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Expression and Characterization of Recombinant E2 Protein of Hepatitis C Virus by Insect Cell/Baculovirus Expression System

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Abstract The E2 protein of HCV (hepatitis C virus) is thought to have a potential role in the development of subunit vaccines and diagnostics. To express it by the insect cell/baculovirus expression (Bacu) system, we constructed a recombinant *Autographa californica* nuclear polyhedrosis virus (AcIL3E2), determined the most appropriate expression conditions in terms of host cell line and culture medium, and characterized the expressed HCV E2 protein. A culture system using *Trichoplusia ni* BTI-TN5B1-4 cells and SF 900IISFM medium expressed a relatively high level of HCV E2 protein. It was revealed that its glycosylation properties and subcellular localization were almost the same as the ones in the mammalian cell expression system previously reported, suggesting the recombinant HCV E2 protein derived from our Bacu system can be utilized for development of a subunit vaccine and diagnostics. Interestingly, HCV E2 protein was not degraded at all even at 43 h post-heat shock in the heat shock-induced necrotic cells, probably due to its integration into the microsomal membrane, indicating that heat shock can be employed to purify HCV E2 protein.

Key words: HCV (hepatitis C virus) E2 protein, insect cell/baculovirus expression system, glycosylation property, heat shock

Hepatitis C virus (HCV) is one of the major causative agents of chronic hepatitis and liver disease throughout the world [3, 7] and it has been proposed that it is a new genus of the *Flaviviridae* family [15]. At least 10 protein products have been identified, which are putative structural proteins of C, E1, E2, p7, and nonstructural proteins of NS2, NS3, NS4A, NS4B, NS5A, and NS5B [4, 12]. HCV has a broad cellular tropism, including hepatocyte and peripheral blood mononuclear cells, but an efficient *in vitro* replication system has not been established at present, which makes it difficult to construct and test attenuated viruses for vaccine development. Therefore, much effort has been made in the development of a subunit vaccine using various expression systems.

In particular, more attention has been paid to the E2 protein of HCV since the NS1 protein of flaviviruses and the E2 protein of pestiviruses, which are the counterparts of the E2 protein of HCV, have been reported to induce a protective immune response [1, 20]. Furthermore, Choo *et al.* [2] showed that a purified E1-E2 oligomer from recombinant vaccinia virus-infected HeLa cells could elicit protective immunity against challenges of low doses of homologous virus. However, it has been suggested that vaccine antigens from multiple serotypes may be necessary for global protection because of the presence of a hypervariable region in the N-terminus of the E2 protein, which is thought to be derived from immune-escape variants and to contain an epitope for neutralizing antibody or cytotoxic T cell receptors [20]. Additionally, HCV E2 protein is suggested to have a potential role in the development of next generation diagnostics because the native type E2 protein has been shown to improve the detection of HCV infection in immunosuppressed patients [13].

In this study, we adopted the insect/baculovirus expression (Bacu) system to express recombinant E2 protein of HCV (Korean isolate: HCV 1b type), which offers the advantages of hyperexpression and various authentic post-translational modifications [22] when biologically functional protein is abundantly required [14]. Then, we investigated the effects of host cell line and cell culture medium on the expression of HCV E2 protein, to determine its most appropriate expression condition. We also investigated the characteristics of the expressed HCV E2 protein, in which we examined the property of glycosylation, subcellular localization of the expressed HCV E2 protein, and its degradation pattern

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in the heat shock-induced necrotic cells. Because it has been reported that chitinase (M.W. 58 kDa), which is similar in size to HCV E2 protein (M.W. 68 kDa), was expressed in the late phase of virus replication in insect cells infected with *Autographa californica* nuclear polyhedrosis virus (AcMNPV) [6], the expressions of both HCV E2 protein and chitinase were simultaneously checked when expression levels and characteristics of HCV E2 protein were being analyzed.

MATERIALS AND METHODS

Cells, Viruses, and Transfection

Trichoplusia ni BTI-TN5B1-4 (High 5™, Invitrogen, Carlsbad, CA, U.S.A.) cells were adapted to and grown either in a serum free medium (SF900IISFM medium, Gibco BRL, Gaithersburg, MD, U.S.A.) or in TNM-FH medium supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (FBS) (HyClone, Logan, Utah, U.S.A.). *Spodoptera frugiperda* IPL-Sf9 (*Sf9*) cells were grown in TNM-FH medium with 10% heat-inactivated FBS. These cells were maintained according to the procedures described by King and Possee [9].

