Expression and characterization of glycoprotein gp35 of hepatitis C virus using recombinant vaccinia virus

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Complementary DNA clones corresponding to one of the putative structural regions of the hepatitis C virus (HCV) genome were obtained from sera of non-A non-B hepatitis patients. The putative envelope gene was expressed by using a recombinant vaccinia virus carrying this region of the HCV genome. In cells infected with the recombinant vaccinia virus, a glycosylated protein with an Mr of about 35K (gp35) was specifically detected by convalescent sera from hepatitis C patients. The sera from rabbits immunized with this recombinant vaccinia virus reacted to the gp35 produced in insect cells and also to gp35 which was translated in vitro in the glycosylated and processed form. The gp35 was used to detect antibodies in sera of only 7 to 23% of HCV patients at various stages of HCV disease. These results suggest that the gp35 of HCV may not have high antigenicity in humans.

Introduction

Hepatitis C virus (HCV), the main causative agent of chronic non-A non-B hepatitis (NANBH), is transmitted mainly by blood transfusion (Choo et al., 1989). Chronic NANBH frequently progresses to liver cirrhosis and cancer (Lefkowitch & Apfelbaum, 1987; Muchmore et al., 1988). The nucleotide sequence of the HCV genome has recently been elucidated (Kato et al., 1990; Takamizawa et al., 1991; Choo et al., 1991). The genome of HCV is an ssRNA with positive polarity, consisting of approximately 10000 nucleotides which can be translated into a large single polyprotein of 3010 amino acids. Analysis of the nucleotide and deduced amino acid sequences of different HCV isolates (Okamoto et al., 1990; Kato et al., 1990; Takamizawa et al., 1991; Choo et al., 1991) revealed that HCV has a genome organization similar to those of flaviviruses and pestiviruses (Takeuchi et al., 1990).

By using an in vitro protein synthesis system followed by amino acid sequence analysis of the products, Hijikata et al. (1991) have shown that the structural HCV gene products are arranged in the order: NH2-p22-gp35-gp70-COOH, and have suggested that gp35 and gp70 are the HCV envelope glycoproteins. Chiba et al. (1991) and Matsuura et al. (1992) have reported that p22 and gp35 produced in insect cells infected with recombinant baculovirus carrying cDNAs of the corresponding genome regions were detected by sera from NANBH patients. Thus, it is suggested that p22 and gp35 are the mature viral proteins existing in cells of patients infected with HCV. Considering the similarity between the genome organization of HCV and those of flaviviruses and pestiviruses, it is very possible that p22 and gp35 are the HCV core protein and envelope glycoprotein respectively.

Here we constructed a recombinant vaccinia virus carrying cDNA corresponding to the HCV genome encoding the whole gp35, and demonstrated that gp35 was produced in rabbit cells infected with the recombinant vaccinia virus. Furthermore, sera from rabbits immunized with the recombinant vaccinia virus reacted with gp35 from cells infected with the recombinant baculovirus as reported by Matsuura et al. (1992) and in an in vitro protein synthesis system as reported by Tsukiyama-Kohara et al. (1992). Some sera of humans at different stages of HCV infection were also reactive with the recombinant gp35.

Methods

Materials. Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co.; calf intestinal alkaline phosphatase was from Boehringer-Mannheim. A cDNA cloning system, λgt11 (Amersham) was used for preparing a cDNA library. Labelled
compounds, [3H]glucosamine and [35S]methionine, were purchased from DuPont/NEN and ICN Radiochemicals, respectively. Enzymes and radiochemicals were used according to the instructions of the manufacturers. An electroporation technique was used for DNA transfection (Chu et al., 1987).

**Cells and viruses.** Rabbit kidney (RK13) cells were maintained in Eagle's MEM supplemented with 5% newborn calf serum. Insect Spodoptera frugiperda (Sf9) cells were maintained at 27 °C in Grace's medium (Gibco) containing 10% foetal calf serum.

The attenuated vaccinia virus Lister strain and its related strain were propagated in RK13 cells. The parental baculovirus was Autographa californiae nuclear polyhedrosis virus (AcNPV). Recombinant baculovirus strain Ac816 contained the HCV genome between nucleotide positions 761 and 1652 (encoding gp35), as reported by Matsuura et al. (1992). This recombinant baculovirus was propagated in Sf9 cells and used to express cDNAs encoding the viral structural proteins.

