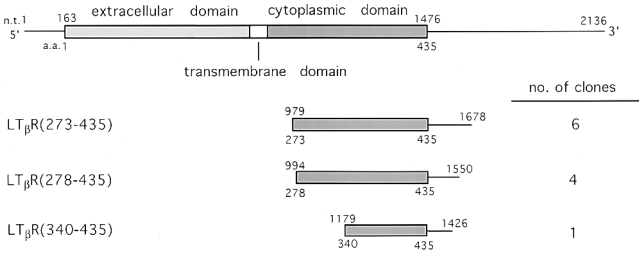

FIG. 1. Schematic representation of the cDNA structure of $LT_\beta R$ mRNA and the clones obtained from HCV-core-interacting yeast two-hybrid cDNA library screening. Boxes in the cDNA clones indicate $LT_\beta R$-encoding regions, and bars indicate untranslated regions of $LT_\beta R$ cDNA. Nucleotide numbers of $LT_\beta R$ cDNA are indicated above boxes, and amino acid numbers are indicated below the boxes. The total numbers of each cDNA clone obtained in the screening are indicated.

sequence analysis revealed that 11 of these clones matched the gene sequence of $LT_\beta R$. These 11 clones were from three independently derived clones, representing different portions of the same region of $LT_\beta R$, which has a total length of 435 aa residues. Six of these clones encode 163 aa residues (aa 273 to 435), four clones encode 158 residues (aa 278 to 435), and one clone encodes 96 residues (aa 340 to 435) of $LT_\beta R$ (Fig. 1), all of which are from the extreme C terminus of $LT_\beta R$ that was fused in frame to the GAL4 activation domain. The findings that these clones constituted more than half of the positive clones and that they represented three independently derived cDNA clones which encode the same region of $LT_\beta R$ indicated the specificity of the interactions between HCV core and $LT_\beta R$ in this yeast two-hybrid assay.

The specificity of these interactions was further tested by cotransforming one of the pGAD/$LT_\beta R$ clones with the GAL4 DNA-binding domain fused to two other unrelated proteins, which have been previously shown to bind to their respective interacting clones in the yeast two-hybrid system. One was E12 protein (45), which interacted with myogenin. The other was *ras*, which interacted with neurofibromatosis type 1 (NF1) protein (40). The E12-myogenin and *ras*-NF1 interactions were reproduced in our assay (Table 1). In contrast, the pGAD10/$LT_\beta R$ clone did not interact with *ras* or E12, nor did they interact with the vector plasmid (pGBT9) alone (Table 1). Likewise, pGBT9/core(1-115) did not interact with NF1 or myogenin. Therefore, HCV core protein appears to interact specifically with the cytoplasmic domain of $LT_\beta R$.

To determine whether these interactions were unique to the particular core protein used (derived from the Taiwan isolate [9]), we also tested the core protein sequences of two other HCV isolates, including RH (a Southern California isolate) (39) and the prototype HCV isolate (12), fused to the GAL4 DNA-binding domain. These two core proteins differ from that of the Taiwan isolate by 4 and 7 aa, respectively, within the N-terminal 115 aa that were used as bait in the two-hybrid screening. Table 1 shows that these two isolates have a very similar binding specificity with $LT_\beta R$ and did not bind to NF1, myogenin, or GAL4-activation-domain vector. Therefore, we conclude that the interaction between HCV core protein and $LT_\beta R$ is a general phenomenon common to many HCV isolates.

TABLE 1. Specificity of interactions of the HCV core protein with $LT_\beta R$ in a yeast two-hybrid system

DNA-binding domain hybrid	% β-Gal induction for activation domain hybrid[a]			
	$LT_\beta R$ (273–435)	NF1	Myogenin	Vector alone
Core (T, 1-115)	100	0	0	0
Core (C, Arg, 1-115)	100	0	0	0
Core (RH, 1-115)	100	0	0	0
RAS	0	100	NT	NT
E12	0	NT	100	NT
Vector alone	0	NT	NT	0

[a] Percentage of blue colonies among transformants. NT, not tested; Core (T, 1-115), Taiwan isolate (9); Core (C, Arg, 1-115), prototype HCV with an Arg substitution at aa 9 (12, 38); Core (RH, 1-115), Southern California isolate of HCV (39).

