Hepatitis C Virus Envelope Proteins Bind Lactoferrin

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Hepatitis C virus (HCV) has two envelope proteins, E1 and E2, which form a heterooligomer. During dissection of interacting regions of HCV E1 and E2, we found the presence of an interfering compound or compounds in skim milk. Here we report that human as well as bovine lactoferrin, a multifunctional immunomodulator, binds two HCV envelope proteins. As demonstrated by far-Western blotting, the baculovirus-expressed E1 and E2 could bind lactoferrin in human milk directly separated or immunopurified and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The bindings of lactoferrin and HCV envelope proteins in vitro were confirmed by another method, the pull-down assay, with immunoprecipitated lactoferrin-bound protein A resin. By the same assay, mammal-expressed recombinant E1 and E2 were also demonstrated to bind human lactoferrin efficiently in vitro. Direct interaction between E2 and lactoferrin was proved in vivo, since anti-human lactoferrin antibody efficiently coimmunoprecipitated with secreted and intracellular forms of the E2 protein, but not glutathione S-transferase (GST), from lysates of HepG2 cells transiently cotransfected with the expression plasmids of human lactoferrin and gE2t-GST (the N-terminal two-thirds of E2 fused to GST) or GST. The N-terminal loop of lactoferrin, the region important for the antibacterial activity, has only a little role in the binding ability to E2 but affected the secretion or stability of lactoferrin. Taken together, these results indicate the specific interaction between lactoferrin and HCV envelope proteins in vivo and in vitro.

MATERIALS AND METHODS

Plasmid constructions. The bacterial expression vectors of HCV E1 and E2 were constructed with a glutathione S-transferase (GST) expression vector, pGENK1, which has an artificial phosphorylation site and which can be used for in vitro phosphorylation by a kinase, as reported previously (21, 28, 30). The transmembrane portions of E1 and E2 at termini were truncated because the full-length constructs of E1 and E2 did not give a detectable level of protein expression in GST-fused forms in Escherichia coli (30). The mammalian expression vectors gE1t-GST and gE2t-GST were constructed with pSG5UTPL as reported previously (21, 30), and the regions derived from HCV JKI (10) are shown in the figures. A mammalian expression vector of lactoferrin was constructed by inserting a cDNA of human lactoferrin (GenBank accession no. HSU07643) into the EcoRI and BamHI sites of pSG5UTPL (21). To construct a deletion mutant of human lactoferrin (deletion of 10 to 46 amino acids [aa] of mature lactoferrin), upstream and downstream fragments of the lactoferrin cDNA were prepared by PCR with two sets of primer pairs: T7 (sense primer of upstream sequence of pSG5UTPL vector) and 5'-GCTTCAGCCACACTGAAACTCTTCTCTTTGAC-3' (antisense) and 5'-GGCTTGGACCACTGAAACTCTTCTCTTAC-3' (sense)/SG3 (antisense primer of downstream sequence of pSG5UTPL vector). After digestion of the fragments with EcoRI/XhoI and XhoI/BamHI, respectively, the fragments were inserted into pSG5UTPL digested with EcoRI and BamHI.

Protein preparation. Expression and induction of bacterial GST-fused proteins were carried out as described previously (21). Washed pellets of E. coli transformant cells were washed once with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (PBST) and lysed on ice by mild sonication in a 1/10 culture volume of PBST. Sonicated pellets, recovered by centrifugation at 3,000 × g for 10 min, were resuspended in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), heated at 100°C for 5 min, fractionated by SDS-polyacrylamide gel electrophoresis (PAGE [12.5% polyacrylamide]), and then electrically transferred to nitrocellulose membranes (Schleicher & Schuell Co., Ltd.) in 192 mM glycine–25 mM Tris–20% methanol (21, 30) and used for far-Western blotting. GST-fused proteins were further purified with glutathione-Sepharose 4B according to the manufacturer’s procedures (Pharmacia LKB Co., Ltd.), and the purified proteins were used for the pull-down assay with protein A-Sepharose prebound with human lactoferrin and anti-human lactoferrin antibody (DAKO AS [1:10,000 dilution]).

