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Direct Interaction of Hepatitis C Virus Core Protein with the Cellular Lymphotoxin-β Receptor Modulates the Signal Pathway of the Lymphotoxin-β Receptor

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Previous studies suggest that the core protein of hepatitis C virus (HCV) has a pleiotropic function in the replication cycle of the virus. To understand the role of this protein in HCV pathogenesis, we used a yeast two-hybrid protein interaction cloning system to search for cellular proteins physically interacting with the HCV core protein. One such cellular gene was isolated and characterized as the gene encoding the lymphotoxin-β receptor (LT-βR). In vitro binding analysis demonstrated that the HCV core protein binds to the C-terminal 98 amino acids within the intracellular domain of the LT-βR that is involved in signal transduction, although the binding affinity of the full-length HCV core protein was weaker than that of its C-terminally truncated form. Our results also indicated that the N-terminal 40-amino-acid segment of the HCV core protein was sufficient for interaction with LT-βR and that the core protein could form complexes with the oligomeric form of the intracellular domain of LT-βR, which is a prerequisite for downstream signaling of this receptor. Similar to other members of the tumor necrosis factor (TNF) receptor superfamily, LT-βR is involved in the cytotoxic effect of the signaling pathway, and thus we have elucidated the biological consequence of interaction between the HCV core protein and LT-βR. Our results indicated that in the presence of the synergizing agent gamma interferon, the HCV core protein enhances the cytotoxic effects of recombinant forms of LT-βR ligand in HeLa cells but not in hepatoma cells. Furthermore, this enhancement of the cytolytic activity was cytokine specific, since in the presence of cycloheximide, the expression of the HCV core protein did not elicit an increase in the cytolytic activity of TNF in both HeLa and hepatoma cells. In summary, the HCV core protein can associate with LT-βR, and this protein-protein interaction has a modulatory effect on the signaling pathway of LT-βR in certain cell types. Given the known roles of LT-βR/LT-$\alpha_1\beta_2$ receptor-ligand interactions in the normal development of peripheral lymphoid organs and in triggering cytolytic activity and NF-κB activation in certain cell types, our finding implies that the HCV core protein may aggravate these biological functions of LT-βR, resulting in pathogenesis in HCV-infected cells.

Hepatitis C virus (HCV) is a positive-strand RNA virus with a 9.5-kb viral genome which encodes at least 10 viral proteins generated from proteolytic processing of a precursor polyprotein (3,010 to 3,033 amino acids) (8, 32, 36, 49, 77, 86). This virus has been identified as the major causative agent of post-transfusion non-A, non-B hepatitis (19, 47) and differs from other RNA viruses in its prolonged, persistent infection, which eventually causes hepatocellular carcinoma (15, 20, 43, 73). In addition to its association with liver disease, chronic HCV infection has also been associated with autoimmune syndromes and immune complex disorder (2, 3, 34, 42, 54, 87). Intriguingly, HCV persists despite the presence of virus-specific cytotoxic T lymphocytes (9, 16, 45, 66), which probably play an important role in chronic HCV infection with respect to mediating both tissue injury and viral clearance (61, 67). The reason for the failure of the host immune response to resolve HCV infection is not known. It is possible that cytotoxic T lymphocytes may not be sufficient to eliminate the virus completely. Alternatively, this failure could be in part due to the effect of viral gene products on the host immune defense system, as has been noted for several viruses (27). The most likely candidate to play such a role in HCV is its core protein. The core protein is the structural protein of the virus. However, evidence is emerging which suggests that this protein has a pleiotropic nature. In addition to having a packaging function, the core protein has been shown to act in *trans* on the viral and cellular promoters (63, 79, 80), and it is also capable of transformation of rat embryonic fibroblasts through cooperation with the *ras* oncogene (64). In order to understand the pathogenesis of HCV infection, we examined the possibility that the HCV core protein interacts with cellular proteins. One such candidate, as reported in this study, was found to be the lymphotoxin-β (LT-β) receptor (LT-βR). A similar finding of interaction between the HCV core protein and LT-βR was also reported by Matsumoto et al. (56).

LT-βR was originally designated tumor necrosis factor receptor (TNFR)-related protein (TNFRrp) because of its amino acid sequence homology to the human TNFR (4, 11). Recently, this TNFRrp receptor has been designated the LT-β-specific receptor for a cell surface heterotrimeric LT ligand (LT-$\alpha_1\beta_2$) that is composed of LT-α and LT-β subunits (13, 21, 90). Unlike the case for TNF/TNFR, which is involved in mediating a wide spectrum of biological activities, including cytotoxicity, proliferation, and antiviral response (reviewed in references 1, 35, 84, and 89), knowledge of the function of LT-βR is only emerging. The aberrant development of lymphoid organs, including lymph nodes and the spleen, observed in LTα knockout mice (23) but not in TNFR genetic knockout mice (26, 62, 68) strongly suggests an essential function for

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LT-βR/LT-$\alpha_1\beta_2$ receptor-ligand interactions in the normal development of peripheral lymphoid organs. More recently, several reports indicated that like members of the TNF ligand receptor family, LT-βR is also engaged in cytolytic and NF-κB activation in certain cell types (14, 55, 60). In this study, our results demonstrated that the interaction of the HCV core protein and LT-βR can potentiate cytolytic effects elicited by the LT-βR ligand in some cell types. This finding presumably can partially explain the molecular mechanism of HCV pathogenesis.

MATERIALS AND METHODS

Bacterial and yeast strains. All yeast strains and plasmids for two-hybrid experiments were obtained from Clontech (Palo Alto, Calif.) as components of the MATCHMAKER Two Hybrid System. Yeast strain HF7c (*MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 can*^{r} *gal4-542 gal80-538 LYS2*::*GAL1-HIS3 URA3*::(*GAL4 17-mers*)$_3$-*CYC1-lacZ*) was used for library screening and to assay for protein and protein interactions. HF7c has the upstream activating sequence and TATA sequences of the *GAL1* promoter fused to the *HIS3* gene. In addition, three copies of a 17-mer *GAL4* consensus sequence and the TATA sequence of the *CYC1* promoter were fused to the *lacZ* gene in HF7c. Both *HIS3* and *lacZ* are responsive to the GAL4 transcriptional activator.

