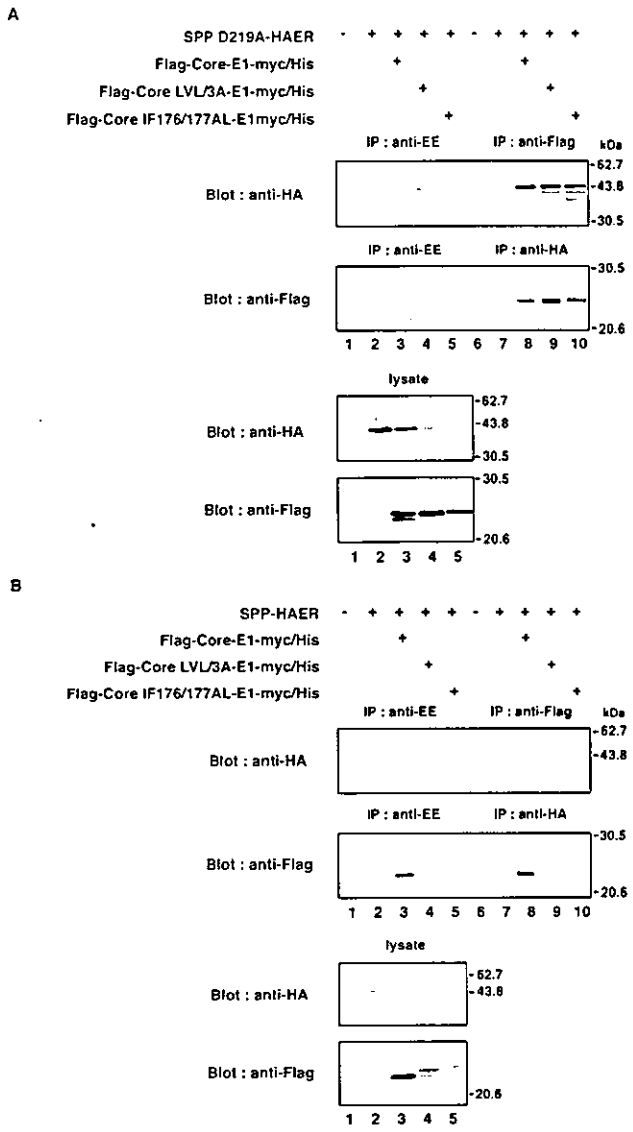

FIG. 6. Interaction of HCV core protein with SPP. SPP D219A-HAER (A) or SPP-HAER (B) was coexpressed with Flag-Core-E1-myc/His, Flag-Core LVL/3A-E1-myc/His, or Flag-Core IF176/177AL-E1-myc/His in 293T cells and immunoprecipitated (IP) with anti-Flag or anti-HA antibody. The immunoprecipitates were analyzed by immunoblotting with anti-HA or anti-Flag antibody. As a control, immunoprecipitation was carried out with anti-EE antibody. + and −, presence or absence of each plasmid, respectively.

core mutants suppress the expression of the SPP mutant. Clear reduction or elimination of the processing of the HCV core protein signal sequence was observed in cells coexpressing SPP D219A-HAER in comparison with those coexpressing wild-type SPP (Fig. 6A and B, bottom panels, lanes 3 to 5). Conversely, no interaction of HCV core protein with wild-type SPP was observed in cells coexpressing SPP-HAER and the HCV polyprotein substrates (Fig. 6B, upper panels, lanes 8 to 10). Broad bands were detected in immunoprecipitates with anti-Flag or anti-EE antibody by immunoblotting with the anti-Flag antibody, probably due to nonspecific binding of the processed

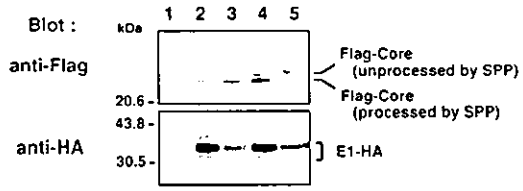

FIG. 7. Processing of HCV core-E1 polyprotein in the human hepatoma cell line FLC-4. Flag-Core-E1-HA (lane 2), Flag-Core ASC/VLV-E1-HA (lane 3), Flag-Core LVL/3A-E1-HA (lane 4), or Flag-Core IF176/177AL-E1-HA (lane 5) was expressed in FLC-4 cells and analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. Cells transfected with an empty plasmid were used as a negative control (lane 1).

core protein to protein G-Sepharose (Fig. 6B, second panel, lanes 3 and 8). These results indicate that a direct interaction of SPP with HCV core protein only between the unprocessed core protein and the loss-of-function mutant of SPP is verifiable. SPP should bind to the signal sequence of HCV core protein and release it after proteolysis, whereas SPP D219A cannot liberate the substrate after binding due to lack of the catalytic activity, suggesting that the SPP mutant may possess dominant negative effects.

Processing of the signal sequence of HCV polyprotein in a human hepatoma cell line. To confirm the data obtained for 293T cells with human liver cells, processing of core-E1 polyprotein in FLC4 cells, a human hepatoma cell line, was examined (Fig. 7). Processing by signal peptidase and SPP was evident in cells expressing Flag-Core-E1-HA or Flag-Core ASC/VLV-E1-HA (lanes 2 and 3), whereas clear processing by signal peptidase, but not complete cleavage by SPP, was observed in FLC-4 cells expressing Flag-Core LVL/3A-E1-HA or Flag-Core IF176/177AL-E1-HA (lanes 4 and 5). These results are consistent with data obtained with 293T cells, suggesting that the processing of the signal sequence of HCV core protein is not cell type dependent or an artifact of the techniques used in this study.

Localization of mutant HCV core proteins. To determine the effect of mutations on the localization of HCV core protein, EGFP-Core 191and its mutants that are defective in cleavage by SPP were expressed in HeLa cells (Fig. 8A). EGFP-Core 191 was processed by SPP and colocalized with an ER marker. EGFP-Core IF176/177AL, which bears a mutation that confers α-helix structure to the signal sequences, was diffusely distributed but did not completely colocalize with ER-DsRed as seen with EGFP-Core 191. EGFP-Core LVL/3A was localized mainly to the nucleus and, to a lesser extent, the cytoplasm, and EGFP-Core Δ128-151 exhibited complete nuclear localization. To confirm the subcellular localization of mutant HCV core proteins, cells were transfected with expression plasmids encoding N-terminally Flag-tagged and C-terminally HA-tagged core proteins to minimize the effect of fusion protein and fractionated, as described in Materials and Methods (Fig. 8B). Consistent with the subcellular localization of EGFP-Core proteins, Flag-Core 191-HA was detected mainly in the membrane-organelle fraction and Flag-Core LVL/3A-HA and Flag-Core Δ128-151-HA were localized mainly in the nuclear fraction. Although EGFP-Core IF176/177AL did not completely colocalize with the ER marker, 55% of Flag-

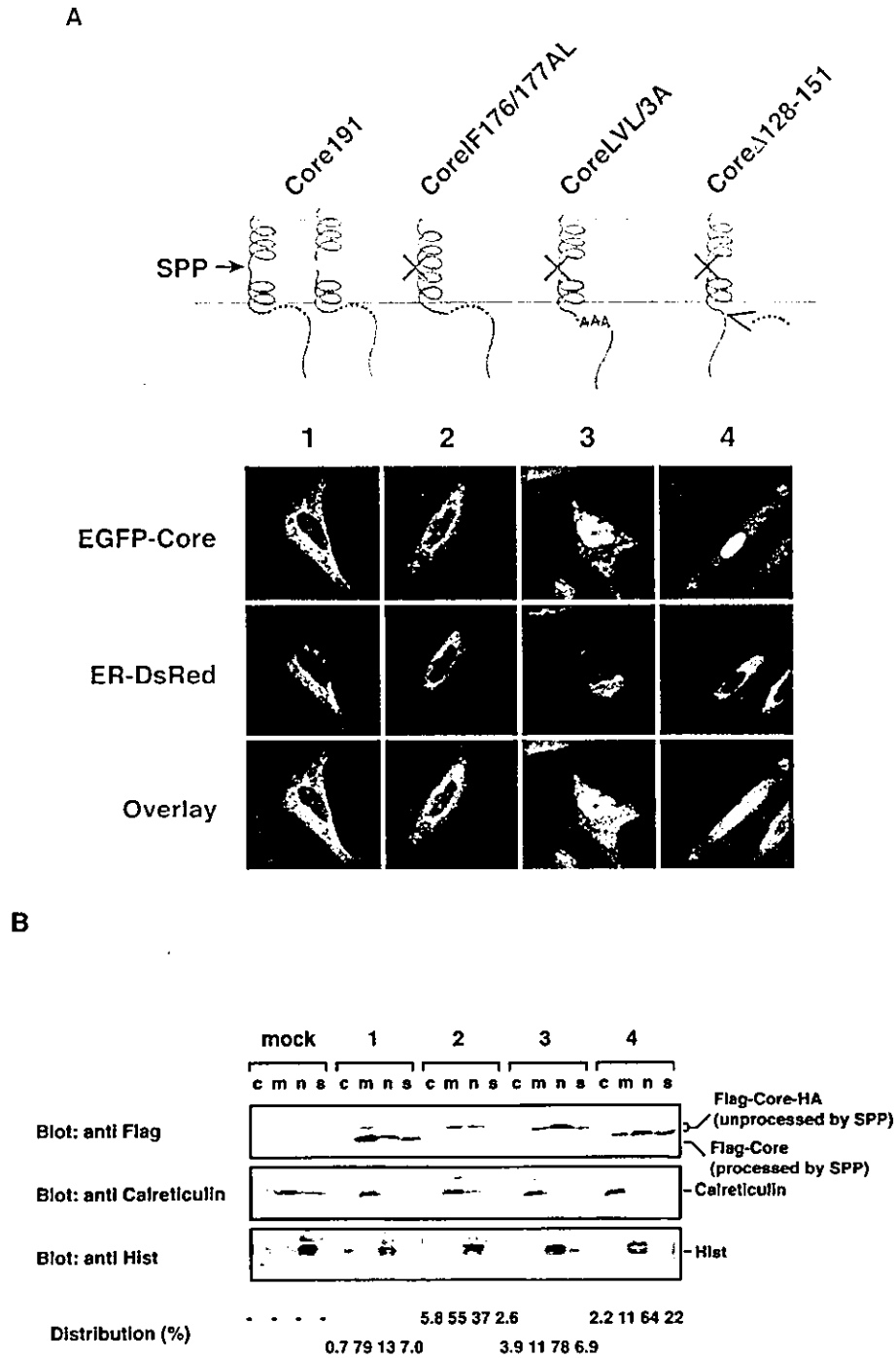

FIG. 8. Localization of mutant HCV core proteins. (A) Putative processing mechanisms of wild-type and mutant core proteins is illustrated at the top. EGFP-Core-191 (column 1), EGFP-Core IF176/177AL (column 2), EGFP-Core LVL/3A (column 3), and EGFP-Core Δ128-151 (column 4) were coexpressed with ER-DsRed in HeLa cells, and subcellular localization of core proteins was examined by confocal microscopy. (B) Subcellular fractionation of HeLa cells transfected with plasmids encoding Flag-Core-HA polyproteins. Cells transfected with an empty plasmid (lane M) or plasmid encoding Flag-Core 191-HA (lanes 1), Flag-Core IF176/177AL-HA (lanes 2), Flag-Core LVL/3A-HA (lanes 3), or Flag-Core Δ128-151-HA (lanes 4) were extracted into four fractions, as described in Materials and Methods. Each fraction was concentrated and subjected to immunoblotting with anti-Flag antibody (upper panel). Lanes c, m, n and s, cytosol, membrane-organelle, nuclear, and cytoskeleton fractions, respectively. Calreticulin and histone (His) were used as markers for membrane-organelle and nuclear fractions, respectively. To determine the distributed ratio of processed and unprocessed core proteins in each fraction, the density of core protein in each fraction was measured and is indicated as a percentage at each bottom of lane.

