Scavenger Receptor Class B Type I and Hepatitis C Virus Infection of Primary *Tupaia* Hepatocytes

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. The study of early steps during HCV infection has been hampered by the lack of suitable *in vitro* or *in vivo* models. Primary *Tupaia* hepatocytes (PTH) have been shown to be susceptible to HCV infection *in vitro* and *in vivo*. Human scavenger receptor class B type I (SR-BI) represents an HCV receptor candidate mediating the cellular binding of E2 glycoprotein to HepG2 hepatoma cells. However, the function of SR-BI for viral infection of hepatocytes is unknown. In this study, we used PTH to assess the functional role of SR-BI as a putative HCV receptor. Sequence analysis of cloned tupaia SR-BI revealed a high homology between tupaia and human SR-BI. Transfection of CHO cells with human or tupaia SR-BI but not mouse SR-BI cDNA resulted in cellular E2 binding, suggesting that E2-binding domains between human and tupaia SR-BI are highly conserved. Preincubation of PTH with anti-SR-BI antibodies resulted in marked inhibition of E2 or HCV-like particle binding. However, anti-SR-BI antibodies were not able to block HCV infection of PTH. In conclusion, our results demonstrate that SR-BI represents an important cell surface molecule for the binding of the HCV envelope to hepatocytes and suggest that other or additional cell surface molecules are required for the initiation of HCV infection. Furthermore, the structural and functional similarities between human and tupaia SR-BI indicate that PTH represent a useful model system to characterize the molecular interaction of the HCV envelope and SR-BI on primary hepatocytes.

Hepatitis C virus (HCV) is a major cause of posttransfusion and community-acquired hepatitis in the world (8, 24, 25). The majority of HCV-infected individuals develop chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (15). Treatment options for chronic HCV infection are limited, and a vaccine to prevent HCV infection is not available (11, 15, 17).

HCV has been classified in a separate genus (*Hepacivirus*) of the *Flaviviridae* family. The virion contains a positive-stranded RNA genome of approximately 9.6 kb in length (25). The genome consists of a highly conserved 5' noncoding region followed by a long open reading frame of 9,030 to 9,099 nucleotides that is translated into a single polyprotein of 3,010 to 3,030 amino acids. Processing of the polyprotein occurs by a combination of host and viral proteases. The HCV structural proteins comprise the putative nucleocapsid or core protein and the two envelope glycoproteins E1 and E2 (25). The E2 glycoprotein is thought to be responsible for initiating virus attachment due to its ability to bind to human cells (32).

HCV purified from plasma has been reported to exist in association with plasma lipoproteins, suggesting that the virus

may use the low-density lipoprotein receptor for uptake (2). In the absence of highly purified native infectious HCV particles as a tool for the study of virus-cell interaction, recombinant HCV glycoprotein E2, E1E2 liposomes (22), infectious HCV pseudotype particles expressing E1 and E2 (HCVpp) (4, 16), and HCV-like particles (HCV-LPs) (3, 39, 41) have been used to analyze virus-cell membrane interaction. Based on these experimental *in vitro* studies, CD81 (5, 32), dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin (33) and highly sulfated heparan sulfate (3) have been proposed to play a role in mediating E2 binding and/or HCV internalization.

Recently, the scavenger receptor class B type I (SR-BI) has been proposed as a putative HCV receptor candidate (36). SR-BI, a high-density lipoprotein-binding molecule, plays a functional role in lipid metabolism and is highly expressed in hepatocytes and steroidogenic tissues (34). SR-BI is a 509-residue glycoprotein with a large extracellular loop (LEL) anchored to the plasma membrane at both the N and C termini by transmembrane domains with short extensions into the cytoplasm (21). SR-BI has been shown to play a role in mediating the binding of recombinant E2 to HepG2 hepatoma cells and the entry of recombinant HCVpp into Huh-7 hepatoma cells (5, 36). Due to the lack of convenient *in vitro* or *in vivo* models for the study of HCV infection, the