Plasmids. Yeast-*Escherichia coli* shuttle plasmids containing the GAL4 DNA binding domain (pGBT9) and the GAL4 activation domain (pGAD424) as well as the control plasmids pVA3 (murine p53$_{72-390}$ in pGBT9), pTD1 (simian virus 40 [SV40] large T antigen$_{84-708}$ in pGAD3F) were from Clontech. Plasmids pGBT/HCVc195, pGBT/HCVc122, and pGBT/HCVc101 were derivatives of pGBT9 harboring various lengths (195, 122, or 101 amino acids, respectively) of the HCV core gene fused in frame to the GAL4 DNA binding domain. They were constructed by inserting a 0.6-kb *Acc*I-*Fsp*I fragment (Klenow filled in), a 0.38-kb *Acc*I-*Cla*I fragment (Klenow filled in), or a 0.3-kb *Acc*I-*Sac*II fragment (Klenow filled in and T4 DNA polymerase digested), respectively, of the HCV core gene (80) into the *Sma*I site of pGBT9, respectively. Plasmid pGST/LTβR(338-435), which can direct the expression of a glutathione *S*-transferase (GST) fusion protein containing the cytoplasmic region amino acids 338 to 435 of LT-βR, was constructed by insertion of the DNA fragment spanning from nucleotide 1179 to 1700 of the LT-βR gene (4) (GenBank Accession number L04270) into *Bam*HI-digested pGEX-2KS (Pharmacia). The T7 epitope tag construct pET23a/LTβRdN, which can express the T7-tagged LT-βR coding region lacking the N-terminal 99 amino acid residues (designated T7-LTβRdN), was constructed by insertion of the DNA fragment spanning from nucleotide 465 to 1700 of the LT-βR gene into *Bam*HI-filled-in, *Eco*RI-digested pET23a (Novagen). Similarly, plasmid pET23a/HCVc, a derivative of pET23a harboring the 0.6-kb full-length HCV core gene (*Acc*I-*Fsp*I) was constructed by insertion of the HCV core gene fragment (Klenow filled in) into the *Sal*I site (Klenow filled in) of the vector pET-23a. When linearized with *Hin*dIII or *Cla*I and transcribed with T7 RNA polymerase, the transcripts encoded the T7-tagged 195 (T7-C195) or 122 (T7-C122) amino acid residues of the HCV core protein, respectively. Plasmid pGST/HCVc122Δ(41-107), containing an in-frame deletion of amino acid residues 41 to 107 of the HCV core protein portion in the GST-HCVc122 fusion protein, was constructed by partial digestion of pGST/HCVc122 (80) with *Apa*I and religation of the vector. The plasmid pHβlacINLSneo (50), which carries a G418 selection marker and utilizes the human β-actin promoter to direct transcription of the *lacINLS*-containing gene, was kindly provided by H.-S. Liu. The plasmid pSVlacOC195 was constructed by insertion of the *Bam*HI (filled in)-*Hin*dIII fragment of the HCV core gene derived from pECE/HCVcKF (80) into the *Xho*I (filled in)-*Hin*dIII-digested pSVlacOZ vector (30). In this construct the expression of the HCV core protein is under *lac* operator negative regulation, and the expression of HCV core protein is inducible in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) (final concentration, 20 mM).

Yeast two-hybrid cloning system. DNAs encoding various lengths of the HCV core protein were cloned into the yeast GAL4 DNA binding domain vector pGBT9. The resulting plasmids pGBT/HCVc195, pGBT/HCVc122, and pGBT/HCVc101 were used as baits in two-hybrid screens of human liver cDNA libraries (Clontech) by the MATCHMAKER Two Hybrid System protocol (Clontech). Positive yeast clones were selected by prototrophy for histidine and expression of β-galactosidase. A total of 2×10^6 transformed colonies were plated. The LacZ$^+$ His$^+$ clones were isolated and retransformed alone or with the pGBT/HCVc195, pGBT/HCVc122, or pGBT/HCVc101 derivative. The library clones that activate the *lacZ* reporter gene only in the presence of the pGBT9 derivative of the HCV core gene were chosen for sequencing.

cDNA cloning. The LT-βR cDNA insert of approximately 0.5 kb from the yeast two-hybrid clone CAP12 was used as a probe to screen human HepG2 cDNA libraries in λgt11 (Stratagene) by standard methods (75). The nucleotide sequence of the positive clone containing the 1.3-kb LT-βR cDNA insert was determined by standard methods (75).

Assay for β-galactosidase activity. Yeast transformants harboring both GAL4 binding and activation domain fusion proteins were assayed for β-galactosidase activity by the filter and liquid assay methods. For the filter assay, yeast transformants were transferred to nitrocellulose filters, permeabilized in liquid nitrogen, and placed on Whatman no. 1 filter paper that had been soaked with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) containing 0.33 mg of 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-Gal) per ml at 30°C. For the liquid culture assay, cultures were grown overnight to mid-log phase. Cells were then snap frozen in liquid nitrogen, thawed at 37°C, and then assayed for β-galactosidase activity with *o*-nitrophenylgalactoside as the substrate. The measurement of β-galactosidase activity was by the method of Miller (57).

In vitro transcription and translation. In vitro transcription of RNA templates for translation of LT-βR or HCV core protein in rabbit reticulocyte lysates was performed under standard in vitro transcription conditions (Promega). The plasmids pET23a/LTβRdN and pET23a/HCVc were derived from the pET23a plasmid (Novagen) and served as DNA templates for transcription with T7 RNA polymerase. RNA templates were purified by using phenol-chloroform extraction and ethanol precipitation. Optimal amounts of synthetic RNAs (4 to 8 μg for HCV core protein translation and 0.5 to 1 μg for LT-βR translation) were subjected to in vitro translation by incubating the RNA with rabbit reticulocyte lysate (Promega) that contained [^{35}S]methionine (>1,000 Ci/mmol; 10 mCi/ml) at a final concentration of 0.7 mCi/ml.

Cell culture. Human hepatocellular carcinoma cell lines HuH-7 and HepG2 and human cervical carcinoma cell line HeLa were cultured at 37°C under 5% CO_2 and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 mM nonessential amino acids, 2 mM L-glutamine, penicillin-streptomycin (100 mg/ml), and amphotericin B (0.25 mg/ml). The human adenocarcinoma cell line HT-29 obtained from the Cell Bank of Verterans General Hospital-Taipei, was cultured in modified Eagle's medium with Earle's salts, 10% fetal calf serum, glutamine, penicillin-streptomycin, and nonessential amino acids. Cells were grown to confluence, scraped from plates, and harvested for preparation of cell extracts. In brief, cells were washed with 2 volumes of phosphate-buffered saline and lysed in NETNT (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 7.5], 0.5% Nonidet P-40, 0.5% Tween 20) with gentle rocking at 4°C for 3 h. After cells lysed, chromosomal DNA was fragmented by using a 1-ml syringe with a 25-gauge needle. Fragmented DNA and cell debris were removed from the cell extract by centrifugation at $15,000 \times g$ for 15 min at 4°C.

Establishment of HCV core protein-producing cell lines. HepG2 cells harboring the inducible full-length HCV core gene fragment were established according to methods described previously (50). HepG2 cells (seeded at 1.8×10^6 cells per 100-mm-diameter plate) were cotransfected with plasmids pSVlacOC195 (20 μg) and pHβlacINLSneo (2 μg) by the calcium phosphate coprecipitation method (31). Geneticin (G418; Sigma) at 1 mg/ml was added to the culture medium at 48 h posttransfection, and stable transfectants were then selected for 2 weeks. Four clones were isolated and expanded in the absence of IPTG. One of the stable transfectants, designated HepG2/C195, expressed the HCV core protein after induction with 20 mM IPTG for 48 h and was chosen for this study. HeLa and HuH-7 cells constitutively expressing the HCV core protein (designated HeLa/C190 and HuH-7/C190, respectively) were established by using the retrovirus vector containing the full length of the HCV core gene fragment (38).

Expression of GST/LTβR(338-435) and GST/HCV core fusion proteins and preparation of antiserum against GST/LTβR(338-435). LT-βR and HCV core protein were expressed individually as GST fusion proteins from the expression vector pGST/LTβR(338-435), pGST/HCVc122, or pGST/HCVc122Δ(41-107). Expression and purification of the GST fusion proteins were performed as described previously (80). The GST/LTβR(338-435) hybrid protein, after being purified by affinity chromatography on a glutathione-Sepharose 4B (Pharmacia) column, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel), and the purified proteins were used for immunization of rabbits (Fig. 1A). The immunospecificities of antisera against GST/LTβR(338-435) for detection of LT-βR were examined by immunoprecipitation of in vitro-translated T7-LTβRdN, as shown in Fig. 1B.