Skim milk (Difco Co., Ltd.) was dissolved in PBST and heated at 100°C in SDS sample buffer and fractionated by SDS-PAGE (12.5% polyacrylamide). Bovine lactoferrin (Sigma Co., Ltd.) was also dissolved in PBST and fractionated. Human milk donated by volunteer mothers was diluted with SDS sample buffer, heated denatured, and fractionated by SDS-PAGE (12.5% polyacrylamide). To purify the gE1t-GST and gE2t-GST expressed in HepG2 cells, PBS-washed transfected cells were lysed in lysis buffer T (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100), and the supernatant was recovered by centrifugation at 12,000 × g for 10 min. The proteins were purified by glutathione-Sepharose.

Far-Western blotting. 32P-labeled GST fusion proteins were prepared as described previously (21, 30). Briefly, proteins purified with glutathione-Sepharose were resuspended in 1× HMK buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 12

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5997
Antibody or antilactoferrin antibody. In nontransfected HepG2 lysates and by Western blotting with either anti-GST antibody alone, or by using anti-human lactoferrin antibody, nonspecific moieties were detected, since similar bands were also detected in HepG2-transfected lysates detected by antilactoferrin antibody. Anti-human lactoferrin antibody was directly added to the cleared cell lysates, and proteins were visualized with an enhanced chemiluminescence reagent kit (Amersham International plc.). The commercially available purified protein was eluted by addition of SDS sample buffer, heated at 100°C for 5 min, fractionated by SDS-PAGE (12.5% polyacrylamide), and then transferred to nitrocellulose membranes (Schleicher & Schuell Co., Ltd.), and subjected to Western blotting with anti-human lactoferrin antibody or anti-GST antibody (21) (1:5,000 dilution) for 1 h, and proteins were visualized with an enhanced chemiluminescence reagent kit (Amersham International plc.). The commercially available purified protein (Sigma Co., Ltd.) is species specific and could not recognize bovine lactoferrin.

Immunoprecipitation and pull-down assay. Human lactoferrin in human milk was immunoprecipitated with anti-human lactoferrin antibody (DAKO A/S [1:10,000 dilution]) in N buffer. For the pull-down assay, purified GST-fused proteins were incubated with protein A resin and immunoprecipitates of human lactoferrin and anti-human lactoferrin antibody. After overnight binding at room temperature, the membranes were washed four times with N buffer. Retained proteins were eluted by addition of SDS sample buffer, heated at 100°C for 5 min, fractionated by SDS-PAGE (12.5% polyacrylamide), and then transferred to nitrocellulose membranes (Schleicher & Schuell Co., Ltd.), and subjected to Western blotting with anti-human lactoferrin antibody or anti-GST antibody (21) (1:5,000 dilution). In mammal-expressed 32P-labeled E1 and E2 proteins, fractionated gels were dried and exposed to imaging plates (Fuji Co., Ltd.) or X-ray films (XAR-Omat; Kodak Co., Ltd.).

FIG. 1. Lactoferrin in milk binds the bacterial recombinant HCV E1 and E2 proteins in vitro. (A) Rat or rabbit anti-lactoferrin antibody (DAKO A/S [1:10,000 dilution]) were incubated with horseradish peroxidase-conjugated protein A (1:4,000 dilution) for 1 h, and proteins were visualized with an enhanced chemiluminescence reagent kit (Amersham International plc.). The commercially available purified protein (Sigma Co., Ltd.) is species specific and could not recognize bovine lactoferrin.

RESULTS

Bacterially expressed E1 and E2 bind lactoferrin. During delineation of the E1 and E2 binding regions by far-Western blotting (30), a method to detect interaction of protein and protein in situ, the specific binding signals were barely detected when skim milk (DIFCO Co. Ltd.) instead of BSA was used as a blocking agent (data not shown). To identify the inhibitory compound or compounds, skim milk proteins were separated by SDS-PAGE (12.5% polyacrylamide) (Fig. 1A), transferred to nitrocellulose membrane, and subjected to far-Western blotting with 32P-labeled E1 probe (Fig. 1B) (Materials and Methods). One band approximately 80 kDa in size was specifically detected in skim milk by the E1 probe (Fig. 1B). The reported electrophoresis data from bovine skim milk (12) strongly suggested that an 80-kDa protein among the major components of skim milk (Fig. 1A, lane 2) corresponds to bovine lactoferrin. To examine the possibility, the commercially available purified bovine lactoferrin (Sigma Co., Ltd.), human milk from volunteer mothers, and immunopurified human lactoferrin derived from the human milk were separated (Fig. 1C) and subjected to far-Western blotting (Fig. 1E and F). The result confirmed the speculation, since the E1 and E2 probes both specifically bind to bovine lactoferrin but not to BSA. Furthermore, human lactoferrin in milk was bound by the probes with an intensity similar to that of bovine lactoferrin (lanes 3 and 4 in Fig. 1E and F). These results indicate that human as well as bovine lactoferrin specifically binds the HCV E1 and E2 proteins in vitro.