The middle domain of the hydrophilic region of HCV core protein interacts with $LT_\beta R$. To determine the sequence of the HCV core protein responsible for its interaction with $LT_\beta R$, we performed a two-hybrid β-Gal assay using several truncation mutants of HCV core protein, which represent various regions of the hydrophilic domain of the protein (41), to interact with the $LT_\beta R$ clones. Table 2 shows that several truncated forms of HCV core protein (aa 1 to 115, 1 to 191, 36 to 115, and 36 to 91) interacted with all three pGAD10/$LT_\beta R$ clones; only one remaining truncated clone (aa 1 to 25) did not. This result suggests that the minimum binding domain is aa 36 to 91 of the HCV core protein. However, the aa 36 to 91 alone interacted relatively poorly with the $LT_\beta R$ clones; particularly, this clone almost failed to interact with the smallest $LT_\beta R$ clone (aa 340 to 435 of $LT_\beta R$), which contains only 96 aa from the C terminus of $LT_\beta R$. These findings suggest that, although the aa 36 to 91 region of HCV core protein contains the minimum sequence for the interaction with $LT_\beta R$, the neighboring sequences within the HCV core protein also contributed to efficient binding. The aa 36 to 91 domain contains a highly conserved region (aa 45 to 59) and a highly variable region (aa 68 to 78) among different HCV isolates (data not shown). A computer analysis of the core protein sequence predicts that this interacting domain contains two potential β-sheet structures, which span aa 30 to 41 from the N terminus (data not shown). Whether any of these structural features are involved in binding to $LT_\beta R$ is not known. It is notable that the full-length (aa 1 to 191) core protein did not show β-Gal activity in the two-hybrid assay (Table 2). This was probably due to the presence of the hydrophobic sequences at the C terminus (41), which may have interfered with the transport of the fusion protein into the nucleus of the yeast, thereby preventing its potential interaction with pGTAD10/$LT_\beta R$ in the nucleus. The extreme C terminus of the core protein contains a transmembrane α-helix. Indeed, the full-length HCV core protein could bind to $LT_\beta R$ in an in vitro binding assay (see below).

HCV core protein and $LT_\beta R$ can interact directly in vitro. To confirm that HCV core protein can directly interact with $LT_\beta R$, we first performed a Far Western protein-protein blotting assay. *E. coli*-expressed core protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane and incubated with in vitro-translated, [^{35}S]methionine-labeled, full-length $LT_\beta R$. As a negative control, baculovirus-expressed delta antigen of hepatitis delta virus (27) was similarly processed. The results showed that $LT_\beta R$ bound to HCV core protein but not to HDAg (Fig. 2). It is notable that HDAg has biochemical properties very similar to those of HCV core protein, i.e., it is hydrophilic in the N-terminal two-thirds of the protein, while noncharged in the C terminus, and both are comparable in size (7). This result thus indicates that the interaction between HCV core protein and $LT_\beta R$ was specific and not merely due to the basic charge of the core protein. This

TABLE 2. Interaction between $LT_\beta R$ and various domains of HCV core protein by yeast two-hybrid system

DNA-binding domain hybrid	% β-Gal induction for activation domain hybrid[a]			
	$LT_\beta R$ 273–435	$LT_\beta R$ 278–435	$LT_\beta R$ 340–435	Vector alone
Core 1-191	0 (0/211)	0 (0/285)	0 (0/231)	0 (0/86)
Core 1-115	100 (340/340)	97.2 (292/301)	100 (278/278)	0 (0/63)
Core 1-91	87.9 (240/273)	95.9 (213/222)	72.7 (189/265)	0 (0/106)
Core 1-25	0 (0/209)	0 (0/150)	0 (0/290)	0 (0/79)
Core 36-115	40.0 (66/165)	80.6 (116/144)	82.1 (271/330)	0 (0/71)
Core 36-91	35.7 (100/280)	46.7 (63/135)	1.0 (3/280)	0 (0/120)
Vector alone	0 (0/172)	0 (0/180)	0 (0/141)	0 (0/105)

[a] Values in parentheses are number of blue colonies/number of scored transformants.

result also shows that the full-length HCV core protein, including the hydrophobic domain, can bind to $LT_\beta R$.

