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Identification of a Lactoferrin-derived Peptide Possessing Binding Activity to Hepatitis C Virus E2 Envelope Protein*

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Bovine and human lactoferrins (LF) prevent hepatitis C virus (HCV) infection in cultured human hepatocytes; the preventive mechanism is thought to be the direct interaction between LF and HCV. To clarify this hypothesis, we have characterized the binding activity of LF to HCV E2 envelope protein and have endeavored to determine which region(s) of LF are important for this binding activity. Several regions of human LF have been expressed and purified as thioredoxin-fused proteins in *Escherichia coli*. Far-Western blot analysis using these LF fragments and the E2 protein, expressed in Chinese hamster ovary cells, revealed that the 93 carboxyl amino acids of LF specifically bound to the E2 protein. The 93 carboxyl amino acids of LFs derived from bovine and horse cells also possessed similar binding activity to the E2 protein. In addition, the amino acid sequences of these carboxyl regions appeared to show partial homology to CD81, a candidate receptor for HCV, and the binding activity of these carboxyl regions was also comparable with that of CD81. Further deletion analysis identified 33 amino acid residues as the minimum binding site in the carboxyl region of LF, and the binding specificity of these 33 amino acids was also confirmed by using 33 maltose-binding protein-fused amino acids. Furthermore, we demonstrated that the 33 maltose-binding protein-fused amino acids prevented HCV infection in cultured human hepatocytes. In addition, the site-directed mutagenesis to an Ala residue in both terminal residues of the 33 amino acids revealed that Cys at amino acid 628 was determined to be critical for binding to the E2 protein. These results led us to consider the development of an effective anti-HCV peptide. This is the first identification of a natural protein-derived peptide that specifically binds to HCV E2 protein and prevents HCV infection.

Hepatitis C virus (HCV)[1] infection frequently causes chronic hepatitis (1, 2) and frequently progresses to liver cirrhosis and hepatocellular carcinoma (3, 4). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the *Flaviviridae* (5–7). The HCV genome encodes a large polyprotein precursor of about 3,000 amino acid (aa) residues, which is cleaved by the host and viral proteases to generate at least ten proteins: the core, E1 (envelope 1), E2, p7, NS2 (nonstructural protein 2), NS3, NS4A, NS4B, NS5A, and NS5B (8–12). The most characteristic feature of the HCV genome is its remarkable sequence heterogeneities and variations, and to date at least six major HCV genotypes, which have been further grouped into more than 50 subtypes, have been identified (13–16). The genetic complexity of HCV is thus a major hindrance to the development of the vaccines.

To date, interferon has been the sole effective antiviral reagent used in the clinical therapy of hepatitis C, but its effectiveness is limited to about 30% of the reported cases (17). Combined treatment of interferon and ribavirin has been shown to be more effective than treatment with interferon alone (18). The side effects of interferon are also in some cases severe enough to lead to treatment cessation.

Although the entry mechanism of HCV, as well as that of hepatitis B virus, remains unclear, it was reported recently that human CD81 (19) and scavenger receptor class B type I (20) could be bound by a truncated, soluble form of the E2 protein; such findings suggest that these proteins may act as receptors for HCV on the cell surface. Low density lipoprotein receptor (21) was also reported as a putative HCV receptor in endocytosis experiments using isolated HCV-lipoprotein complexes. However, because of the lack of a reproducible and efficient HCV proliferation system, it is not known whether these candidate receptors for HCV serve as the functional receptor on human hepatocytes (22).

We previously reported that non-neoplastic human hepatocyte-derived PH5CH8 cells supported HCV replication, although HCV proliferation was at a fairly low level; in that study, we also demonstrated the antiviral effects of interferon-α in HCV-infected PH5CH8 cells (23). Using a PH5CH8 cell culture system, we found that bovine and human lactoferrin (LF), a milk glycoprotein belonging to the iron transporter family, specifically prevented HCV infection in the cells (24). Recently, Matsuura *et al.* (25) also showed that bovine LF specifically inhibited infection by the pseudotype vesicular stomatitis virus possessing chimeric HCV E1 and E2 glycoproteins.

LF has a molecular mass of 80 kDa and consists of two

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[1] The abbreviations used are: HCV, hepatitis C virus; LF, lactoferrin; aa, amino acid; TF, transferrin; BSA, bovine serum albumin; PNGaseF, peptide-*N*-glycosidase F; RT, reverse transcription; LEL, large extracellular loop; TRX, thioredoxin; MBP, maltose-binding protein; ELISA, enzyme-linked immunosorbent assay; HBS, HEPES-buffered saline.

homologous globular lobes (an N-lobe and a C-lobe), each with a single iron (Fe^{3+}) binding site. There is a notable degree of internal homology between the two lobes, *i.e.* ~35% identical amino acid residues have been identified in the corresponding portions (26). The three-dimensional structures of human and bovine LFs have been clarified by crystallographic studies (27, 28). Although the overall structure of LF is similar to that of transferrin (TF) (~60% amino acid sequence homology to LF), LF has two distinct features that may be functionally important. First, the association constant of LF for iron is 300 times that of TF (29). Second, in contrast to TF, LF possesses strong inhibitory activity against bacterial growth. The antimicrobial activity of LF has been ascribed to the basic N-terminal region ("lactoferricin") (30). Lactoferricin (24 aa residues) shows activity against a wide range of microorganisms including bacteria and fungi (30). LF is present in the milk of most mammals, and the LF content in milk changes substantially during the lactation period. The concentrations of LF in mature milk are 0.1–0.4 mg/ml in bovines and 1–3 mg/ml in humans, and LF is especially enriched in the colostrum (0.8 mg/ml in bovines and 10 mg/ml in humans) (31, 32). It is well established that LF plays an important role in the newborn as the primary nonspecific defense against pathogenic microorganisms (26). Also, it has been reported that rats fed a 2% bovine LF diet displayed no significant side effects (33). This low risk of severe side effects presents a major clinical advantage of bovine LF; hence, clinical pilot studies have been performed recently. The results have shown that bovine LF was effective in some patients with chronic hepatitis C (34, 35).

A recent study by our group (36) suggested that the prevention of HCV infection in these cells was because of interactions of LF with HCV rather than with the cells themselves; our study demonstrated that LF inhibited viral entry into the cells by interacting directly with HCV (36). On the other hand, Yi *et al.* (37) independently demonstrated that HCV envelope proteins (E1 and E2) could bind to human and bovine LFs, although their binding specificities have not been clarified. E2 protein expressed in mammalian cells specifically binds to human target cells (38), and such binding is associated with HCV particles binding to target cells *in vitro*, as well as with HCV infection *in vivo* (38). In addition, the level of antibody response to E2 protein has been shown to correlate with protection against HCV in animal models (39) and with occasional clearance of HCV in cases of natural infection (40), suggesting that the E2 protein is the major receptor-binding protein. For these reasons, we focused on the interactions between LF and E2 proteins to understand the mechanism by which LF prevents HCV infection of target cells. In this study, we have characterized the binding activity of LF to the E2 protein and have endeavored to determine which region(s) of LF are important for this binding activity. Here, we report the finding of 33 human LF-derived amino acids possessing binding activity to the E2 protein of HCV, which leads to inhibition of HCV infection in target cells.

EXPERIMENTAL PROCEDURES

Far-Western Blot Analysis—Far-Western blot analysis was carried out according to a method described previously (37) with some modifications. Briefly, 0.5 μg of human and bovine LFs (Sigma) and various recombinant LF fragments were resolved by 10 or 12% SDS-PAGE and were transferred to polyvinylidene difluoride membranes. The membranes were then blocked in N-buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.25% gelatin) with 2% bovine serum albumin (BSA) for 30 min at room temperature. The binding reaction was carried out at room temperature in N-buffer containing 2% BSA, and the secreted form of E2 protein (E2–681) expressed in Chinese hamster ovary cells was used as a probe (41). After 1 h of incubation, the membranes were washed with N-buffer three times for 10 min at room temperature, blocked once again with N-buffer with 2% BSA, and incubated at room temperature with rat monoclonal antibody, MO-2 or MO-12, against E2 protein (42). Normal rat serum (Invitrogen) was used as a control instead of MO-2 or MO-12. After 1 h of incubation at room temperature, the membranes were washed with 0.1% Tris saline three times for 5 min at room temperature. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; PerkinElmer Life Sciences).