In vitro binding analysis. For each in vitro binding assay, 20 μl of glutathione-Sepharose 4B beads (Pharmacia) bound to the appropriate GST fusion protein (4 μg) was incubated with in vitro-translated mixtures, or with cell extracts (500 μg) prepared from the HepG2 cells, at 4°C for overnight with gentle rotation. The beads were washed four times with 1 ml of NETNT. Proteins bound on the beads were eluted with sample buffer (48), fractionated by SDS-PAGE, and detected either by autoradiography or by Western blot analysis. Detection of LT-βR was performed with rabbit anti-GST/LTβR(338-435) serum (1:1,000 dilution) as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (DAKO) (1:2,000 dilution) as the secondary antibody by the enhanced-chemiluminescence detection method (Amersham).

Cytotoxicity assays. The cytotoxicities of LT-$\alpha_1\beta_2$ in various cell lines were determined essentially as described previously (14). In brief, cells were seeded into 96-well microtiter plates at 5×10^3 cells/well, and serial dilutions of soluble forms of the recombinant ligand LT-$\alpha_1\beta_2$ (kindly provided by J. L. Browning [Biogen]) were added to cell medium containing 50 U of gamma interferon (R & D Systems) per ml. After 3 days, the surviving cells were quantitated by the MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] conversion assay (Boehringer Mannheim Biochemicals) for HT29, HepG2, and HuH-7 cells (14). In the case of HeLa cells, cell viability was determined by the crystal violet staining method (20% methanol and 1% crystal violet) (92). In brief, the dye

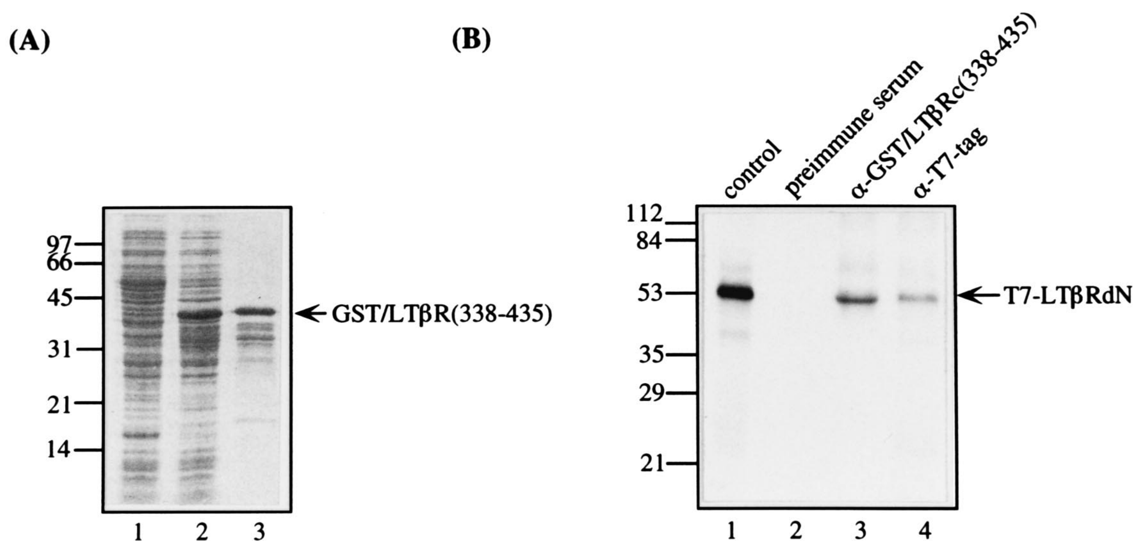

FIG. 1. Immunospecificity of antisera against GST/LTβR(338-435). (A) Expression and purification of GST/LTβR(338-435). *E. coli* DH5α harboring pGST/LTβR(338-435) was cultured in the absence (lane 1) or presence (lane 2) of IPTG (see Materials and Methods), and cell lysates were prepared by solubilization in sample buffer (48), subsequently analyzed by SDS-PAGE (12% gel), and stained with Coomassie brilliant blue. The GST/LTβR(338-435) hybrid protein (lane 3) was purified from cell extracts by using glutathione-Sepharose beads. (B) Characterization of the immunospecificity of antisera against GST/LTβR(338-435). In vitro-translated T7-LTβRdN was immunoprecipitated with preimmune serum (lane 2), rabbit antiserum against GST/LTβR(338-435) [α-GST/LTβR(338-435)] (lane 3), and anti-T7-tag monoclonal antibody (α-T7-tag) (lane 4). The precipitates were analyzed by SDS-PAGE (12% gel) and autoradiography. The control (lane 1) was in vitro-translated T7-LTβRdN before immunoprecipitation. The positions of prestained molecular mass markers are indicated (in kilodaltons) on the left of each panel.

from the stained cells was eluted with methanol for 15 min, and the optical density at 550 nm was measured with an enzyme-linked immunosorbent assay plate reader. The cytotoxicity of TNF alpha (TNF-α) in various cell lines was examined as previously described (92). Briefly, cells were seeded into 96-well microtiter plates at 10^4 cells/well and allowed to grow overnight, and subsequently, serial dilutions of TNF-α in the presence of cycloheximide (10 μg/ml) were added. After 20 to 24 h of incubation at 37°C, the viable cells were quantitated either by the MTT assay or by the crystal violet staining method.

RESULTS

Identification of LT-βR interacting with HCV core protein by use of the yeast two-hybrid system. To identify cellular factors that directly interact with the HCV core protein, the yeast two-hybrid system was used. The GAL4 DNA binding domain fused with full-length (pGBT/HCVc195) or truncated (pGBT/HCVc122 and pGBT/HCVc101) versions of the core protein was used as bait for yeast two-hybrid screening of a human liver cDNA library (MATCHMAKER; Clontech) (see Materials and Methods). Approximate 2×10^6 yeast strain HF7c transformants were screened for the $LacZ^+$ His^+ phenotype. Ten positive clones (as pGAD fusion constructs) were obtained by using truncated versions of the core protein, but not the full-length core protein, as bait. The cDNA sequences of positive clones were determined and aligned with the EMBL data bank. Six of 10 cDNA clones were found to encode the cytoplasmic domain (amino acid residues 338 through 435) of LT-βR (initially referred as TNFRrp) (4) (GenBank Accession number L04270). This cDNA clone, designated CAP12 (for core association protein 12), displayed β-galactosidase activity only when cotransformed with HCV constructs (pGBT/HCVc122 and pGBT/HCVc101) (Table 1). The β-galactosidase activity assay also suggested that the core–LT-βR interaction is weaker than the interactions observed for SV40 T antigen (pTD1) and p53 (pVA3) in the yeast two-hybrid system (Table 1). This result, which suggests interaction between the cytoplasmic tail of LT-βR and the truncated forms of the HCV core protein but not the full-length protein, is consistent with the previous finding of Matsumoto et al. (56).

