Hepatitis C Virus Core Protein Regulates Cell Growth and Signal Transduction Pathway Transmitting Growth Stimuli

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Received January 20, 1999, returned to author for revision February 17, 1999, accepted March 10, 1999

To investigate the transforming potential of hepatitis C virus (HCV), HCV core protein was produced in BALB/3T3 A31-I-1 cells. The cells expressing HCV core gene cooperatively with the v-H-ras gene showed loss of contact inhibition, morphological alterations, and anchorage-independent and serum-independent growth. The cells producing HCV core protein showed enhanced growth against stimulus of growth factor. In addition, antisense oligodeoxynucleotides against mRNA encoding HCV core protein suppressed the growth of HCV core-producing cells. Furthermore, HCV core protein activated mitogen-activated protein kinase and serum response element, which respond to growth stimuli. From these results, we concluded that HCV core protein is involved in the acquisition of cell growth advantage.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis and has been implicated in the etiology of hepatocellular carcinoma. HCV, a member of the Flaviviridae family, has a positive-strand RNA genome containing a large open reading frame (ORF) composed of over 9000 nucleotides. The precursor protein encoded in the ORF is cleaved by viral and cellular proteases and results in production of structural and nonstructural proteins. HCV core protein is encoded at the most 5'-terminal region of the ORF and is produced by cleavage between amino acids 191 and 192 of the precursor polyprotein by host signal peptidase (Clarke, 1997). In addition to the encapsidation of viral RNA, recent in vitro studies suggested that HCV core protein has other biological properties. It was reported that the promoter activity of c-myc, RSV LTR, SV-40 early promoter, and AP-1 is up-regulated in HCV core gene expressing cell lines (Ray et al., 1995; Chang et al., 1998; Shrivastava et al., 1998). It was also reported that HCV core protein trans-suppresses some cellular promoters, i.e., p53 and p21 (Ray et al., 1997, 1998a). Though the mechanisms of these regulations are still unclear, it is possible that these properties of HCV core protein influence host cell growth, survival, and carcinogenesis. Recently, pro- or anti-apoptotic properties of HCV core protein were reported (Zhu et al., 1998; Ray et al., 1998b). It was also shown that HCV core protein has transforming potential in rat embryo fibroblasts with or without active ras gene products (Ray et al., 1996; Chang et al., 1998), and transgenic mice expressing HCV core gene developed hepatocellular carcinoma (Moriya et al., 1998). However, the precise role of HCV core protein in these transformation processes remains to be elucidated. Therefore, we examined the contribution of HCV core protein during the development of transformation. In this study, we found that HCV core protein transformed a mouse fibroblast-derived cell line cooperatively with v-H-ras gene product. These transformed cells acquired a growth advantage in serum-free medium, and antisense oligodeoxynucleotides (ODN) that were designed to reduce the production of HCV core protein suppressed the growth of these cells. Furthermore, we found that HCV core protein enhanced the signal transduction pathway that mediates growth stimuli. These results suggested that HCV core protein probably affects the system of cell growth.

RESULTS AND DISCUSSION

Cooperative transformation of BALB/3T3 A31-I-1 cells by HCV core protein and v-H-ras gene products

Since our preliminary studies and a previous report showed that murine fibroblasts expressing the HCV core gene alone did not show any transformed features, we supposed that the transforming ability of HCV core protein is rather weak (data not shown; and Ray et al., 1996). To detect such weak transforming activity, we coexpressed the HCV core gene with another oncogene, v-H-ras. In many established murine fibroblasts, the ac-
tive ras gene product alone induces transformation. However, one of the subclones of BALB/3T3 cells, A31-I-1, is relatively resistant to ras-induced transformation and requires the presence of another oncogene, e.g., c-myc or MTG8, for transformation (Table 1; and Kakuand Crow, 1980; Sueoka et al., 1998). BALB/3T3 A31-I-1 cells were transfected with pLXSH-core191, which is an expression plasmid of the HCV core gene encoding 191 amino acids, with or without v-H-ras expression plasmid, pLXSN-ras. Several focus-forming colonies were obtained at 21 days posttransfection in three independent experiments as shown in Table 1. The number of foci was equivalent to that observed in experiments performed with myc and ras genes, while transfection with pLXSH-core191 alone did not show any foci (Table 1). From each cell population transfected with HCV core and ras, and with myc and ras, 20 foci were cloned for further analysis and were named Bcr and Bmr, respectively. The production of HCV core protein in the clones of Bcr cells was confirmed by immunoblotting using anti-HCV core monoclonal antibody. The HCV core protein was found in all these clones as a single band of 21 kDa, and no other form was detected (data not shown).