**Cloning of HCV cDNA and construction of recombinant vaccinia virus.** A cDNA clone, C10-E12 (see Fig. 1), was isolated by immunoscreening from the Ag11 cDNA library prepared from RNAs obtained from sera of NANBH patients (Tsukiyama-Kohara et al., 1991). The HCV cDNA C10-E12 was excised by digestion with KpnI at the cleavage site which exists in an EcoRI adapter used for the cDNA cloning, and subcloned into the KpnI site of the vaccinia virus transfer vector pVR-1 (Tsukiyama et al., 1989). The KpnI cleavage site of pVR-1 resides downstream of the P7.5 promoter within the haemagglutinin (HA) gene of vaccinia virus (see Fig. 2). The plasmid thus obtained was designated p7.5-E12. RK13 cells were infected with the Lister strain of vaccinia virus at a multiplicity of 10 followed by transfection with plasmid p7.5-E12 by electroporation (Chu et al., 1987). A recombinant virus (HA-) was selected by HA assay and gene hybridization as previously described (Tsukiyama et al., 1989). The recombinant vaccinia virus strain thus constructed was designated the RLV strain.

**Nucleotide sequence analysis.** Clone C10-E12 was subcloned into pUC119 and the nucleotide sequence was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a 7-deaza Sequenase kit (United States Biochemical).

**Preparation of anti-gp35 antibody.** Japanese white rabbits were used to raise anti-gp35 antibodies. The first immunization was performed by intradermal inoculation of the back with 1 x 10^6 p.f.u. of the recombinant vaccinia virus RLV strain. At 2 months after the first immunization, the rabbits were immunized again with an intravenous injection of the same amount of the RLV strain. Endpoint titres of anti-gp35 antibodies in rabbit sera were determined by Western blot analysis using recombinant baculovirus gp35 (Matsuura et al., 1992) as the antigen at 1, 2 and 3 months after the first immunization.

**Indirect immunofluorescence assay (IFA).** RK13 cells infected with the Lister or RLV strain of vaccinia virus at a multiplicity of 0-1 and grown in culture at 37 °C for 15 h, were fixed with cold methanol at -20 °C for 10 min, and then incubated at 37 °C for 40 min with serum of a normal human (as a negative control) or a convalescent HCV patient (1:30 dilution in PBS). The cells, after being washed with PBS, were further incubated at 37 °C for 30 min with the second antibody, fluorescein-conjugated rabbit anti-human IgG (1:200 dilution) (Dako Patts), and were observed with a Zeiss fluorescence microscope.

**Immunoprecipitation.** RK13 cells (5 x 10^5 cells) infected with the Lister or RLV strain of vaccinia virus at a multiplicity of 10 were labelled with [3H]glucosamine (3.7 MBq/dish) at 37 °C for 14 h. The labelled cells were lysed with radioimmunoprecipitation buffer containing 10 mM-Tris–HCl pH 7.4, 1% Triton X-100, 1 mM sodium deoxycholate, 0.1% SDS, 0.15 M-NaCl, and 1 mM-PMSF, and incubated at 4 °C overnight with HCV patient serum or normal human serum which had been adsorbed with a lysate of RK13 cells infected with the Lister strain. To the mixture, Affi-Gel Protein A (Bio-Rad) was added and incubated at 4 °C for another 1 h with gentle shaking. Materials precipitated with Affi-Gel Protein A were analysed by 10% PAGE in Laemmli's buffer system (Laemmlli, 1970).

For in vitro products labelled with [35S]methionine, the products were incubated with rabbit sera against gp35, and then with Affi-Gel Protein A. The precipitates were analysed by electrophoresis as above.

**Western blotting.** Insect Sf9 cells infected with recombinant baculovirus cloned Ac816 were harvested 48 h after infection (Matsuura et al., 1992). Cell lysates were prepared and separated by electrophoresis on a 15% polyacrylamide gel according to the methods of Laemmlli (1970), and transferred to nitrocellulose filters. Strips of the filter were incubated with the RLV-infected rabbit or HCV patient serum (1:10 dilution) at 4 °C for 14 h. The strips were then treated with biotinylated anti-rabbit or anti-human IgG and peroxidase-conjugated avidin (Amersham), followed by incubation with the substrate (Bio-Rad).