Deglycosylation of LF—Human and bovine LFs (10 μg each; Sigma) were denatured with 0.5% SDS, 1% 2-mercaptoethanol for 10 min at 100 °C and were then treated with 2,000 units of peptide-*N*-glycosidase F (PNGaseF; New England Biolabs) in 50 mM sodium phosphate, pH 7.5, 1% Nonidet P-40 for 4 h at 37 °C. After incubation, the samples were immediately used for the Far-Western blot analysis.

Isolation of LF, TF, and CD81 cDNAs—Total RNAs (2 μg each) from human cancer breast tissue (43), normal bovine breast tissue, and normal horse peripheral blood mononuclear cells were used as templates for reverse transcription (RT)-PCR to obtain the full-length LF cDNAs. The total RNA (2 μg) from PH5CH8 cells was also used as a template for RT-PCR to obtain the TF cDNA encoding aa 587–679 and the CD81 cDNA encoding the large extracellular loop (LEL; aa 113–201). Oligo(dT) was used to prime the cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). Amplification by PCR with a highly efficient proofreading DNA polymerase, KOD-plus (Toyobo) was performed for 20 cycles using each primer set (see Table I) arranged from the nucleotide sequences of human LF (X52941), bovine LF (M63502), horse LF (AJ010930), human TF (S95936), and human CD81 (M33680) cDNAs. The PCR product containing the coding region of full-length human LF was cloned into the *Hin*dIII and *Bam*HI sites of the pHook™-2 (Invitrogen), as described previously (44). The PCR products containing the coding region of full-length bovine and horse LFs were also cloned into the *Not*I and *Hpa*I sites of pCXbsr (45), as described previously (46). The PCR products containing the human TF fragment (aa 587–678) and the CD81 LEL were cloned into the *Bam*HI and *Hin*dIII sites of the pET32a (Novagen), respectively. The nucleotide sequences of obtained cDNA clones encoding human, bovine, and horse LF, and human TF and CD81 were determined by Big Dye terminator-cycle sequencing on an Applied Biosystem 310 automated sequencer (Applied Biosystems, Norwalk, CT). It was confirmed that these cDNAs had identical nucleotide sequences to those in the databases.

Construction of Expression Plasmids for Escherichia coli—The pET32a was used for the production of LF, TF, or CD81 fragments as thioredoxin (TRX)-fused proteins in *E. coli*. For the expression of human, bovine, and horse LFs, the inserts of obtained cDNA clones were transferred into the *Bam*HI and *Hin*dIII sites of the pET32a. Based on these pET32a plasmids containing the coding regions of the full-length LFs, the pET32a expression plasmids encoding the various regions of LF were constructed by inserting the PCR product amplified using the primer sets listed in Table I into the *Bam*HI-*Hin*dIII site of the pET32a. The pMAL-c2X (New England Biolabs, Beverly, MA) was used for the production of human LF fragment (aa 600–632) as maltose-binding protein (MBP)-fused protein in *E. coli*. The DNA fragment encoding the human LF fragment (aa 600–632), which was obtained from the pET32a expression vector by digestion with *Bam*HI and *Hin*dIII, was transferred into the *Bam*HI-*Hin*dIII sites of the pMAL-c2X. The single-amino acid substitutions (*i.e.* changed to an Ala residue) were introduced into pET32a containing the coding region of human LF fragment (aa 600–632) by site-directed mutagenesis, as described previously (47), using the mutation primer sets. The obtained pET32a mutants were confirmed by nucleotide sequencing.

Expression and Purification of TRX-fused LF Fragments, TF Fragment, and CD81 LEL—Expression plasmids for TRX-fused human LF fragments were transformed into the *E. coli* strain AD494(DE3) (Novagen). The transformants were cultured in 10 ml of LB medium containing ampicillin (50 μg/ml) and kanamycin (15 μg/ml) at 37 °C overnight and were then transferred to 200 ml of LB medium; the culture period was then for 4 h at 37 °C. One mM isopropyl-β-D-thiogalactopyranoside was added to the culture and then continued for 4–5 h at 37 °C. After centrifugation at 3,400 × *g* for 10 min, the harvested cells were suspended in 10 ml of B-PER™ (Pierce) by pipetting until the cell suspension became homogenous and was incubated for 15 min at room temperature. After centrifugation at 27,000 × *g* for 15 min, the supernatant obtained as the soluble fraction was used for the purification of LF fragments and CD81 LEL using a His-Bind purification kit (Novagen) according to the manufacturer's protocol, because these TRX-fused proteins possesses His tag sequences. On the other hand, the pellets obtained as the insoluble fraction were suspended in 10 ml of B-PER™; 200 μl of lysozyme (10 mg/ml; Sigma) was then added, and the samples were incubated for 5 min at room temperature. After centrifugation at

TABLE I
Oligonucleotides used for construction of expression plasmids

Oligonucleotide	Sequence	Direction	Expressed proteins
hLF(B)	attatGGATCCGGCCGTAGGAGAAGGAGTGTTCAG	Forward	Full-length human LF
hLFCRH	taataAAGCTTTTACTTCCTGAGGAATTCACAGGC	Reverse	Full-length human LF, lactoferricin-truncated human LF, human LF C-lobe and C-s3, human LF (aa 610–692, 624–692, 640–692, 653–692)
hLFB1	tgataGGATCCCAGGCCATTGCGGAAAACAGGGCC	Forward	Lactoferricin-truncated human LF human LF N-lobe and N-s1
hLFH1	taataAAGCTTGGGCTCAGGTGGACCCG	Reverse	Human LF N-s1
hLFB2	tgataGGATCCATTGAGGCAGCTGTGGCCAGGTTC	Forward	Human LF N-s2
hLFH2	taataAAGCTTGGCATGAGAAGGGACCCGGG	Reverse	Human LF N-s2
hLFB3	tgataGGATCCGTTGTGGCACGAAGTGTGTGAATGGC	Forward	Human LF N-s3
hLFNRH	tgataAAGCTTAGCCACTTCCTCCTCACTTTTCCTC	Reverse	Human LF N-lobe and N-s3
hLFCB	tgataGGATCCGCCCGGCGTGCGCGGGTCGTGTGG	Forward	Human LF C-lobe and C-s1
hLFH3	taataAAGGTTAAATTTGCAGGAGCCCGTCTG	Reverse	Human LF C-s1
hLFB5	tgataGGATCCGATGAATATTTCAGTCAAAGCTG	Forward	Human LF C-s2
hLFH4	taataAAGCTTGGCATGATTCGGGGCCATGG	Reverse	Human LF C-s2
hLFB6	tgataGGATCCGTGGTGTCTCGGATGATAAGG	Forward	Human LF C-s3, human LF (aa 600–622, 600–627, 600–632, 600–637, 600–642, 600–647, 600–652, 600–670)
C3ABR	taataAAGCTTTTATGCGACATACTGTGGTCCC	Reverse	Human LF (aa 600–670)
C3ARZ	taataAAGCTTTTAGGCCAGACACTCAGTGTTGTC	Reverse	Human LF (aa 590–652, 600–652, 605–652, 610–652)
C3HF	tgataGGATCCAGACTCCATGGCAAAACAACATATG	Forward	Human LF (aa 653–692)
C3HFA	tgataGGATCCCTGAAACAGGTGCTGCTCCAC	Forward	Human LF (aa 610–692, 610–652)
C3HFB	tgataGGATCCAATGGATCTGACTGCCCGGAC	Forward	Human LF (aa 624–692)
C3HFC	tgataGGATCCAAAAACCTTCTGTTCAATGAC	Forward	Human LF (aa 640–692)
590F	tgataGGATCCCTGCCATCTTGCCATGGCCCCG	Forward	Human LF (aa 590–652)
605F	tgataGGATCCGATAAGGTGGAACGCCTGAAACAGG	Forward	Human LF (aa 605–652, 605–632)
622R	taataAAGCTTTTACCCAAATTTAGCCTGTTGGTGG	Reverse	Human LF (aa 600–622)
627R	taataAAGCTTTTAGTCAGATCCATTTCTCCCAAATTTAGC	Reverse	Human LF (aa 600–627)
632R	taataAAGCTTTTAAAACTTGTCCGGGCAGTCAGATCC	Reverse	Human LF (aa 600–632, 605–632)
637R	taataAAGCTTTTAAGACTGGAATAAGCAAAACTTGTCC	Reverse	Human LF (aa 600–637)
642R	taataAAGCTTTTAAAGGTTTTTGGTTTCAGACTGGAATAA	Reverse	Human LF (aa 600–642)
647R	taataAAGCTTTTAGTTGTCATTGAACAGAAGGTTTTTGG	Reverse	Human LF (aa 600–647)
CD81B	tgataGGATCCTTTGTCAACAAGGACCAG	Forward	Human CD81 LEL (aa 113–201)
CD81RbH	taataAAGCTTCTTCCCGGAGAAGAGGTCATCG	Reverse	Human CD81 LEL (aa 113–201)
bLF(N)	attatGCGGCCGCCACCATGAAGCTCTTCGTCCCCGCCCT	Forward	Full-length bovine LF
bLFR(H)	attatGTTAACTCATTACCTCGTCAGGAAGGCGCAGG	Reverse	Full-length bovine LF
bLFC3F	tgataGGATCCGTGGTGTCTGGAGCGATAGGG	Forward	Bovine LF (aa 597–689, 597–629)
bLFC3R	taataAAGCTTTTACCTCGTCAGGAAGGCGCAGGC	Reverse	Bovine LF (aa 597–689)
bLF632R	taataAAGCTTTTAAAACTTGTCCGGGCAGTTTTTTCC	Reverse	Bovine LF (aa 597–629)
hoLF(N)	attatGCGGCCGCCACCATGGCCCCTAGGAAAAGCGTTCG	Forward	Full-length horse LF
hoLF(H)	attatGTTAACTCATTATGCCCTCAGGAAGGCGCAGGC	Reverse	Full-length horse LF
hoLFC3F	tgataGGATCCGTGGTATCTCAGAGTGATAGGGC	Forward	Horse LF (aa 597–689)
hoLFC3R	taataAAGCTTTTATGCCCTCAGGAAGGCGCAGGC	Reverse	Horse LF (aa 597–689)
C3TF	tgataGGATCCGTGGTCACACGGAAAGATAAGG	Forward	Human TF (aa 587–679)
C3TR	taataAAGCTTTTAAGGTCTACGGAAAGTGCAGGC	Reverse	Human TF (aa 587–679)