A recombinant baculovirus (BacPAK6) encoding β-galactosidase protein (Clontech, Palo Alto, CA, U.S.A.) was used as a negative control. Stock viruses of AcIL3E2 and BacPAK6 were obtained by the standard procedure using *Sf9* cells. Briefly, *Sf9* cells in the exponential growth phase were infected for 1 h with 1 PFU $cell^{-1}$ of AcIL3E2 or BacPAK6. The virus solution was removed and fresh cell culture medium was added. Progeny viruses were harvested at 5 days postinfection (dpi) by centrifugation at 600 × g for 10 min and stored at 4°C. Virus titer was determined using the end-point dilution method [19].

Transfection of *Sf9* cells was undertaken as described by King and Possee [9] using the Lipofectin™ (Gibco BRL, Gaithersburg, MD, U.S.A.). The recombinant baculovirus expressing the HCV E2 protein, AcIL3E2, was isolated through three cycles of plaque formation.

Plasmid Construction

The HCV E2 gene was PCR amplified from the pUCE2 with sense (5'-CACACCCACGTGACAGGGGG-3') and antisense (5'-GTTCTCTAAGGTGGCCTCAG-3') primers [9]. The oligonucleotide primer for the sense strand of E2 was designed not to have the ATG initiation codon since it was provided by the IL-3 signal sequence. The oligonucleotide primer for the antisense strand of E2 was designed to have the TGA termination codon. The resulting E2 gene was subcloned into the *Bam*HI site of the pAcIL3ss vector which was treated with the Mung bean nuclease to conserve the reading frame

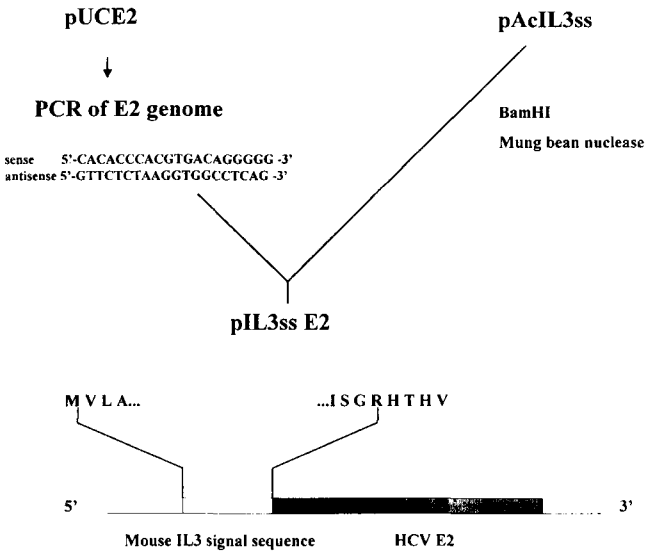

Fig. 1. Schematic diagram of a recombinant baculovirus vector and strategy for expression of HCV E2 protein.
Mouse interleukine 3 signal sequence and HCV E2 structural gene are indicated as the white and gray box, respectively.

and designated pIL3ssE2 (Fig. 1). The pAcIL3ss, kindly provided by Dr. C. Y. Kang at the University of Western Ontario, Canada, is a polyhedrin promoter based baculovirus transfer vector with the mouse IL-3 signal sequence inserted upstream of the cloning site. Dideoxy nucleotide chain termination sequence analyses were undertaken to verify the sequence between the promoter and E2 coding region.

HCV$^+$ Human Sera and Anti-chitinase Antibody

Sera were collected from patients with chronic HCV infection. These patients were devoid of serological markers of hepatitis B infection. Guinea pig-derived anti-chitinase polyclonal antibody was kindly provided by Dr. R. D. Possee (NERC Institute of Virology and Environmental Microbiology, Oxford, U.K.).

Suspension Culture

All suspension cultures were performed in 250 ml spinner flasks (Corning, NY, U.S.A.) in a humidified 27°C incubator. The cell culture volume was 100 ml and the agitation speed was set at 80 rpm.