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		1	70
Tupaia	(1)	MGGRARARWVAVAVGTGLLCAVLGAVMIVMVPSLIKQQLKNVRIDP	TSLSFNMWKEIPVPFYSVYFF
Mouse	(1)	MGGSSRARWVALGLGALGLLFAALGVVMILMVPSLIKQQLKNVRIDP	SSLSFGMWKEIPVPFYSVYFF
Human	(1)	MGCSAKARWAAGALGVAAGLLCAVLGAVMIVMVPSLIKQQLKNVRIDP	SSLSFNMWKEIPVPFYSVYFF
Consensus	(1)	MGGSARARWVALALGV	GLLCAVLGAVMIVMVPSLIKQQLKNVRIDPSSLSFNMWKEIPVPFYSVYFF
		71	140
Tupaia	(71)	DVLNPNEILKGEKQVRERGPYVYREFRHKSNITFNDNDTVSYLEYRSFR	FQPAKSRGLESYIVMPNIL
Mouse	(71)	EVVNPNEVLNGQKPVVRERGPYVYREFRQKVNITFNDNDTVSFVENRSLHF	FQPKSHGSESDYIVLPNIL
Human	(71)	DVMNPSEILKGEKQVRERGPYVYREFRHKSNITFNNNDTVSFLEYRTFQ	FQPSKSHGSESDYIVMPNIL
Consensus	(71)	DVLNPNEILKGEKQVRERGPYVYREFRHKSNITFNDNDTVSFLEYRSF	FQPAKSHGSESDYIVMPNIL
		141	210
Tupaia	(141)	VLGAAIMMENKPMCLKLMTLAFSTLGERAFMNRTVAEIMWGYEDPLVSLINKY	FPDMLPFKGFGLFAE
Mouse	(141)	VLGGSILMESKPVSLKLMTLALVTMGQRAFMRNTVGEILWGYDDPFVHFLNTYL	PDMLPIKGFGLFVG
Human	(141)	VLGAAVMMENKPMCLKLIMTLAFTTLGERAFMNRTVGEIMWGYKDPLVNLINKY	FPGMFPFKDKFGLFAE
Consensus	(141)	VLGAAIMMENKPMCLKLMTLAFSTLGERAFMNRTVGEIMWGYDDPLV	LINKYFPDMLPFKGFGLFAE
		211	280
Tupaia	(211)	LNNNSGLFTVFTGVKDFQRIHLVDKWNGLSKVNFHWS	DQCNMINTSGQMWPFTPESSLEFYSPEAC
Mouse	(211)	MNNSSGVFTVFTGVQNFRIHLVDKWNGLSKIDYNHSE	QCNMINTSGQMWPFTPESSLEFFSPEAC
Human	(211)	LNNDSGLFTVFTGVQNISRIHLVDKWNGLSKVDFHWS	DQCNMINTSGQMWPFTPESSLEFYSPEAC
Consensus	(211)	LNNNSGLFTVFTGVQNFRIHLVDKWNGLSKVDFHWS	DQCNMINTSGQMWPFTPESSLEFYSPEAC
		281	350
Tupaia	(281)	RSMKLIYQEPGVYEGIPTYRFVAPKTLFANGSDYPPNEGFCPCLES	GVQNVSTCRFSAPLFLSHPHFYNA
Mouse	(281)	RSMKLTYNESRVFEGIPTYRFTAPDTL FANGSVYPPNEGFCPCRES	GIQNVSTCRFGAPLFLSHPHFYNA
Human	(281)	RSMKLMYKESGVFEGIPTYRFVAPKTLFANGSIYPPNEGFCPCLES	GIQNVSTCRFSAPLFLSHPHFLNA
Consensus	(281)	RSMKLIYNESGVFEGIPTYRFVAPKTLFANGSIYPPNEGFCPCLES	GIQNVSTCRFSAPLFLSHPHFYNA
		351	420
Tupaia	(351)	DPVLAEAVIGLHPNQEEHSLFLDIHPVTGIPMNCVKLQLSLYIKAVK	GIGQTGKIEPVVLPPLLWFGESE
Mouse	(351)	DPVLSEAVLGLNPNPKEHSLFLDIHPVTGIPMNCVKMLQLSLYIKSV	KIGQTGKIEPVVLPPLLWFEQSG
Human	(351)	DPVLAEAVTGLHPNQEAHSLFLDIHPVTGIPMNCVKLQLSLYIKSV	AGIGQTGKIEPVVLPPLLWFAESG
Consensus	(351)	DPVLAEAVIGLHPNQEEHSLFLDIHPVTGIPMNCVKLQLSLYIKSV	KIGQTGKIEPVVLPPLLWFAESG
		421	490
Tupaia	(421)	AMEGETLRTFTYTQVLVLLPAVLHYAQYVLLALGCLLLLVPVHH	IRSQEKCYLFWSSSKKGPDKKEAIQAY
Mouse	(421)	AMGGKPLSTFTYTQVLVLPQVLHYAQYVLLGLGCLLLLVPVICQ	IRLSQEKCFLFWSSSKKGSQDKKEAIQAY
Human	(421)	AMEGETLHTFTYTQVLVLPKVMHYAQYVLLALGCVLLLVPVICQ	IRSQEKCYLFWSSSKKGSQDKKEAIQAY
Consensus	(421)	AMEGETLTFYTQVLVLPVLHYAQYVLLALGCLLLLVPVICQ	IRLSQEKCYLFWSSSKKGSQDKKEAIQAY
		491	510
Tupaia	(491)	SESLMTPAPRGTVLQEARL-	
Mouse	(491)	SESLMSPAAGTVLQEAKL-	
Human	(491)	SESLMTSAPKGSVLQEAKL-	
Consensus	(491)	SESLMTPAPKGTVLQEAKL	

FIG. 1. Alignment of amino acid sequences of tupaia, mouse, and human SR-BI. Tupaia SR-BI cDNA was cloned and sequenced by RT-PCR of tupaia mRNA with human SR-BI-specific primers as described in Materials and Methods. SR-BI amino acid sequences of mouse and human SR-BI are depicted according to a previous report (1). Amino acid homology and differences between species are indicated by different colors.

functional role of SR-BI as a (co)receptor for binding of the viral envelope to primary host cells and infection with native virus is unknown.

The chimpanzee (*Pan troglodytes*) is the only nonhuman host serving as a model for HCV infection (7, 38). While HCV infection can be successfully studied in chimpanzees, these animal experiments are expensive and raise ethical issues. An alternative model for HCV infection is the tree shrew *Tupaia belangeri*, a small, squirrel-like mammal closely related to primates (29). *T. belangeri* has been shown to be susceptible to a variety of human viruses including herpes simplex, hepatitis B, and rotavirus (18, 19, 30, 31, 40, 43). Two studies have demonstrated that *T. belangeri* can be infected in vivo with HCV (20, 42). We have recently shown that primary *Tupaia* hepatocytes (PTH) can be successfully infected with serum or plasma derived from chronically HCV-infected humans (46). Incubation of PTH with native HCV from chronically HCV-

infected patients resulted in the production of infectious virus in vitro, indicating that PTH provide a model for the study of HCV infection and the functional assessment of HCV receptor candidates (46).

In this study, we analyzed the functional role of SR-BI for HCV binding and infection of PTH. We demonstrate that SR-BI represents an important cell surface molecule mediating binding of the HCV envelope to hepatocytes. Since inhibition of E2-SR-BI interaction did not block viral infection, our data suggest that other or additional cell surface molecules are required for HCV infection.

(Results of this study were presented in part in abstract form at the 10th International Meeting on Hepatitis C Virus and Related Viruses in Kyoto, Japan, 2 to 6 December 2003, and at the 55th Annual Meeting of the American Society for the Study of Liver Disease [AASLD] in Boston, Mass., 28 October to 2 November 2004.)