27,000 × *g* for 15 min, the pellets were resuspended in 30 ml of 10-fold diluted B-PER™, and the treatment was repeated two times. The pellets obtained by this method were then dissolved in 10 ml of binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, and 6 M urea) as purified inclusion bodies. The inclusion bodies thus obtained were also used for the purification of several LF fragments using a His-Bind purification kit in the presence of 6 M urea. The purity of obtained LF fragments and CD81 LEL was evaluated to be more than 95%, as determined by electrophoresis on 12% SDS-PAGE gels. The protein concentration was determined by using Coomassie protein assay reagent (Pierce). TRX (22 kDa) produced from the pET32a vector, with the linker sequence-derived 26 amino acids in the C-terminal portion, was used as a control protein.

Expression and Purification of the MBP-fused LF Fragment—The expression plasmid for the MBP-fused human LF fragment (aa 600–632) was transformed into the *E. coli* strain JM109. The transformants were cultured in 100 ml of LB medium containing ampicillin (100 μg/ml) and glucose (2 mg/ml) at 37 °C until an optical density of 0.6 at 600 nm was reached. At this point, 1 mM of isopropyl-β-D-thiogalactopyranoside was added to the culture, and the culture was continued for 4 h at 37 °C. After centrifugation at 3,400 × *g* for 10 min, the harvested cells were resuspended in 5 ml of column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM EDTA) and were then disrupted by sonication for 2 min with short pulses. Insoluble cellular debris was removed by centrifugation at 10,000 × *g* for 30 min at 4 °C. The supernatant obtained as the soluble fraction was applied onto an amylose resin affinity column (New England Biolabs, Beverly, MA). The column was washed with 10 column volumes of column buffer to remove the unbound proteins. The bound protein was eluted with 10 mM maltose under conditions recommended by the manufacturer. The purity of the obtained MBP-fused protein was evaluated to be more than 95% by electrophoresis on 12% SDS-PAGE gels. The concentration of the purified MBP-fused protein was determined by using Coomassie protein assay reagent (Pierce). The MBP2 (43 kDa) produced from the pMAL-c2X with a stop codon inserted into the *Xmn*I site was used as a control protein.

Enzyme-linked Immunosorbent Assay (ELISA)-based Binding Assay—A previously described ELISA-based binding assay was used to examine the binding affinity between the E2 protein and MBP-fused human LF fragment (48). Briefly, 96-well microtiter immunoplates (Maxisorp; Nunc) were coated overnight at 4 °C with the secreted form of the E2 protein (E2–681) (50 μl/well, 10 μg/ml) in HEPES-buffered saline (HBS) (10 mM HEPES, pH 7.0, 150 mM NaCl, 3.4 mM EDTA) (41). Both BSA and human TF (each: 50 μl/well, 10 μg/ml in HBS) were also used as negative controls. Rat monoclonal antibodies, MO-2 and MO-12, against E2 protein were used to determine the appropriate concentration of the E2 protein. 96-well microtiter plates were washed three times with HBS containing 0.05% Tween 20 and treated with 0.15% BSA in HBS (100 μl/well) to reduce the incidence of nonspecific binding. Following incubation on a shaking platform for 1 h at room temperature, MBP-fused human LF fragment (aa 600–632) or MBP2 (each: 5, 25, and 50 ng, 50 μl/well) was added. Following further incubation on a

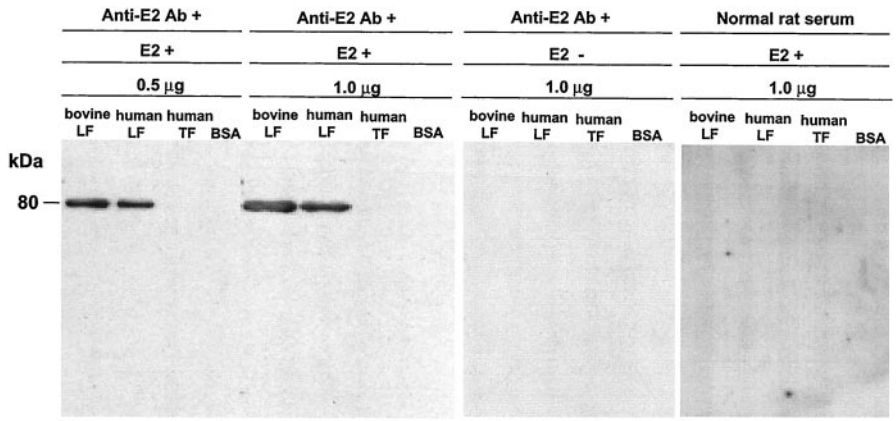

FIG. 1. **Direct interaction between LF and HCV E2 envelope protein.** Human and bovine LFs, and human TF and BSA (0.5 or 1 μg each; each sample was more than 90% pure) were resolved by 10% SDS-PAGE. Far-Western blot analysis using E2 protein expressed in Chinese hamster ovary cells (41) as a probe was performed as described under "Experimental Procedures." Rat monoclonal antibody MO-12 (42) against E2 protein (*Anti-E2 Ab*) was used for the detection of E2 protein bound to LF. Far-Western blot analyses in the absence of the E2 protein and using normal rat serum instead of rat monoclonal antibody MO-12 against E2 protein were also performed. The other rat monoclonal antibody, MO-2, which recognizes a different epitope from that recognized by MO-12, was also used for the detection, and the same results were obtained as those observed with MO-12 (data not shown).

shaking platform for 3 h at room temperature, the plates were washed as described above, and rabbit anti-MBP primary (New England Biolabs, Beverly, MA) and horseradish peroxidase-conjugated anti-rabbit IgG secondary (Amersham Biosciences) antibodies were used for the detection of binding between MBP-fused protein and the E2 protein. After the addition of the substrate, TMB One solution (Promega), A_{450} was measured using a Benchmark microplate reader (Bio-Rad).