Core IF176/177AL-HA was detected in membrane-organelle fraction. Since we could not separate ER and Golgi fractions by the fractionation method used, it is possible that Flag-Core IF176/177AL-HA localizes mainly in the Golgi rather than the ER. These results indicate that not only the C-terminal signal sequence but also the hydrophobic region from amino acid 139 to 144 in domain 2 and proper processing by SPP are involved in the ER retention of HCV core protein.

DISCUSSION

Hope and McLauchlan identified three regions in the HCV core protein, including two hydrophobic regions in the C-terminal one-third of the protein and the region from amino acid 119 to 174, which was designated domain 2 (12). Domain 2 was hypothesized to interact with lipid droplets and confer stability to the HCV core protein (12). Although a mutation in the signal sequence of core protein that renders it resistant to SPP proteolysis restored retention on lipid droplets and overall stability (30), deletion of domain 2 from HCV core protein leads to diffusion in the cytoplasm and degradation after processing by signal peptidase (30). Indeed, these data suggest that mature HCV core protein is retained on lipid droplets via domain 2, but they do not necessarily indicate that the region is required for the ER retention of HCV core protein. The intramembrane proteolysis of the signal sequence of HCV core protein by SPP is abolished when helix-breaking and -bending residues in the C-terminal signal-anchor sequence are replaced by basic amino acids (30). However, the involvement of other regions of HCV core protein in processing by SPP is not known. In this study, we could demonstrate that not only the C-terminal signal-anchor domain but also three hydrophobic amino acids Leu^{139}, Val^{140}, and Leu^{144} in domain 2 are required for intramembrane proteolysis by SPP. However, this domain is not essential for the cleavage of the core-E1 junction by signal peptidase or for translocation of E1 into the ER. Furthermore, the nuclear localization of domain 2 core mutants possessing an unprocessed C-terminal signal-anchor sequence indicates that association with the ER membrane through domain 2 is required for ER retention of HCV core protein.

Martoglio and colleagues demonstrated, by using a Semliki Forest virus expression system, that Ala^{180}, Ser^{183}, and Cys^{184} break the α-helical structure within the signal sequence and are essential for the intramembrane proteolysis of HCV core protein of the type 1a Glasgow strain by SPP in BHK and Huh7 cell lines (23, 30). However, mutation of Ala^{180}, Ser^{183}, and Cys^{184} in core proteins of the type 1b J1 and type 1a H77 strains could not inhibit signal sequence processing by SPP in the BHK and 293T cell lines by expression with plasmid. HCV core protein of the type 1a Glasgow strain shares 96.3 and 95.8% amino acid homology to those of the H77c and J1 strains, respectively. Furthermore, the signal sequences of the core proteins of these three strains are almost identical, and therefore the observed differences in cleavability by SPP might be attributable to sequence other than the signal sequence or expression system used. We reexamined the HCV core protein signal sequence and, using the method of Garnier et al. (9), chose to further examine Ile^{176} and Phe^{177} as residues that may interfere with the assumption of a compact α-helix structure and allow for intramembrane proteolysis by SPP. Mutation of Ile^{176} and Phe^{177} (Core IF176/177AL) of genotype 1a and 1b strains, which is predicted to confer α-helical structure to the signal sequences, inhibited processing by SPP. EGFP-Core IF176/177AL exhibited no colocalization with an ER marker; this differs from the case for the wild-type core protein. These data suggest that the processing of the signal sequence by SPP may play a role in the ER retention of HCV core protein.

Precursor HCV core protein consists of 191 amino acids and is processed by signal peptidase from a polyprotein after translocation of the C-terminal signal-anchor sequence into the ER. This is then cleaved by SPP into the mature core protein and localizes primarily to the ER. The mature core protein, further processed by an unidentified protease, is composed of amino acids 151 to 153 and is detected in the nucleus (33, 47). Actually, HCV core protein is observed in the cytoplasm, nucleus, and nucleoli in transgenic mice expressing HCV core protein (35). Under normal conditions, the precursor core protein is processed by SPP to 173 to 179 amino acids and localizes to the ER. In contrast, a 179-amino-acid construct containing a limited C-terminal anchor-signal sequence, Core 179, localizes primarily to the nucleus and to the ER to a lesser extent. This striking difference in the subcellular localizations of Core 191 and Core 179, in conjunction with data from the Core IF176/177AL construct, indicates that the presence of the full-length signal-anchor sequence and proper processing by SPP is required for retention of HCV core protein on the ER membrane.

We also demonstrated that the reduction in hydrophobicity in domain 2 affects proteolysis of the signal sequence by SPP and localization of HCV core protein. It was suggested that HCV core protein interacts with lipid droplets containing triacylglycerol and/or ER membrane through domain 2 irrespective of intramembrane proteolysis of the signal sequence (30). A mutant HCV core protein in domain 2, EGFP-Core LVL/3A with Leu^{139}, Val^{140}, and Leu^{144} replaced by Ala, was processed by signal peptidase but not by SPP and localized to the nucleus in spite of the presence of an unprocessed hydrophobic signal sequence in the C terminus. This result suggests that penetration of the HCV core protein signal sequence into the ER membrane is necessary, but not sufficient, for ER retention of HCV core protein. Insertion of the C-terminal signal-anchor sequence of core protein into the ER may induce conformational changes in domain 2 to render it accessible to the ER membrane and/or lipid droplets by exposure of hydrophobic residues in the domain, residues that are well conserved among various genotypes of HCV. Although it was suggested that processed HCV core protein was retained on the ER membrane via an interaction with unprocessed core protein (25) or with the C-terminal transmembrane region of E1 (26), our data provide a new model of the ER retention of HCV core protein. HCV core protein is a structural protein that forms the nucleocapsid, and virus particles are thought to be released into ER. Therefore, retention of HCV core protein on the ER membrane should be essential for the assembly of HCV.

Intramembrane-cleaving proteases have been shown to play pivotal roles in cell regulation and signaling and are involved in diseases such as Alzheimer's disease (52). SPP belongs to a family of aspartic proteases family and has two aspartic acid residues, Asp^{219} and Asp^{265}, in the enzyme active site (50).

Signal peptidase II also belongs to this aspartic protease family and cleaves the signal sequence by attacking a proton of a water molecule via an aspartic acid of the enzyme (38). Mutant SPP bearing an Asp^{265}-to-Ala substitution was deficient in the processing of HLA-A but retained binding activity to the SPP substrate analogue TBL_4K (50). We could demonstrate a direct interaction by immunoprecipitation of unprocessed HCV core proteins with mutant SPP lacking catalytic and substrate-releasing activities by replacement of Asp^{219} with Ala. Binding of the loss-of-function SPP mutants with unprocessed core proteins irrespective of mutation or deletion in domain 2 indicates that the domain is not directly involved in the interaction.

It has been demonstrated that expression of HCV core protein alone is sufficient for the induction of hepatic steatosis and hepatocellular carcinoma in transgenic mice (24, 34, 35). Furthermore, we demonstrated that nuclear localization and degradation of HCV core protein is regulated by PA28γ-dependent proteolysis (33). These findings suggest that HCV core protein plays a pivotal role in the development of hepatocellular carcinoma and that intramembrane proteolysis may regulate the subcellular localization of HCV core protein. Although the SPP inhibitor $(Z\text{-}LL)_2$-keton suppresses cleavage of signal sequence essential for homeostasis, host defense, etc., a specific inhibitor against the intramembrane proteolysis of HCV core protein, such as antagonists for the binding of HCV core protein to ER membrane via domain 2, will be an effective antiviral drug for patients with chronic hepatitis C. Furthermore, involvement of intramembrane proteolysis by SPP in the processing of other HCV proteins and the fates of the peptides cleaved by SPP in the replication and pathogenesis of hepatitis C are subjects of future studies.

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Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin

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Abstract

Dendritic cells (DC) are the most potent antigen-presenting cells that regulate immune responses. One of the mechanisms for hepatitis C virus (HCV) persistence is the ability of HCV to suppress DC function. Direct HCV infection to blood DC has been implicated for DC dysfunction. To clarify the susceptibility of each DC subset to HCV, we used pseudotype vesicular stomatitis virus (VSV) coated with chimeric HCV envelope glycoproteins (E1 and E2). We demonstrate that pseudotype VSV enters myeloid DC (MDC) but not plasmacytoid DC (PDC). The highest efficiency of pseudotype VSV entry to MDC was observed when MDC were cultured with GM-CSF. Such efficiency decreased when MDC are matured with the treatment of IL-4, CpG oligodeoxynucleotide, or CD40 ligand. Mannan inhibited pseudotype VSV entry to MDC, but Ca^{2+} chelators failed to do so. These results show that pseudotype VSV possessing HCV-E1 and E2 enters immature MDC through the interaction with lectins in a Ca^{2+}-independent manner.

Keywords: Hepatitis C virus; Envelope proteins; Pseudotype virus; Dendritic cells; Lectin

Introduction

Hepatitis C virus (HCV), a single-stranded plus-sense RNA virus belonging to the flaviviridae family (Miller and Purcell, 1990), causes persistent infection in more than 70% of infected patients. The most important feature of HCV persistence is the potential for liver disease progression from mild hepatitis to liver cirrhosis and hepatocellular carcinoma (HCC) (Alter et al., 1989). Chronic HCV infection is a serious health problem because the total number of HCV-positive HCC patients is growing worldwide. One of the mechanisms for HCV persistence is the ability of HCV to escape from the host cellular immune response (Farci et al., 1992; Weiner et al., 1995). Cumulative studies show that functional impairment of immunocompetent cells is found in patients with chronic HCV infection (Corado et al., 1997; Wedemeyer et al., 2002), suggesting that HCV has various arms for suppressing the immune response.