FIG. 2. Expression of SR-BI in PTH and human hepatoma cells. (A) PTH, human hepatoma HepG2 cells, and Sf9 insect cell lysates were subjected to SDS-PAGE. Following gel transfer to polyvinylidene difluoride membranes, immunoblotting was performed using rabbit anti-SR-BI polyclonal antibody (NB 400-104) and horseradish peroxidase-conjugated anti-rabbit IgG. The presence of SR-BI is indicated on the left, and molecular weight (MW) is indicated on the right. (B) Analysis of SR-BI expression on freshly isolated PTH by flow cytometry is shown. Following fixation and permeabilization, cells were incubated with rabbit anti-SR-BI polyclonal antibody (NB 400-104 at a 1:500 dilution) and subsequently stained with FITC-conjugated goat anti-rabbit IgG. Negative control (NC) represents PTH incubated with control antibody. *x* and *y* axes show mean fluorescence intensity and relative number of stained cells, respectively.

MATERIALS AND METHODS

Recombinant proteins, antibodies, and cells. Truncated soluble recombinant HCV glycoprotein E2 (amino acids 384 through 661 of the HCV polyprotein) of genotype 1a (isolate H77) and genotype 1b (isolate BK) harboring a C-terminal histidine tag were produced in 293 cells as described previously (28, 35). HCV-LPs (derived from H77-cDNA, genotype 1a) were synthesized in Sf9 insect cells

as described previously (6, 41). HCV-LP E2 concentration was determined as described previously (9, 41). Mouse monoclonal anti-E2 antibody 16A6 (41) and chimpanzee anti-E2 monoclonal antibody 49F3 were generously provided by E. Depla, Innogenetics, N.V., Ghent, Belgium. Both monoclonal anti-E2 antibodies (16A6 and 49F3) bind to an E2 epitope located between HCV amino acids 516 and 530 (E. Depla, personal communication). Anti-SR-BI polyclonal serum was raised by genetic immunization of BALB/c mice with plasmids pVJ-SR-BI harboring the full-length tupaia and human SR-BI cDNAs, respectively. Immunization protocol and plasmid vector have been previously described (47). Preimmune control serum was collected from the same mice bled before immunization. R-phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin G (IgG) antibody, PE-conjugated goat anti-human IgG antibody, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa.). Rabbit anti-SR-BI polyclonal serum (NB 400-104) was obtained from Novus Biologicals (Littleton, Colo.) and used as previously described (36). This antibody recognizes a defined epitope (CSPAAGTTLQEAAL, corresponding to amino acids 496 through 509) in the SR-BI cytoplasmic C-terminal domain. Horseradish peroxidase-conjugated anti-rabbit IgG was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Penta-His-biotin conjugate and streptavidin-R-PE were purchased from QIAGEN (Hilden, Germany). Origin and maintenance of HepG2, Sf9 insect and CHO cells have been described previously (36, 41).

Isolation, culture, and HCV infection of PHT. *T. belangeri* was obtained from the German Primate Center, Göttingen, Germany. The animals were bred and maintained at the animal facilities of University Hospital Freiburg in accordance with institutionally approved protocols and the National Institutes of Health guidelines for the use of experimental animals. PTH were isolated from adult *T. belangeri* shrews (male and female, 10 to 12 weeks old, 180 to 200 g of body weight) and cultured as previously described (18, 19, 46). Briefly, freshly isolated PTH were seeded at a density of $\sim 3 \times 10^5$ cells/ml of medium (6×10^5 cells/well) on collagen-coated six-well plates. Confluence after plating was 80 to 90%, with a hepatocyte viability of $>90\%$. For infection of hepatocytes, plasma samples from patients with chronic hepatitis C (genotypes 1 and 4) were used. Plasma-derived HCV used for infection studies was characterized by genotyping and quantitation of viral load. HCV genotype was determined using the VER SANT HCV genotype assay (LiPA; Bayer, Tarrytown, N.Y.). Quantitation of plasma HCV RNAs was performed using the VERSANT HCV RNA 3.0 assay (Bayer). Multiplicity of infection (MOI) was calculated as the number of HCV genomic equivalents (determined as described above) present in the inoculum divided by the number of hepatocytes used for infection. PTH were infected with

FIG. 3. E2 binding to CHO cells transfected with mouse, human, and tupaia SR-BI. CHO cells were transfected with expression constructs containing the cDNA for mouse (pcDNA3/mSR-BI), human (pcDNA3/hSR-BI), tupaia SR-BI (pcDNA3/tSR-BI), or control vector (pcDNA3) as described previously (36). (A) FACS analysis of anti-SR-BI binding (using polyclonal rabbit anti-SR-BI interacting with both human and mouse SR-BI in permeabilized CHO cells [36]) in transfected CHO cells indicates that approximately 30% of cells transfected express the receptor on the cell surface. (B) FACS analysis of E2 binding to transfected CHO cells shows comparable E2 binding to human and tupaia SR-BI but not to mouse SR-BI.