Mapping of the interacting domain on the $LT_\beta R$. To determine the region of $LT_\beta R$ responsible for its binding to HCV core protein, different regions of the cytoplasmic domain of $LT_\beta R$ were fused to GST protein (Fig. 3A). Figure 3B shows that similar amounts of the various GST-$LT_\beta R$ fusion proteins were used in these assays. These proteins were incubated with the in vitro-translated, [^{35}S]methionine-labeled core protein of the prototype HCV, which migrated as a p16 protein because of the proteolytic cleavage of the C-terminal sequences (39) (Fig. 3D). The labeled HCV core proteins bound to the various GST-$LT_\beta R$ fusion proteins were eluted and analyzed by SDS-PAGE. The results showed that all of the truncation mutants of the cytoplasmic domain of $LT_\beta R$ tested, except the aa 248 to 337 mutant, bound HCV core protein (Fig. 3D). GST by itself did not bind. These results suggested that the minimum binding domain on the $LT_\beta R$ is aa 338 to 395.

The core proteins derived from two other HCV isolates were also tested. One of them (the Taiwan isolate) (9) separated into two protein species, p21 and p16, the latter being a truncation product of the former. Figure 3C shows that both p21 and p16 have similar binding specificity as that of the core protein of the prototype HCV isolate (Fig. 3D), although the truncated form appears to have a stronger binding activity. The core protein of the RH (Southern California) (39) isolate, which yielded a single protein species of 21 kDa, also has a similar binding pattern, although it bound to aa 276 to 395 to a much lower extent (Fig. 3E). It is noted in these assays that the different truncation forms of $LT_\beta R$-GST fusion proteins bound at various efficiencies to different HCV core proteins. The significance of these variations is not clear. Nevertheless, these binding patterns also indicate that the binding sequence resides in aa 338 to 395.

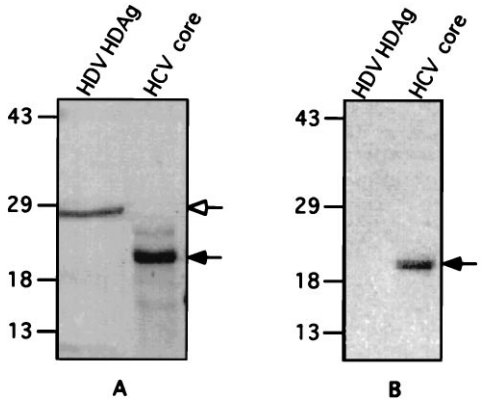

FIG. 2. Interaction of HCV core protein with $LT_\beta R$ in Far Western protein blotting. *E. coli*-expressed HCV core protein and baculovirus-expressed HDAg (HDV HDAg) were separated by SDS-PAGE on a 15% polyacrylamide gel and stained with Coomassie brilliant blue (A) or electrotransferred onto a nitrocellulose membrane (B). The membrane was denatured-renatured by guanidine HCl and then incubated with in vitro-translated, [^{35}S]methionine-labeled $LT_\beta R$. Protein binding was detected by autoradiography. Marker, molecular size marker (in kilodaltons). HCV core is indicated by solid arrows. HDAg is indicated by an unfilled arrow.