SDS-polyacrylamide Gel Electrophoresis (PAGE) and Immunoblot Analysis

Extracts of AcIL3E2- or BacPAK6-infected cells were prepared by standard methods [9] and subjected to electrophoresis on 12% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane electrophoretically for immunoblot analysis (Bio-Rad Laboratories, CA, U.S.A.). The blots were

incubated sequentially with 1:1,000 diluted HCV patient serum and with a goat-derived peroxidase-labeled antihuman IgG (heavy and light) (Kirkegaard and Perry Laboratories, MD, U.S.A.) at 1:2,500 dilution. Then, 100 ml DAB substrate developing solution [60 mg 3,3-diaminobenzidine (Sigma, St Louis, MO, U.S.A.) and 0.1 ml 30% H_2O_2] were added. The reaction was stopped by rinsing thoroughly with distilled water. Relative expression level of the HCV E2 protein was determined by densitometric analysis of immunoblots.

In order to check the progression of the viral infection process of both AcIL3E2 and BacPAK6, expression of chitinase was investigated as an internal control using immunoblot analysis. For immunoblot analysis of chitinase, guinea pig-derived polyclonal anti-chitinase antibody was used in 1:100,000 dilution.

Characterization of the Expressed HCV E2

AcIL3E2- or BacPAK6-infected *Trichoplusia ni* BTI-TN5B1-4 cells cultured in a spinner flask were harvested at 3 days post-infection (dpi). The cells were dissolved in the binding buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.4) and disrupted in a Dounce homogenizer with 25 strokes. The extracts were incubated overnight at 4°C with Concanavalin A (ConA)-Sepharose beads (Pharmacia) or agarose beads linked with various lectins (Sigma, St Louis, MO, U.S.A.) including *Galanthus nivalus* (GNA), *Arachis hypogaea* (PNA), and *Wheat germ agglutinin* (WGA) in an eppendorf tube in the presence of 2% Triton X-100 (Sigma, St Louis, MO, U.S.A.). After washing with the binding buffer at 10 times the resin volume, the bound proteins were eluted in the SDS-sample buffer with 1% (w/v) β-mercaptoethanol and subjected to the immunoblot analysis.

To gauge the subcelluar localization of the expressed E2 protein in the insect cells, a subcellular fractionation study was undertaken. 10^7 *Trichoplusia ni* BTI-TN5B1-4 cells infected with either AcIL3E2 or BacPAK6 were harvested at 3 dpi and washed twice with PBS. The cells were suspended and incubated in STM buffer [250 mM sucrose, 10 mM Tris (pH 8.0), 10 mM $MgCl_2$, 10 mg ml^{-1} of aprotinin] on ice for 10 min and disrupted in a Dounce homogenizer with 25 strokes. The nuclear fraction from the lysate was pelleted at 500×g for 10 min and the microsomal fraction and the cytoplasmic fraction in the supernatant were separated by centrifugation at 100,000×g for 30 min. A portion of each fraction was analyzed by 12% SDS-PAGE and immunoblot analysis as described above.

To assess the possibility that HCV E2 protein is integrated into the microsomal membrane, the microsomal membrane fraction was treated with the binding buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.4) in the presence or absence of 2% Triton X-100. The HCV E2 protein in the extracts was isolated by using the Concanavalin A (ConA)-Sepharose beads (Pharmacia, Uppsala, Sweden) in an eppendorf tube. The bound HCV E2 protein was eluted with binding buffer containing 1 M α-methyl-D-mannoside (Sigma, St Louis, MO, U.S.A.). Proteins of each fraction were separated on 12% SDS-PAGE and subjected to immunoblot analysis. Integration into the microsomal membrane of the expressed HCV E2 protein was confirmed by investigation of the stability of the protein in heat-induced necrotic cells. The temperature for insect cell culture was increased to 43°C at 3 dpi and maintained thereafter. As a reference, another insect cell culture was performed at 27°C in parallel. Status of the HCV E2 protein and chitinase in the heat shock-induced necrotic cells were analyzed with immunoblot analysis in comparison with other cytoplasmic proteins.