Western Blot Analysis—SDS-PAGE and immunoblotting analysis were performed with polyvinylidene difluoride membranes as described previously (9). The rabbit polyclonal antibody (A0186; DAKO) against human LF and mouse polyclonal antibody (Pharmingen) against human CD81 were used for the detection of LF or CD81 by Western blot analysis. Rabbit anti-MBP antibody was also used for the detection of MBP by Western blot analysis. Immunocomplexes on the filters were detected by enhanced chemiluminescence assay (Renaissance; PerkinElmer Life Sciences).

Anti-HCV Activity of the MBP-fused LF Fragment—An assay for anti-HCV activity of the MBP-fused LF fragment was performed by a method described previously (36, 49) using LightCycler real-time PCR technology. Briefly, 2 μl (2×10^4 HCV) of HCV-positive serum 1B-2 (genotype 1b), described previously (23), and the MBP-fused human LF fragment (aa 600–632) (final concentration, 0.5–2.0 mg/ml) were preincubated in 100 μl of medium for 60 min at 4 °C and then the mixture of HCV and the LF fragment was added to the PH5CH8 cells (1.5×10^4 cells were cultured for 2 days at 37 °C before viral inoculation on a 96-well plate). The mixture was then incubated for 90 min at 37 °C. The cells were washed three times with 100 μl of PBS and cultured for 1 day at 32 °C. The cellular RNA was prepared using an ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan), and 0.5 μg of RNA was used for the quantitative analysis of HCV RNA using LightCycler PCR (49). As the positive and negative controls for anti-HCV activity, human LF and MBP2, respectively, were used. All experiments in this assay system were performed in triplicate at least three times.

RESULTS

Direct Interaction between Human and Bovine LFs and HCV E2 Envelope Protein—We found previously (23, 24) that human and bovine LFs prevented HCV infection in human hepatocyte PH5CH8 cells (24) that were susceptible to HCV infection (23). Using LightCycler real-time PCR technology, we showed recently (49) that IC_{50} doses of human and bovine LFs were 5 and 1.5 μM, respectively, for HCV infection of hepatocyte cells. Our previous data (36) showed that the HCV-inhibiting activity of LF is because of the direct interaction between LF and HCV, suggesting that LF directly binds to HCV envelope proteins. This hypothesis is compatible with a previous report by another group (37) that HCV envelope proteins could bind human and bovine LFs, although their binding specificities have not been clarified. Therefore, we first examined the specificity of the direct interaction between LF and the E2 protein, which is thought to play a major role in HCV binding to target cells (38). Far-Western blot analysis was performed using the secreted form (aa 384–681) of E2 protein expressed in Chinese hamster ovary cells as a probe (41). As shown in Fig. 1, human and bovine LFs, which show ~80% aa homology, bound to E2 protein with similar intensity, whereas E2 protein binding activities of human TF showing ~60% aa homology to LF and BSA were not observed after a brief exposure. However, the long period of exposure clarified that human TF (see Fig. 4) and bovine TF (data not shown) did slightly bind to the E2 protein. This phenomenon suggests that the E2 protein binding activity of LF is overwhelmingly greater than that of TF. To exclude the possibility of cross-reactions between LF and anti-E2 antibody, we performed a Far-Western blot analysis in the absence of the E2 protein, as well as an analysis using normal rat serum instead of the anti-E2 antibody. As shown in Fig. 1, no significant bands were obtained in these control experiments, indicating that the bands obtained in this Far-Western blot analysis were derived from the interaction between LF and the E2 protein. Although the E2 proteins used by our group and Yi *et al.* (37) showed an ~14% amino acid difference because of the different HCV strains, bovine and human LFs were equally able to bind to these E2 proteins. These results suggest that LF binds specifically to the E2 protein, regardless of particular HCV strains.

Deglycosylation of LF Strengthens the Interaction between LF and E2 Protein—Both human and bovine LFs are glycoproteins possessing three and five *N*-linked oligosaccharide chains, respectively. These chains are composed of galactose, mannose, fucose, *N*-acetylglucosamine, and sialic acid (50). To examine whether these oligosaccharide chains reflect the E2 protein binding activity of LF, human and bovine LFs were deglycosylated with PNGaseF, which is preferred for the complete removal of the *N*-linked oligosaccharide chain. As shown in Fig. 2*A*, the molecular masses of human and bovine LFs decreased from 80 to 72 kDa, respectively, after treatment with PNGaseF, thereby indicating that the *N*-linked oligosaccharide chains of LF were removed. Using the deglycosylated and natural forms of human and bovine LFs, we compared the binding abilities of LFs to E2 protein. Far-Western blot analysis revealed that deglycosylation of human and bovine LFs resulted in intensified E2 protein binding activity (Fig. 2*B*). This result suggests that the interaction between LF and the E2 protein is

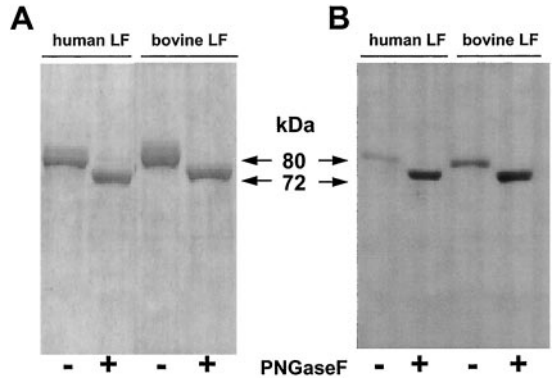

FIG. 2. **Deglycosylation of LF resulted in intensified binding to E2 protein.** *A*, human and bovine LFs deglycosylated with PNGaseF (each 0.5 μg) were resolved by 12% SDS-PAGE and were detected by Coomassie Brilliant Blue staining. *B*, effect of deglycosylation of LFs on the E2 protein binding ability. Far-Western blot analysis using E2 protein as a probe was carried out as indicated in Fig. 1.

not mediated through the *N*-linked oligosaccharides of LF and that at least one oligosaccharide interferes with the direct interaction between LF and the E2 protein. These results led us to undertake the preparation of various recombinant LF fragments using an *E. coli* expression system in which glycosylation does not occur.

Expression in E. coli and Purification of Human LF Fragments—We chose human LF for the preparation of the recombinant LF to investigate which region(s) of LF is (are) important for direct binding to the E2 protein, because the natural host of HCV is human, and a human source would be advantageous in the context of future medical applications. We obtained by RT-PCR a full-length LF cDNA using the total RNA (43) from human breast cancer tissue. Sequence analysis of the obtained LF cDNA clone confirmed that the deduced amino acid sequence was identical to the previously reported human LF (X52941) sequence (51).

To identify the region(s) of LF that bind to the E2 protein (Fig. 3*A*), we divided LF into eight fragments, namely, the N-lobe (N-terminal half without lactoferricin; aa 48–340), N-s1 (aa 48–123), N-s2 (aa 146–255), N-s3 (aa 256–340), the C-lobe (C-terminal half; aa 341–692), C-s1 (aa 341–486), C-s2 (aa 487–599), and C-s3 (aa 600–692), according to the well characterized three-dimensional domain of human LF (27). These LF fragments were successfully expressed as TRX-fused proteins in AD494 cells using the pET32a expression vector; it is already known that the solubility of an expressed protein increases in cells (52). Four LF fragments (N-s2, N-s3, C-s2, and C-s3) were successfully expressed in the soluble form in the cells, although the solubility of the remaining four LF fragments (N-lobe, N-s1, C-lobe, and C-s1) was still at a low level. All of the LF fragments expressed either as soluble or insoluble fractions were purified by affinity column chromatography using His-Bind resin, as indicated under "Experimental Procedures." As a result, eight TRX-fused LF fragments were purified as a single staining band with the expected molecular size after separation with SDS-PAGE (Fig. 3*B*).