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) that regulate various immune responses (Banchereau and Steinman, 1998; Hart, 1997). Blood DC mainly consist of two subsets, that is, myeloid and plasmacytoid DC (Liu, 2001). Myeloid DC (MDC) are characterized by their potent immunostimulatory properties for both primary and secondary T-cell responses against virus. Plasmacytoid DC (PDC), previously known as interferon (IFN)-producing cells, produce a large amount of type I IFN upon virus infection (Liu, 2001). However, some viruses such as

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measles virus or HIV have been shown to infect monocyte-derived DC (MoDC) or PDC and subsequently inhibit their immunostimulatory functions (Grosjean et al., 1997; Patterson et al., 2001; Schnorr et al., 1997). Previous studies including our own have shown that MoDC from patients with chronic HCV infection are functionally impaired (Bain et al., 2001; Kanto et al., 1999). In addition, our recent investigation revealed that the function of both types of blood DC is suppressed as well in HCV-infected patients (Kanto T. et al., unpublished data). These results led us to hypothesize that HCV infection to DC is one of the mechanism for DC dysfunction in chronic hepatitis C patients.

The existence of the HCV genome in blood cells including DC has been shown in several studies by means of reverse transcription (RT)-PCR (Bain et al., 2001; Lerat et al., 1996, 1998; Navas et al., 2002). The detection of the positive strand of HCV-RNA does not enable to define whether HCV enters cells or only adheres to their surface. Alternatively, the negative strand of HCV-RNA has been used as a surrogate marker of HCV replication (Navas et al., 2002). Recently, Matsuura et al. (2001) established the pseudotype vesicular stomatitis virus (VSV) having chimeric HCV E1 and E2 protein as an envelope (VSV-E1E2). Because it has a green fluorescent protein (GFP) reporter gene in its genome, the infected cells can be viewed under fluorescence. Using this system, we tried to clarify the susceptibility of each DC to HCV. Consequently, we demonstrate that MDC is susceptible to VSV-E1E2 but PDC is not. Furthermore, we showed that the lectin-containing molecules on MDC are critically involved in VSV-E1E2 entry. Our study provides useful information for the exploration of target molecules that efficiently block HCV entry to DC.

Results

Immature MDC are susceptible to VSV-E1E2

We inoculated pseudotype VSV on various cells separated from PBMC or cord blood. Because no positive fluorescence was obtained from CD4 T cells, CD8 T cells, B cells, NK cells, and fresh PDC inoculated with VSV ΔG-G which is complemented with the VSV G protein, the susceptibility of these cells to VSV-E1E2 could not be estimated. In contrast, VSV ΔG-G entered fresh MDC, monocytes, and $CD34^+$ hematopoietic precursor cells on the day of separation, whereas VSV-E1E2 and VSV ΔG did not. Thus, fresh MDC as well as DC precursors are not susceptible to VSV-E1E2 (Fig. 1).

To examine the influence of differentiation or maturation of DC on the susceptibility to the pseudotype VSV, MDC were cultured in the presence of GM-CSF with or without IL-4. Phenotypic analysis revealed that day 4 MDC cultured with GM-CSF and IL-4 had higher expression of CD1a and CD86 than those cultured with GM-CSF only (Fig. 2), showing the role of IL-4 in DC maturation. No significant difference was observed in the expression of CD11c, CD40, CD80, CD83, and HLA-DR between these MDC (Fig. 2). On day 4 of culture in the presence of IL-3, PDC showed higher expressions of CD40, CD80, CD83, and CD86 when compared to the day of separation (data not shown).

After the inoculation with VSV ΔG-G, positive signals were obtained from day 4 MDC and day 4 PDC, regardless of the difference of cytokines used (Figs. 3A and B). No significant signals were detected from day 4 MDC and day 4 PDC inoculated with VSV ΔG. With respect to VSV-E1E2, GFP^+ cells were observed in day 4 MDC but not in day 4 PDC (Figs. 3A and B). In

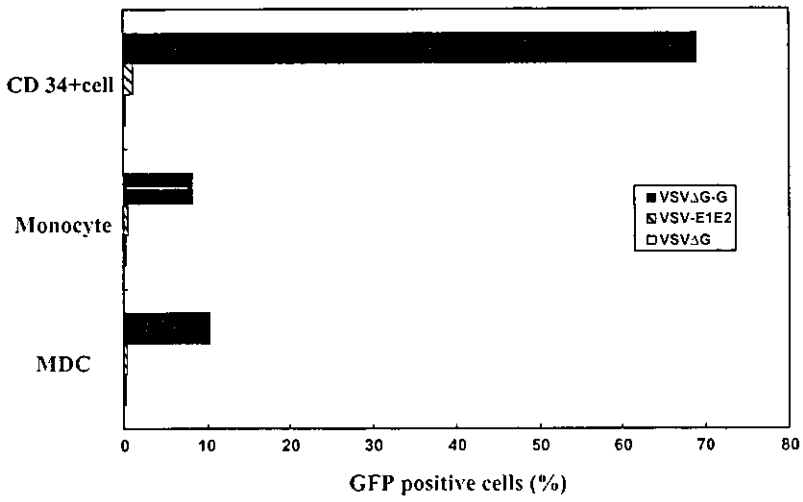

Fig. 1. Freshly isolated DC are not susceptible to VSV-E1E2. Freshly isolated $CD34^+$ hematopoietic precursor cells, monocytes, or MDC were inoculated with pseudotype VSVs and the percentages of GFP^+ cells were determined by flow cytometry. Representative results from three experiments are shown.

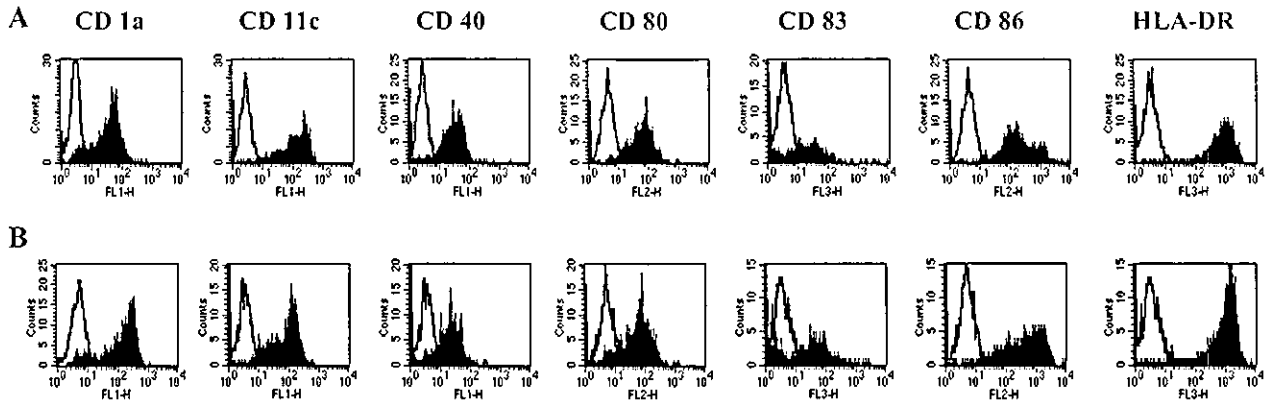

Fig. 2. Phenotypes of MDC cultured with GM-CSF or GM-CSF and IL-4. Flow cytometric analyses of surface molecules on day 4 MDC, obtained from healthy volunteers, cultured with GM-CSF (A) or with GM-CSF and IL-4 (B). Representative results from three subjects are shown. Open histograms represent the results with isotype Ab and filled ones represent those with relevant Abs. Fluorescence intensity is shown in the x-axis and the number of cells is shown in the y-axis.

comparison of the culture conditions for MDC, higher percentage of GFP^+ cells was observed in day 4 MDC cultured with only GM-CSF than those with GM-CSF and IL-4 (Fig. 3B).

To confirm the reliability of the pseudotype VSV system in the assessment of E1E2-mediated virus entry, we quantified HCV-RNA in day 4 cultured MDC or PDC after inoculation of a window-period serum from a hepatitis C patient. Among the cells examined, the highest HCV RNA titer was detected in day 4 MDC cultured with GM-CSF (Fig. 3C), which was compatible with the results obtained with the pseudotype VSV. In contrast with the results of VSV-E1E2 inoculation, low-level HCV RNA were detected by quantitative RT-PCR in PDC inoculated with authentic HCV (Fig. 3C). To further investigate whether HCV replicates in each DC subset after HCV inoculation, we performed strand-specific RT-PCR for the detection of negative-strand HCV-RNA as a surrogate marker of HCV replication. Positive strand of HCV-RNA was detected both in MDC cultured with GM-CSF and PDC with IL-3, whereas negative strand was detected in GM-CSF-MDC but not in IL-3-PDC (Fig. 3D). These results suggest that HCV replicates in GM-CSF-MDC but not in IL-3-PDC. Therefore, the data with the pseudotype VSV system correctly reflect the susceptibility of cells to authentic HCV.

Maturation stimuli protect MDC from VSV-E1E2

Based on the findings described above, we hypothesized that the more MDC mature, the less susceptible they are to VSV-E1E2. To find the substances protecting DC from HCV infection, we treated MDC with various maturation factors for the inoculation study. In MDC cultured with GM-CSF, the addition of IL-4, CpG oligodeoxynucleotide (ODN) 2006, or CD40 ligand (CD 40L) to the culture significantly reduced the percentage of GFP^+ cells with VSV-E1E2 without influencing their susceptibility to VSV ΔG-G (Fig. 4). On the other hand, IFN-α, polyI:C, TNF-α, and lipopolysaccharide (LPS) reduced the percentage of GFP^+ cells with both VSV-E1E2 and VSV ΔG-G (Fig. 4). Phenotypic analysis revealed that IL-4 up-regulated the expression of CD1a and CD86 on MDC cultured with GM-CSF (Fig. 2). CpG ODN or CD40L also up-regulated the expression of CD1a, CD83, and CD86 on MDC cultured with GM-CSF (data not shown). Therefore, immature DC lose their susceptibility to VSV-E1E2 as they develop to be more mature state.