RESULTS

Effect of Host Cell Line and Culture Medium on the Expression of HCV E2 Protein

To determine the most appropriate expression condition for HCV E2 protein in the insect cell/baculovirus expression (Bacu) system, effects of the host cell line and culture medium on its expression level were examined. All the cultures, using the three suspension culture systems described above, were performed by the same strategy; cells were taken from the mid-exponential growth phase, resuspended in fresh medium, and infected with 10 PFU $cell^{-1}$ of AcIL3E2. Of the three culture systems, the *Trichoplusia ni* BTI-TN5B1-4 cells and SF900IISFM medium system supported the highest expression level of HCV E2 protein (Fig. 2A and 2B, lanes 1 and 4), even though its secretion was not detectable (data not shown) possibly due to the C-terminal hydrophobic transmembrane domain, which is consistent with previous reports [5, 11, 16]. The molecular weight of HCV E2 protein expressed by *Trichoplusia ni* BTI-TN5B1-4 cells was approximately 68 kDa, which is consistent with the reports for the HCV E2 protein expressed in eukaryotic cell systems [4, 8, 17]. In the case of chitinase, however, similar expression levels were obtained in all the culture systems. Therefore, we envisaged that the viral infection process progressed similarly at least up to the late phase in the life cycle of the AcIL3E2. The expression level of the HCV E2 protein was very low when *Trichoplusia ni* BTI-TN5B1-4 and *Sf*9 cells were grown and infected in TNM-FH medium with 10% FBS instead of SF 900IISFM medium (Fig. 2A and 2B, lanes 2, 3, 5, and 6), even though similar expression levels of chitinase were observed in both conditions.

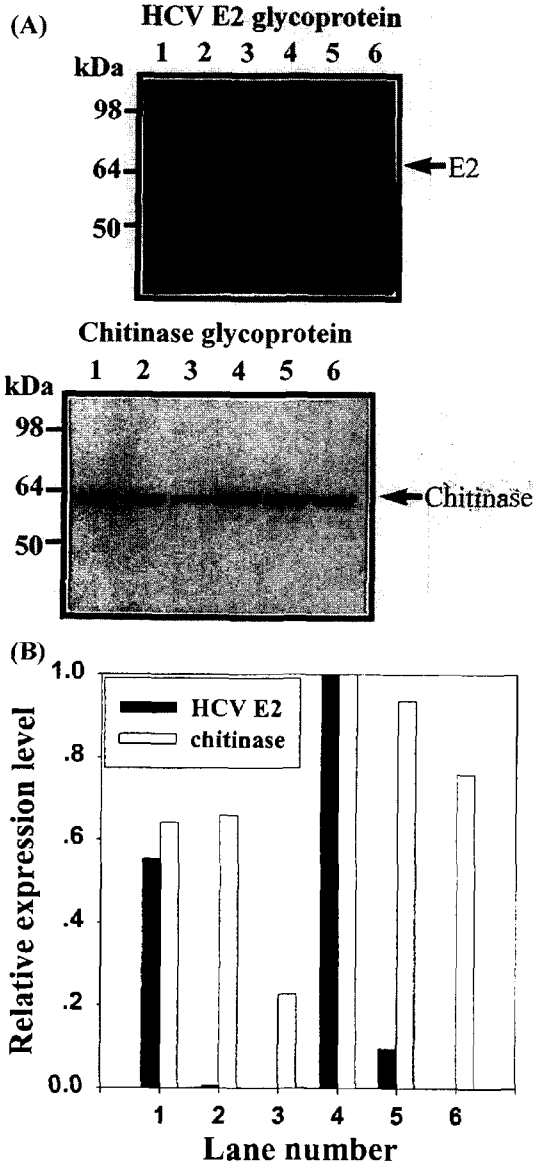

Fig. 2. Effect of host insect cell lines and cell culture media on the expression of HCV E2 protein.

(A) *Trichoplusia ni* BTI-TN5B1-4 cells were grown in SF900IISFM (lanes 1 and 4) or TNM-FH medium with 10% FBS (lanes 2 and 5). *Sf* 9 cells were grown in TNM-FH medium with 10% FBS (lanes 3 and 6). Cells were taken at 1×10^6 cells ml^{-1} in the mid-exponential growth phase and resuspended in the respective fresh media. Then, they were infected with 10 PFU $cell^{-1}$ of AcIL3E2. Cell extracts were taken at 1 dpi (lanes 1~3) and 3 dpi (4~6) and analyzed by immunoblot analysis with HCV^+ patient human serum (upper) and goat-derived anti-chitinase antibody (lower). (B) Relative expression levels of HCV E2 protein (■) and chitinase (□) were determined by densitometric analysis of the immunoblots above.