**Transcription and translation in vitro.** DNA corresponding to HCV genome nucleotide positions 261 to 1764 was inserted into the Bluescript KS vector (Stratagene) in which the cDNA was designed to be expressed under the control of the phage T7 promoter. Transcription reactions were performed after digestion of the plasmid with HindIII as reported by Kaminski et al. (1990). Synthetic RNAs were then translated in rabbit reticulocyte lysates (Amersham N150) in the presence of [35S]methionine at 30 °C for 60 min. The translation products were processed by the addition of canine microsomal membranes (Amersham).

## Results

### Isolation of cDNA clone

Nucleotide sequence analysis of the cDNA clone C10-E12 followed by comparison with that of the HCV genome reported by Kato et al. (1990) revealed that the cDNA corresponded to genome nucleotide positions 676 to 1607. The nucleotide sequence of the cDNA and the deduced amino acid sequence are shown in Fig. 1. According to data reported by Hijikata et al. (1991), this region of the genome encodes a C-terminal part of the p22 protein (the putative core protein), the whole gp35 glycoprotein (the putative envelope glycoprotein) and a N-terminal part of the gp70 glycoprotein (another putative envelope glycoprotein) (Fig. 2). Identity values of the deduced amino acid sequence within gp35 with those reported by Kato et al. (1990), Takamizawa et al. (1991) and Choo et al. (1991) were calculated to be 94.3%, 91.1% and 78.6%, respectively. Thus, higher levels of similarity were observed in the amino acid sequences of gp35 among Japanese HCV isolates.

### Expression of HCV cDNA in RLV-infected cells

A monolayer of RK13 cells was infected with the RLV or the parental Lister strain of vaccinia virus. An indirect IFA was performed by using serum from a normal human or a convalescent HCV patient as described in
HCV gp35: recombinant expression

Methods. Fluorescence was observed in the cytoplasm of cells infected with the RLV strain of vaccinia virus by patient serum (Fig. 3), but not by normal human serum (data not shown) nor in cells infected with the parental Lister strain. The result indicates that the HCV cDNA is expressed in cells infected with the RLV strain.

Cells infected with the Lister or RLV strain of vaccinia virus were labelled with [3H]glucosamine and the materials reacted with either patient or normal human sera were analysed by electrophoresis on a 10% polyacrylamide gel (Fig. 4). A band of approximately 35K Mr was specifically detected by the patient serum in the RLV-infected cells. Since the Mr is similar to that of gp35 (Hijikata et al., 1991), it is possible that the mature gp35 glycoprotein is produced by specific protein processing and by glycosylation in the infected cells. This may in turn indicate that antibodies against gp35, the putative virus envelope protein, exist in convalescent sera of HCV patients.

Reactivity of anti-gp35 antibody

Anti-gp35 antibodies are elicited in rabbits at titres of 10^2 to 10^4 and 10^3 to 10^5 at 2 and 3 months after the first immunization, respectively (Table 1). The reactivity of each serum was verified by its specific reaction with two kinds of products directed by the HCV genome region encoding gp35, i.e. materials produced by using the in vitro translation system (Fig. 5) and the baculovirus expression vector system (Fig. 6).

For the first set of experiments, capped HCV RNA representing nucleotide positions 267 to 1764 was synthesized under the control of the T7 promoter, and the transcript was used as a template in an in vitro translation system prepared from rabbit reticulocytes.
Fig. 4. Immunoprecipitation. RK13 cells were infected with the Lister strain (lanes 1 and 2) or RLV strain (lanes 3 and 4) and labelled with [3H]glucosamine as described in Methods. The major bands in lanes 1 and 2 are vaccinia virus HA protein. The cell lysates were mixed with an NANBH patient's (lanes 2 and 4) or normal human (lanes 1 and 3) sera. Immunoprecipitation and PAGE were as described in Methods.

Table 1. Production of anti-gp35 antibody

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Antibody titre*</th>
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<tr>
<td></td>
<td>1†</td>
</tr>
<tr>
<td>A</td>
<td>$10^2$</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>$10^2$</td>
</tr>
<tr>
<td>D</td>
<td>$10^2$</td>
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* Titres by Western blotting analysis.
† No. of months post-infection.

(Tsukiyama-Kohara et al., 1992). As shown in Fig. 5, a polypeptide of the expected size ($M_r$, 52K) was synthesized (Fig. 5, lane 1). The peptide was processed into two different proteins with $M_r$ values of 22K and 35K on addition of canine microsomal membranes (Fig. 5, lane 4). This observation is compatible with that of Hijikata et al. (1991), and therefore indicates that these products are p22 and gp35, respectively. An immunoprecipitation experiment involving the rabbit serum was performed as described in Methods and precipitates were analysed by SDS–PAGE (Fig. 5, lanes 2, 3, 5 and 6). The rabbit serum reacted with gp35 but not with unprocessed products or p22 (Fig. 5, lane 6). These data indicate that the rabbit serum contains antibodies specific to HCV gp35, particularly to the glycosylated, properly processed protein.