Lectin on DC is involved in VSV-E1E2 entry to DC

The C-type lectins expressed on DC are reported to interact with various viruses as well as microbial agents (Geijtenbeek et al., 2000; Tailleux et al., 2003). These studies led us to consider the involvement of lectins in VSV-E1E2 entry to DC. Thus, we first used mannan to examine whether it inhibits VSV-E1E2 entry to MDC. The pretreatment of MDC with mannan reduced the percentage of GFP^+ cells with VSV-E1E2 in a dose-dependent manner without having any impact on VSV ΔG-G entry (Fig. 5A). Such an inhibitory effect of mannan was confirmed with MDC inoculated with authentic HCV (data not shown). A D-mannose-specific lectin, methyl α-D-mannopyranoside (Kaku et al., 1991), also inhibited VSV-E1E2 entry to MDC in a dose-dependent fashion at concentrations from 10 to 40 μg/ml (data not shown). In contrast, galactose had no effect on the infection with either VSV-E1E2 or VSV ΔG-G in MDC (Fig. 5A). Interestingly, EDTA did not reduce the infectivity of VSV-E1E2, whereas it completely abolished that of VSV ΔG-G (Fig. 5A). These data demonstrate that mannose-type carbohydrate is involved in the interaction of DC with VSV-E1E2 in a Ca^{2+}-independent manner. The treatment of MDC with antihuman DC-SIGN Ab,

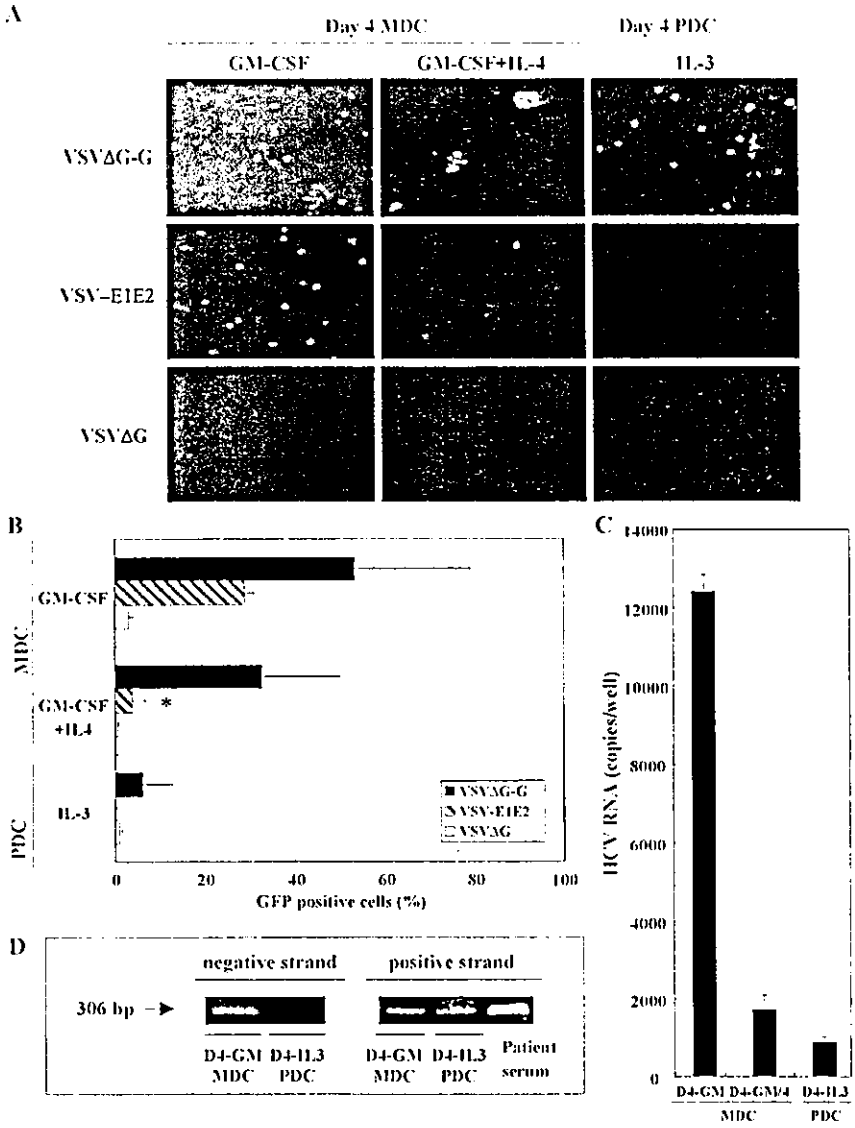

Fig. 3. Myeloid DC cultured with GM-CSF are susceptible to VSV-E1E2 or authentic HCV from patient serum. Day 4 MDC cultured with GM-CSF or with GM-CSF and IL-4 or day 4 PDC cultured with IL-3 were inoculated with VSV ΔG, VSV ΔG-G, or VSV-E1E2. They were viewed under fluorescence microscopy (A) and the percentages of GFP^+ cells were analyzed by flow cytometric analysis (B). The results of fluorescence microscopy are the representative ones from three subjects. The results of flow cytometric analysis are expressed as the mean + SD from three representative experiments. $^*P < 0.001$ vs. VSV-E1E2-inoculated day 4 MDC cultured with GM-CSF. (C) Quantitative analysis of HCV RNA in DC inoculated with HCV-positive patient serum was performed as described in Materials and method. D4-GM or D4-GM/4 represents MDC cultured with GM-CSF or GM-CSF and IL-4 for 4 days. D4-IL 3 represents PDC cultured with IL-3 for 4 days. The results are expressed as the mean + SD of triplicate wells from three representative experiments. (D) The detection of positive and negative strand of HCV-RNA in DC inoculated with HCV-positive patient serum. Strand-specific RT-PCR was performed with samples from MDC, PDC, and patient serum used as inoculum, as described in Materials and method. D4-GM and D4-IL 3 represent as the same as above.

which is able to block the binding of DC-SIGN to ICAM-3 (Wu et al., 2002), did not inhibit the entry of either VSV-E1E2 or VSV ΔG-G (Fig. 5B). To see whether the expression of DC-SIGN on MDC parallels their susceptibility to VSV-E1E2, we compared the expression of DC-SIGN between MDC cultured with GM-CSF and those with a combination of GM-CSF and IL-4. The expression of DC-SIGN was higher on MDC cultured with GM-CSF and IL-4 than on those with GM-CSF (Fig. 5C), which is contrary to their susceptibility to VSV-E1E2. These results show that DC-SIGN is less likely to be involved in the VSV-E1E2 entry to MDC. Human hepatoblastoma cell line, HepG2, is one of the most sensitive cells to pseudotype VSV (Matsuura et al., 2001). To compare the machinery of VSV-E1E2 entry between MDC and HepG2, we inoculated it to mannan-treated HepG2. In contrast to MDC, mannan did not inhibit the VSV-E1E2 entry to HepG2 (Fig. 5D), suggest-

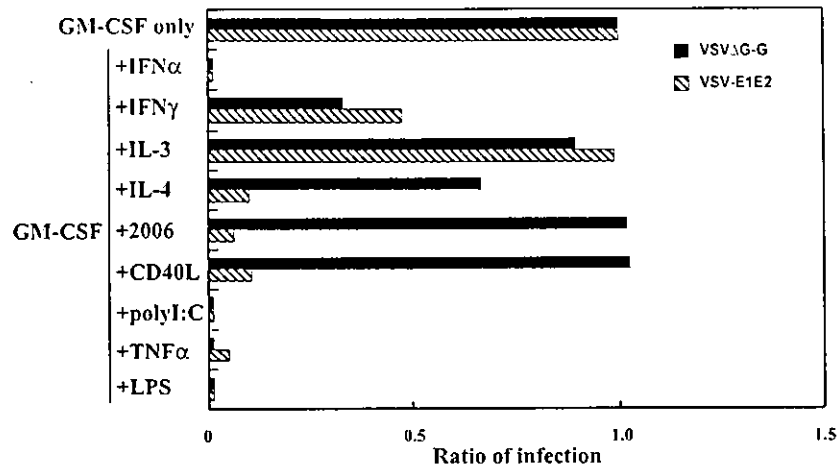

Fig. 4. IL-4, CpG ODN, or CD40L protects MDC from VSV-E1E2. Various immunomodulators were added to MDC cultured with GM-CSF, and the ratio of infection was determined between the cells treated with or without the reagents. IFN-α (100 U/ml), IFN-γ (100 U/ml), IL-3 (50 ng/ml), or IL-4 (10 ng/ml) was added on the day of MDC separation. CpG ODN 2006 (10 μM), CD40L (1 μg/ml), polyI:C (50 μg/ml), TNFα (20 ng/ml), or LPS (10 μg/ml) was added to MDC 24 hrs before the pseudovirus inoculation. Representative results are shown from three independent experiments.

ing that the molecules responsible for VSV-E1E2 entry differ between HepG2 and MDC. Furthermore, it also shows that mannan affects the molecules on MDC but not those on VSV-E1E2.

Discussion

Using the pseudotype VSV system, we have demonstrated that each DC subset has distinct susceptibility to HCV. First, VSV-E1E2 enters MDC but not PDC, which is in sharp contrast with PDC susceptibility to HIV (Patterson et al., 2001). Second, MDC cultured with GM-CSF are more susceptible to VSV-E1E2 than freshly prepared MDC or those cultured with GM-CSF and IL-4, showing that HCV targets immature MDC. Third, certain molecules containing the lectin domain on MDC are involved in the interaction with VSV-E1E2.

One of the suggested mechanisms of persistent HCV infection is the functional suppression of immunocompetent cells, including NK cells, T cells, and DC (Bain et al., 2001; Corado et al., 1997; Kanto et al., 1999; Wedemeyer et al., 2002). The possibility being raised for such immunological impairment is that HCV directly infects these blood cells. To elucidate this issue, investigators have used RT-PCR to examine whether the HCV genome is detectable or not in blood cells recovered from HCV-infected patients (Bain et al., 2001; Lerat et al., 1996, 1998). However, the existence of HCV-RNA does not enable to define whether HCV enters cells or only adheres to their surface. Instead of qualitative RT-PCR, we used the pseudotype VSV system to study the HCV E1E2-mediated virus entry to each DC subset. The pseudotype VSV system is a valid model for investigating the early steps of HCV infection, that is, viral attachment, receptor binding, and membrane fusion. Also, it enables us to estimate the efficiency of HCV E1E2-mediated virus entry to target cells. However, there are several limitations in this system. First, the positive results with pseudotype VSV do not indicate the replicative ability of HCV in the relevant cells. Because pseudotype VSV is constructed from VSV genome, their replication capacity is not exactly the same as HCV. Second, the evaluation of pseudotype VSV entry is possible only in cells that permit VSV replication. In other words, it cannot be used to determine the entry of VSV-E1E2 in the cells that suppress VSV replication. In this study, we could not evaluate the susceptibility of T, B, NK cells or fresh PDC to VSV-E1E2.

Alternatively, we performed an inoculation experiment with authentic HCV particle to confirm the reliability of the pseudotype VSV system. Quantitative RT-PCR assay showed that the highest titer of HCV-RNA was detected in MDC cultured with GM-CSF; however, low titer of HCV-RNA was detected in PDC. We hypothesized that the reason such discrepancy occurs between two assays is that RT-PCR amplified HCV genome from HCV attached to the surface of PDC. Strand-specific RT-PCR showed that negative strand of HCV-RNA, a surrogate marker of HCV replication, was detected in MDC cultured with GM-CSF but not in PDC. These results indicate that HCV enters and replicates in MDC but not in PDC, which are well correlated with those of pseudotype VSV entry.