To further establish the identity of the proteins bound to the GST-$LT_\beta R$ fusion proteins, an alternative approach was taken. The various GST-$LT_\beta R$ fusion proteins were mixed with the purified recombinant HCV core protein (RH isolate) expressed in *E. coli* (39). The bound proteins were separated by SDS-PAGE and detected by immunoblotting using rabbit polyclonal antibody against HCV core protein (28). Figure 3F shows that the proteins bound to the GST-$LT_\beta R$ fusion constructs, except aa 248 to 337, were indeed the HCV core protein. These results also confirmed that the minimum binding domain on the $LT_\beta R$ resides in the aa 338 to 395. However, it is noted that the binding of aa 338 to 395 to the HCV core protein in this assay was weaker than that of the other constructs, suggesting that the other regions of the $LT_\beta R$ may also contribute to the binding. Computer analysis of aa 338 to 395 of the $LT_\beta R$ sequence suggests that the structure of this region is poorly ordered but may contain a β-sheet structure within aa 360 to 378. Since HCV core protein has been shown to undergo homotypic interactions and multimerize (41), we also compared the strength of HCV core-$LT_\beta R$ binding with that of core-core binding. Figure 3F shows that HCV core protein bound the GST-HCV core fusion protein to an extent similar to that of core-$LT_\beta R$ binding. It is also notable that the HCV core protein expressed in *E. coli* separated into two species of slightly different electrophoretic mobilities. The separation of HCV core protein into multiple species is frequently observed, the species probably representing processing of the core protein expressed in the cells. The precise condition for the processing of the HCV core protein is not yet clear.

The association of HCV core protein with the membrane-bound $LT_\beta R$ in mammalian cells. To demonstrate that HCV core protein can bind to $LT_\beta R$ expressed in mammalian cells, we used a membrane flotation method that is highly sensitive in detecting interactions between membrane-bound and soluble proteins (51). This method did not employ detergents for cellular disruption, thus avoiding disruption of the weak protein-protein or protein-lipid interactions. It has been successfully used for detection of protein-protein interactions in several viruses, which could not otherwise be demonstrated (17, 51). The plasmids encoding $LT_\beta R$ and a C-terminus-truncated form of HCV core protein (aa 1 to 115) were transfected under a CMV immediate-early promoter into COS cells, which did not express a detectable level of endogenous $LT_\beta R$. $LT_\beta R$ is