FIG. 4. (A) Dose-dependent and saturable binding of E2 to PTH. Hepatocytes were incubated with His-tagged E2 at the concentrations indicated. Cellular E2 binding (corresponding to net mean fluorescence intensity [Δ MFI]) was determined by FACS analysis using an anti-His-biotinylated mouse antibody and streptavidin-R-PE as described in Materials and Methods. (B) E2 binding in the presence of anti-tupaia SR-BI antibody. PTH were incubated with anti-tupaia SR-BI antiserum (black shadowed graph) or preimmune serum (grey shadowed graph) 1 h prior to the addition of recombinant E2 (E2 concentration of ~ 4 μ g/ml, antiserum dilution of 1:10). Negative control (NC) representing PTH incubated with preimmune serum (1:10 dilution), anti-His-biotinylated mouse antibody, and streptavidin-R-PE in the absence of E2 protein is shown. Cellular E2 binding was analyzed by FACS analysis as described above. (C) Dose-dependent inhibition of E2 binding to PTH by anti-tupaia SR-BI antiserum. PTH were preincubated with different dilutions of anti-tupaia SR-BI (squares) or preimmune serum (circles). After washing with PBS, PTH were incubated with recombinant E2 (E2 concentration of ~ 1.5 μ g/ml) and cellular E2 binding was analyzed by FACS analysis as described for panel A. Data are shown as percent binding compared to binding of E2 in the presence of PBS (at 100%) of a representative experiment. (D) Inhibition of cellular E2 binding by anti-tupaia SR-BI and anti-human SR-BI antibodies. PTH were preincubated with anti-tupaia SR-BI, anti-human SR-BI, or control antibody (preimmune serum, all diluted 1:10 in PBS) and subsequently analyzed for E2 binding (E2 concentration ≈ 1.5 μ g/ml) as described for panel A. Data are shown as percent binding (mean $\pm$ standard deviation of a representative experiment performed in triplicate) in the presence of antibody compared to binding of E2 in the presence of PBS (at 100%).

plasma-derived HCV on day 2 after hepatocyte plating by incubation for 6 h with plasma by using an MOI between 0.05 and 0.25 as described previously (46). At days 1 and 5 postinfection, PTH were collected and analyzed for the presence of positive- and negative-strand HCV RNAs by a highly specific strand-specific reverse transcription (RT)-PCR (23, 46). Mock-infected PTH served as negative controls. Total RNA from cells and medium was isolated twice by guanidinium isothiocyanate-acid-phenol based extraction (RNeasy; QIAGEN). Positive- and negative-strand HCV RNAs were then analyzed in 1/50 of total RNAs by strand-

specific RT-PCR using rTth polymerase and 5'-UTR-specific primers (23, 46). For the synthesis of control HCV positive- and negative-strand RNAs, plasmids pCV-H77C (44) and pGEMT-H77UTRC (46) were linearized by digestion with XbaI or SpeI, respectively. In vitro transcription and purification of HCV RNAs from linearized gel-purified cDNAs (10 μ g) was performed as described previously (44). The absence of residual cDNA template was confirmed by PCR using HCV-specific primers.

For the analysis of SR-BI-dependent viral infection, PTH were preincubated

with anti-tupaia SR-BI or preimmune serum (1:10 dilution) at 37°C for 1 h. After washing with phosphate-buffered saline (PBS), cells were infected by the addition of plasma and processed as described above. To exclude that SR-BI molecules are accessible for HCV-E2 at the hepatocyte cell surface via de novo expression or intracellular pool redistribution of SR-BI during the time of viral infection, we performed a second series of experiments in the presence of anti-SR-BI antiserum or preimmune serum (1:10 dilution) in the tissue culture medium before and during the time of infection.

Analysis of tupaia SR-BI. Tupaia SR-BI cDNA was obtained by RT-PCR (kit Superscript II; Invitrogen) of purified PTH RNA (RNeasy; QIAGEN) with human SR-BI-specific primers (forward, 5'ATG GGG CCC CAG GCG CGC AGA CAT GGG C 3', and reverse, 5'AGC GGG GTG TAG GGG CTG GGG GGC CGG 3'). The PCR products from two independent reactions were cloned (pcRZero Blunt kit; Invitrogen) and sequenced. Full-length tupaia SR-BI cDNA was cloned into expression vector pcDNA3 (Invitrogen) and transfected in CHO cells by liposome-mediated gene transfer (Lipofectamine; Invitrogen) as previously described for the human SR-BI (36). Tupaia SR-BI amino acid sequence was aligned with mouse and human SR-BI sequences (1). For the analysis of SR-BI expression, HepG2 cells, Sf9 cells, and PTH were lysed in a buffer containing 50 mM Tris-HCl, 1% NP-40, 50 mM NaCl, and 5 mM EDTA, pH 7.4. Following removal of nuclei and cell debris by low-speed centrifugation, lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were detected by immunoblotting using rabbit anti-SR-BI polyclonal serum (NB 400-104 at a 1:1,500 dilution in PBS containing 1% Tween 20) and horseradish peroxidase-conjugated anti-rabbit IgG (1:1,000 dilution). Cell surface expression of SR-BI on freshly isolated PTH and transfected CHO cells was analyzed by using a fluorescence-activated cell sorter (FACS) with mouse anti-tupaia or anti-human SR-BI polyclonal serum (various dilutions in PBS) and R-PE-conjugated goat anti-mouse IgG antibody (1:100 dilution in PBS). For FACS analysis of SR-BI with polyclonal rabbit anti-SR-BI (NB 400-104 directed against an epitope in the C-terminal intracellular domain of SR-BI), freshly isolated PTH and transfected CHO cells were permeabilized with 0.1% saponin prior to incubation with rabbit anti-SR-BI (1:500 dilution in PBS) and FITC-conjugated goat anti-rabbit IgG antibody (1:100 dilution in PBS).

Analysis of cellular E2 and HCV-LP binding. Binding of recombinant soluble E2 to PTH was performed as described recently (35, 36). Briefly, freshly isolated PTH (10^5 cells per assay) were incubated with E2 for 1 h at room temperature. Cells were washed two times and incubated with a biotinylated anti-penta-His mouse antibody directed against the His tag of recombinant E2 (1:50 dilution) for 1 h at room temperature. The cells were washed again, and cell-bound antibodies were detected by the addition of streptavidin-R-PE (1:100 dilution) binding to biotin residues of the primary antibody. Flow cytometry was performed using FACSCalibur (Becton Dickinson) and analyzed with CellQuest software. For the measurement of HCV-LP binding, freshly isolated PTH (10^5 cells per assay) were incubated with HCV-LPs or insect cell control preparation (derived from insect cells infected with a recombinant baculovirus containing the cDNA for β -glucuronidase) in PBS for 1 h at 4°C. Cell bound HCV-LPs were analyzed by FACS as described previously using either mouse monoclonal anti-E2 (16A6) and R-PE-conjugated goat anti-mouse IgG antibodies or chimpanzee monoclonal anti-E2 (49F3) and R-PE-conjugated goat anti-human IgG antibodies (41). To study whether cellular binding of recombinant E2 was inhibited by anti-SR-BI antibodies, cells were preincubated with different dilutions of