Delineation of the lactoferrin binding regions in E1 and E2. Several deletion-containing versions of E1 and E2 proteins in GST-fused forms were expressed in E. coli, partially purified (Fig. 2A and B), and then examined for their ability to bind to protein A resin prebound with human lactoferrin and anti-human lactoferrin antibody (pull-down assay). The E1 and E2 proteins in GST-fused forms, but not GST, were efficiently pulled down (Fig. 2C, lanes 2 and 5 in the upper panel). The C-terminal part of E1 (E1-t2 [aa 239 to 340]) seems to be important to the lactoferrin binding, since both E1t and E1-t2 could be efficiently pulled down (Fig. 2C). Two parts of E2 seem to contribute to lactoferrin binding, since the truncated proteins of E2, E2-t1 and E2-t3, but not E2-t2, were recovered in the bound fractions (Fig. 2C, lanes 5 to 8). Anti-human lactoferrin antibody alone could not retain the E1 and E2 proteins in the pull-down assay (data not shown). Therefore, the specific interaction between human lactoferrin and HCV E1 or E2 was shown in vitro by two different methods, far-Western blotting (Fig. 1E and F) and pull-down assay (Fig. 2B and C), with protein A resin prebound with human lactoferrin and anti-human lactoferrin antibody.

Mammal-expressed E1 and E2 bind human lactoferrin in vitro. Since HCV E1 and E2 are glycoproteins, we next ad-
dressed whether glycosylated forms of E1 and E2 expressed in mammalian cells bind human lactoferrin. HepG2 cells were transiently transfected with the eukaryotic expression vectors of E1 and E2 derived from pSG5UTPL (21), gE1t-GST and gE2t-GST, respectively (Fig. 3A). The expressed recombinant proteins have the GST portion at the C terminus which facilitates purification with glutathione resin and labeling in vitro with [γ-32P]ATP (Materials and Methods). The gE2t-GST protein could be efficiently expressed in HepG2 cells, although the level of expression of gE1t-GST was extremely poor (30). The mammal-expressed E1 and E2 were purified with glutathione resin, in vitro labeled, and subjected to the pull-down assay with the protein A resin prebound with human lactoferrin. The resin efficiently pulled down both of the glycosylated forms of E1 and E2 but not GST (Fig. 3B, lanes 6 and 9 compared to lane 3). This result clearly indicates the specific interaction between lactoferrin and the glycosylated forms of HCV envelope proteins expressed in mammalian cells.

Expression of recombinant human lactoferrin and E2 proteins in mammalian cells. The interactions of lactoferrin and HCV envelope proteins in vitro raised the possibility that such interactions may happen in vivo in mammalian cell culture systems. At first, expression of recombinant lactoferrin was examined. We constructed two mammalian expression vectors of human lactoferrin derived from a cDNA clone, a full-size lactoferrin (LF) and a deletion mutant (Lfdl) lacking the N-terminal loop that has been reported to interact with several proteins. A. GST

FIG. 2. Delineation of lactoferrin binding regions of HCV E1 and E2. (A) Several truncated versions of the HCV E1 and E2 expression vectors were constructed with pGENK1 as described previously (21, 30). The region included in each construct is shown by the amino acid numbers of the HCV JK1 polypeptide (10). The estimated molecular masses of GST-fused proteins derived from constructs 1 to 8 are 26, 43, 32, 38, 58, 40, 37, and 34 kDa, respectively. (B) GST-fused proteins were expressed in E. coli, partially purified, fractionated by SDS-PAGE (12.5% polyacrylamide), and detected by Coomassie brilliant blue (CBB) staining. (C) Partially purified GST-fused proteins were incubated with protein A resin-prebound immunoprecipitates of anti-human lactoferrin antibody and human lactoferrin (pull-down assay). The resin was recovered and washed as described in Materials and Methods. (Upper) Total bound fractions were solubilized, separated by SDS-PAGE (12.5% polyacrylamide), and detected by anti-GST antibody. (Lower) One-twentieth the amount of unbound fractions was separated and detected by anti-GST antibody.