A C-terminal Fragment of Human LF Predominantly Interacts with E2 Protein—Using the eight human LF fragments, we performed Far-Western blot analysis to examine which region(s) of LF possess the capacity to bind to E2 protein. The results revealed that two LF fragments, *i.e.* N-lobe (aa 48–340) and C-s3 (aa 600–692), specifically bound to the E2 protein, suggesting that at least two regions of human LF are involved in the direct interaction with the E2 protein (Fig. 3*C*). We noticed that the binding activity of C-s3 was similar to that of human LF, in contrast to the weak binding activity of the N-lobe. To avoid bias in the reactivity of the MO-12 rat monoclonal antibody, used for the detection of the E2 protein in the Far-Western blot analysis, the other rat monoclonal antibody, MO-2, which recognizes a different epitope from that recognized by MO-12, was also used. The obtained result was the same as that observed with MO-12 (data not shown). Because the C-s3 containing aa 600–692 of human LF was considered to be a major binding region, we focused on C-s3 to further characterize the E2 protein binding activity.

C-s3-relevant Fragments of Bovine and Horse LFs also Bind to E2 Protein—It is already known that the aa sequences of mammalian LFs show ~80% sequence homology with each other (53). We demonstrated that human and bovine LFs bind equally well to E2 protein (Fig. 1). Regarding C-s3, 74, 71, 58, and 53% aa sequence homology was demonstrated with the C-s3-relevant fragment of bovine and horse LFs and human and bovine TFs, respectively. To evaluate the E2 protein binding specificity of the C-s3, we examined the E2 protein binding activities of C-s3-relevant fragments of bovine and horse LF and human TF and compared their activities with that of C-s3. We first obtained bovine and horse LF cDNAs from bovine breast tissue and horse peripheral blood mononuclear cells, respectively, by RT-PCR. Human TF cDNA was also obtained by RT-PCR from the cultured PH5CH8 human hepatocytes. Using the obtained LF and TF cDNAs, three C-s3-relevant fragments of bovine and horse LFs and human TF were expressed as TRX-fused proteins in *E. coli* and were purified along with TRX-fused C-s3. Far-Western blot analysis showed that the C-s3-relevant fragments of bovine and horse LF also bound to the E2 protein, as well as C-s3, but the binding activity of the C-s3-relevant fragment of human TF was very weak (Fig. 4). This result suggests that the conserved amino acids among the three LF fragments were involved in the binding to the E2 protein. Comparison with the amino acid sequences of LFs and TFs of human, bovine, and horse revealed that LF-specific amino acids are mainly located in the first half of C-s3 (data not shown). This observation led us to carry out a homology search regarding the first half of the C-s3 fragments using the amino acid sequence databases. We were unable to obtain any interesting proteins showing high homology to this region, although a number of LF- and TF-related proteins were obtained in this survey. However, during the course of the survey, we did notice that the latter half (aa 146–201) of the LEL of the putative HCV receptor CD81 (19) showed partial homology with the first half (aa 600–647) of C-s3, although several gaps were necessary to make an alignment (Fig. 5*A*). This observation prompted us to compare the E2 protein binding activities of C-s3 and CD81 LEL.

E2 Protein Binding Activity of C-s3 Is Comparable with That of CD81 LEL—To compare the binding activities of C-s3 (93 aa) and CD81 LEL (89 aa), we obtained a cDNA encoding a CD81 LEL from PH5CH8 cells by RT-PCR. We confirmed that the nucleotide sequences of the obtained CD81 LEL cDNA were identical to that of previously reported human CD81 LEL (54). CD81 LEL was also expressed as a TRX-fused protein in *E. coli* using a pET-32a expression vector and was purified with the same efficiency as C-s3 (Fig. 5*B*). To confirm that the purified C-s3 and CD81 LEL are actually parts of human LF and CD81, respectively, we examined their immunological specificities by Western blot analysis. As shown in Fig. 5, *C* and *D*, anti-human LF and anti-human CD81 polyclonal antibodies specifically recognized TRX-fused C-s3 and CD81 LEL, indicating the successful preparation of recombinant C-s3 and CD81 LEL. Far-Western blot analysis revealed that both C-s3 and CD81 LEL bound to E2 protein, as shown in Fig. 5*E*. Interestingly, the binding activity of C-s3 was equal to or stronger than that of

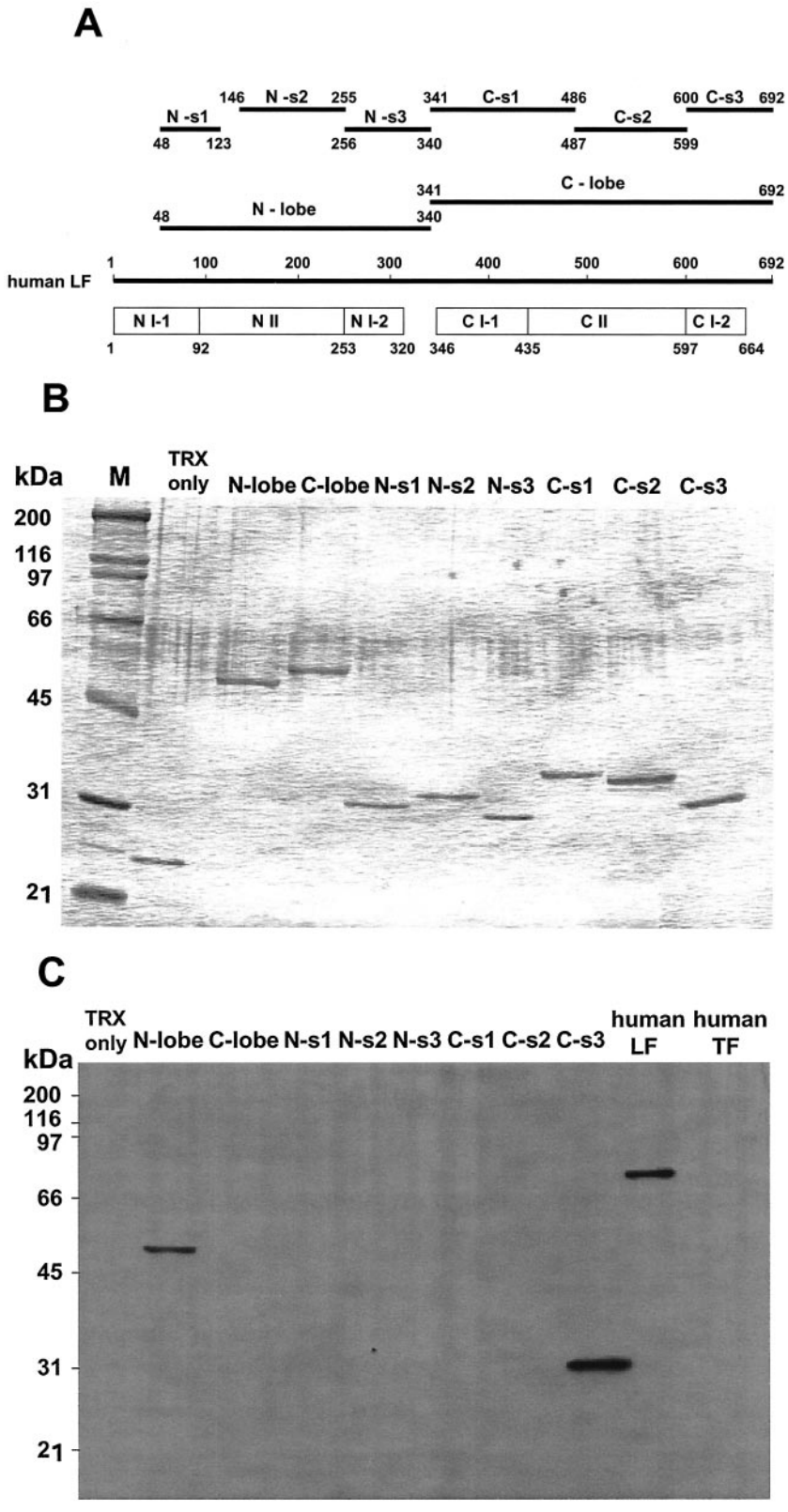

FIG. 3. **Expression of human LF fragments in *E. coli* and their E2 protein binding activities.** *A*, localization map of human LF fragments expressed in *E. coli*. *Numbers* indicate the aa positions of eliminated N-terminal signal sequences (19 amino acids) in LF. *NI-1*, *NII*, *NI-2*, *CI-1*, *CII*, and *CI-2* indicate the three-dimensional domain of human LF (27). *B*, purification of TRX-fused human LF fragments. Each 0.5 μg of LF fragments was resolved by 12% SDS-PAGE and was detected by Coomassie Brilliant Blue staining. *Lane M*, molecular marker of proteins. *C*, E2 protein binding activities of human LF fragments. The purified TRX-fused human LF fragments, human LF, and human TF were resolved by 12% SDS-PAGE and then Far-Western blot analysis using E2 protein as a probe was performed as indicated in Fig. 1. MO-12, a rat monoclonal antibody (42) against E2 protein, was used for the detection of E2 protein bound to LF or to LF fragments.