In the second set of studies, Ac816-infected Sf9 cells produced 24K to 35K proteins that reacted to the sera from rabbits infected with the RLV strain (Fig. 6, lane 1). The difference in the size of the proteins (24K to 35K) was considered to be due to the different extent of glycosylation in the insect cells (Matsuura et al., 1992). The proteins of 24K to 35K reacted with the convalescent hepatitis C patient's serum (Matsuura et al., 1992) (Fig. 6, lane 3). The results strongly suggest that the immunized rabbit serum contains antibodies against gp35 in its native form.

Detection of anti-gp35 antibodies in HCV patients

Sera from various NANBH patients were examined for the existence of antibodies reactive with gp35 that had been produced from recombinant vaccinia virus (Table 2). The results obtained by ELISA and IFA are shown in...
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These observations indicate that the gp35 of HCV has a lower antigenicity than the core and NS3 proteins in human infection and that the immune systems of individuals are different in their abilities to recognize HCV proteins.

Discussion

An HCV cDNA clone, C10-E12 which encodes gp35 (the putative virus envelope protein), was expressed in a vaccinia virus vector. A glycoprotein of the expected Mr of 35K was detected in RK13 cells infected with the recombinant vaccinia virus. Rabbits immunized by infection with the recombinant vaccinia virus produced antibodies against gp35. The results indicate that the recombinant vaccinia virus strain RLV produced natural, properly processed HCV glycoprotein gp35 in rabbits.

The gp35 described here has an amino acid sequence very similar to those of Japanese HCV isolates (Takamizawa et al., 1991; Kato et al., 1990), but considerably different from that of HCV-1 (Choo et al., 1991). Possible N-glycosylation sites at amino acid positions 5, 18, 43, 59, 104 and 134 of the gp35 of the Japanese HCV isolates are observed in the amino acid sequence encoded by C10-E12 whereas the site at position 59 is missing in the gp35 of HCV-1. A new possible N-glycosylation site at position 42 was observed in our gp35. It is possible that these genetic variations cause an altered antigenicity of gp35.

It is of particular interest that glycosylated and processed forms of recombinant gp35 were recognized by convalescent sera of an acute HCV patient, and that sera of both immunized rabbits and a convalescent HCV patient showed patterns very similar to each other in their recognition of gp24-35 produced in the baculovirus expression vector system (Fig. 6). It is important for future vaccine development that the rabbit sera contain antibodies of the same specificity as those in convalescent HCV patients, since it is possible that the HCV

**Table 2. Detection of HCV antibody in NANBH patients' sera**

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<tr>
<th>Antigen</th>
<th>Condition (no. of cases detected)</th>
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<tr>
<td></td>
<td>HD*</td>
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<tr>
<td>Imu C11/C7‖</td>
<td>0/24</td>
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<tr>
<td>gp35/RLV¶</td>
<td>0/24</td>
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* Healthy donor.  
† Chronic hepatitis.  
‡ Liver cirrhosis.  
§ Hepatocellular carcinoma.  
‖ Core and NS3 antigen: Saito et al. (1992).  
¶ Sera examined by indirect IFA using RLV-infected RK13 cells.

Table 2. Anti-gp35 antibodies were detected in only a limited number of NANBH patients' sera (7 to 23%), although anti-core and anti-NS3 (Saito et al., 1992) antibodies were in 90 to 95% of the patient sera. All patients who had been found to carry antibodies against gp35 were positive for antibodies against the core and NS3 proteins. These observations indicate that the gp35 of HCV has a lower antigenicity than the core and NS3 proteins in human infection and that the immune systems of individuals are different in their abilities to recognize HCV proteins.
patient's serum contains antibodies active in neutralizing HCV. Only experiments involving chimpanzees have so far shown the detection of neutralizing antibodies against HCV. However, an in vitro culture cell system partially permissive for HCV infection was developed very recently (Shimizu et al., 1992). Detection of neutralizing antibodies against HCV in immunized rabbit sera and in convalescent sera of an acute HCV patient is currently being investigated by the use of this system.

References


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