Cloned Bcr cells showed altered morphology and a disoriented pattern in culture dishes (Fig. 1A). We tested whether these cells could grow in semisolid medium of soft agar to determine their ability to undergo anchorage-independent growth. All the Bcr clones (20/20) formed colonies (Fig. 1A). We observed similar results with Bmr clones (data not shown).

In this series of experiments, we showed that HCV core- and ras-transfected cells lost contact inhibition of growth, showed altered morphology, and acquired anchorage-independent growth. These results suggested that HCV core protein transformed BALB/3T3 A31-I-1 cells cooperatively with the v-H-ras gene product.

**HCV core protein contributes to the acquisition of growth advantages of cells**

The growth advantages of the cells producing HCV core protein were examined. First, we prepared other BALB/3T3-derived cell lines, Bmock, Bc, and Br, stably transfected with LXSH, LXSH-core191, and LXSN-ras, respectively. Then, we compared the growth rates of Bcr and Bmr cells with those of Bmock, Bc, and Br cells. To exclude the possible effect of clonal selection, we examined mixed populations consisting of 10 clones from each cell. Cell proliferation was measured by MTT assay at 24-h intervals. As shown in Fig. 1B, the Bcr cells proliferated in serum-free medium as efficiently as Bmr cells. Bmock, Bc, and Br cells did not show any growth under the same conditions. We supposed that HCV core protein contributed to the acquisition of growth advantages of BALB/3T3 cells cooperatively with active Ras.

Since Ras is activated by the stimuli of growth factors, we examined whether HCV core protein enhances cell growth in growth factor-supplemented medium. Bc and Bmock cells were cultured in serum-free medium supplemented with 0, 0.1, and 1.0 μg/ml of epidermal growth factor (EGF) for 72 h and proliferation of cells under each condition was measured by MTT assay. Bc and Bmock cells did not proliferate in serum-free medium, and Bmock cells did not respond to EGF stimuli as well as the parental BALB/3T3 A31-I-1 clone (Fig. 1C; and Tatsuka et al., 1989). Bc cells were sensitive to EGF stimuli and grew effectively (Fig. 1C). It is suggested that HCV core does not stimulate cell proliferation without growth stimuli, but cooperates with growth factors or their intracellular downstream signals and enhances cell growth, at least in BALB/3T3 A31-I-1 cells. Previous reports showed that stably and inducibly expressed HCV core protein had no effect on cell growth (Moradpour et al., 1996; Tokushige et al., 1997). However, cell growth was assessed in serum-supplemented medium in these reports. Bc cells did not show any growth advantages in medium supplemented with 10% FBS (data not shown). They might have missed the property of HCV core protein in the acquisition of cell growth advantage under these conditions.

An antisense ODN against HCV core mRNA suppressed proliferation of the HCV core-producing cells