anti-SR-BI for 1 h at room temperature prior to the addition of recombinant E2 and E2 binding was detected using biotinylated anti-penta-His mouse antibody and streptavidin-R-PE (1:100 dilution). Since this assay does not use dye-conjugated secondary antibodies for the detection of E2, it can easily distinguish between bound nonbiotinylated mouse anti-SR-BI (not reacting with streptavidin-PE) and biotinylated anti-His-E2 (specifically interacting with streptavidin-PE). To study whether cellular binding of HCV-LPs was inhibited by anti-SR-BI antibodies, cells were preincubated with different dilutions of mouse anti-SR-BI for 1 h at room temperature prior to the addition of recombinant HCV-LPs, and HCV-LP binding was detected using chimpanzee monoclonal anti-E2 (49F3) and R-PE-conjugated goat anti-human IgG antibodies.

Nucleotide sequence accession number. The sequence of *T. belangeri* SR-BI was deposited in the GenBank database under GenBank accession number AY428553.

RESULTS

Isolation and characterization of tupaia SR-BI. To assess the function of SR-BI for HCV binding, entry, and infection of PTH, we first identified and characterized tupaia SR-BI. To analyze the tupaia SR-BI primary structure, SR-BI cDNA was cloned from tupaia hepatocyte mRNA with human SR-BI-specific primers. Sequence analysis revealed a marked homology (88%) between human and tupaia SR-BI. In contrast, homology of mouse and human SR-BI reached only 82% (Fig. 1). These findings are consistent with the close phylogenetic relationship between the respective species. The LEL of tupaia SR-BI, containing the putative SR-BI HCV E2-binding domain, exhibited 44 amino acid changes compared to human SR-BI. In contrast, mouse SR-BI LEL differed by 73 amino acids from human SR-BI LEL.

For the analysis of SR-BI expression in PTH, lysates of cultured PTH were subjected to SDS-PAGE and immunoblotting using rabbit anti-SR-BI polyclonal antibody. Tupaia SR-BI exhibited a similar molecular mass (approximately 85 to 90 kDa) as human SR-BI expressed in HepG2 cells. By contrast, polyclonal anti-SR-BI did not interact with an SR-BI-like molecule in lysates of Sf9 insect cells (Fig. 2A). Expression of SR-BI on PTH was confirmed by FACS analysis using rabbit polyclonal anti-SR-BI (Fig. 2B).

SR-BI-dependent binding of HCV envelope glycoprotein E2 to PTH. To assess whether tupaia SR-BI was able to bind recombinant His-tagged viral envelope glycoprotein E2 (derived from an HCV genotype 1a-cDNA) recently used for the study of SR-BI interaction on human hepatoma cells (36),

FIG. 5. Specific and dose-dependent binding of anti-tupaia and human SR-BI antibodies to tupaia SR-BI. (A) Specific binding of rabbit anti-SR-BI (NB 400-104, left panel), mouse anti-tupaia SR-BI (middle panel), and mouse anti-human SR-BI (right panel) to tupaia SR-BI expressed in CHO cells. CHO cells were transfected with expression constructs containing control vector (pcDNA3) or the cDNA for tupaia SR-BI (pcDNA3/tSR-BI) as described previously (36). Flow cytometry of transfected cells incubated with anti-SR-BI and R-PE or FITC-conjugated secondary antibodies demonstrated specific interaction of anti-SR-BI antibodies with tupaia SR-BI (bottom panels). In contrast, no interaction was present in CHO cells transfected with control vector and incubated with anti-SR-BI antibodies (upper panels). Numbers inside the panel represent the percentage of positively stained cells in relationship to the total number of cells. For staining with anti-SR-BI NB 400-104, cells were permeabilized prior to incubation with antibody. (B) Dose-dependent interaction of anti-SR-BI with tupaia SR-BI expressed in CHO cells. CHO cells were transfected with expression constructs containing control vector (pcDNA3) or cDNA for tupaia SR-BI (pcDNA3/tSR-BI) as described for panel A. Cells were then incubated with anti-tupaia SR-BI in increasing dilutions, and anti-SR-BI binding was determined on nonpermeabilized cells as shown for panel A. The percentages of SR-BI-positive cells specifically recognized by anti-tupaia SR-BI were determined by subtracting the number of positive cells in pcDNA3-transfected cells stained with anti-SR-BI (negative control) from the number of positive cells in pcDNA3/tSR-BI-transfected cells stained with anti-SR-BI. Histograms (left panel) and percentages of cells (right panel) recognized by anti-tupaia SR-BI are shown in relationship to antibody dose. (C) Detection of cell surface SR-BI on PTH by using mouse anti-tupaia and human SR-BI antibodies. Nonpermeabilized PTH were incubated with mouse anti-tupaia (left panel) and human SR-BI (right panel) sera (1:500 dilution) or mouse preimmune serum (1:500 dilution) and subsequently stained with R-PE-conjugated goat anti-mouse IgG. x and y axes show mean fluorescence intensity and relative number of stained cells, respectively.