FIG. 3. E1 and E2 expressed in HepG2 cells bind lactoferrin in vitro. (A) Mammalian expression constructs gE1t-GST and gE2t-GST were reported previously (30). The nucleotide numbers within HCV JK1 are indicated for the E1 and E2 constructs. The processing sites of E1 and E2 by the host signalase (filled box) and the artificial consensus kinasin site (21) (arrow) are shown in the lower panel. (B) Mammal-expressed E1 and E2 were recovered by pull-down assay with protein A resin prebound with human lactoferrin and anti-human lactoferrin antibody. After purification of the proteins expressed in HepG2 cells by glutathione resin, each protein was labeled with [γ-32P]ATP through the kinase site. The labeled proteins were pulled down and were recovered as described in the legend to Fig. 2. I, U, and B indicate 10% of labeled input proteins, 10% of unbound proteins, and total bound proteins, respectively. The labeled input proteins of gE2t-GST and gE1t-GST are from the 1 × 10^7 to 2 × 10^7 transfected cells and from the 1 × 10^7 to 2 × 10^7 transfected cells, respectively.
bioactive compounds (2, 5, 15, 16, 18, 29) (Fig. 4A). The human lactoferrin (hLF), which is shown as a band of approximately 80 kDa, was expressed efficiently in transiently transfected HepG2 cells, and the majority was recovered in the cell culture supernatants, as detected by immunoprecipitation and subsequent Western blotting (Fig. 4B, lanes 1 and 2). The mutant protein (hLFdl), slightly smaller than hLF, was detected in cell lysates and very weakly in the supernatant (Fig. 4B, lanes 3 and 4). The reduced amount of secreted forms of hLFdl suggests that the N-terminal loop affects the secretion or stability of human lactoferrin.

The GST-fused E2 protein, gE2t-GST, was expressed in transiently transfected HepG2 cells, purified with glutathione resin or by immunoprecipitation with anti-GST antibody, and finally detected by anti-GST antibody (Fig. 4C and D). The E2 protein was recovered in the cell lysates and also in the supernatants as secreted forms. The specific gE2t-GST bands are close to 80 kDa in the cell lysates and around 100 kDa in the supernatants, respectively, both of which are much higher than the expected molecular mass of gE2t-GST (60 kDa) (Fig. 4C, lanes 7 and 8, and D). These phenomena might be due to different modifications of oligosaccharide during maturation into secreted forms, since these bands were reduced in size and a band corresponding to the expected size appeared by tunicamycin treatment as reported previously (data not shown) (30). Interestingly, the secreted forms of gE2t-GST were less efficiently recovered by glutathione resin (Fig. 4C, lane 8, and D lanes 3 and 4).

Complex formation of human lactoferrin and E2 proteins in vivo. Next we addressed whether human lactoferrin binds HCV envelope proteins in vivo. HepG2 cells were cotransfected with the expression plasmids of gE2t-GST and hLF or hLFdl. The gE2t-GST protein was efficiently recovered in coimmunoprecipitates with anti-human lactoferrin antibody from the cell lysates as well as from the cell culture supernatants (Fig. 5A, lanes 1 to 4). The complexed forms of gE2t-GST were much more efficiently recovered from the supernatants than those from the cell lysates. The majority of the intracellular forms of gE2t-GST seem not to be complexed with hLF, since hLF escaped from coimmunoprecipitation with antilactoferrin antibody and recovered by glutathione resin (lanes 1 to 4 in Fig. 5A and C). The gE2t-GST proteins bound to hLFdl lacking the N-terminal loop were also recovered from the cell lysates and the supernatants (Fig. 5A, lanes 5 to 8), although the amount from the supernatants is small in proportion to the decreased amount of the secreted hLFdl (Fig. 4A and 5C). The result indicates that the N-terminal loop of human lactoferrin has little effect on E2-binding ability, although it is necessary for its secretion or the stability of human lactoferrin. The distribution profiles of these mammalian-expressed proteins in the cells and in the supernatants are affected by different detection methods, since glutathione resin inefficiently recovered the secreted gE2t-GST (Fig. 4D) and could not recover gE2t-GST complexed with hLF in the supernatant (data not shown). This result suggests that the conformation of the secreted gE2t-GST may be different from that in the cells, so that the ability to bind to glutathione was affected. Even with such a detection bias, it is plausible that lactoferrin interacts efficiently with intracellular as well as secreted HCV E2 expressed in cotransfected HepG2 cells.