To investigate whether the HCV core protein actually contributes to the enhancement of cell proliferation, we attempted to specifically reduce the production of HCV core protein. Previous studies showed that antisense ODN targeting of the translational initiation site of the HCV core gene reduced HCV core protein production (Hanecak et al., 1996; Wakita and Wands, 1994). According to these results, a phosphorothioate ODN, AS(−)5-15, was designed complementary to the nucleotide sequence from 5 nucleotides upstream to 15 nucleotides downstream of the predicted translational initiation site of the HCV core gene. First, we verified the suppressive effect of AS(−)5-15 on HCV core protein production. When Bcr cells were incubated with 50 μM AS(−)5-15,
FIG. 1. Transformation of BALB/3T3 A31-I-1 cells by v-H-ras and HCV core protein. (A) Morphology on culture dishes (left column) and anchorage-independent growth in soft agar (right column). Magnification, ×40. (B) Serum-independent growth. A total of $5 \times 10^3$ transformed and control cells were cultured in the absence of serum for 72 h. Cell growth was assayed by MTT assay. "Control" was the absorbance at 570 nm at 0 h. The data show the means of three independent experiments. Closed triangles, BALB/3T3/A31-I-1 transfected with pLXSH-core191 and pLXSN-ras (Bcr); open triangles, transfected with pLXSH-core191 (Bc); closed circles, transfected with pLXSH-myc and pLXSN-ras (Bmr); open circles, transfected with pLXSN-ras (Br); open squares, transfected with pLXSH (Bmock). (C) EGF-dependent growth. A total of $5 \times 10^3$ Bc and Bmock cells were cultured in serum-free medium supplemented with 0, 0.1, and 1.0 μg/ml of EGF for 72 h and MTT assay was performed. "Control" was the absorbance at 570 nm at a concentration 0 μg/ml. The data show the means of three independent experiments.
production of HCV core protein was reduced compared with cells treated with the sense ODN, S(−)5-15, as a negative control (Fig. 2A). This reduction of protein production was likely to be specific for HCV core protein because the production of actin protein was not affected by the antisense ODN (Fig. 2A). To examine the effects of the antisense ODN on cell proliferation, Bcr cells were incubated in serum-supplemented medium with various concentrations of AS(−)5-15 for 72 h and cell growth was assessed by MTT assay. AS(−)5-15 inhibited cell proliferation in a dose-dependent manner. In contrast, S(−)5-15 showed no effect at concentrations up to 20 μM and showed slightly reduced cell proliferation at 50 μM possibly because of a nonspecific cytotoxic effect (Fig. 2B). This inhibition was specific for HCV core protein-producing cells. The proliferation of control BALB/3T3 A31-I-1 cells and Bmr cells was not affected by treatment with 20 μM AS(−)5-15 for 72 h and cell growth was assessed by MTT assay. AS(−)5-15 inhibited cell proliferation in a dose-dependent manner. In contrast, S(−)5-15 showed no effect at concentrations up to 20 μM and showed slightly reduced cell proliferation at 50 μM possibly because of a nonspecific cytotoxic effect (Fig. 2B). This inhibition was specific for HCV core protein-producing cells. The proliferation of control BALB/3T3 A31-I-1 cells and Bmr cells was not affected by treatment with 20 μM AS(−)5-15 (Fig. 2C). These findings were consistent with the observation that the HCV core protein producing cells showed growth advantages cooperatively with a growth factor or active Ras. Transiently expressed HCV core gene product also activated SRE activity as well as the stable transfectant. SRE activity in HCV core gene expressing cells is almost equivalent to that of the cells that expressed constitutive active MEK1, an upstream kinase of Erk (Fig. 3B). Similar results were observed with COS-7 cells (data not shown). We supposed that HCV core protein enhances SRE activity cooperatively with intracellular signals downstream of the EGF receptor or active ras gene products.

Though a previous report showed that HCV core protein activates some cellular and viral promoters (Ray et al., 1995; Chang et al., 1998; Shrivastava et al., 1998). It is possible that the core protein activates cellular promoters involved in signal transduction of growth stimuli and contributes to the acquisition of a growth advantage of HCV core protein producing cells. A serum response element (SRE) was identified beside the promoter of c-fos, and stimuli of growth factors activates this element (Wasylyk et al., 1998). We examined whether HCV core protein activates the SRE. Bcr, Bc, and Bmock cells were cotransfected with SRE/luciferase reporter plasmid and β-galactosidase reporter plasmid driven by the cytomegalovirus minimum promoter. After transfection, cells were cultured in medium supplemented with 0.5% FCS for 48 h and then treated with 100 ng/ml of epidermal growth factor (EGF) for 15 min. Luciferase activity of these cells was normalized relative to β-galactosidase activity. The SRE activity of Bc cells without EGF treatment was nearly equivalent to that of Bmock cells. On the other hand, the basal activity of Bcr cells was higher than that of the other cells. Following treatment with EGF, transcription from the reporter plasmid of Bc cells was induced to a level similar to that of Bcr cells. No such definite activation was observed in Bmock cells (Fig. 3A). These findings were consistent with the observation that the HCV core protein producing cells showed growth advantages cooperatively with a growth factor or active Ras. Transiently expressed HCV core gene product also activated SRE activity as well as the stable transfectant. SRE activity in HCV core gene expressing cells is almost equivalent to that of the cells that expressed constitutive active MEK1, an upstream kinase of Erk (Fig. 3B). Similar results were observed with COS-7 cells (data not shown). We supposed that HCV core protein enhances SRE activity cooperatively with intracellular signals downstream of the EGF receptor or active ras gene products.