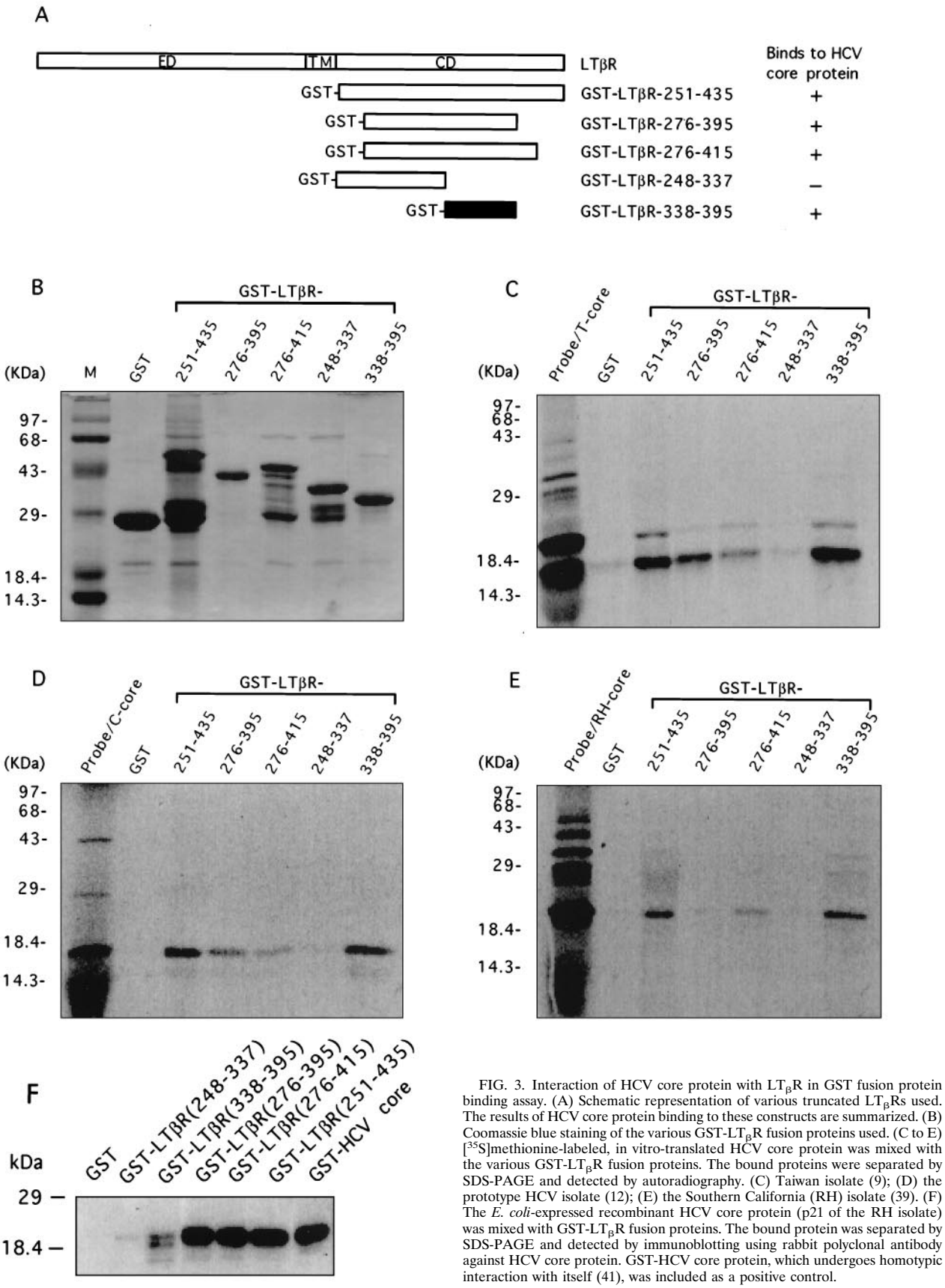

FIG. 3. Interaction of HCV core protein with $LT_{\beta}R$ in GST fusion protein binding assay. (A) Schematic representation of various truncated $LT_{\beta}Rs$ used. The results of HCV core protein binding to these constructs are summarized. (B) Coomassie blue staining of the various GST-$LT_{\beta}R$ fusion proteins used. (C to E) [^{35}S]methionine-labeled, in vitro-translated HCV core protein was mixed with the various GST-$LT_{\beta}R$ fusion proteins. The bound proteins were separated by SDS-PAGE and detected by autoradiography. (C) Taiwan isolate (9); (D) the prototype HCV isolate (12); (E) the Southern California (RH) isolate (39). (F) The *E. coli*-expressed recombinant HCV core protein (p21 of the RH isolate) was mixed with GST-$LT_{\beta}R$ fusion proteins. The bound protein was separated by SDS-PAGE and detected by immunoblotting using rabbit polyclonal antibody against HCV core protein. GST-HCV core protein, which undergoes homotypic interaction with itself (41), was included as a positive control.