The LT-βR cDNA fragment (0.5 kb) of the CAP12 clone was used as a probe to further screen for other cDNA clones of LT-βR in a human HepG2 cDNA library (see Materials and Methods). One such cDNA clone, encoding amino acid residues 100 to 435 of LT-βR, was obtained. For further biochemical studies, this LT-βR fragment was fused in frame with a T7 tag (designated T7-LTβRdN). Figure 1 shows that this in vitro-translated T7-LTβRdN could be immunoprecipitated by the anti-T7 tag antibody or by the antibody prepared against the fusion protein between GST and the C-terminal residues 338 to 435 of LT-βR [designated GST/LTβR(338-435)]. Notably, this in vitro-translated T7-LTβRdN migrated as a 53-kDa species in SDS-PAGE, which is rather different from the expected molecular mass of 38 kDa. Since this recombinant protein contains the cysteine-rich region and transmembrane domain of the receptor (90), these properties may contribute to its abnormal migration in SDS-PAGE.

In vitro binding analysis demonstrates that the full-length HCV core protein can associate with the C terminus of LT-βR. To confirm the results from the yeast two-hybrid system, the in

TABLE 1. Yeast two-hybrid system for analysis of the interaction between HCV core protein and CAP12

pGBT fusion	pGAD fusion	X-Gal filter assay result[a]	β-Galactosidase activity (U)[a]
pVA3	pTD1	Blue	13.5
pGBT/HCVc195	—[b]	White	<0.1
pGBT/HCVc122	—	White	<0.1
pGBT/HCVc101	—	White	<0.1
pGBT/HCVc195	CAP12	White	<0.1
pGBT/HCVc122	CAP12	Blue	7.7
pGBT/HCVc101	CAP12	Blue	ND[c]
—	CAP12	White	<0.1

[a] See Materials and Methods.
[b] —, none.
[c] ND, not determined.

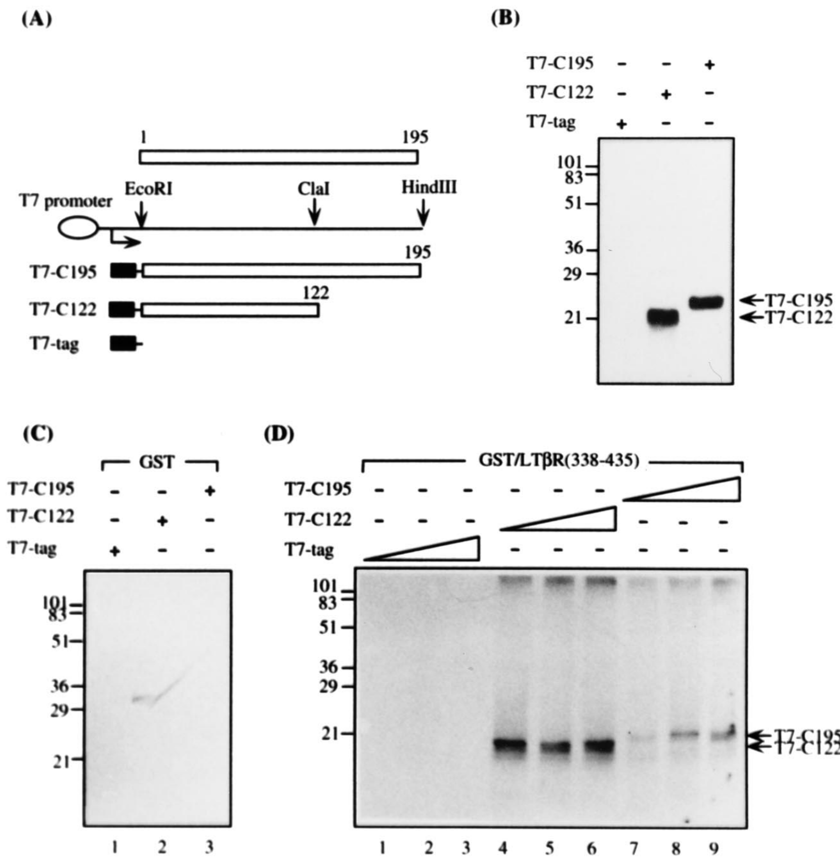

FIG. 2. In vitro binding analysis of HCV core protein and the C-terminal region of LT-βR. (A) Schematic diagram of the in vitro-translated, T7-tagged products of HCV core protein used in the binding assay (B) The ^{35}S-labeled T7 tag or T7-tagged HCV core proteins (T7-C195 and T7-C122) prepared by in vitro transcription and translation reactions were analyzed by SDS-PAGE (13.5% gel) and autoradiography. (C) Analysis of the binding between the T7-tagged HCV core proteins and GST. The in vitro-translated, T7-tagged HCV core protein. (T7-C195 or T7-C122) or T7 tag (30 μl each) was incubated with GST-prebound glutathione-Sepharose beads, and the bound fractions were analyzed by SDS-PAGE (13.5% gel) and autoradiography. (D) Analysis of the binding between the T7-tagged HCV core proteins and GST/LTβR(338-435). Various amounts (7.5, 15, or 30 μl) of in vitro-translated mixtures of T7-tagged HCV core proteins or T7 tag were incubated with GST/LTβR(338-435)-prebound glutathione-Sepharose beads, and the bound fractions were analyzed by SDS-PAGE (13.5% gel) and autoradiography. The positions of T7-C195 and T7-C122 are indicated. Molecular masses (in kilodaltons) are indicated on the left in panels B to D.

vitro binding properties of the HCV core protein and LT-βReceptor were examined. The fusion protein between GST and the C-terminal residues 338 to 435 of LT-βR, GST/LTβR(338-435), was affinity purified on glutathione-Sepharose and tested for binding to a ^{35}S-labeled, T7-tagged full-length HCV core protein (T7-C195) or the truncated version of the HCV core protein (T7-C122) (Fig. 2A and B). Both the full-length (T7-C195) and the truncated version (T7-C122) of the in vitro-translated HCV core protein could associate with GST/LTβR(338-435) (Fig. 2D, lanes 4 to 9). This association is rather specific, since neither of these two forms of HCV core protein could associate with GST protein (Fig. 2C, lanes 2 and 3) and since no binding between the in vitro-translated T7 tag and GST or GST/LTβR(338-435) occurred (Fig. 2C, lane 1, and D, lanes 1 to 3). The binding between the full-length HCV core protein and the LT-βR(338-435) protein is weaker than that for the truncated version of the HCV core protein, as reflected from the relative intensities of the HCV core protein precipitated by the glutathione beads (Fig. 2D, compare lanes 4 to 6 with lanes 7 to 9). Therefore, our in vitro binding assay suggested that both the full-length and truncated forms of the HCV core protein can associate with the C-terminal 98-amino-acid segment of LT-βR but with different affinities. This lower binding affinity of the full-length HCV core protein to the LT-βR cytoplasmic domain may account for the failure to detect their interaction in the yeast two-hybrid assay (Table 1), although it can be also due to the in vivo compartmentalization of the full-length HCV core protein in the endoplasmic reticulum membrane, as pointed out previously (52, 56, 76, 77).