The Th1 response is thought to be needed to eradicate HCV from hosts (Gerlach et al., 1999). Myeloid DC potentially activate CD4+T cells to support Th1 differentiation (Liu, 2001). We found that MDC from HCV-infected patients are less able to induce the Th1 response than the normal counterpart (Kanto T., unpublished data). It has been reported that MoDC expressing HCV protein were impaired in the stimulation of allogeneic T cells and IL-12 production, indicating an inhibitory capacity of HCV

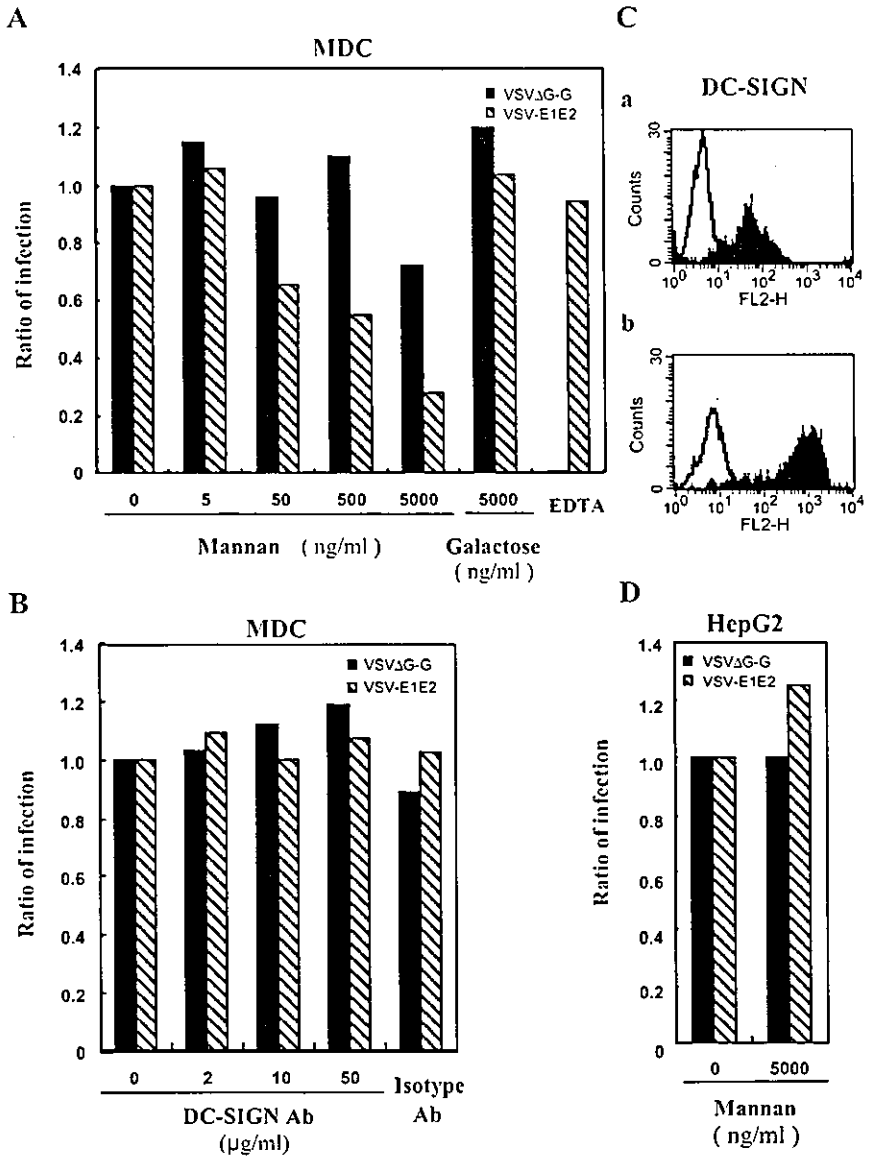

Fig. 5. Mannan inhibits VSV-E1E2 entry to MDC, but not to HepG2. Various concentrations of mannan, galactose, or 5 mM EDTA were added to day 4 MDC cultured with GM-CSF before the pseudotype VSV inoculation. Alternatively, day 4 MDC were treated with 2, 10, or 50 μg/ml antihuman DC-SIGN Ab or 10 μg/ml isotype IgG_{2B} for 30 min before the pseudotype VSV inoculation. HepG2 was treated with 5 μg/ml mannan before the addition of pseudotype VSV. The ratio of infection with pseudotype VSV in MDC (A, B) or HepG2 (D) was determined as described in Materials and method. Representative results are shown from three independent experiments. (C) Flow cytometric analyses were done for DC-SIGN expression on day 4 MDC cultured with GM-CSF (a) or with GM-CSF and IL-4 (b) generated from a healthy volunteer. Representative results from three subjects are shown. Open histograms represent the results with isotype Ab and filled ones represent those with anti-DC-SIGN Abs. Fluorescence intensity is shown in the *x*-axis and the number of cells is shown in the *y*-axis.

protein on DC function (Sarobe et al., 2002). These data suggest that direct HCV infection to myeloid DC suppress their function. Thus, the protection of DC from HCV infection is a rational approach to improve DC-mediated anti-HCV immune response. In the present study, we demonstrate that some of the maturation stimuli are capable of protecting DC from VSV-E1E2 entry. However, MoDC from HCV-infected patients are reported to be resistant to maturation stimuli, such as TNF-α (Auffermann-Gretzinger et al., 2001). Thus, further investigation is necessary to determine the effective modulation that allows MDC to mature in HCV infection. As shown in this study, CpG ODN or CD40L stimulated MDC to mature and become less susceptible to VSV-E1E2. The potent ability of CpG ODN to stimulate a DC-inducing Th1 response has been demonstrated in vivo tumor treatment models (Heckelsmiller et al., 2002). Therefore, CpG ODN are promising as a DC adjuvant in HCV-infected patients that potentially leads to MDC maturation as well as boosting Th1 response.

It is arguably necessary to identify molecules that are responsible for HCV entry to protect DC. Previously, tetraspanin CD81 has drawn much attention as a presumed HCV receptor due to its high affinity to HCV-E2 (Pileri et al., 1998). However, its involvement in VSV-E1E2 entry is unlikely because the CD81 is equally expressed on both MDC and PDC but is lacking on the VSV-E1E2-sensitive cell line HepG2 (Flint et al., 1999). In this study, we showed some of the characteristics of the molecules on DC involved in VSV-E1E2 entry. They are myeloid-lineage specific, inducible by GM-CSF, down-regulatable by IL-4 or other maturation stimuli. In addition, they possess some lectin domain, as evidenced by the inhibition of VSV-E1E2 as well as authentic HCV entry with mannan. These results raised the possibility that such molecules are categorized as members of C-type lectins, such as DC-SIGN, mannose receptor (MR), Langerin, DEC205, BDCA2, or asialoglycoprotein receptor. (Figdor et al., 2002) Recently, two independent studies have demonstrated that HCV E1 and E2 glycoproteins efficiently bind to DC-SIGN (Lozach et al., 2003; Pohlmann et al., 2003). However, the involvement of DC-SIGN in VSV-E1E2 entry is less likely because its expression on MDC did not parallel the susceptibility to VSV-E1E2. In addition, the treatment with anti-DC-SIGN Ab did not inhibit VSV-E1E2 entry. Furthermore, the treatment with EDTA failed to block VSV-E1E2 entry to MDC, showing that the VSV-E1E2 entry occurs in a Ca^{2+}-independent manner. It is still obscure in which step lectins are involved in VSV-E1E2 entry to MDC. From an analogy with the interaction of HIV with DC-SIGN (Geijtenbeek et al., 2000), it is conceivable that lectins are essential for HCV attachment to MDC. Nevertheless, the possibility remains that HCV entry receptors or co-receptors, which may be other than lectins, exist on MDC. With the aid of the pseudotype VSV system, exploration has been underway to identify the molecules on MDC that are critically involved in HCV infection.

Materials and method

Reagents

Recombinant human IL-4 and GM-CSF were purchased from PeproTech (London, UK). Recombinant human soluble CD40L, human TNF-α and IL-3, and mouse monoclonal antihuman DC-SIGN (CD209/DC-SIGN1) Ab (12507) were from R & D Systems (Minneapolis, MN). LPS, polyI:C, mannan, galactose, and methyl α-D-mannnopyranoside were from Sigma (St. Louis, MO). Recombinant human IFN-γ was from Strathman Biotech GmbH (Hamburg, Germany). Human lymphoblastoid IFN-α was provided by Sumitomo Pharmaceuticals (Osaka, Japan). Unmethylated CpG ODN 2006 (Krug et al., 2001) was synthesized at and purchased from Sigma Genosys (Hokkaido, Japan). Isotype IgG (mouse IgG_{2B}) for the blocking experiments was kindly provided from the JT laboratory (Osaka, Japan).

Separation of DC precursors and other cells from PBMC

After informed consent had been obtained from healthy volunteers, buffy coats were isolated from venous blood drawn from them at the Osaka Red Cross Blood Center (Osaka, Japan). PBMC were collected from buffy coats by Ficoll–Hypaque density-gradient centrifugation. B cells, MDC, and PDC were magnetically isolated by using CD19 microbeads, BDCA-1, or BDCA-4 DC isolation kits from Miltenyi Biotec (Bergish-Gladbach, Germany), respectively. BDCA-1^+ and BDCA-4^+ cells are phenotypically compatible with MDC and PDC, respectively (Dzionek et al., 2000). CD4, CD8 T cells, and NK cells were separated from PBMC by using the relevant Stem-Sep kits (Stem Cell Technologies Inc, Vancouver, BC). $CD34^+$ hematopoietic precursor cells were isolated from cord blood mononuclear cells by using CD34-microbeads from Miltenyi. The purity of all isolated cells was more than 90% as determined by FACS Caliber (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Culture of DC

Isolated MDC were cultured for 4 days in IMDM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10 mM Hepes buffer, and 10 μM nonessential amino acid (Complete medium, CM) containing 50 ng/ml GM-CSF with or without 10 ng/ml IL-4. PDC were cultured for 4 days in CM in the presence of 50 ng/ml of IL-3.

Flow cytometry

The expression of surface molecules on DC was analyzed by FACS Caliber (Becton Dickinson). For the staining, DC were stored with specific Abs or isotype Abs for 30 min at 4 °C in PBS containing 2% of BSA and 0.1% of sodium azide. The following FITC-, PE-, PerCP-, or PC5-conjugated antihuman mAbs were used: CD1a (NA1/34; DAKO, Glostrup, Denmark), CD11c (KB90; DAKO), CD14 (M5E2; Becton Dickinson), CD40 (5C3; BD Pharmingen, San Diego, CA), CD80 (L307.4; BD Pharmingen), CD83 (HB15a; Immunotech, Marseille, France), CD86 (IT2.2; B70/B7-2, BD Pharmingen), CDw123 (7G3; IL-3 receptorα chain, BD Pharmingen), DC-SIGN (120507; R & D Systems), and HLA-DR (L243; Becton Dickinson).

Assessment of pseudotype VSV entry into cells

To find which blood cells are susceptible to HCV infection, we used pseudotype VSV possessing chimeric HCV E1 and E2 protein which was generated as described

previously (Matsuura et al., 2001). The pseudotype VSV consists of recombinant VSV in which glycoprotein (G) gene is replaced with a reporter gene encoding GFP.