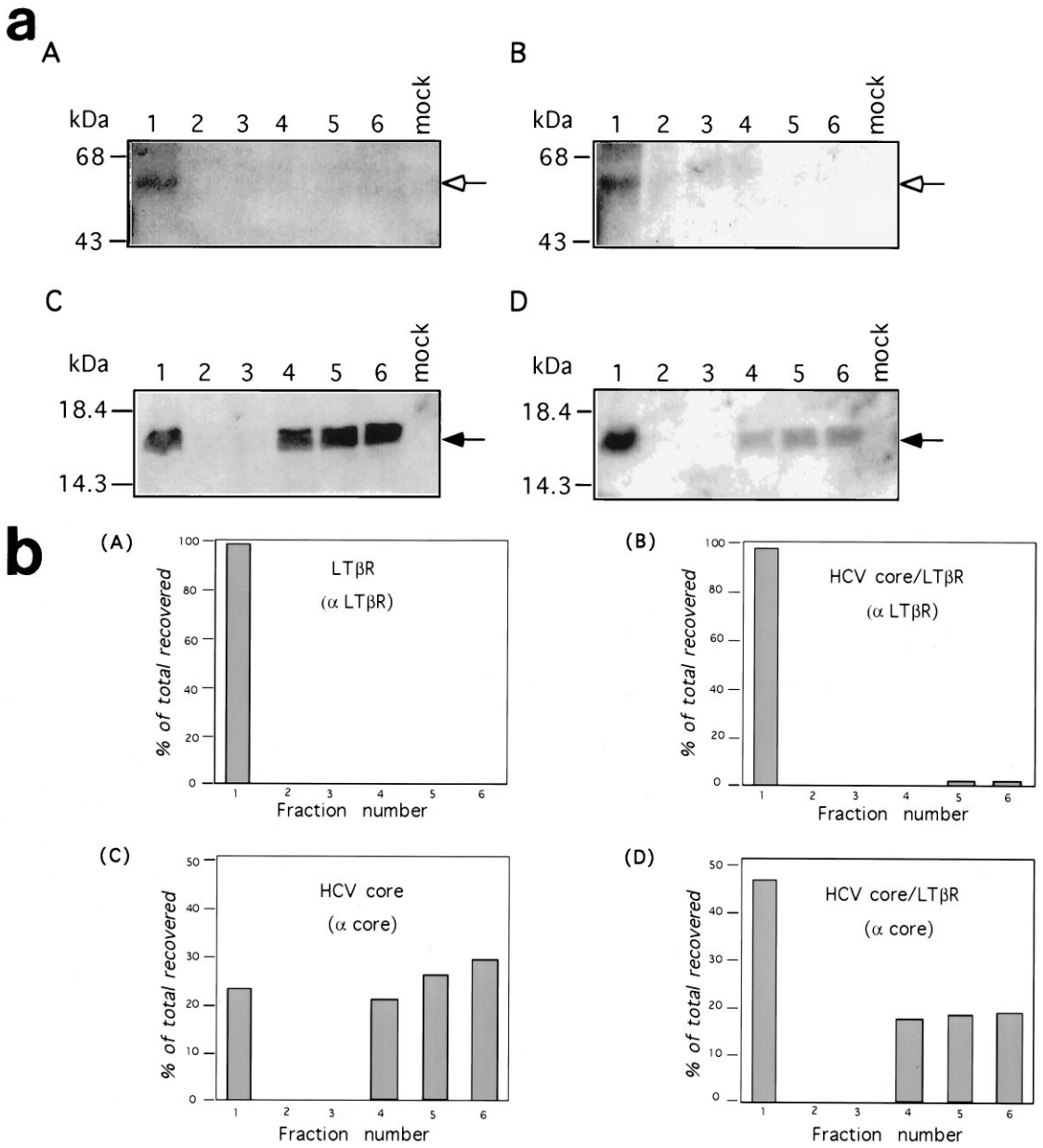

FIG. 4. Membrane flotation analysis of the interaction of HCV core protein with $LT_\beta R$ in mammalian cells. (a) COS 7 cells were transfected with expression plasmids pCMV/$LT_\beta R$ (A), pCMV/core(1-115) (C), or pCMV/$LT_\beta R$ plus pCMV/core(1-115) (ratio 3:1) (B and D). Cells were harvested at 48 h after transfection. Cell lysates were fractionated by equilibrium sucrose gradient centrifugation (see Materials and Methods), and fractions were collected from the top of the gradient. The pelleted proteins were resuspended in buffer and designated fraction 6. The remaining sucrose fractions were numbered from 1 to 5 in the order of top to bottom (light to heavy) fractions. Recovered proteins were separated on 10% (A and B) or 12.5% (C and D) polyacrylamide gels and detected by immunoblotting using a polyclonal antibody against $LT_\beta R$ (A and B) or HCV core (C and D). Unfilled arrows indicate $LT_\beta R$, and solid arrows indicate HCV core protein. Mock, transfected with vector plasmid. (b) Quantitation of the proteins in various fractions by densitometry.