FIG. 6. (A) Dose-dependent and saturable binding of HCV-LPs to PTH and human HepG2 cells. Cells were incubated with increasing concentrations of HCV-LPs. After washing with PBS, cellular binding of HCV-LPs was analyzed by flow cytometry using a mouse monoclonal anti-E2 antibody and PE-conjugated anti-mouse IgG. On the y axis, net mean fluorescence intensity (Δ MFI) values for each HCV-LP E2 concentration were calculated by subtracting the MFI of the negative control (control insect cell preparation) with anti-E2 and PE-conjugated anti-mouse IgG antibodies from that obtained with the respective HCV-LP E2 concentration (x axis). Squares, binding of HCV-LPs to PTH; triangles, binding of HCV-LPs to HepG2 cells. (B) SR-BI-dependent binding of HCV-LPs to PTH. PTH were preincubated with anti-tupaia SR-BI or control antibody (in preimmune serum at a 1:10 dilution). After washing with PBS, PTH were incubated with HCV-LPs in subsaturating concentrations (HCV-LP E2 concentration of ~ 1 μ g/ml) and cellular HCV-LP binding was assessed using a chimpanzee monoclonal anti-E2 antibody as described in Materials and Methods.

CHO cells were transfected with human, tupaia, or mouse SR-BI cDNA. Interestingly, transfection of CHO cells with human or tupaia SR-BI cDNA resulted in E2 binding, whereas CHO cells transfected with mouse SR-BI cDNA did not bind E2 (Fig. 3). These findings suggested that tupaia SR-BI but not mouse SR-BI binds E2 similar to human SR-BI.

To analyze whether PTH interact with recombinant E2, cellular binding of E2 to PTH was measured by flow cytometry. Soluble recombinant E2, previously shown to interact with HepG2 cells in a dose-dependent manner (36), strongly bound to PTH. E2 binding to PTH was dose dependent and saturable and reached a plateau at an E2 concentration of approximately 4 μ g/ml (Fig. 4A). E2 derived from genotype 1b demonstrated a similar binding profile, suggesting that binding of E2 to PTH is not genotype or subtype dependent (data not shown).

To investigate whether HCV envelope binding was mediated by PTH cell surface SR-BI, we performed competitive assays using anti-SR-BI antibodies. Since commercially available antibodies are directed against epitopes of the SR-BI intracellular cytoplasmic C-terminal domain, these antibodies do not interact with the SR-BI extracellular loop and are therefore not suitable for the study of inhibition of HCV envelope-SR-BI interaction on the cell surface. Thus, we generated novel antibodies directed against epitopes of the extracellular loop of human and tupaia SR-BI by the genetic immunization of mice by using full-length tupaia and human SR-BI cDNAs, respectively. To demonstrate that mouse anti-tupaia and human SR-BI antibodies generated by genetic immunization specifically interact with tupaia SR-BI, we studied the binding of antibodies to tupaia SR-BI expressed on the cell surfaces of transfected CHO cells. As shown in Fig. 5, incubation of CHO cells expressing tupaia SR-BI with mouse polyclonal anti-SR-BI antibodies resulted in a specific interaction of anti-tupaia and human SR-BI antibodies with tupaia SR-BI (Fig. 5A, bottom panels). In contrast, no interaction was present in CHO cells transfected with the pcDNA3 control vector and incubated with anti-SR-BI (Fig. 5A, upper panels) or in CHO cells transfected with tupaia SR-BI cDNA and incubated with preimmune serum (data not shown). Interaction of anti-tupaia SR-BI (Fig. 5B) and anti-human SR-BI (data not shown) with tupaia SR-BI expressed in CHO cells was dose dependent. Both antibodies interacted with SR-BI on the cell surfaces of PTH without the need of cell permeabilization, thus confirming the interaction of these antibodies with epitopes of the SR-BI extracellular loop on PTH (Fig. 5C). Saturation of PTH

Data are shown as percent binding (mean $\pm$ standard deviation [SD] of a representative experiment performed in triplicate) in the presence of antibody compared to binding of HCV-LPs in the presence of PBS (at 100%). (C) SR-BI-dependent binding of HCV-LPs to human HepG2 hepatoma cells. HepG2 cells were incubated with anti-human SR-BI or control antibody (in preimmune serum at a 1:10 dilution) 1 h prior to the addition of HCV-LPs. After washing with PBS, HepG2 cells were incubated with HCV-LPs in subsaturating concentrations (HCV-LP E2 concentration of ~ 1 μ g/ml) and cellular HCV-LP binding was assessed as described for panel B. Data are shown as percent binding (mean $\pm$ SD of a representative experiment performed in triplicate) in the presence of antibody compared to binding of E2 in the presence of PBS (at 100%).

SR-BI by polyclonal anti-tupaia SR-BI antibody was achieved at an antibody dilution of 1:10 to 1:100 (data not shown).

Preincubation of PTH with anti-tupaia SR-BI antibody resulted in a marked and concentration-dependent inhibition of E2 binding (Fig. 4B and C). Inhibition of cellular E2 binding by anti-tupaia SR-BI antibodies was also observed when E2 derived from genotype 1b was used as a ligand, suggesting that antibody-mediated inhibition of cellular E2 binding is genotype independent (data not shown). Inhibition of cellular binding reached a plateau at an antibody dilution of 1:10 (Fig. 4C) corresponding to the antibody concentration saturating SR-BI on PTH. Interestingly, preincubation of PTH with anti-human SR-BI also resulted in a marked inhibition of E2 binding, suggesting that the E2-binding domains of tupaia and human SR-BI LEL are highly conserved (Fig. 4D). Polyclonal anti-human SR-BI antibody used in the experiment shown in Fig. 4D had been previously shown to inhibit cellular binding of E2 to HepG2 hepatoma cells and viral entry of HCV pseudoparticles into Huh-7 hepatoma cells in a concentration-dependent manner (5, 36).

SR-BI and cellular binding of virus-like particles. As an alternative to purified HCV particles, truncated forms of glycoprotein E2 are useful to study virus-cell interactions. However, C-truncated recombinant E2 proteins mimic only partially the properties of HCV from infected patients. Since HCV-LPs, by comparison, share common features with virions in their cellular binding profiles (39, 41), we analyzed the interaction of HCV-LPs with SR-BI on human HepG2 cells and PTH. As a prerequisite for these studies, we analyzed whether HCV-LPs interact with PTH. Similar to findings observed for recombinant E2, binding of HCV-LPs demonstrated a dose-dependent and saturable binding to PTH. Saturation of binding was reached at an E2 concentration of approximately 4 $\mu\text{g/ml}$ (Fig. 6A). A side-by-side comparison of HCV-LP binding to HepG2 hepatoma cells revealed that saturation of HCV-LP binding is achieved at a similar E2 concentration (Fig. 6A).