As an envelope, it possesses chimeric HCV E1 and E2 proteins (VSV-E1E2). The viruses were purified by centrifugation at 25000 rpm for 2 h at 4 °C in SW28 rotor (Beckman Coulter Inc., Fullerton, CA) through 20% (v/w) and 60% (v/w) discontinuous sucrose gradient and were stored at −80 °C. To determine RNA copy numbers in the viral samples, TaqMan EZ RT-PCR kit (PE Applied Biosystems, Foster City, CA) was used. We used forward and reverse primers (5′-cattattatcattaaaaggctc-3′ and 5′-gatacaaggtcaaatattccg-3′) that amplify a 323-bp segment of the pseudotyped VSV RNA and also used a dual fluorophore-labeled probe 5′-(6-carboxy-fluorescein)-atccagtggaatacccggcagattac-(6-carboxy-tetramethyl-rhodamine)-3′. The sequence detector (ABI Prism 7000, PE Applied Biosystems) allows measurement of the amplified products in indirect proportion to the increase in fluorescence emission continuously during the PCR amplification. The copy numbers in samples were determined based on the standard curve drawn by a known amount of in vitro synthesized pseudotyped VSV RNA. Because VSV efficiently replicates in a wide range of mammalian cells, we are able to determine the cells exhibiting susceptibility to pseudotype VSV by the expression of GFP. We used VSV ΔG which has no envelope protein as a negative control. Similarly, VSV ΔG-G was used as a positive control which is complemented with the VSV G protein. Various separated blood cells were prepared in CM at 5×10^4 cells/well on 96-well culture plates. Next, they were inoculated with the pseudotype viruses, VSV-E1E2 (1×10^{12} RNA copies/well), VSV ΔG (1×10^{12} RNA copies/well), or VSV ΔG-G (1×10^{11} RNA copies/well) and incubated for 16 h at 37 °C. The infected cells (GFP^+ cells) were observed under fluorescence microscopy, and their positive percentages were determined by FACS analysis. The net percentage of infected cells was expressed as % infection = (% of GFP^+ cells with VSV-E1E2 or VSV ΔG-G) − (% of GFP^+ cells with VSV ΔG).

To find the substances which potentially protect DC from HCV infection, we examined IFNα, IFNγ, IL-3, IL-4, CpG ODN 2006, CD40L, polyI:C, TNFα, or LPS for this purpose. The appropriate concentrations of these reagents were determined in a separate series of experiments. IFNα, IFNγ, IL-3, or IL-4 was added to DC on the day of separation. CpG ODN 2006, CD40L, polyI:C, TNFα, or LPS was added to DC 24 h before the inoculation of pseudovirus. To compare the inhibitory effect of reagents in VSV-E1E2 entry into cells, we determined the ratio of infection of cells with and without treatment.

DC express various molecules containing the lectin domain, some of which are reported to be essential for the attachment to virus (Figdor et al., 2002). To examine whether lectin-containing molecules on DC are involved in HCV infection, we tested mannan, methyl α-D-mannopyranoside, and galactose for the inhibition of VSV-E1E2 entry. Day 4 MDC cultured with GM-CSF were preincubated with various concentrations of mannan, methyl α-D-mannopyranoside, or galactose at 37 °C for 180 min and inoculated with the pseudotype VSV. We also treated DC with EDTA (5 mM), monoclonal antihuman DC-SIGN Ab (50, 10, or 2 μg/ml), or isotype IgG_{2B} before the pseudotype VSV inoculation. To compare DC with hepatoblastoma cell line, HepG2, we treated HepG2 with mannan before the inoculation.

Quantitative analysis of HCV RNA in cells inoculated with HCV particles from patient serum

To test the susceptibility of each DC subset to authentic HCV, we quantified HCV RNA in cells that had been inoculated with patient serum by means of real-time PCR. We used the commercial HCV seroconversion panel as an inoculum, which contains high HCV RNA titer (1×10^5 copies/μl) and no anti-HCV antibody (BioClinical Partners, Inc, USA). We added 3 μl/well of inoculum to DC on 96-well plates and incubated them at 37 °C for 24 h. DC were harvested and washed three times with IMDM supplemented with 1% FCS and then total RNA was extracted from DC using RNeasy Mini Kit (QIAGEN, Germany). To measure HCV RNA, TaqMan EZ RT-PCR kit (PE Applied Biosystems) was used. We used forward and reverse primers [5′-cgggagagccatagtgg-3′ (positions 130–146) and 5′-agtaccacaaggcctttcg-3′ (positions to 272 to 290)] that amplify a 161-bp segment of the 5′ noncoding region of HCV RNA and also used a dual fluorophore-labeled probe [5′-(6-carboxy-fluorescein)-ctgcggaaccggtgagtacac (positions 148–168)- (6-carboxy-tetramethyl-rhodamine) -3′]. The sequence detector (ABI Prism 7000) allows measurement of the amplified products in indirect proportion to the increase in fluorescence emission continuously during the PCR amplification. The copy number in the samples was determined based on the standard curve drawn by a known amount of in vitro synthesized HCV RNA.

The strand-specific RT-PCR assay for HCV-RNA in cells inoculated with authentic HCV particles

To detect negative-strand HCV-RNA that is indicative of RNA replication, we performed the strand-specific RT-PCR assay referring to the methods described by Navas et al. (2002) with some modifications. We used the same batch of HCV seroconversion panel as an inoculum as described in the above section. We added 3 μl/well of inoculum to DC on 96-well plates and incubated them at 37 °C for 24 h. After the washing of DC for three times, total RNA was extracted from DC as the same way as we did in quantitative RT-PCR. As a control for the detection of HCV-RNA, 9 μl of inoculum was used. We used sense and anti-sense primers [5′-cactcccctgtgaggaactactgtc-3′ (positions 38–62) and 5′-atggtgcacggtctacgagacctcc-3′ (positions 319–343) that amplify a 306-bp segment of the 5′ noncoding region of HCV

genome. Ten microliters of purified RNA was used for reverse transcription (RT) with 10 μl of RT reaction mixture containing the thermostable recombinant *Thermus thermophilus* (rTth) enzyme (PE Applied Biosystems). Synthesis of cDNA was carried out with strand-specific primers, sense primer was used to obtain negative-strand RNA, and antisense primer was used to obtain positive-strand RNA, respectively. Reverse transcription was carried out at 70 °C for 15 min. Subsequently, the same primers were used in reverse order in the PCR rounds. Thirty-five cycles of PCR (94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s) followed by 7 min of extension at 72 °C were carried out on GeneAmp PCR System (PE Applied Biosystems). One-fifth of the PCR products was subjected to electrophoresis on 2% agarose gel and stained with ethidium bromide for observation under UV light. The expected molecular size of PCR products derived from target HCV RNA was 306 bp.

Statistical analysis

The paired *t* test was used to test the significance of the pseudotype VSV entry to MDC. Statistical analyses were performed with the Statview version 4.5 software (Abacus Concepts, Berkeley, CA). A *P* value of less than 0.05 was considered statistically significant.

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Evidence for a polytopic form of the E1 envelope glycoprotein of Hepatitis C virus

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Abstract

The polyprotein precursor of the Hepatitis C virus (HCV) contains multiple membrane-spanning domains that define the membrane topology and subsequent maturation of the viral structural proteins. In order to examine the biogenesis of the E1–E2 heterodimeric complex, we inserted an affinity tag (S-peptide) at specific locations within the envelope glycoproteins. In particular, and based on the prediction that the E1 glycoprotein may be able to assume a polytopic topology containing two membrane-spanning domains, we inserted the affinity tag within a putative cytoplasmic loop of the E1 glycoprotein. The HCV structural polyprotein containing this tag (at amino acids 295/296) was highly expressed and able to form a properly processed and noncovalently associated E1–E2 complex. This complex was bound by murine and conformation-dependent human monoclonal antibodies (MAbs) comparably to the native untagged complex. In addition, MAb recognition was retained upon reconstituting the tagged E1–E2 complex in lipid membrane as topologically constrained proteoliposomes. Our findings are consistent with the model of a topologically flexible E1 glycoprotein that is able to adopt a polytopic form. This form of the E1–E2 complex may be important in the HCV life cycle and in pathogenesis.

Keywords: Hepatitis C virus; Envelope glycoprotein; E1–E2 complex; Membrane topology; Affinity tag; Proteoliposome

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis and liver cirrhosis worldwide. It is estimated that over 170 million persons are infected with HCV and at risk for hepatocellular carcinoma (World Health Organization, 1999). HCV is an enveloped RNA virus and the sole member of the *Hepacivirus* genus within the Flaviviridae family. The single-stranded, positive-sense RNA genome of HCV contains a single open-reading frame that encodes a viral polyprotein precursor of approximately 3000 amino acids (Grakoui et al., 1993; Selby et al., 1993). Membrane-spanning domains within the polyprotein force threadings through the membrane of the rough endoplasmic reticulum (ER) and the mature viral proteins are liberated from the precursor polyprotein by the action of viral and cellular proteases ((Dubuisson, 2000; Reed and Rice, 2000) and references therein). The structural proteins of the virus are contained in the N-terminal portion of the polyprotein (in order: Core and the E1 and E2 envelope glycoproteins) and are generated by signal peptidase cleavage in the lumen of the ER. A small membrane protein of unknown function, p7, is encoded downstream of the E2 glycoprotein and is also produced by cellular signal peptidase. The nonstructural proteins of the virus (NS2, NS3, NS4A and B and NS5A and B) are contained in the C-terminal portion of the polyprotein and lie on the cytosolic side of the ER membrane. The mature NS proteins are generated by viral proteases in the cytoplasm. Because there is no robust cell culture system for HCV propagation (reviewed by (Bartenschlager and Lohmann, 2001)), details of envelope glycoprotein biogenesis and virion assembly have not been studied under conditions of productive infection.

Multiple membrane-spanning domains define the topology of the HCV polyprotein precursor during biogenesis. Biochemical and genetic studies indicate that the E1 and E2 glycoproteins adopt a Type I (von Heijne, 1988) topol-

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ogy in the ER membrane: each glycoprotein is anchored by a single C-terminal transmembrane domain and presents an N-terminal ectodomain ((Op De Beeck et al., 2001) and references therein). The C-terminal membrane-spanning domains of the HCV E1 and E2 glycoproteins also encode the signal peptide sequences of the downstream proteins (E2 and p7, respectively); the signal peptide of the E1 glycoprotein is contained within the C-terminal region of Core. These transmembrane domains are additionally important for the formation of the properly folded, noncovalently associated E1–E2 complex (Michalak et al., 1997; Op De Beeck et al., 2000; Patel et al., 2001). During biosynthesis, the HCV envelope glycoproteins are slow to fold properly (Choukhi et al., 1998; Dubuisson and Rice, 1996; Merola et al., 2001), and misfolding is thought to result in the isolation of predominantly disulfide-crosslinked complexes. In addition, the transmembrane domains also function as ER retention signals that largely prevent progress of the E1–E2 complex through the exocytic pathway (Cocquerel et al., 1998; Cocquerel et al., 1999; Dubuisson et al., 1994; Duvet et al., 1998; Flint and McKeating, 1999). Recent studies indicate that some E1–E2 complex may be transported to the plasma membrane (Bartosch et al., 2003; Drummer et al., 2003; Hsu et al., 2003).