The interaction domain for HCV core protein and LT-βR maps to the N-terminal 40-amino-acid segment of HCV core protein. To examine whether the HCV core protein could interact with endogenous full-length LT-βR and to further delineate the functional domain of the HCV core protein responsible for this interaction, affinity chromatography in combination with Western blot analysis was performed. As shown in Fig. 3B, when HepG2 cell lysates were loaded onto glutathione beads that were prebound with GST, GST/HCVc122, or the internal deletion construct GST/HCVc122Δ(41-107) and the bound fractions were examined by immunoblotting with antiserum against GST/LTβR(338-435), two immunoreactive species of 75 and 60 kDa were found to associate with GST/HCVc122 and GST/HCVc122Δ(41-107) beads but not with GST beads. Additional immunoreactive bands, which presumably corresponded to the GST/HCV core fusion proteins or GST, were also present in the eluants, since the antibody was raised against the GST fusion protein. The 75-kDa immunoreactive species, which was also detected in total cell lysates

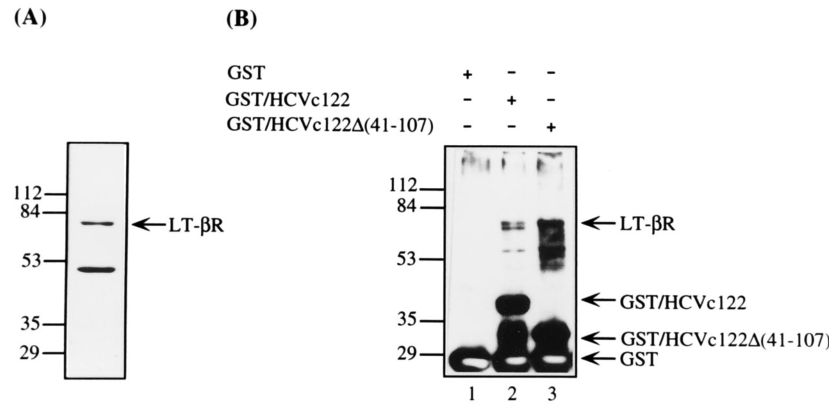

FIG. 3. In vitro binding analysis of HCV core protein and endogenous LT-βR. (A) Endogenous LT-βR in HepG2 cells was detected by Western blotting with antiserum against GST/LTβR(338-435) (see Materials and Methods). (B) In vitro binding analysis of endogenous LT-βR and the various truncated forms of HCV core protein. HepG2 cell lysates retained on GST-, GST/HCVc122-, or GST/HCVc122Δ(41-107)-prebound glutathione-Sepharose were immunoblotted with antiserum against GST/LT-βR(338-435) (see Materials and Methods). The positions of the endogenous LT-βR, GST/HCVc122, GST, and GST/HCVc122Δ(41-107) are indicated. Molecular masses (in kilodaltons) are indicated on the left in each panel.

of HepG2 cells (Fig. 3A), probably represents a full-length LT-βR possessing the glycosylation modification, cysteine-rich region, and transmembrane domain and therefore displays abnormal migration (33). Another 50-kDa immunoreactive protein species was also detected in the HepG2 cell extracts (Fig. 3A). The exact molecular nature of these smaller immunoreactive species (60 and 50 kDa) is unknown. They probably represent the shed form of this receptor released by proteolytic cleavage, a property commonly observed in most members of the TNFR family (1, 33, 90). If this is the case, our results suggest that both the N-terminal 40- and 122-amino-acid fragments of the HCV core protein possess binding affinity to the endogenous full-length and truncated forms of LT-βR. Interestingly, the results also suggested that the N-terminal 40-amino-acid segment of the HCV core protein has much stronger binding affinity than the larger 122-amino-acid segment, implying that the major interaction domain of the HCV protein with LT-βR lies in its N-terminal 40-amino-acid segment.

The HCV core protein can associate with the dimeric or oligomeric form of the LT-βR cytoplasmic domain. Since, similar to the case for the TNFR family, the cytoplasmic domain of LT-βReceptor is very likely the oligomerization domain of the receptor (12, 82) and is probably also the interacting region for cellular association factors engaged in the receptor's signaling pathway (6), it would be interesting to know whether the core protein of HCV could interfere with the receptor's self-interaction or perturb its interaction with other cellular factors. By using yeast two-hybrid screening, 45 positive clones (as pGAD fusion constructs) from a human liver cDNA library were identified as candidates that could associate with the cytoplasmic domain residues 347 to 435 of LT-βR [as pGBT fusion construct pGBT/LTβR(347-435)] (Table 2). Surprisingly, all of these positive clones identified by this method contained the C terminus of LT-βR itself, encompassing amino acid residues 271 to 435, 277 to 435, or 338 to 435, indicating a strong tendency for homotypic association. No cDNA clones encoding other cellular factor was found in this screening assay. The β-galactosidase activity assay indicated that the interaction strengths of these different C-terminal portions of LT-βR were comparable to that of the interaction between SV40 T antigen (pVA3) and p53 (pTD1) but at least two to four times stronger than the interaction between the HCV core protein (pGBT/HCVc122 construct) and LT-βR(338-435) [pLTβR(338-435)] (Table 2). Therefore, the self-association affinity between the cytoplasmic domains of LT-βR is stronger than its affinity to the HCV core protein. To further confirm the self-association of LT-βR in yeast, the in vitro glutathione bead pull-down binding assay was also performed. As shown in Fig. 4, the in vitro-translated T7-LTβRdN could associate with GST/LTβR(338-435) but not with GST. Taken together, these results demonstrated that the minimal region for receptor self-interaction is located at the C-terminal amino acid residues 347 to 435 of LT-βR, which is within the region (residues 338 to 435) interacting with the HCV core protein as defined by the in vitro binding assay (Fig. 2).

We next examined whether the HCV core protein can associate with the oligomeric forms of the receptor. In vitro-translated, ^{35}S-labeled, T7-tagged HCV core proteins, T7-C122 and T7-C195 (Fig. 2B), were used to address this question. As shown in Fig. 5, when increasing amounts of T7 tag (lanes 2 and 3), T7-C122 (lanes 4 and 5), and T7-C195 (lanes 6 and 7) were added to GST/LTβR(338-435) beads that were prebound with radiolabeled T7-LTβRdN, the T7-C122 and T7-C195 signals, but not the T7 tag, were retained on the GST/LTβR(338-435) beads. These results indicated that the HCV core protein also can associate with the dimeric or oligomeric form of the LT-βR cytoplasmic domain. Additionally, since similar intensities of T7-LTβRdN were retained in the complex in the presence or absence of the HCV core protein (compares lane 1 with lanes 4 to 7 in Fig. 5), this implies that the HCV core

TABLE 2. Self-interaction of LT-βR and interaction of LT-βR with HCV core protein

pGBT fusion	pGAD fusion	X-Gal filter assay result[a]	β-Galactosidase activity (U)[a]
pVA3	pTD1	Blue	13.5
pGBT/LTβR(347-435)	—[b]	White	<0.1
pGBT/LTβR(347-435)	pLTβR(338-435)	Blue	30.5
pGBT/LTβR(347-435)	pLTβR(277-435)	Blue	19.5
pGBT/LTβR(347-435)	pLTβR(271-435)	Blue	17.2
—	pLTβR(338-435)	White	<0.1
—	pLTβR(277-435)	White	<0.1
—	pLTβR(271-435)	White	<0.1
pGBT/HCVc122	pLTβR(338-435)	Blue	7.5

[a] See Materials and Methods.
[b] —, none.