Though a previous report showed that HCV core protein activates several promoters, the mechanism of transactivation is still unclear (Ray et al., 1995). We attempted to show that HCV core protein regulates the signaling pathway upstream of the promoter. EGF sig-
naling pathways act through Ras, which stimulates downstream kinases that ultimately activate Erk1/2. Phosphorylated and activated Erk phosphorylates ternary complex factors. Phosphorylated TCF interact with serum response factor as well as SRE to form ternary complexes, and then the SRE is activated (Wasylyk et al., 1998). To investigate the mechanism of SRE activation by HCV core protein, we examined the phosphorylation pattern of endogenous Erk1/2 in Bc cells. Bc and Bmock cells were cultured in medium supplemented with 0.5% FCS for 48 h and treated with 0.1 μg/ml of EGF for 5, 10, 15, and 30 min. In Bc cells, phosphorylation of Erk1/2 was detectable within 5 min of addition of EGF, although only slight phosphorylation of Erk1/2 was observed in Bmock cells (Fig. 3C). These results suggested that HCV core protein stimulates activation of the Erk–SRE pathway under the control of EGF or active Ras in BALB/3T3 A31-l-1 cells. We are currently engaged in further investigations to clarify the mechanism. Recently, it was reported that HCV core protein constitutively activates one of the other MAPK superfamily members, JNK in MCF-7 cells stably producing core protein (Shrivastava et al., 1998). It is likely that HCV core protein has a common mechanism that activates MAPK family kinases.

Liver is persistently regenerating following hepatic injury and growth factors stimulate this liver regeneration in hepatitis (Masuhara et al., 1996). It is possible that HCV core protein in regenerating hepatocytes enhances

**FIG. 2.** Effects of antisense oligodeoxynucleotides. (A) Immunoblot determination of HCV core protein levels in Bcr cells exposed to the antisense or sense oligodeoxynucleotide against pLXSH-core191 at 50 μM for 72 h. Total cell lysates were electrophoresed and HCV core protein was detected by immunoblotting. Results of immunoblotting for actin protein are shown as internal controls. (B) Dose-dependent inhibition of cell proliferation by antisense oligodeoxynucleotide against the HCV core gene. Bc cr cells were incubated for 72 h with various concentrations of antisense (closed circles) or sense (open circles) oligodeoxynucleotides. Cell proliferation was assessed by MTT assay. *Control* was the absorbance at 570 nm at a concentration 0 μM. The data show the means of three independent experiments. (C) HCV core-specific inhibition of cell proliferation by antisense oligodeoxynucleotides against the HCV core gene. BALB/3T3 A31-l-1 cells transfected with (i) HCV core and v-H-ras (Bcr) or (ii) c-myc and v-H-ras (Bmr), or (iii) not transfected were incubated for 72 h with antisense (AS(−)5-15) or sense (S(−)5-15) oligodeoxynucleotide at 20 μM (see text), and the MTT assay was performed. *Control* was the absorbance at 570 nm of oligodeoxynucleotide-free samples. The data show the means of three independent experiments.
growth stimuli and repeated hepatocyte proliferation may cause a disorder of genes in hepatocytes, thus causing hepatocellular carcinoma. In addition, we showed that reduction of HCV core protein production cancels the growth advantage. These observations will help to elucidate the viral pathogenesis and to develop treatment regimens for hepatocellular carcinoma following HCV infection.

FIG. 3. Activation of Erk-SRE signaling pathway by HCV core protein. (A) Bc, Bcr, and Bmock cells were transfected with SRE/luciferase reporter plasmid. Cells were cultured in serum-reduced medium (0.5% FBS) for 48 h (open bar) and stimulated by EGF (solid bar). Luciferase activity was normalized relative to cotransfected β-galactosidase activity. (B) BALB/3T3 A31-I-1 cells were cotransfected with reporter plasmid and HCV core expressing plasmid, constitutive active MEK1 expressing plasmid as a positive control, or empty vector. After EGF treatment, luciferase activity was measured. (C) Bc and Bmock cells were treated with 0.1 μg/ml of EGF for 0–30 min and activated Erk1/2 was detected using anti-phospho-MAPK antibody.
MATERIALS AND METHODS