We also tried to examine the interaction of lactoferrin and E1 in vivo, but we could not get clear results practically because of the extremely low level of expression of gE1t-GST, the detected overlapping of molecular sizes of the E1 protein and the nonspecific proteins that appeared in immunoprecipitates with anti-human lactoferrin antibody (Fig. 4 and 5), and the low sensitivity of antilactoferrin antibody in Western blotting.

**DISCUSSION**

Lactoferrin, a member of the transferrin family of iron-binding glycoproteins, is present mainly in breast milk and in other exocrine secretions. However, lactoferrin is also found in plasma derived from predominantly neutrophil secondary granules, release of which increases during inflammation. Several biological activities of lactoferrin have been addressed, including well-known bacteriostatic, bactericidal, fungicidal, and, especially, antiviral effects in addition to regulation of iron homeostasis (2, 16, 18). Lactoferrin was also assumed to be a putative transcriptional modulator because of its ability to bind a certain DNA element, although the biological meaning of this binding remains unclear (9). The antiviral function of
lactoferrin for HCV has not been addressed until now; however, lactoferrin may interfere with infection of HCV, as is the case with herpes simplex virus type 1, cytomegalovirus, and human immunodeficiency virus (3, 6, 8).

Here we demonstrated that human as well as bovine lactoferrin binds the bacterial and mammal-expressed HCV envelope proteins in vivo and in vitro. The lactoferrin binding regions of HCV envelope proteins were confined in the C-terminal part of an E1 construct lacking the transmembrane domain (aa 239 to 340) and the two parts of E2 at the C-terminal one-third (aa 601 to 661) and N terminus (aa 384 to 500), in which the region spanning aa 384 to 454 is mainly contributing to the lactoferrin binding (data not shown). We recently determined the heteromeric binding regions of E1 and E2 (aa 192 to 238 in E1 and 441 to 500 in E2) (30), which seem not to be overlapped by the lactoferrin binding regions. Therefore, heteromeric complex formation between E1 and E2 may not affect the abilities to bind to lactoferrin. The structural features of lactoferrin that are required for most of the biological functions remain unclear, but the significance of the loop region at the N-terminal portion has been elucidated. The N-terminal loop, especially residues 20 to 37 of human lactoferrin and residues 17 to 41 of bovine lactoferrin, was reported to be important for the antibacterial activities, binding to lipopolysaccharide and glycoproteins (2, 5, 15, 16, 18, 29). Therefore we addressed the role of the N-terminal loop of lactoferrin in the ability to bind to HCV E2. The mutant lactoferrin (missing 10 to 46 residues of the mature lactoferrin) retains the ability to bind to gE2t-GST in cotransfected HepG2 cells, suggesting that the N-terminal loop does not have a role in E2 binding, although the deletion affected the secretion or stability of lactoferrin in HepG2 cells.

The high concentration of lactoferrin in breast milk and the increased concentration of lactoferrin in plasma during pregnancy may explain the unexpectedly rare rate of vertical transmission of HCV compared to that of hepatitis B virus (1, 17, 25, 27). The biological relevance of the interaction of lactoferrin and the HCV envelope proteins remains to be explored, since the liver is the target organ of HCV infection and is also the main clearance site of lactoferrin in the blood (20). Lactoferrin may interfere positively or negatively with infection or maturation of HCV propagation.

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