Expression of HCV E2 Protein by the AcIL3E2 in *Trichoplusia ni* BTI-TN5B1-4 Cells

Immunoblot analysis of the extracts of AcIL3E2-infected *Trichoplusia ni* BTI-TN5B1-4 cells with HCV^+ patient serum showed a strong band as shown in Fig. 2A, whereas no bands were detected in the BacPAK6-infected cell extract (Fig. 3B, lane 2). However, in the Coomassie blue-stained gel, the HCV E2 protein could not be distinguished from the other bands (Fig 3A, lane 1) while a very thick band corresponding to β-galactosidase was detected in the BacPAK6-infected cell extract (Fig. 3A, lane 2). Since similar expression levels of chitinase were obtained in both AcIL3E2- and BacPAK6-infected cell extracts (Fig. 3C), and β-galactosidase under the control of the polyhedrin promoter was expressed abundantly in the BacPAK6-infected cells, we concluded that the HCV E2 protein was also being expressed under the efficient progression of the infection process.

Glycosylation Properties of Expressed HCV E2 Protein

Since HCV E2 protein is known to be heavily N-glycosylated when expressed in the eukaryotic expression system [5, 11, 17], we characterized glycosylation properties of the HCV E2 protein by using various lectins, including ConA-sepharose, terminal mannose-reactive GNA, *N*-acetlyglucosamine-reactive WGA, and galβ(1,3)galNAc-reactive PNA. The expressed HCV E2 protein was found to be bound to ConA-sepharose, GNA and WGA but not to PNA (Fig. 4Aa, Fig. 4Ba). From this experiment, we concluded that the HCV E2 protein has α-mannose residues and hybrid-type glycan with a mannose-terminus and *N*-acetlyglucosamine residue but has no O-linked carbohydrate. Our results indicate that HCV E2 protein expressed by our Bacu system is similar to that expressed in recombinant vaccinia virus-infected HeLa cells in terms of glycosylation properties [17]. We also found another band, which was smaller than that of HCV E2 protein, probably due to the false cross-reaction by the use of HCV^+ patient sera as a source of antibody, or the degraded HCV E2 protein. No band was detected at the corresponding position of the negative control cell lysate.

Subcellular Localization of the Expressed HCV E2 Protein

Analysis of subcellular localization of the expressed HCV E2 protein showed that it was localized predominantly in the microsomal fraction and the nuclear fraction but not in the cytoplasmic fraction (Fig. 5Aa). HCV E2 protein detected in the nuclear fraction is probably due to its localization in the endoplasmic reticulum (ER) on the peri-nuclear membrane. In contrast, chitinase was detected in every fraction (Fig. 5Ab). To assess whether the HCV E2 protein localized in the microsomal fraction is integrated into the microsomal membrane due to its C-terminal hydrophobic region, extraction of HCV E2 protein with or without Triton X-100 was carried out. As seen in Fig. 5B, the detergent extracted most of the HCV E2

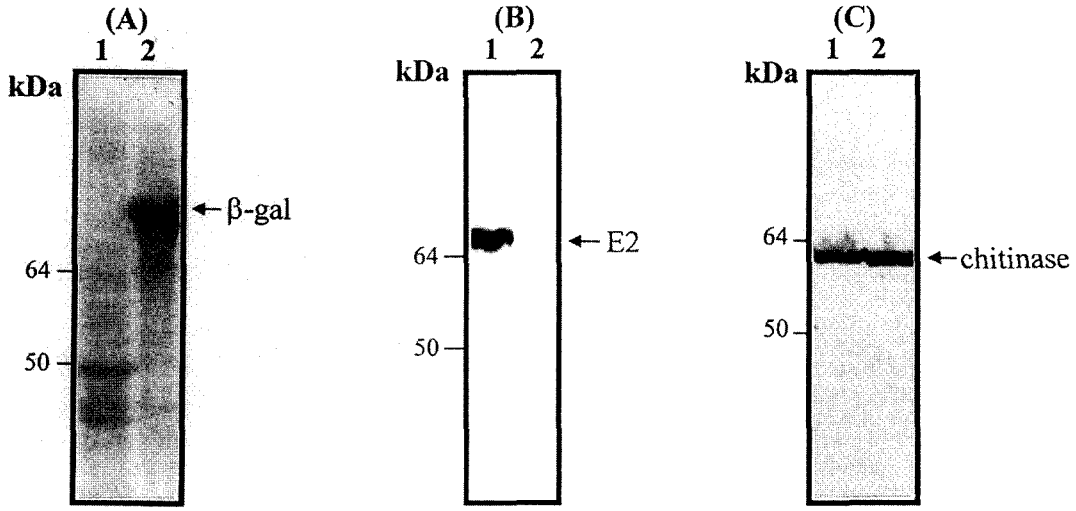

Fig. 3. Immunoblot analysis of HCV E2 protein expressed in *Trichoplusia ni* BTI-TN5B1-4 cells.