CD81 LEL. Because it appears that the E2 protein binding affinity of C-s3 is comparable with that of CD81 LEL, we proceeded to narrow down the potential binding domain to an area within the C-s3.

Identification of a Minimum E2 Protein Binding Domain within C-s3—To identify the E2 protein binding domain within C-s3, we first generated several truncated forms of C-s3, as shown in Fig. 6*A*. Each truncated form of C-s3 was expressed as a TRX-fused protein in *E. coli* and was purified as described under "Experimental Procedures." Far-Western blot analysis using these truncated C-s3 forms revealed that aa 600–652 retained the E2 protein binding activity (Fig. 6*B*). In addition, the binding activity of the C-s3 fragment (aa 610–692) was rather weak; the C-s3 fragment (aa 624–692) failed entirely at binding to the E2 protein (Fig. 6*B*, *lanes 4* and *5*). These results indicated that the N-terminal half of C-s3 suffices for binding

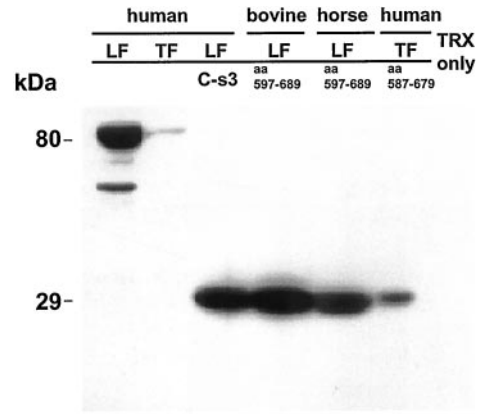

FIG. 4. **Binding to E2 protein of C-s3 and C-s3-relevant fragments of bovine and horse LFs and human TF.** Human LF and TF and purified TRX-fused C-s3 and C-s3-relevant fragments of bovine LF, horse LF, and human TF (0.5 μg each) were resolved by 12% SDS-PAGE. Far-Western blot analysis was carried out as indicated in Fig. 1.

to the E2 protein. Moreover, it was suggested that aa 600–624 of human LF, overlapping with the CD81 homology region (aa 615–644), is necessary for binding to the E2 protein. To further narrow down the binding domain from the C-s3 fragment (aa 600–652), we systematically created a series of truncated forms based on the C-s3 fragment (aa 600–652), as shown in Fig. 7*A*. These truncated forms were then expressed as TRX-fused proteins and were purified along with the other TRX-fused proteins. In this series of experiments, we created an additional TRX-fused LF fragment consisting of aa 590–652 to clarify whether aa 600 is an N-terminal limit for E2 protein binding activity. As shown in Fig. 7*B*, the results showed that the E2 protein binding activity of human LF (aa 590–652) was almost equal to that of the C-s3 fragment (aa 600–652), suggesting that the region of aa 590–599 did not contribute to binding to the E2 protein. Although the C-s3 fragment (aa 600–652) possessed such binding activity, the C-s3 fragment (aa 605–652) had almost no ability to bind to the E2 protein. Regarding the carboxyl-truncated forms of the C-s3 fragment (aa 600–652), it appeared that the C-s3 fragment (aa 600–627) did not bind to the E2 protein and that the C-s3 fragment (aa 600–632) bound equally well to the E2 protein as the C-s3 fragment (aa 600–652) (Fig. 7*B*). Therefore, aa 600–632 of human LF appeared to be the minimum region required for E2 protein binding activity. These results suggest that both aa 600–604 and aa 628–632 contain critical amino acid residues required for binding to the E2 protein.

MBP-fused C-s3 Fragment (aa 600–632) also Binds to the E2 Protein—To exclude the possibility that the E2 protein binding activity of the C-s3 fragment (aa 600–632) was an experimental artifact in the presence of TRX, we prepared the MBP-fused C-s3 fragment (aa 600–632) using the *E. coli* expression system and examined its ability to bind to the E2 protein. We confirmed the purity of the MBP-fused C-s3 fragment (aa 600–632) with Coomassie Brilliant Blue staining (Fig. 8*A*), and we demonstrated its quality with Western blot analysis using anti-human LF antibody (Fig. 8*B*) and anti-MBP antibody (Fig. 8*C*). As shown in Fig. 8*D*, it became apparent that the MBP-fused C-s3 fragment (aa 600–632) and the TRX-fused C-s3 fragment (aa 600–632) bound equally well to the E2 protein, whereas MBP2 (control protein without LF) or TRX (control protein without LF) alone did not bind to the E2 protein. To verify the E2 protein binding activity of the C-s3 fragment (aa 600–632), we performed an ELISA-based binding assay using the MBP-fused C-s3 fragment (aa 600–632) and the E2 protein. As shown in Fig. 9*A*, the MBP-fused C-s3 fragment (aa 600–632) bound to the E2 protein in a dose-dependent manner, whereas the binding activity of MBP2 (control protein without LF) alone was at a significantly low level. Furthermore, an ELISA-based binding assay was used to confirm the E2 protein binding specificity of the MBP-fused C-s3 fragment (aa 600–632). As shown in Fig. 9*B*, the MBP-fused C-s3 fragment (aa 600–632) strongly bound to the E2 protein, whereas the MBP-fused C-s3 fragment (aa 600–632) showed low binding affinity against human TF and BSA. The binding levels of MBP2 to human TF and BSA, as well as that to the E2 protein, were also fairly low. In addition, using this ELISA assay, we confirmed that human LF also bound to the E2 protein in a dose-dependent manner (data not shown). Taken together, these results indicate that the C-s3 fragment (aa 600–632) is directly involved in binding to the E2 protein. We observed that bovine LF (aa 597–629) corresponding to the C-s3 fragment (aa 600–632) and the C-s3 fragment (aa 600–632) were able to bind equally well to the E2 protein (data not shown). It is also noteworthy that the region of the C-s3 fragment (aa 600–632) was located on a surface portion of the C-lobe domain in the three-dimensional structure of human LF (27); the region of aa 606–622 has an α-helix structure, and both terminal ends (five aa each) of the C-s3 fragment (aa 600–632), thought to be important for the E2 protein binding activity, are highly conserved between human and bovine LFs (Fig. 10).

The MBP-fused C-s3 Fragment (aa 600–632) prevents HCV infection in the cells—To evaluate whether the C-s3 fragment (aa 600–632), identified as the minimum binding site to the E2 protein, can prevent HCV infection, we initially examined the anti-HCV activity of the TRX-fused C-s3 fragment (aa 600–632) in our HCV infection system using PH5CH8 cells. Although we carried out a quantitative analysis of HCV RNA using LightCycler PCR, we did not successfully demonstrate the anti-HCV activity of the TRX-fused C-s3 fragment (aa 600–632), because TRX itself showed some cell toxicity in our viral infection system. As regards this cell toxicity, we considered that it might have been because of detergent remaining in the sample after the preparation of the TRX-fused proteins. However, no detergents were used during the process of the purification of MBP-fused protein; we next examined the anti-HCV activity of the MBP-fused C-s3 fragment (aa 600–632) using the same assay system described above. As shown in Fig. 11, the obtained results revealed that the MBP-fused C-s3 fragment (aa 600–632) was able to inhibit HCV infection in PH5CH8 cells in a dose-dependent manner, whereas MBP2 was not able to inhibit HCV infection of the cells. In addition, the IC_{50} dose of the MBP-fused C-s3 fragment (aa 600–632) was estimated to be ~20 μM (1.0 mg/ml), although the IC_{50} dose of human LF was 5 μM (0.4 mg/ml). These results suggest that E2 protein binding activity of the MBP-fused C-s3 fragment (aa 600–632) contributes to the inhibition of HCV infection of these cells. Furthermore, the findings suggest that aa 600–632 of human LF possess anti-HCV activity, although this type of anti-HCV activity was somewhat weaker than that of human LF.