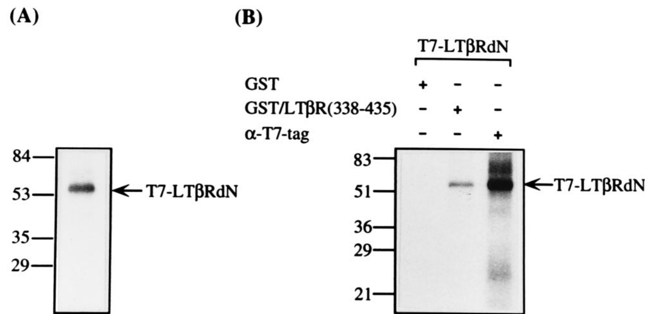

FIG. 4. Self-association of the intracellular domain of LT-βR in vitro. (A) In vitro-translated T7-LTβRdN (1 μl), used for the self-interaction binding assay, was analyzed by SDS-PAGE and autoradiography. The in vitro-translated T7-LTβRdN was prepared essentially as described in the legend to Fig. 1. (B) In vitro binding assay of GST/LTβR(338-435) and T7-LTβRdN. Glutathione-Sepharose beads (20μl) bound to GST or GST/LTβR(338-435) (4 μg) were incubated with in vitro-translated T7-LTβRdN (5 μl). The beads were then washed, and proteins on the beads were eluted with sampling buffer and analyzed by SDS-PAGE (12% gel) and autoradiography (see Materials and Methods). The T7-LTβRdN immunoprecipitated with T7-tag monoclonal antibody (α-T7-tag) served as a positive control. Molecular masses (in kilodaltons) are indicated on the left of each panel.

protein cannot interfere with the self-interaction between GST/LTβR(338-435) and T7-LTβRdN. More likely, the HCV core protein can form a ternary complex with the oligomeric form of the LT-βR cytoplasmic domain.

The HCV core protein can modulate the cytotoxic effect of the LT-βR signaling pathway in HeLa cells. According to recent reports, LT-βR is involved in cytotoxic effects in certain cell lines, including the human adenocarcinoma cell line HT29, when treated with recombinant ligand LT-$\alpha_1\beta_2$ (13, 14). To determine whether the complex association between the HCV core protein and LT-βR can modulate the cytotoxic effects of LT-βR, several HCV core protein-producing cell lines was treated with various amounts of LT-$\alpha_1\beta_2$ ligand in the presence of the synergizing agent gamma interferon (see Materials and Methods). These stable HCV core protein-producing cell lines examined in this study included the HepG2/C195, HuH-7/C190, and HeLa/C190 cell lines (see Materials and Methods for procedures used for cell line establishment). Immunoblot analysis confirmed the expression of the full-length HCV core protein in these cell lines (Fig. 6A). Notably, immunoblots also suggested that the production of HCV core protein did not alter the expression level of the full-length LT-βR in various cell lines, including the stable transfectants HeLa/C190 and HuH-7/C190 and the inducibly expressing cell line HepG2/C195, compared to their parental cell lines (Fig. 6B). Examination of the cytotoxic effect of the LT-$\alpha_1\beta_2$ ligand indicated that although the LT-$\alpha_1\beta_2$ ligand was cytotoxic to HT29 cells when added at 100 ng/ml (more than 20% killing effect) or 1 μg/ml (more than 70% killing effect), as reported previously (14), no significant cytotoxic effect was detected in the hepatoma cells (HuH-7 and HepG2) with or without expression of the HCV core protein, even at the highest concentration tested (1 μg/ml) (Fig. 7A). However, the LT-$\alpha_1\beta_2$ ligand at 100 ng/ml or 1 μg/ml was cytotoxic to the HeLa cells, and the expression of HCV core protein enhanced the cytolytic effects of the ligand by about 10 to 24% (Fig. 7B). These results suggested that the HCV core protein can modulate the cytotoxic effect of LT-βR in a cell line-specific manner.

To distinguish whether this enhancement of the cytotoxic effect exerted by the HCV core protein is cytokine specific, a similar cytotoxicity test with TNF-α was performed on these cell lines. Our results suggested that in the presence of cycloheximide (10 μg/ml), although TNF-α could elicit a cytolytic effect on HeLa or hepatoma cell lines at a concentration of around 0.1 to 100 ng/ml, the expression of the HCV core protein did not cause any significant increase in the cytotoxic effect (Fig. 8). Thus, the modulation of the cytotoxic effect of LT-$\alpha_1\beta_2$ by the HCV core protein is LT-βR specific.

DISCUSSION

The core protein of HCV is a multifunctional protein involved in several processes; it is phosphorylated (79) and has both cytoplasmic and nuclear localization (52, 76, 80), and thus it may play multiple roles in the viral life cycle. Several studies also suggested that it has regulatory roles for viral and cellular genes and possesses transformation activity (63, 64, 79, 80). More recent studies revealed that the core protein can interact with viral envelope protein 1 (E1) (53) and also forms a complex with apolipoprotein AII of the lipid droplet (7). In this study, we likewise observed interaction between the HCV core protein and LT-βR, as reported by another group (56). Moreover, we further demonstrated that this heteromeric protein interaction is weaker than the self-association of the receptor cytoplasmic domain (Table 2) and thus does not disrupt the homotypic interaction of LT-βR itself (Fig. 5), which presumably is important for initiation of the signal pathway of the receptor following the ligand stimulation. Additionally, our results suggested that the interaction of these two proteins modulates the cytolytic activity of LT-βR triggered by its recombinant ligand in HeLa cells. This finding adds another property to those of the HCV core protein and reinforces the notion that the core protein of HCV has a pleiotropic effect on virus-infected cells.

Most animal viruses have evolved strategies to evade or delay apoptosis to allow viral replication and assembly (reviewed in references 78 and 83). For example, many viruses have evolved genes encoding proteins which effectively sup-

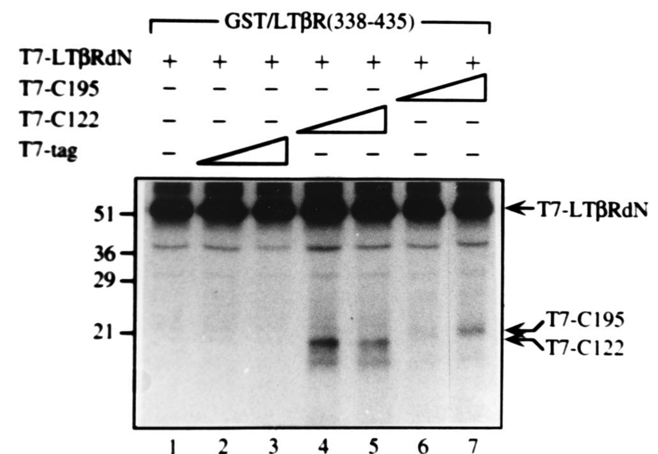

FIG. 5. Analysis of complex formation between dimeric or oligomeric LT-βR and HCV core protein. In vitro-translated T7 tag (lanes 2 and 3), T7-C122 (lanes 4 and 5), and T7-C195 (lanes 6 and 7) in the presence of T7-LTβRdN (5 μl) (lanes 2 to 7) were loaded onto glutathione-Sepharose beads (20 μl) that were prebound with GST/LTβR(338-435) (4 μg). The amounts of T7-tag, T7-C121, and T7-C195 used were 15 μl of in vitro-translated mixtures in lanes 2, 4, and 6 and 30 μl in lanes 3, 5, and 7. Lane 1, no addition of T7-tagged protein. The precipitates of the glutathione-Sepharose beads were analyzed by SDS-PAGE (13.5% gel) and autoradiography. The positions of T7-C195, T7-C122, and T7-LTβRdN are indicated. The loading amount in lane 4 was slightly higher than that in lane 5 as reflected by the individual intensities of the unspecific band. Molecular masses (in kilodaltons) are indicated on the left.