an integral membrane protein, and the full-length HCV core protein is associated with cellular membranes, most notably the endoplasmic reticulum (53); however, the truncated form of HCV core protein is localized mainly in the cytosol (41). If these two proteins interact, the truncated HCV core protein is expected to become associated with the cellular membranes. The lysates of the cells expressing either or both of the two proteins were separated into membrane and soluble fractions by the membrane flotation method, and the HCV core protein or $LT_\beta R$ was detected by immunoblotting. Figure 4a, A, shows that, when $LT_\beta R$ was expressed in the absence of the HCV core protein, all of the $LT_\beta R$ was present in the membrane fraction (fraction 1); none was detected in the soluble fractions (fractions 4, 5, and 6), consistent with its properties as an integral membrane protein. In contrast, when the truncated form of the HCV core protein was expressed alone, in the absence of $LT_\beta R$, the majority (80%) of the truncated HCV core protein was present in the soluble fractions (fractions 4 to 6); only a small fraction (20%) of the proteins was associated with the cellular membrane (fraction 1) (Fig. 4a, C). However, when the cells were cotransfected with $LT_\beta R$ and the truncated HCV core proteins, an increased fraction of the HCV

core protein was detected in the membrane fraction, which accounts for nearly 50% of the total core protein in the cells (Fig. 4a, D). The distribution of $LT_\beta R$ was not affected by the coexpression of HCV core protein (Fig. 4a, B). It should be noted that the total amount of the HCV core protein expressed in the cells was reduced when it was cotransfected with $LT_\beta R$, probably as a result of the reduction of transfection efficiency or the interference of plasmid expression. In this system, the HCV core protein was also separated into two protein species of slightly different electrophoretic mobility, similar to that expressed in *E. coli* (Fig. 3F). The shift of the HCV core protein from the soluble fractions to the membrane fraction when it was coexpressed with $LT_\beta R$ suggests that HCV core protein associates with $LT_\beta R$ on the cellular membrane, although it cannot be determined whether this association is direct or mediated through other factors.

DISCUSSION

The data presented in this report demonstrated that HCV core protein interacts with $LT_\beta R$. This interaction expands the list of potential functions of this viral protein. The primary function of the HCV core protein is to encapsulate the viral RNA to form a viral nucleocapsid. It has RNA-binding (28, 53) and multimerization (41) properties, both of which are probably required for nucleocapsid formation. In addition, the core protein has been shown to bind to ribosome and endoplasmic reticulum (53), but the significance of these two properties is not yet known. The core protein also possesses a nuclear localization signal (55), and some forms of HCV core protein were detected in the nuclei (36, 38, 55), suggesting its potential role in gene regulation. Indeed, HCV core protein can suppress hepatitis B virus gene expression and replication (54, 55) and regulate the promoter activities of certain cellular and viral genes (47). Furthermore, it may cooperate with some cellular oncogenes to transform cells (46).

The finding of the interaction between HCV core protein and $LT_\beta R$ in this study suggests an additional potential function for this protein in disrupting the host's immune defense. It should be noted that the full-length HCV core protein binds to the cytoplasmic side of the endoplasmic reticulum (53), and our findings here showed that the core protein interacts with the cytoplasmic tail of $LT_\beta R$, which is also exposed to the cytoplasmic side of the endoplasmic reticulum. Thus, these two interacting proteins are located in the same compartment within the cells, further indicating that their interactions are physiologically relevant. Indeed, using the membrane flotation method, we demonstrated that the core protein could bind directly or indirectly to $LT_\beta R$ associated with the cellular membrane, although, at the present time, we cannot determine whether this interaction occurs on the endoplasmic reticulum or the plasma membrane. So far, the interaction between the HCV core protein and $LT_\beta R$ in the mammalian cells could be demonstrated only by the membrane flotation method, which did not employ detergents in the analytical procedure. All other methods utilizing detergents for disruption of cells have resulted in the disruption of these interactions (unpublished observation). Thus, this interaction may be weak under normal conditions in the tissue culture cells.