After the cells were grown to 1×10^6 cells ml^{-1} in suspension culture with SF900IISFM medium, they were resuspended in fresh medium and infected with 10 PFU $cell^{-1}$ of AcIL3E2 or BacPAK6. At 3 dpi, AcIL3E2-infected cell extracts (lane 1) and BacPAK6-infected cell extracts (lane 2) were separated on SDS-PAGE and stained with Coomassie blue (A) or subjected to immunoblot analysis using either HCV^+ patient serum (B), or guinea pig-derived anti-chitinase antibody (C).

protein population into a soluble fraction. HCV E2 protein did not bind to ConA-sepharose in the absence of the detergent, probably due to steric hindrance by the surrounding membrane. Therefore, our result indicates that the expressed HCV E2 protein was integrated into the microsomal membrane, though further confirmation by immunofluorescence might be necessary.

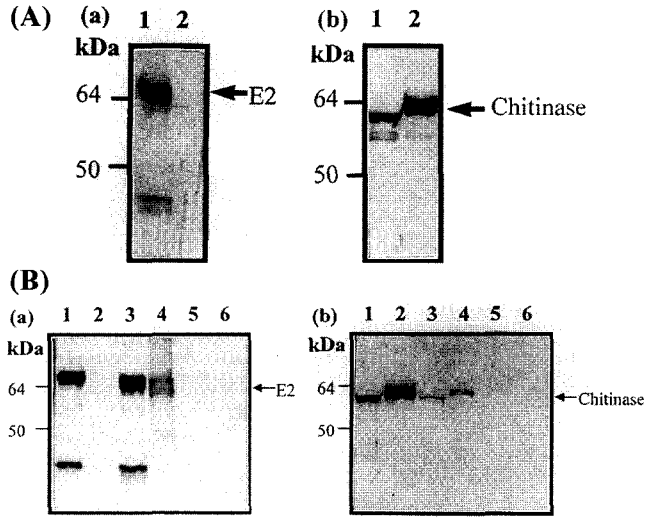

Fig. 4. Analysis of glycosylation properties of HCV E2 protein.

(A) Extracts of AcIL3E2-infected cells (lane 1) and BacPAK6-infected cells (lane 2) were precipitated with ConA-sepharose. (B) Extracts of AcIL3E2-infected cells (lanes 1, 3, and 5) and BacPAK6-infected cells (lanes 2, 4, and 6) were precipitated with agarose beads linked with various lectins including WGA (lanes 1 and 2), GNA (lanes 3 and 4), or PNA (lanes 5 and 6). Precipitated proteins were analyzed by immunoblot analysis with either HCV^+ patient serum (a) or guinea pig-derived anti-chitinase antibody (b).

Expression of HCV E2 Protein in the Heat Shock-induced Necrotic Cells

The integration into the microsomal membrane of the HCV E2 protein seemed to protect it from degradation by cellular proteases because its intracellular expression level was negligibly decreased even at very late post-infection, 9 dpi (data not shown). Comparative degradation rates of HCV E2 protein with that of chitinase were examined in heat shock-induced necrotic cells, where many intracellular proteins are known to be rapidly degraded by cellular proteases released from lysosomes. We observed that cell viability decreased from 91% (at 3 dpi, 0 hour post-heat shock (hphs)) to 0% (at 17 hphs) while cell viability was not changed significantly in the culture maintained at 27°C throughout the experiment. Chitinase expressed at 3 dpi degraded very rapidly and a low level of the protein was detected at 17 hphs. In contrast, HCV E2 protein did not significantly decrease, even at 43 hphs (Fig. 6). The relative expression level of HCV E2 protein at 43 hphs (lane 8) was almost the same (about 1.2 times higher) as the one at the corresponding time in the culture maintained at 27°C (lane 9), indicating that HCV E2 protein expressed at 3 dpi was not affected at all in the heat shock-induced necrotic cells. The difference in the degradation patterns of the two glycoproteins in the heat shock-induced necrotic cells might be primarily due to the differences in the degree of membrane association as shown in Fig. 5.