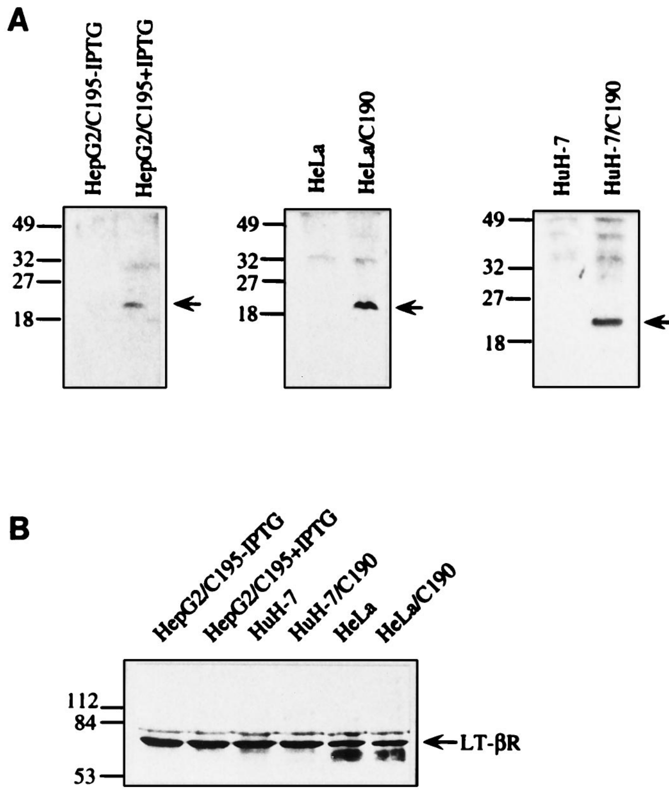

FIG. 6. Expression of LT-βR in HCV core protein-producing cell lnes. Total cell extracts (60 μg) prepared from HeLa, HeLa/C190, HuH-7, HuH-7/C190, or HepG2/C195 cells in the absence or presence of IPTG induction (20 mM) were examined for the expression level of HCV core protein (A) or LT-βR (B) immunoblotting. The antibodies used for detection of HCV core protein in panel A are either from human patient sera for HepG2/C195 cells or rabbit antisera against HCV core protein (80) for HeLa and HuH-7 cells. For detection of LT-βR, rabbit anti-GST/LTβR(338/435) serum was used as the primary antibody (see Materials and Methods). The expected molecular size of the full-length LT-βR (LT-βR) is indicated. Molecular masses (in kilodaltons) are indicated on the left.

press apoptosis long enough for the production of high yields of progeny virus. In addition, several viruses are known to induce apoptosis actively at later stages of infection (83). This process may represent a very efficient mechanism by which the virus can disseminate progeny to neighboring cells while also evading induction of host inflammatory and immune responses. Such virus-induced apoptosis may also contribute to clinical manifestations of viral disease. In view of these facts, in order to understand the pathogenesis of HCV, it seems imperative to know which viral gene products contribute to the protection from cell death to establish lifelong persistence of HCV and which products may induce apoptotic cell death at the late stage of HCV infection. Since the core protein is the first HCV protein to be expressed after infection, presumably the virus adapts this protein to antagonize apoptosis before an antiviral defense is instituted by the host cell. A recent study by Ray et al. (65) indicated that the HCV core protein can suppress the cisplatin- and c-Myc-mediated apoptotic effect, supporting its role in the establishment of persistent HCV infection. However, our present work and the work from another laboratory (71) indicate that the core protein also has the ability to enhance cell death triggered by LT-βR ligand or anti-Fas monoclonal antibody. Based on these findings, it is clear that the core protein of HCV may employ different modes of action to either inhibit or induce cell death mediated by different inducers acting through multiple pathways in different cell types or at different stages of viral infection. It remains unclear whether the HCV core protein is the only viral protein that may regulate the cellular death response in the HCV life cycle during infection. Considering that the protease domain of NS3 protease has transformation ability (74), it is very likely that this viral protein may also have a role in regulation of cellular growth or death events in HCV-infected cells. The use of multiple viral proteins that either induce or inhibit cell death is not unique for HCV (78, 83). Adenovirus has several such proteins (83). The E1A protein can trigger apoptosis by repressing the transcription of genes that are required for sustained cell growth (22), and the E1B proteins (19 and 55 kDa) counteract apoptosis by stimulating expression of a subset of these cellular genes (22, 72, 83). The E1A protein also renders cells susceptible to lysis by the inflammatory cytokine TNF (17, 25); nevertheless, this TNF-induced cytolysis is inhibited independently by the E1B 19-kDa protein and several E3 proteins to allow virus replication (24, 28, 29, 46, 91).

The molecular mechanism by which the HCV core protein enhances the cytolytic activity of LT-βR is still unknown. Several possibilities have emerged. Given the facts that the intracellular region of LT-βR, like those of TNFR and Fas, probably does not possess any domain with intrinsic catalytic activity and that the signaling pathway of LT-βR is mediated through receptor-associated factors (6, 55, 60, 69, 70, 89, 91), presumably the association of the core protein and the LT-βR cytoplasmic domain potentiates but does not disrupt the interaction of these signal transducers. Alternatively, since the core protein has the ability to regulate gene expression, one may speculate that it enhances apoptosis by inducing the expression of cellular factors that favor death or by repressing the expression of a subset of protective cellular gene products that favor survival. The latter possibility may likewise explain the opposite effect of the HCV core protein on the cytolytic activity stimulated by different cytokines or inducers, as mentioned in the previous section.

With so many cytokines involved in regulation of the cellular response to viral infection, the question whether the core protein may also modulate the cytolytic activity of TNF deserves attention. TNF is a pleiotropic cytokine involved in cellular growth, differentiation, and inflammatory and antiviral effects in virus-infected cells (reviewed in references 1, 35, 81, and 84). Our finding that the HCV core protein did not elicit an enhancement of TNF-induced cytolytic activity (Fig. 8) suggests that the molecular mechanism for the enhancement of the cytolytic activity of LT-βR by the HCV core protein (Fig. 7) probably is not mediated through the same pathway used by the TNFR signaling. This explanation is justified since the cytoplasmic region of LT-βR has little sequence similarity with other members of the receptor family (21, 81, 90), suggesting that the mechanism used to signal cellular responses by LT-βR may diverge from that for TNFR. In accordance with this hypothesis, it is well established that TNF signaling of cell death is mediated mainly by the association of TNFR type I with the cellular factor TRADD (41), which then recruits the FADD molecule of the Fas-associated factor (18, 40) or the RIP molecule (39) and activates the downstream ICE-like caspase activity to generate signals which are partially distinct from the Fas-triggered cell killing (6, 59, 84, 89, 92). In the case of LT-βR, the exact molecules responsible for signaling its killing effect have not yet been determined. However, it is well known that the cell death pathways triggered by Fas ligand, TNF, and LT-βR ligand do not overlap completely, since TNF and LT-βR ligand but not Fas ligand can trigger another sig-