Cys on aa 628 Is Critical for the Binding to E2 Proteins—Because both aa 600–604 and aa 628–632 of human LF were estimated to be important for binding to the E2 protein, and five (aa 602, 604, 629, 630, and 631) of these 10 positions show aa differences between human LF and TF, we considered that the specificity of amino acids also provides E2 protein binding activity. To gain further insight into the roles of these aa sequences, a series of 14 point-substitution mutations (aa 600–605 and 625–632) of the C-s3 fragment (aa 600–632) were constructed by site-directed mutagenesis to Ala. Far-Western blot analysis using these Ala mutants revealed that the Cys of

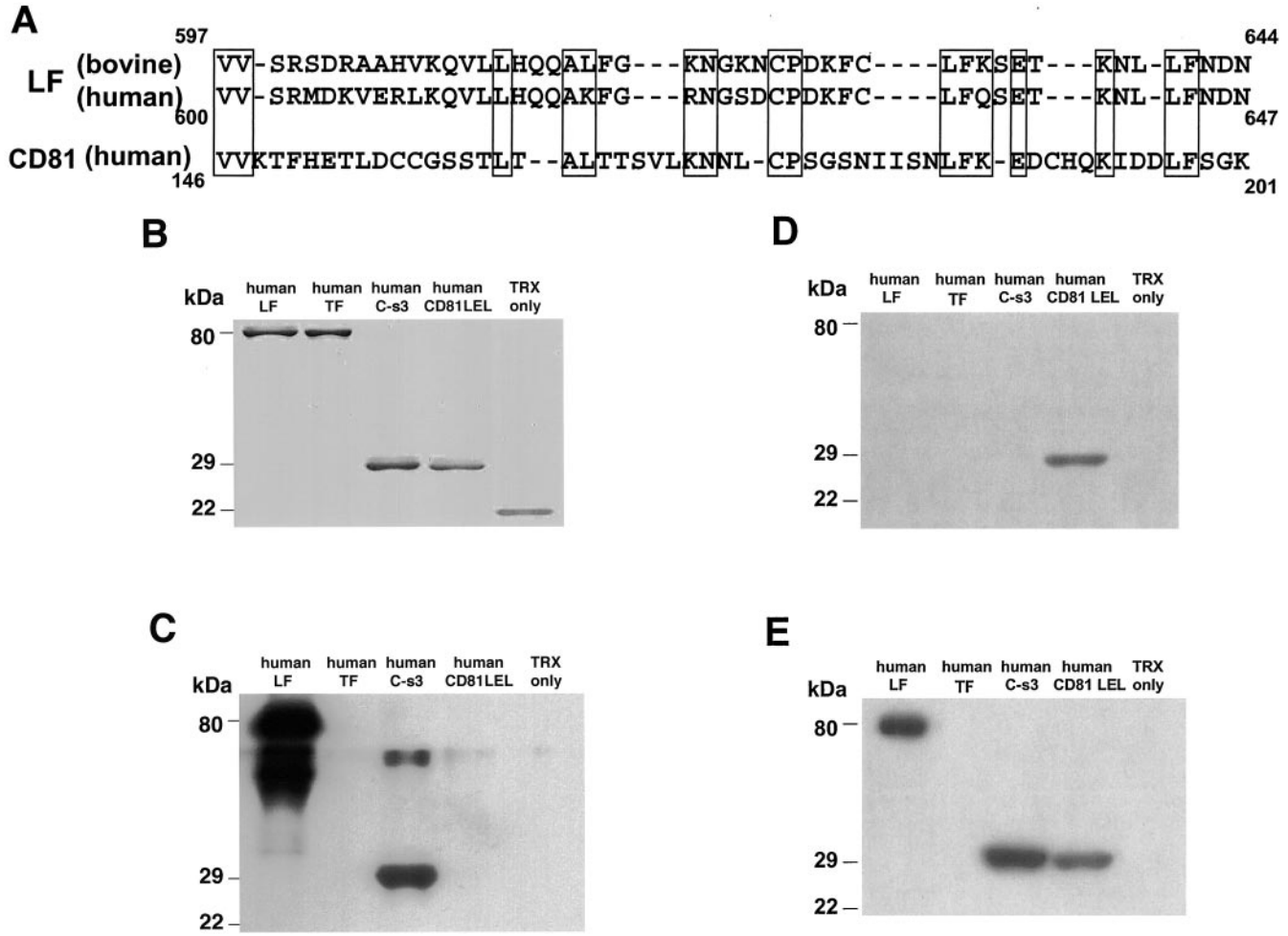

FIG. 5. **Comparison of E2 protein binding abilities of C-s3 and CD81 LEL.** *A*, amino acid sequence alignment of the first half of C-s3 and C-s3-relevant regions of bovine LF and the latter half of CD81 LEL. The same amino acids are *boxed* in each case. *Hyphens* indicate gaps. *B*, purification of TRX-fused human CD81 LEL. Human LF, human TF, TRX-fused C-s3, and TRX-fused CD81 LEL (0.5 μg each) were resolved by 12% SDS-PAGE and were detected by Coomassie Brilliant Blue staining. *C*, immunological specificity of TRX-fused C-s3. Western blot analysis using rabbit anti-human LF polyclonal antibody was carried out using the same proteins as those shown in *B*. *D*, immunological specificity of TRX-fused CD81 LEL to E2 protein. Western blot analysis using anti-human CD81 polyclonal antibody was carried out using the same proteins as those shown in *B*. *E*, E2 protein binding activities of C-s3 and CD81 LEL. Far-Western blot analysis using the E2 protein as a probe was carried out using the same proteins as those shown in *B*.

aa 628 is quite important for binding to the E2 protein, because a Cys to Ala substitution on aa 628 completely abolished E2 protein binding activity (Fig. 12). In addition, four Ala mutants (aa 626, 627, 629, and 630) increased binding activity, suggesting that the binding affinity of the C-s3 fragment (aa 600–632) to the E2 protein was not optimal.

DISCUSSION

Based on the our studies (24, 36) and those of other groups (37), we undertook observations regarding the direct interactions between LF and HCV envelope proteins. In this study, we demonstrated the binding specificity between LF and the E2 protein and identified 33 aa residues from human LF that are primarily responsible for E2 protein binding activity and inhibiting HCV infection of target cells.