Cell line and plasmids

BALB/3T3 A31-i-1 cells (JCRB0601, provided by Dr. Kiyoshi Sasaki, Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan) were maintained in minimal essential medium (MEM, Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum. Plasmid pLXSH-core191 was constructed by inserting the PCR product encoding the entire 191 amino acids of the HCV core gene into the retrovirus vector pLXSH (provided by Dr. Dusty Miller, Fred Hutchinson Cancer Research Center, Seattle, WA). Plasmid pC980 containing the N-terminal 980-amino-acid coding region of HCV-J cDNA was used as a template for PCR (Hijikata et al., 1991). The c-myc fragment (obtained from Japan Cancer Research Resources Bank, YG-CO053) was also cloned into pLXSH and designated pLXSH-myc. The constitutively active ras gene-expressing plasmid pLXSN-ras was constructed by inserting the v-H-ras fragment from the Harvey murine sarcoma virus genome into the retroviral vector pLXSN (Ellis et al., 1980).

In vitro transformation assay

BALB/3T3 cells (2 × 10⁵ cells per 100-mm diameter plate) were transfected with 1 μg of pLXSH-core191 gene with or without 1 μg of pLXSN-ras using Lipofectamine Plus reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. The cells were maintained in MEM supplemented with 5% fetal calf serum and were fed every 4 days. Transformed foci were counted at 21 days posttransfection. pLXSH-myc and pLXSN-ras were used in cotransfection experiments as positive controls. From each set of transfections, 20 foci were picked and subcloned. Anchorage-independent growth was assessed by soft agar assay. Briefly, 3 ml of base medium containing 5% fetal calf medium with 1% SEAPLACE agarose (FMC, ME) in 60-mm culture dishes was used. Cells (10⁵ cells/dish) were seeded in 4 ml of medium containing 5% fetal calf serum with 0.33% agar and layered onto the base. Colonies were observed after 10 days. Cell growth was measured by MTT assay with a CellTiter 96 Non-radioactive Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer’s protocol.

Antisense oligodeoxynucleotides

Phosphorothioate oligonucleotides were purchased from GlinerJapan Corp. (Tokyo, Japan). AS(−)5-15 (AGGATTTTGGCTCATGATGC) targeted the initiation site of the HCV core gene in pLXSH-core191. S(−)5-15 (GCATCATGAGCACAATCCT) was complementary to AS(−)5-15. These oligonucleotides were added to the culture medium at various concentrations and subsequent assays were performed.

Immune detection of HCV core protein

The protein samples were separated by 12.5% SDS-PAGE. The electrophoresed proteins were transferred onto PVDF membranes. Membranes were incubated with blocking buffer (5% dried milk, 5% fetal bovine serum, 0.05% Tween 20, phosphate-buffered saline) and then incubated with anti-HCV core monoclonal antibody (615S, provided by Dr. Michinori Kohara, The Metropolitan Institute of Medical Science, Tokyo, Japan) at 1 μg/ml in blocking buffer for 60 min. Membranes were subsequently treated with horseradish peroxidase-conjugated anti-mouse antibody at a 1:3000 dilution for 45 min. Protein binding was detected with Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA).

SRE reporter enzyme assay

SRE activity was assayed with the PathDetect Cis-Reporting System (Stratagene, San Diego, CA). Transfected cells were maintained in MEM supplemented with 0.5% fetal bovine serum for 48 h. Then, 100 ng/ml of epidermal growth factor (Toyobo, Osaka, Japan) was added to the medium and cells were incubated at 37°C for 15 min. Luciferase activity was measured with Luciferase Assay System (Promega) and Lumat LB9507 (EG&G Berthold, Bad Wildbad, Germany) according to the manufacturer’s protocol.

Immune detection of phosphorylated Erk

The protein samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked and incubated with rabbit anti-phospho-p42/44 MAP kinase polyclonal antibody or rabbit anti-MAPK antibody (NEB, Beverly, MA) at a 1:1000 dilution in blocking buffer for 60 min. Membranes were subsequently treated with HRP-conjugated anti-rabbit antibody at a 1:1000 dilution for 45 min.

ACKNOWLEDGMENTS

We thank Dr. Kiyoshi Sasaki for helpful discussions and technical advice. This work was supported by grants-in-aid for cancer research and for a second-term comprehensive 10-year strategy for cancer control from the Ministry of Health and Welfare, by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, and by the program for the promotion of fundamental studies in health sciences of the Organization for Drug ADR Relief, R&D Promotion, and Product Review of Japan.

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