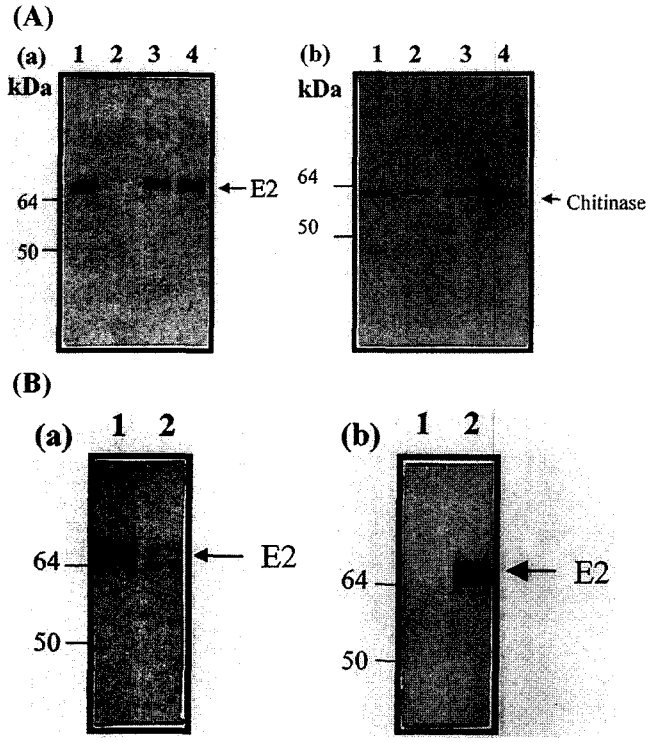

Fig. 5. Analysis of cellular localization of the expressed HCV E2 protein.

(A) Nuclear fraction (lane 1), cytosolic fraction (lane 2), microsomal fraction (lane 3), and unfractionated cell extract (lane 4) were analyzed by immunoblot analysis with either HCV^+ patient human serum (a) or guinea pig-derived anti-chitinase antibody (b). (B) Cell lysates were extracted with 2% Triton X-100 (a) or without (b) and incubated overnight at 4°C with ConA-sepharose. The suspensions were centrifuged at 500×g for 5 min to sediment the beads (lane 1) and the supernatants (lane 2) were removed. The bound proteins were washed and eluted with 1 M α-methyl-D-mannoside.

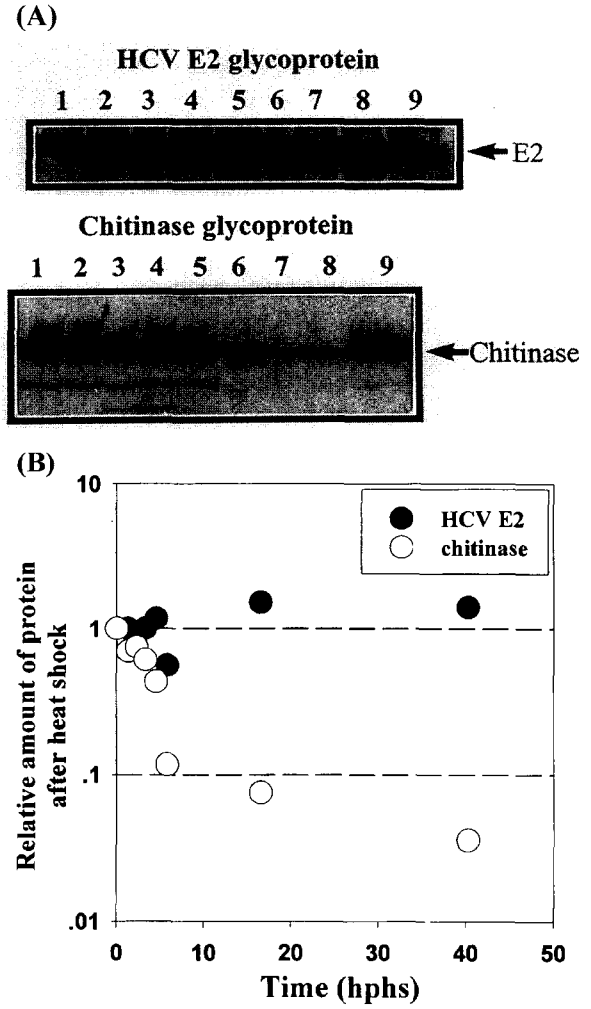

Fig. 6. Degradation of HCV E2 protein and chitinase in the heat shock-induced necrotic cells.