The smallest $LT_\beta R$ cDNA clone obtained from the yeast two-hybrid screening encodes the extreme C terminus (amino acid residues 340 to 435) of $LT_\beta R$, suggesting that the protein-binding domain is localized at the C terminus of the cytoplasmic tail of $LT_\beta R$. GST fusion protein binding assays further narrowed the protein-binding domain to within aa 338 to 395 of the protein. However, the binding of the core protein to this domain was weaker than to the larger fragments of $LT_\beta R$, particularly when only a short stretch of HCV core protein was used for interaction. Therefore, the remaining sequences of the cytoplasmic domain of $LT_\beta R$ probably also contribute to the stabilization of the protein-protein interaction. Preliminary data demonstrated that the $LT_\beta R$-associated protein TRAF3 also binds to the same C-terminal region of $LT_\beta R$. Since TRAF3 is related to TRAF2 (44), which mediates signaling of the 80-kDa TNFR (50), TRAF3 may participate in the signal transduction of $LT_\beta R$. Therefore, it is possible that the binding of the HCV core protein to $LT_\beta R$ can affect the binding of TRAF3, thus potentially disrupting the signal transduction of $LT_\beta R$. Interestingly, Epstein-Barr virus transforming protein LMP-1 binds to TRAF3 (previously referred to as LAP-1) (44). Thus, HCV and Epstein-Barr virus might affect the same signaling pathway of $LT_\beta R$, though via different mechanisms.

Our studies indicated that the domain of HCV core protein responsible for its binding to $LT_\beta R$ resides in aa 36 to 91. This domain has previously been shown to mediate homotypic interactions of the HCV core protein (41). It is unclear whether multimerization of HCV core protein can affect its binding to $LT_\beta R$ and possible perturbation of $LT_\beta R$ signaling. It has been shown that the clustering of TNFR/TRAF molecules activates downstream signaling, such as NFκB activation (50). The multimerization of HCV core protein may have similar effects. The predicted structures of the interacting domains of both HCV core protein (aa 36 to 91) and $LT_\beta R$ (aa 338 to 395) are relatively poorly ordered but do contain several potential β-sheet structures. It is not known whether these structures are involved in protein-protein interactions.

At the present time, the precise function of the $LT_\beta R$ is not fully understood; however, emerging evidence indicates dual roles for $LT_\beta R$ in germinal center formation in the adult (42) and as a control element for peripheral lymph node development during embryonic life (13, 15). The $LT_\beta R$ is expressed in most cell types and tissues, including liver, but not in lymphocytes (18), whereas the ligand is expressed by cytotoxic T cells, activated B cells, and NK cells (62). Like several other receptors in this family, including Fas and both TNFRs, the $LT_\beta R$ also induces cell death in certain types of tumor cells (6). The interaction of TRAF3 with $LT_\beta R$ suggests a role for this member of the emerging family of zinc RING finger proteins as a signaling molecule. Thus, HCV core protein could potentially interfere with $LT_\beta R$-TRAF3 signaling pathway(s), resulting in disruption of host immune defenses, such as germinal center formation. This outcome may result from two possible mechanisms. (i) The binding of the HCV core protein to $LT_\beta R$ may occur at the endoplasmic reticulum, thus interfering with the translocation of $LT_\beta R$ to the cell surface and resulting in the reduction of surface expression and inefficient signaling of $LT_\beta R$. (ii) The core protein could bind to the $LT_\beta R$ expressed on the plasma membrane and block the signaling of $LT_\beta R$, perhaps by competitive blockade of TRAF3, in response to the $LT_\beta R$ ligand, without affecting the surface expression of $LT_\beta R$. Both possibilities could explain how HCV-infected cells escape the host's immune defense mechanism, resulting in persistent infection. An additional possibility is that the binding of the HCV core protein may result in constitutive activation of the $LT_\beta R$, which may contribute to the inflammation and death of the virus-infected cells.

HCV probably uses multiple mechanisms for persistence, one of which may be mutations in the epitopes of the hypervariable region of the viral E2 glycoprotein, rendering the virus unrecognizable by existing virus-specific antibodies (31). However, the significance of the HCV-specific antibodies in disease prevention is still questionable. Mutations in the epitopes rec-

ognized by HCV-specific cytotoxic T cells may also account for the viral persistence (63). The finding here that HCV core protein binds to $LT_\beta R$ could provide an alternative mechanism, one directed at the effector mechanism rather than antigen recognition, by which HCV escapes host defense. It is interesting to note that HCV infection has been associated with various types of autoimmune diseases (2, 3, 23, 29). Whether the putative perturbation of immune functions by the binding of HCV core protein to $LT_\beta R$ contributes to the occurrence of these HCV-associated autoimmune diseases remains to be addressed. The observations described here may provide another clue to the pathology of HCV.

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