To analyze whether cellular HCV-LP binding to PTH is SR-BI dependent, we studied cellular HCV-LP binding in the presence of anti-SR-BI antibodies. Anti-tupaia SR-BI inhibited binding of HCV-LPs to PTH compared to control antibody up to 56% (HCV-LP E2 concentration of 1 $\mu\text{g/ml}$, anti-SR-BI dilution of 1:10). Although the antibody concentration required for inhibition of HCV-LP binding was high, the anti-SR-BI-mediated inhibition of HCV-LP binding was specific, since side-by-side experiments using preimmune serum at the same concentration did not modify cellular HCV-LP binding (Fig. 6B). These observations suggested that SR-BI represents a cell surface molecule mediating the binding of HCV-LPs to PTH. To compare results obtained in PTH with human cell lines, we studied the effect of anti-SR-BI antibody on cellular HCV-LP binding to the human hepatoma cell line HepG2. Preincubation of HepG2 cells with anti-human SR-BI antibody (previously shown to block binding of soluble E2 glycoprotein and entry of HCVpp in Huh-7 hepatoma cells [5]) partially reduced the binding of HCV-LPs to HepG2 hepatoma cells (Fig. 6C) similar to PTH (Fig. 6B).

HCV infection of tupaia hepatocytes and SR-BI. To study whether SR-BI mediates the HCV infection of hepatocytes, PTH were infected with HCV RNA-positive plasma from pa-

tients with chronic HCV infection. Successful infection of PTH was determined by a time-dependent increase of positive-strand HCV RNA and the detection of negative-strand HCV RNA in hepatocytes by a highly strand-specific RT-PCR (Fig. 7A and B). To study whether HCV infection could be inhibited by anti-SR-BI antibodies, PTH were preincubated with anti-SR-BI prior to HCV infection. As shown in Fig. 7C (upper left panel), anti-tupaia SR-BI was not able to block the viral infection of PTH at concentrations shown to inhibit E2 binding to PTH and saturating SR-BI. Similarly, the incubation of PTH with preimmune serum did not result in a measurable modification of HCV infection (Fig. 7C, upper right panel). To exclude that SR-BI molecules were accessible for HCV-E2 at the hepatocyte cell surface via de novo expression or the intracellular pool redistribution of SR-BI during the time of viral infection, we performed a second series of experiments in the presence of anti-SR-BI antiserum or preimmune serum (1:10 dilution) in the tissue culture medium before and during the time of infection (Fig. 7C, lower panel). Again, no antibody-mediated inhibition of HCV infection was observed (Fig. 7C, lower panel). Infection experiments were performed at low MOIs (starting from an MOI of 0.05) corresponding to the minimal infectious dose to ensure that a lacking inhibitory effect of anti-SR-BI was not the result of a high virus input competing with antibody-SR-BI interaction. To evaluate a genotype-dependent effect of SR-BI on HCV infection, we incubated PTH with sera and plasma containing different HCV isolates, subtypes, or genotypes. Similar to the results presented in Fig. 7, no inhibition of infection by anti-SR-BI was observed (data not shown).

DISCUSSION

The first step in viral infection is the attachment and entry of the virion to the host cell requiring the interaction between viral and cellular surface proteins, called receptors and coreceptors. Because of the difficulties to propagate HCV *in vitro* and the limited animal tropism, the search for HCV receptor candidates has been performed with binding studies using truncated versions of recombinant HCV glycoprotein E2 and human hepatoma or lymphoma lines (12, 13, 33). Based on such experimental studies, SR-BI has been recently suggested as a novel HCV receptor candidate (36). However, the potential role of SR-BI for the HCV infection of natural target cells, i.e., primary hepatocytes, is unknown. Since PTH can be successfully infected by serum- or plasma-derived native HCV (46), we used this model to assess the functional role of SR-BI as a putative HCV receptor on primary hepatocytes.

Structural and functional characterization of tupaia and human SR-BI validated the tupaia system as a convenient and useful model for the evaluation of HCV receptor candidates. Consistent with our previous study assessing the structure and function of HCV receptor candidate CD81 in PTH (46), HCV receptor candidate SR-BI exhibited a remarkably high degree of homology at the amino acid level between tupaia and humans. This sequence homology reflects the close genetic relationship between tupaia and primates. Interestingly, the degree of homology of SR-BI at the amino acid level between humans, tupaia, and mice (humans versus tupaia, 88%; hu-

A

B

C

mans versus mice, 82%) correlated with the ability of SR-BI to bind HCV envelope glycoprotein E2 (Fig. 3). Alignment of the amino acids of the different species suggests that (i) E2-binding domains between human and tupaia SR-BI are highly conserved and (ii) defined amino acid changes in the extracellular loop of mouse SR-BI abolish E2-SR-BI binding. A detailed comparative and functional analysis of the amino acid differences in the three species will finally allow mapping of the E2-SR-BI-binding domain. Furthermore, the marked structural and functional similarities between human and tupaia SR-BI indicate that PTH represent a useful model system to characterize the molecular interaction of the HCV envelope and SR-BI on primary hepatocytes.