In order to study the biogenesis of the HCV envelope glycoproteins, we have defined conditions for the isolation of noncovalently associated E1–E2 complexes. To probe this structure, we have introduced an affinity tag at specific locations in E1, E2 and p7. Here, we show that the E1 glycoprotein can readily accommodate a tag at position 295/296 — in a region bounded by a predicted transmembrane helix on one side and the C-terminal transmembrane domain on the other. Affinity purification and membrane reconstitution of the tagged E1–E2 complex suggests that the E1 glycoprotein may exhibit topologic flexibility that includes a polytopic form in which the tagged region comprises a cytoplasmic loop joining two membrane-spanning regions. The existence of a topologically distinct form of the E1–E2 complex may have important implications in HCV biology and pathogenesis.

2. Materials and methods

2.1. HCV expression plasmids

The infectious HCV cDNA (genotype 1b) used in these studies was originally isolated from an HCV carrier (Aizaki et al., 1998) and the region encoding Core, the E1 and E2 glycoproteins and p7 was adapted to enable translation of the structural portion of the polyprotein (pCAG HCV aa 1–810, (Takikawa et al., 2000)). The numbering of the HCV amino acids is from the initiating methionine of the polyprotein. The pCAG HCV expression cassette (*Bgl*II–*Bgl*II) was transferred to the *Bam*HI site of pcDNA 3.1 (Invitrogen) and a properly oriented insert was identified. The unique *Kpn*I site within the pcDNA 3.1 polylinker was subsequently destroyed in pHCV–Δ*Kpn* to facilitate subsequent manipulations.

The PHD-htm algorithm (Rost et al., 1995); accessed at (Columbia University Bioinformatics Center, 2002) was used to predict transmembrane helical regions within the HCV structural proteins. Based on these predictions and other considerations described in the text, sites of potential cytosolic exposure in E1, E2 and p7 were identified and engineered to include the 15 amino acid S-peptide (Spep) affinity tag KETAAAKFERQHMDS (Kim and Raines, 1993; Fig. 1A).

Molecular cloning to insert a DNA sequence encoding the affinity tag within the HCV membrane proteins utilized two oligonucleotides. The upstream oligonucleotide included, at its 3′ end, a sequence complementary to that encoding HCV amino acids N-terminal to the Spep insertion site. This was followed (in the 5′ direction on the oligonucleotide primer) by a sequence complementary to that encoding a triple glycine (Gly_3) linker, the Spep, and another Gly_3 linker. The latter Gly_3 was followed by an additional threonine that enabled the inclusion of a *Kpn*I site (GGT ACC) near the 5′ end of the oligonucleotide. The second primer was complementary to the *Kpn*I site and included at its 3′ end a sequence encoding HCV amino acids C-terminal to the Spep insertion site. Using the pHCV–Δ*Kpn* plasmid, these two oligonucleotides were extended for 25 cycles of PCR using Herculase DNA Polymerase (Stratagene). Following *Dpn*I digestion to inactivate the original plasmid, the mixture was digested with *Kpn*I and the newly synthesized plasmid was allowed to recircularize in the presence of T4 DNA ligase. Transformation of *Escherichia coli* resulted in the recovery of plasmids encoding a perfect splice of the Gly_3-Spep-Gly_3-Thr cassette within the respective E1, E2 and p7 coding regions. The insertions were confirmed by DNA sequencing. Three clones of each were carried forward in subsequent expression studies to exclude the possibility of adventitious changes elsewhere. The resulting plasmids encoding Spep-tagged HCV Core-E1-E2-p7 polyprotein are identified as E1Spep, E2Spep and p7Spep, respectively. For some studies, p7 expression was ablated from the HCV and E1Spep constructs by the introduction of a TAA stop codon at the E2–p7 junction (Δp7).

2.2. Monoclonal antibodies

Murine MAbs directed to HCV Core (#c11-7, #c11-10 and #c11-14), E1 (#299 and #384) and E2 (#187) were prepared against HCV aa 1–810 polyprotein expressed in insect cells (Matsuura et al., 1994; Takikawa et al., 2000). These MAbs are reactive in Western blot analysis. Murine anti-E1 MAb #159 (Triyatni et al., 2002) was kindly provided by J. Lau (Ribapharm, Inc.). Human MAbs directed to E1 (H-111 and H-114, unpublished) and E2 (CBH-2, 5, and 7; (Hadlock et al., 2000; Triyatni et al., 2002)) were derived from HCV-infected individuals and were kindly

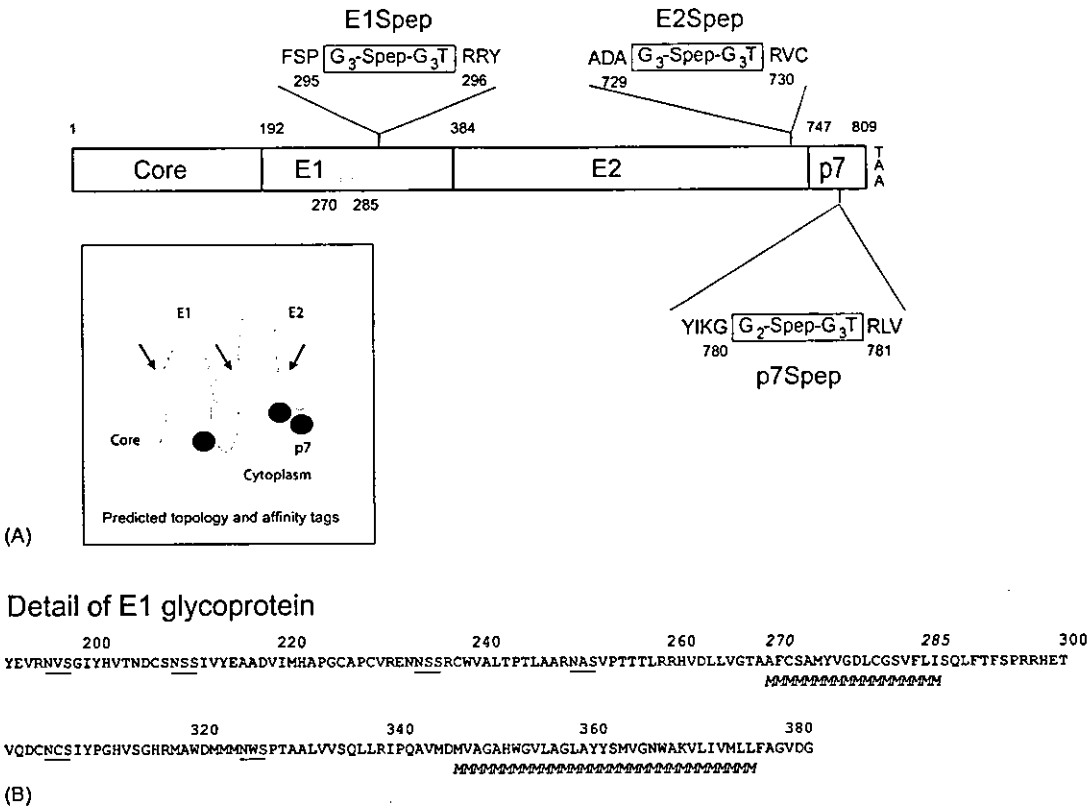

Fig. 1. The HCV structural polyprotein. (A) Schematic representation of the N-terminus encoding the Core, E1, E2 and p7 proteins; translation is terminated by an engineered termination codon (TAA) after p7. Amino acid numbering is shown, and transmembrane helical regions are indicated by shading. The putative internal transmembrane domain in E1 (aa 270–284) is darkened. The positions of the respective Spep tags are shown. The insert (lower left) contains a schematic showing the predicted membrane topology of the Core-E1-E2-p7 polyprotein including the polytopic form of the E1 glycoprotein. The respective Spep tags are shown as black balls. Arrows denote signal peptide cleavage sites in the polyprotein. (B) Amino acid sequence of the E1 glycoprotein. Transmembrane regions predicted by the PHD-htm algorithm (Rost et al., 1995); accessed at (Columbia University Bioinformatics Center, 2002) are indicated by *M*'s under the amino acid sequence. Only extended regions identified with ≥90% probability are shown. Potential glycosylation sites are underlined.

provided by Z.-Y. Keck and S.K.H. Foung (Stanford University). Of these, only H-111 is able to recognize its respective protein in Western blot analysis (Z.-Y. Keck and S.K.H. Foung, unpublished). The other human MAbs recognize conformational determinants (Hadlock et al., 2000). Control studies utilized human MAbs directed against HIV-1 gp120 (447-52D, from S. Zolla-Pazner (Conley et al., 1994)) and gp41 (F240, from M. Posner (Cavacini et al., 1998)) and a murine anti-gp41 MAb (Chessie 8, from G. Lewis (Abacioglu et al., 1994)).

2.3. Characterization of affinity-tagged HCV glycoproteins

HCV expression plasmids were used to transiently transfect monkey COS cell using FuGENE-6 (Roche Biochemicals) reagent (Lu et al., 2001). In some experiments, cells were metabolically labeled for the final 16 h in 5 ml of cysteine- and methionine-free Dulbecco's Modified Eagle Medium containing 2% dialyzed fetal bovine serum and 125 μCi each ^{35}S-methionine and ^{35}S-cysteine (Amersham Life Sciences). Cells were harvested 48 h after transfection and lysed in solubilization buffer comprising 20 mM Tris–HCl, 100 mM ammonium sulfate, 10% glycerol (Mirzabekov et al., 2000), 0.5% cyclohexyl-pentyl-β-D-maltoside (Cymal-5; Anatrace, Maumee, OH) nonionic detergent and protease inhibitors (Complete Protease Inhibitor Tablets; Roche Diagnostics). Preliminary studies explored the use of other nonionic and zwitterionic detergents (Cymal-5, Triton X-100, Brij-97, and CHAPSO; 0.5% and 1%) and 0.5% Cymal-5 was chosen based on its efficiency in extracting noncovalently associated E1–E2 heterodimers and its high critical micellar concentration (to facilitate dialysis in the subsequent formation of proteoliposomes). In many studies, the solubilization buffer also contained 1 mM DTT (Tatu et al., 1993).

Spep-tagged proteins were isolated using S-protein agarose (SAG) beads (Novagen, Inc., Madison, WI). Briefly, cleared cell lysates (300–500 μl) were incubated with SAG beads (100 μl of slurry) at 4 °C for at least 1 h and the beads were subsequently washed in solubilization buffer. The affinity-purified material was typically eluted by boiling in Laemmli SDS-PAGE sample buffer, in the pres-

ence or absence of 100 mM DTT. Deglycosylation of the affinity-purified material using PNGase F (New England Biolabs, Beverly, MA) was performed as recommended by the manufacturer. Immunoprecipitation of Spep-tagged and untagged HCV envelope glycoproteins from cell lysates (500 μl) utilized 5 μg of the human MAbs. Immune complexes were precipitated using protein A–Sepharose (100 μl of a 50% v/v slurry; Sigma).