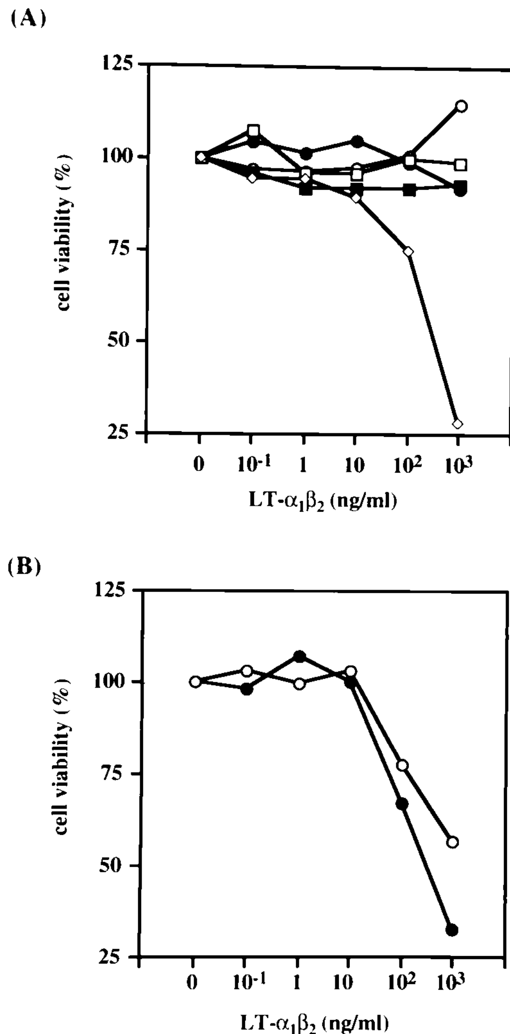

FIG. 7. Cytotoxic effects of LT-βR ligand (LT-$\alpha_1\beta_2$) on various HCV core protein-producing cell lines. (A) Comparison of the cytotoxic effects of various amounts of LT-$\alpha_1\beta_2$ on HT29 cells (◇), HuH-7 cells (□), HuH-7/C190 cells (■) and HepG2/C195 cells in the presence (●) or absence (○) of IPTG induction. Serial dilutions of recombinant LT-$\alpha_1\beta_2$ were added to the cultured cells (5×10^3 cells in a 96-well plate) in medium containing gamma interferon (50 U/ml). After 3 days, the surviving cells were quantitated by the MTT assay method (see Materials and Methods). For induction of HCV core protein expression in HepG2/C195 cells, 20 mM IPTG was added to the culture medium; induction was for 48 h. (B) Comparison of cytotoxic effects of LT-$\alpha_1\beta_2$ on HeLa (○) or HeLa/C190 (●) cells expressing the HCV core protein. Experimental conditions were similar to those described for panel A, except the surviving cells were quantitated by the crystal violet method (see Materials and Methods). The results shown were obtained in two separate experiments. All samples were set up in triplicate, and the values shown represent means.

naling pathway by activation of NF-κB (6, 55, 59, 60, 84, 89), a ubiquitous transcriptional factor involved in the regulation of diverse cellular genes, including antiapoptotic ones (5, 10, 51, 85, 88). Evidence is also emerging that in the activation of the NF-κB pathway, TNF and LT-βR ligand do not mediate through the same cellular-associated factors (TRAFs) (70). A novel TRAF5 factor, in addition to TRAF3, is involved in signaling NF-κB activation following LT-βR ligand stimulation (60), while in TNF signaling of NF-κB activation, TRAF1, TRAF2, and probably TRAF3 molecules, but not TRAF5, are involved (6, 58, 69, 84). Therefore, the molecular mechanism by which the HCV core protein enhances the signaling pathways of LT-βR probably is cytokine specific. Further examination of the effects on NF-κB activation of the HCV core protein in different cytokine-treated cells is needed to clarify this viewpoint.

The cell-type-specific response of the cytolytic activity of the LT-βR signaling exerted by the HCV core protein is rather intriguing. Our results suggest that this difference does not result from the influence on the LT-βR expression level in the HCV core protein-producing cell lines (Fig. 6). The question thus arises whether this lack of response to LT-βR ligand triggering may stem from a defect of the postreceptor-signaling pathway in these two particular hepatoma cell lines or whether it may represent an intrinsic feature of the hepatocyte response to the LT-βR signaling. Notably, previous studies also found that LT-βR triggers cytolytic activity or NF-κB activation following receptor ligation in some but not all LT-βR-positive cells (14). Although knowledge of the biological activity of LT-βR in hepatoma cells or hepatocytes is yet elusive, our observation of the disparity of the cellular responses of HeLa and hepatoma cells to LT-βR signaling presumably indicates the importance of Fas but not LT-βR as having a major role in mediating hepatitis (44). Support for this notion comes from several studies which indicated that Fas is overexpressed in HCV patients (37) and that the HCV core protein can sensitize Fas-induced apoptotic cell death in HepG2 hepatoma cells (71). In light of these findings and in conjunction with our current knowledge that LT-βR is broadly expressed in different tissues (including spleen, thymus, and other major organs), is involved in developmental regulation of peripheral lymphoid organs (23, 90), and participates in signaling cytolytic activity and NF-κB activation (14, 55, 60), one may speculate that the primary action of the HCV core protein mediated through its interaction with LT-βR in chronic HCV infection probably

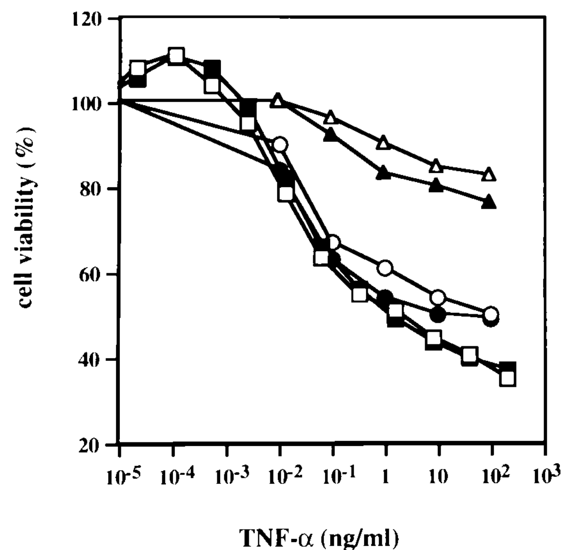

FIG. 8. Cytotoxic effects of TNF-α on various HCV core protein-producing cell lines. Serial dilutions of TNF-α were added to cultured cells (10^4 cells in a 96-well plate) in medium containing cycloheximide (10 μg/ml). After 20 to 24 h, the surviving cells were quantitated by the MTT assay or crystal violet method (see Materials and Methods). For induction of HCV core protein expression in HepG2/C195 cells, 20 mM IPTG was added to the culture medium 48 h prior to the cytotoxicity test. The results shown were obtained in two separate experiments. All samples were set up in triplicate, and the values shown represent means. Symbols: □, HepG2/C195 cells without induction; ■, HepG2/C195 cells with induction; ○, HeLa cells; ●, HeLa/C190 cells; △, HuH-7 cells; ▲, HuH-7/C190 cells.

occurs in extrahepatic tissues, resulting in aggravation of the immune function and consequently the association of immunopathologic syndromes with HCV infection (2, 3, 34, 42, 54, 87). Furthermore, since there is uncertainty about the biological significance of the interaction between the HCV core protein and LT-βR in hepatocytes, knowledge about a role for LT-βR in HCV-associated liver disease, if any, awaits further elucidation.

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