Concentration-dependent inhibition of E2 or HCV-LPs binding by anti-tupaia and anti-human SR-BI antibodies clearly demonstrates that SR-BI can mediate binding of the viral envelope to PTH. These observations are in line with a previous study using HCVpp as a surrogate model for HCV binding and entry (5). Although recombinant E2, HCV-LPs, and HCVpp represent convenient tools to study envelope-glycoprotein-mediated HCV binding and/or entry to or into hepatoma cells, it is still unclear whether these model systems reflect the early steps of natural infection of hepatocytes by native virions. Therefore, studies in primary hepatocytes with native virions are needed to verify findings obtained with HCVpp and other surrogate systems mimicking the early steps of viral infection. Using native plasma-derived HCV and primary hepatocytes, we present evidence that SR-BI is not sufficient to mediate the HCV infection of primary hepatocytes. A polyclonal anti-SR-BI antibody markedly inhibiting the cellular binding of E2 failed to block or modulate the HCV infection of PTH (Fig. 7), although the experimental conditions (blocking antibody in concentrations saturating SR-BI and a study of HCV infection at MOIs corresponding to the minimal infectious dose) had been designed to detect a measurable effect of anti-SR BI on viral infection. Since recombinant proteins (E2 and HCV-LPs) and plasma-derived virions used for detailed studies of envelope binding (Fig. 3 through 6) and HCV infection (Fig. 7) consisted of the same genotype (all genotype 1), it is unlikely that genotype-specific differences

between proteins and virions play a role in the lacking ability of anti-SR-BI to block HCV infection.

As shown for other viruses, several cellular surface molecules serve as receptors for the attachment and entry of viruses (37). In addition, viral entry may require the interaction of viral proteins with coreceptors or entry receptors that are distinct from those that mediate initial virus binding (37). For HCV CD81 (10, 12, 16, 32, 45), dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin (13, 26, 27, 33), the low-density lipoprotein receptor (2), and heparan sulfate (3) have been suggested to play an important role in virus attachment and entry. Based on this and previous observations, HCV binding and entry is most likely a multifactorial process which requires a set of initial binding molecules as well as (co)receptors for viral entry.

The finding of the present study indicates that SR-BI represents an important hepatocyte cell surface molecule mediating binding of the viral envelope to the hepatocyte cell membrane. SR-BI may act in concert with other E2-binding cell surface molecules such as highly sulfated heparan sulfate (3) or CD81 (10, 12, 16, 32, 45). The latter hypothesis is underlined by the finding that preincubation of anti-SR-BI of PTH or HepG2 cells (Fig. 6) resulted only in an incomplete inhibition of HCV-LP binding. It is conceivable that other cell surface molecules such as highly sulfated heparan sulfate (recently shown to mediate the binding of HCV-LPs [3] and E2 [14] to HepG2 cells) may contribute to E2-cell surface attachment. The presence of several different cell surface molecules for the capture of virions may represent a strategy of the virus to secure efficient interaction of the virus with the target cell and escape antibody-mediated virus neutralization. Since the inhibition of E2-SR-BI interaction did not block or modulate viral infection, our data suggest that SR-BI is not sufficient for HCV entry into hepatocytes. Although mechanisms of viral entry may not be identical in tupaia and human hepatocytes due to species-specific factors and SR-BI and CD81 have been shown to represent coreceptors for the entry of HCVpp into Huh-7 cells (5) and human hepatocytes (4), the findings obtained in the HCV-PTH model system suggest that the entry of native virus into primary hepatocytes requires additional or other

FIG. 7. (A) Sensitivity and specificity of HCV RNA detection from hepatocytes by strand-specific RT-PCR. Different amounts of in vitro-synthesized positive- or negative-strand HCV RNAs (synthesized as described in Materials and Methods) were added to the total cellular RNAs of uninfected PTH, respectively, to study the sensitivity and specificity of HCV RNA detection using strand-specific RT-PCR. Positive- and negative-strand HCV RNAs were then analyzed by strand-specific RT-PCR using rTth polymerase 5'-UTR-specific primers (23, 46). Sensitivity for both strands ranged from 0.1 to 1 fg per assay for the correct RNA template, while for the incorrect template (use synthetic negative-strand HCV RNA as template for the detection of positive-strand HCV RNA or vice versa), at least 0.1 pg of the template was needed to give a false-positive signal. (B) Infection of PTH by plasma-derived HCV. PTH were incubated with HCV RNA-positive plasma (samples M1, M2, G3, and G4, all HCV genotype 1) on day 2 after plating as described in Materials and Methods. HCV infection was determined by strand-specific RT-PCR of cellular positive- and negative-strand HCV RNAs on days 1 and 5 postinoculation (p. i.) in PTH as shown for panel A and described in Materials and Methods. (C) HCV infection of PTH in the presence of anti-tupaia SR-BI. PTH were preincubated with anti-tupaia SR-BI (left panel) or preimmune serum (right panel) at a dilution of 1:10 1 h prior to the addition of HCV plasma (samples M2 and G4, both genotype 1). Following the washing of nonbound anti-SR-BI, plasma-derived HCV was added to the cell culture medium. The MOIs (calculated as the number of HCV genomic equivalents present in the inoculum divided by the number of hepatocytes) were 0.05 (3×10^4 genomic equivalents/ 6×10^5 hepatocytes) and 0.25 (1.5×10^5 genomic equivalents/ 6×10^5 hepatocytes) for plasma samples M2 and G4, respectively. HCV infection was determined by strand-specific RT-PCR as described for panel A. To exclude that SR-BI molecules were accessible for HCV-E2 at the hepatocyte cell surface via the de novo expression or intracellular pool redistribution of SR-BI during the time of viral infection, infection experiments were repeated in the presence of anti-tupaia SR-BI or preimmune serum before and during the time of infection (lower panel). For these experiments, PTH were preincubated for 1 h with anti-tupaia SR-BI or preimmune serum (both serum dilutions of 1:10) and plasma-derived HCV (samples and MOI as described for panel B) was added to the cell culture medium without the removal of non-cell-bound anti-SR-BI. Mock-infected PTH served as negative controls.

cellular molecules besides SR-BI (this study) and CD81 (46). Efforts to identify these molecules will be crucial for the understanding of viral infection and potentially allow the development of novel antiviral strategies for the prevention and treatment of HCV infection.

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