Proteins were resolved by SDS-PAGE and detected by phosphorimaging or by Western blot analysis using either S-protein horseradish peroxidase (S-HRP; Novagen, Inc.) or murine MAbs with an HRP-conjugated second antibody. Western blots were visualized by chemifluorescence using ECL-Plus (Amersham Biosciences) and quantitated by fluorescence imaging. Quantitative analyses were performed using the Fuji FLA3000G imager and Image Gauge software (Fuji).

2.4. S-protein paramagnetic beads and proteoliposomes

M-270 carboxylic acid Dynabeads (Dynal AS, Lake Success, NY) were activated using carbodiimide chemistry and coupled to S-protein (RNS2; Biozyme Laboratories, San Diego, CA) using methods recommended by Dynal AS. Spep-tagged HCV glycoproteins were isolated from cell lysates by overnight incubation with beads at 4 °C. Typically, 5×10^7 beads were used per 500 μl of cell lysate in order to obtain a dense coverage. The paramagnetic beads were subsequently washed extensively and maintained for up to 1 month in solubilization buffer at 4 °C. The nonionic detergent was included in all subsequent immunochemical analyses of the beads.

Lipids were obtained as chloroform solutions from Avanti Polar Lipids (Alabaster, AL). A total of 10 mg of lipids {1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and dimyristoylphosphatidic acid (DMPA), mixed in a molar ratio of 6:3:1} were dried in a glass vial under a vacuum until all of the solvent was removed. One milliliter of phosphate-buffered saline (PBS) was added to the tube, and a liposomal suspension was obtained by 1–2-min ultrasonication in an ice bath. A stock of the fluorescent lipid dioleoylphosphoethanolamine–lissamine rhodamine B (Rhodamine–DOPE) was similarly prepared, at a final concentration of 1 mg ml^{-1}, and used as a tracer to assess lipid accretion. All liposomal stocks were kept in liquid N_2 until use.

Proteoliposomes were produced using methods described by Sodroski and colleagues (Mirzabekov et al., 2000) and modified in our laboratory. In brief, the liposomal solution was added to a suspension of paramagnetic beads bearing the affinity-purified HCV envelope glycoproteins, and the Cymal-5 nonionic detergent was slowly removed by dialysis to enable proteoliposome formation. Considerable evidence supports the model that during solubilization, the nonionic detergent binds to transmembrane domains and replaces the membrane; in reconstitution, this process is reversed (see review (Rigaud et al., 1995)). Fifty million paramagnetic beads and 50 μl of the 10 mg ml^{-1} liposome stock were mixed in 0.5 ml of solubilization buffer for 1 h at 4 °C before dialysis was initiated. Dialysis using a 10 kDa molecular weight cutoff cassette (Slide-A-Lyzer 10 K, Pierce, Rockford. IL) was carried out for 24 h at 4 °C against two changes of solubilization buffer lacking both detergent and protease inhibitors. Beads were then isolated magnetically and washed before storage in PBS containing 0.1% bovine serum albumin (BSA). Proteoliposome were stable for over 3 months at 4 °C, and were routinely washed into PBS (lacking BSA) prior to use.

Preliminary optimization and quality control studies for proteoliposome formation utilized the lipid mixture containing a tracer of Rhodamine–DOPE (1% w/w). Lipid was subsequently solubilized from the proteoliposomes and the amount of Rhodamine–DOPE tracer was determined spectrophotometrically. Approximately 60 μg of lipid could be deposited on 10^8 beads containing Spep-tagged transmembrane protein, sufficient to saturate the surface area of the beads (estimated at 50 μg (Mirzabekov et al., 2000)). Preliminary studies demonstrated that lipid accretion onto paramagnetic S-protein beads required that a transmembrane protein be bound (CTM and JHN; unpublished). Reports from numerous laboratories have demonstrated the robust utility of this methodology in reconstituting functional and antigenically intact transmembrane glycoproteins (see Babcock et al., 2001; Grundner et al., 2002; Mirzabekov et al., 2000; Rigaud et al., 1995; Walter et al., 1990).

2.5. Flow cytometric analysis of paramagnetic beads

Flow cytometry was performed using a FACSCalibur instrument (BD Biosciences). Paramagnetic beads ($\approx 5 \times 10^5$) containing affinity-purified HCV envelope glycoproteins in solubilization buffer (with 0.5% Cymal-5) or proteoliposomal beads ($\approx 5 \times 10^5$) in PBS and 0.1% BSA were incubated with 5 μg of the primary murine or human MAb in 50 μl for 1 h on ice and then washed in their respective buffers prior to incubation with the appropriate secondary antibody (CalTag, Burlingame, CA) at room temperature for 30 min. The murine MAbs were detected using a phycoerythrin-conjugated goat antibody, and the human MAbs were detected using a fluorescein-conjugated goat antibody. Both protein and proteoliposomal beads were ultimately washed into 1 ml of PBS for flow cytometric analysis.

3. Results

3.1. Selection of sites for affinity tags in the HCV envelope glycoproteins

In these studies, we sought to identify sites within E1, E2 and p7 that could accommodate the insertion of an affinity

tag without disrupting the biochemical and immunochemical properties of the noncovalently associated E1–E2 complex. We examined three sites with the potential for exposure on the cytosolic face of the membrane.

In the case of the polytopic p7 protein, which contains two hydrophobic membrane-spanning domains separated by a short cytoplasmic region (Carrere-Kremer et al., 2002), the affinity tag was introduced between charged amino acids in the cytoplasmic loop (Fig. 1A). Although the C-terminal regions of the E1 and E2 glycoproteins also contain charged amino acids embedded within hydrophobic transmembrane domains (Cocquerel et al., 2000), these residues are believed to lie within the membrane. It has been proposed that these charged residues play a role in reorienting the C-termini from the lumenal to cytosolic side of the ER following signal peptidase cleavage (Cocquerel et al., 2002; Op De Beeck et al., 2001). To explore this latter model in the E2 glycoprotein, we introduced the affinity tag between charged residues in the transmembrane domain of E2 (Fig. 1A), anticipating that this would severely disrupt biogenesis.

The E1 glycoprotein presented an unusual opportunity to probe structural and topologic aspects of biogenesis. Using predictive computer algorithms trained to identify transmembrane helices (Rost et al., 1995), we found evidence for a potential transmembrane helix internal to the E1 glycoprotein, between amino acid residues 270–284 (Fig. 1B). This raised the possibility that the E1 glycoprotein might contain two membrane-spanning regions — the well-documented C-terminal domain and the predicted internal domain — separated by an intervening cytoplasmic loop. This suggestion was consistent with earlier studies in which an internal hydrophobic region was shown to be involved in membrane association (Matsuura et al., 1994). Cytosolic exposure of the E1 glycoprotein has been suggested as the basis for interaction with the virion Core protein (Lo et al., 1996; Merola et al., 2001). Based on these theoretical and experimental suggestions, we introduced the affinity tag in the predicted cytoplasmic loop, between amino acids 295 and 296 (Fig. 1A).

3.2. Expression of the HCV envelope glycoproteins

The infectious HCV cDNA (genotype 1b) was originally isolated from an infected individual (Aizaki et al., 1998) and the region encoding the N-terminal polyprotein (Core, the E1 and E2 glycoproteins and p7) was used in these studies. The 15 amino acid S-peptide affinity tag (Spep) is derived from the small subtilisin-generated fragment of ribonuclease A and binds with high affinity to its ligand, the larger ribonuclease fragment (S-protein) (Kim and Raines, 1993; Richards and Vithayathil, 1959). This peptide was chosen based on its ability to function as an affinity tag when inserted internally within the protein of interest (JHN, unpublished). When transfected into simian COS-7 cells, the three tagged constructs (E1Spep, E2Spep and p7Spep; Fig. 1A) were expressed at levels comparable to the untagged

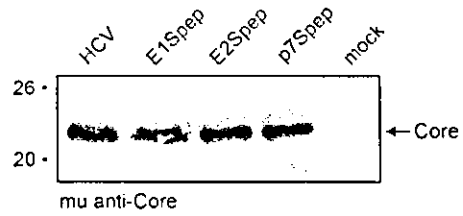

Fig. 2. Expression of HCV Core from Spep-tagged and untagged polyprotein. COS cells expressing the E1Spep, E2Spep or p7Spep polyproteins, or the untagged HCV polyprotein were lysed in solubilization buffer, and proteins were resolved by SDS-PAGE. The Western blot was probed using a pool of murine anti-Core MAbs (#c11-7, #c11-10 and #c11-14; indicated below the image as mu anti-Core). Molecular weight markers are shown at left.

HCV construct and all polyprotein expression resulted in the accumulation of proteolytically processed mature Core protein (Fig. 2).

We then examined the biosynthesis of the envelope glycoproteins to determine the effects of the affinity tag on protein processing, folding and assembly. Cells were lysed using 0.5% Cymal-5 nonionic detergent and the tagged glycoproteins were isolated using S-protein agarose (SAG) beads. In all cases, both E1 and E2 glycoproteins could be co-isolated, regardless of the position of the affinity tag (Fig. 3A). By far the highest recovery of E1–E2 complex was from the E1Spep polyprotein, and lower amounts were isolated from the p7Spep and E2Spep polyproteins, respectively. The expressed glycoproteins were further characterized by using peptide N-glycosidase F (PNGase F) to generate the fully deglycosylated proteins. In all cases, the E1 polypeptide was detected as a single band of the appropriate molecular weight (data not presented). The pattern of E2 polypeptides was, however, more complex (Fig. 3B). In the case of the E1Spep polyprotein, a discrete 40 kDa band corresponding to authentic E2 polypeptide was observed, as well as a series of more slowly migrating species. By contrast, the discrete band was absent in the E2Spep and p7Spep polyprotein products and only the more slowly migrating species were seen. Based on previous reports (Lin et al., 1994; Mizushima et al., 1994), we suspected that one or more of the slowly migrating species might represent unprocessed E2–p7 precursor. To further examine this question, we modified the E1Spep construct by introducing a stop codon at the E2–p7 junction to ablate expression of p7. The slowly migrating species vanished and only the authentic E2 polypeptide remained (Fig. 3C). These results confirm that the more slowly migrating species represent forms of the uncleaved E2–p7 precursor. Therefore, we concluded that the E2Spep and p7Spep polyproteins were largely defective in signal peptidase cleavage at the E2–p7 junction. By contrast, the E1Spep polyprotein was able to undergo proteolytic processing to yield mature E1 and E2 glycoproteins.

To assess the state of protein folding in the E1Spep-E2 complex, we examined the mobility of the E2 glycoprotein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)