(A) At 3 dpi, necrosis was induced by incubating the cells at 43°C. Cells were taken at 0 hphs (hours post-heat shock) (lane 1), 1.25 hphs (lane 2), 2.25 hphs (lane 3), 3.25 hphs (lane 4), 4.5 hphs (lane 5), 5.75 hphs (lane 6), 16.5 hphs (lane 7), and 40.25 hphs (lane 8). Lane 9 is a reference corresponding to cells grown at 27°C throughout the culture and taken at the same time as lane 8. Cell extracts were analyzed by immunoblot analysis with either HCV^+ patient human serum (upper) or goat-derived anti-chitinase antibody (lower). (B) Relative expression levels of the HCV E2 protein (●) and chitinase (○) were determined by densitometric analysis of the immunoblots as described in the text.

DISCUSSION

As shown in Fig. 2, all three insect cell culture systems used in this study expressed chitinase comparably, indicating that the viral infection process was well being processed. When these culture systems were employed for HCV E2 protein expression, only the *Trichoplusia ni* BTI-TN5B1-4 cells and SF900IISFM medium system expressed HVC E2 protein properly. We previously observed that the higher titer of the extracellular virus progenies of both AcIL3E2 and BacPAK6, approximately several hundred times, were obtained from the *Sf9* cell culture system in comparison with the *Trichoplusia ni* BTI-TN5B1-4 cell culture system (data not shown). This observation suggests that the poor expression of HCV E2 protein in the *Sf9* cells is not due to incomplete progression of the viral infection processes but rather to the innate lower expression capacity of the *Sf9* cell culture system. Besides the expression capacity of the *Sf9* cells, the possibility cannot be ruled out that the post-translational glycosylation process of the protein is incomplete in the *Sf9* cells, resulting in expression of unglycosylated or partially glycosylated HCV E2 protein, which is less reactive to the HCV^+ sera due to its low antigenicity.

Our results above therefore indicate that the insect cell line and the cell culture medium appropriate for the expression of HCV E2 protein are not strictly correlated with the one appropriate for the production of extracellular AcIL3E2 virus progeny. Furthermore, the result in Fig. 2, showing different expression levels of HCV E2 protein

in the different culture media, indicates that a nutritional effect is important in the case of the expression of HCV E2 protein. It might be possible that some unidentified nutrients in the SF900IISFM medium were critical for the expression of HCV E2 protein. Further investigation using other recombinant baculoviruses will be necessary to see whether the *Trichoplusia ni* BTI-TN5B1-4 cells and SF900IISFM medium system has a general advantage for the production of other recombinant proteins.

Results obtained in this study provide important information for the production of E2 protein of HCV by the Bacu system. Using the Bacu system with *Trichoplusia ni* BTI-TN5B1-4 cells in SF900IISFM medium, we could express 68 kDa of recombinant HCV E2 protein in a fully glycosylated form. Subcellular localization was not distinguishable from that of HCV E2 protein expressed in the mammalian cell expression system [17]. Therefore, we speculate that HCV E2 protein derived from our expression system can elicit protective immunity in chimpanzees as reported by Ralston *et al.* [17]. The recombinant HCV E2 protein can also be utilized for development of next generation diagnostics for sera of immunosuppressed patients.

It was difficult to separate HCV E2 protein from the other cellular proteins, especially from chitinase using sequential Mono-Q FPLC and hydroxyapatite column chromatography, suggesting that chitinase has a similar glycan structure and electrochemical characteristics to those of the HCV E2 protein. However, they can be differentiated by their subcellular localizations and different degradation patterns in the heat shock-induced necrotic cells, as seen in Figs. 5 and 6. The heat shock process markedly reduced the soluble protein, chitinase glycoprotein, but it had no impact on the membrane-integrated HCV E2 protein. This observation indicates that environmental stresses such as heat shock can be employed to purify HCV E2 protein in the downstream purification process.

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