We observed that the deglycosylation of human and bovine LFs enhanced E2 protein binding activity. This observation suggests that a certain *N*-linked oligosaccharide chain interferes with the interaction between LF and the E2 protein. Comparison with the putative *N*-linked glycosylation sites between human LF (3 sites; aa 138, 479, and 624) and bovine LF (5 sites; aa 233, 281, 368, 476, and 545) revealed that only one glycosylation site (aa 479 for human LF, aa 476 for bovine LF) is conserved in both LFs. Therefore, it is likely that this conserved glycosylation site, which is located in C-s1, weakens the E2 protein binding activity of human or bovine LF. This may also explain why we were able to detect the strong binding affinity of C-s3 for the E2 protein. However, the reason why the C-lobe did not bind to the E2 protein remains unclear. One possibility would be that the refolding of the TRX-fused C-lobe was not successful. Our results indicate that at least two regions of human LF (the N-lobe and C-s3) are involved in the interaction with the E2 protein, although we could not identify the binding region in the N-lobe. Although N-s3 shows ~35% aa homology to C-s3, N-s3 is unable to bind to the E2 protein. In addition, 12 aa are shared in common between the N-s3 fragment (aa 256–287) and the C-s3 fragment (aa 600–632) identified as a critical domain for binding to E2 protein. However, a Cys residue at aa 628 appeared to be essential for binding to the E2 protein; it is of note that this Cys is not present in the corresponding position of N-s3. Therefore, some boundary region between N-s1 and N-s2, or between N-s2 and N-s3, may possess the binding ability to the E2 protein. Interestingly, the C-s3 fragment (aa 600–632) was located close to the boundary region between N-s2 and N-s3 in the three-dimensional structure of human LF (27), suggesting that the E2 protein is able to bind to both

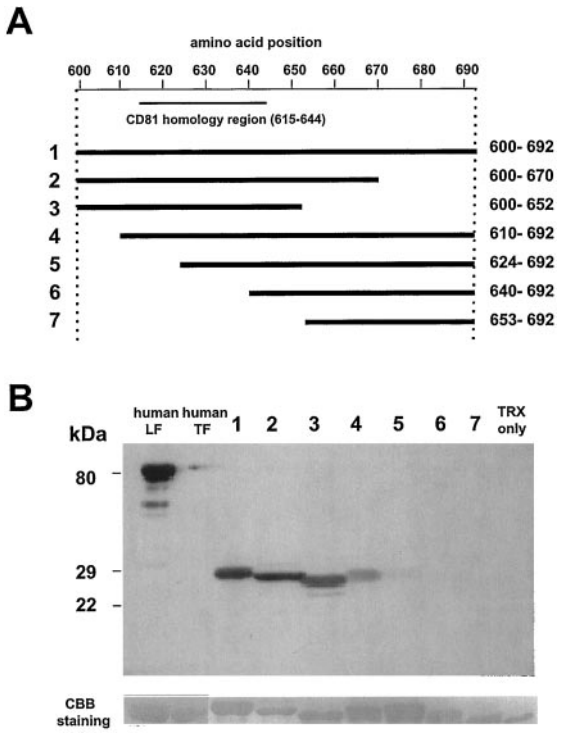

FIG. 6. **Deletion analysis of C-s3 possessing E2 protein binding activity.** *A*, localization map of the C-s3 variants truncated from N-terminal or C-terminal portions of C-s3. These LF fragments were expressed as TRX-fused proteins in *E. coli* and were purified for the Far-Western blot analysis. *Numbers* indicate the aa positions of human LF. *B*, E2 protein binding activities of C-s3 truncated variants. Far-Western blot analysis using the E2 protein as a probe was carried out as indicated in Fig. 1. To indicate that equal amounts of TRX-fused C-s3 variants were resolved by 12% SDS-PAGE, these LF fragments were stained with Coomassie Brilliant Blue (*CBB*; *lower panel*).

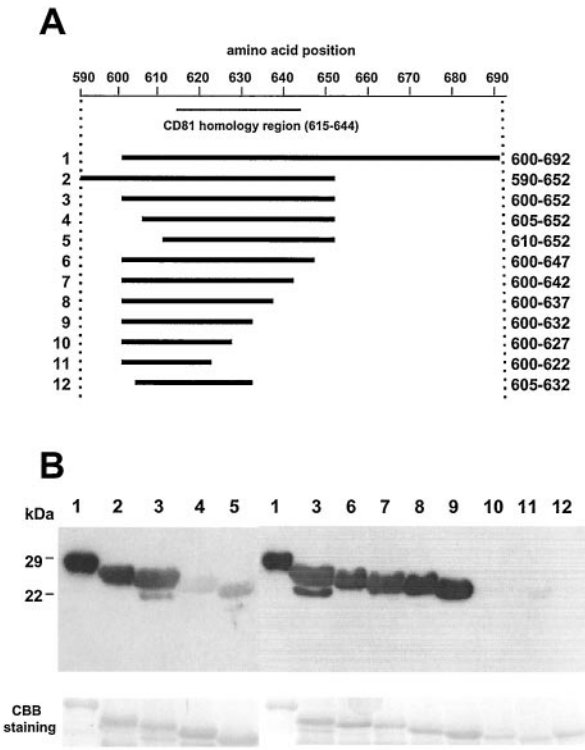

FIG. 7. **Identification of a minimum E2 protein binding domain in C-s3.** *A*, localization maps of the C-s3-truncated variants and a human LF (aa 590–652) were examined regarding E2 protein binding activity. These LF fragments were expressed as TRX-fused proteins in *E. coli* and purified for Far-Western blot analysis. *Numbers* indicate the aa positions of human LF. *B*, E2 protein binding activities of C-s3-truncated variants and a human LF (aa 590–652). Far-Western blot analysis using the E2 protein as a probe was carried out as indicated in Fig. 1. To indicate that equal amounts of TRX-fused C-s3 variants were resolved by 12% SDS-PAGE, these LF fragments were stained by Coomassie Brilliant Blue (*CBB*; *lower panel*).

sites of human LF. To clarify this assumption, further analysis will be needed.

We demonstrated that C-s3-relevant fragments (93 aa) of bovine and horse LFs bound as well to the E2 protein as did C-s3. The C-s3-relevant fragments of bovine and horse LFs show 74 and 71% aa sequence homology to C-s3 of human LF, respectively. These values are significantly higher than those (58 and 53%, respectively) of C-s3-relevant fragments of human and bovine TFs, which possess little binding activity to the E2 protein. The identified critical domain (aa 600–632) of human LF also shows 70% aa sequence homology to bovine LF (aa 597–629), whereas it shows only 42% aa sequence homology to the relevant regions of human or bovine TF (Fig. 10). These data suggest that the binding activity to E2 protein is restricted to the LF family.

During the process of characterization of a C-s3 LF fragment possessing E2 protein binding activity, we noticed that C-s3 showed partial aa homology with LEL of CD81, which can also bind to E2 protein and is considered as a candidate HCV receptor (19). The E2 protein binding activity of CD81 LEL has been well characterized *in vitro* (55) and *in vivo* (56), and the binding specificity of CD81 to E2 protein has been clarified (57). However, it has been reported that CD81 is not directly involved in the cell fusion caused by HCV (58). Because it has been shown that TRX-fused C-s3 (93 aa) has a comparable E2 protein binding ability with that of TRX-fused CD81 LEL (89 aa), C-s3 may interfere with the binding of the E2 protein to CD81. This may be one of the reasons why LF prevents HCV infection in target cells. However, one major contradiction remains. Regarding the interaction between human CD81 LEL and the E2 protein, it has been shown that aa 186 (Phe) of the CD81 is the critical residue for binding to the E2 protein (57). This Phe residue is conserved between human CD81 and LF (aa 635) (Fig. 5*A*). However, in this study, it appeared that the Phe at aa 635 of human and bovine LFs was unimportant for binding to the E2 protein, because the C-s3 fragment (aa 600–632) possessing the E2 protein binding activity does not contain this Phe residue. In addition, it has been reported that the four Cys of CD81 LEL form two disulfide bridges, the integrity of which would be necessary for CD81-E2 interaction (54). However, such a phenomenon was not observed in C-s3 containing five Cys residues, because the C-s3 fragment (aa 600–632) identified as the E2 protein binding domain contains only one Cys. Therefore, these data suggest that the E2 protein region targeted by human LF and CD81 may differ. Preliminarily, our experiment with Far-Western blot analysis showed that the C-s3 fragment (aa 600–632) preferentially bound to aa 411–500 of the E2 protein, which is one of two regions (aa 384–500 and 600–661) identified previously (37) as regions binding to human LF; however, the C-s3 fragment (aa 600–632) did not bind to aa 501–599 of E2 protein (data not shown). Because it has been indicated that both aa 480–493 and aa 544–551 of the E2 protein are involved in the binding to CD81 (56), human LF and CD81 may recognize rather different sites on the E2 protein. Further analysis will be necessary to clarify this point.

Because aa 600–632 of human LF possesses only one Cys at aa 628, it is unlikely that a disulfide bond is required for the E2 protein binding activity of the C-s3 fragment (aa

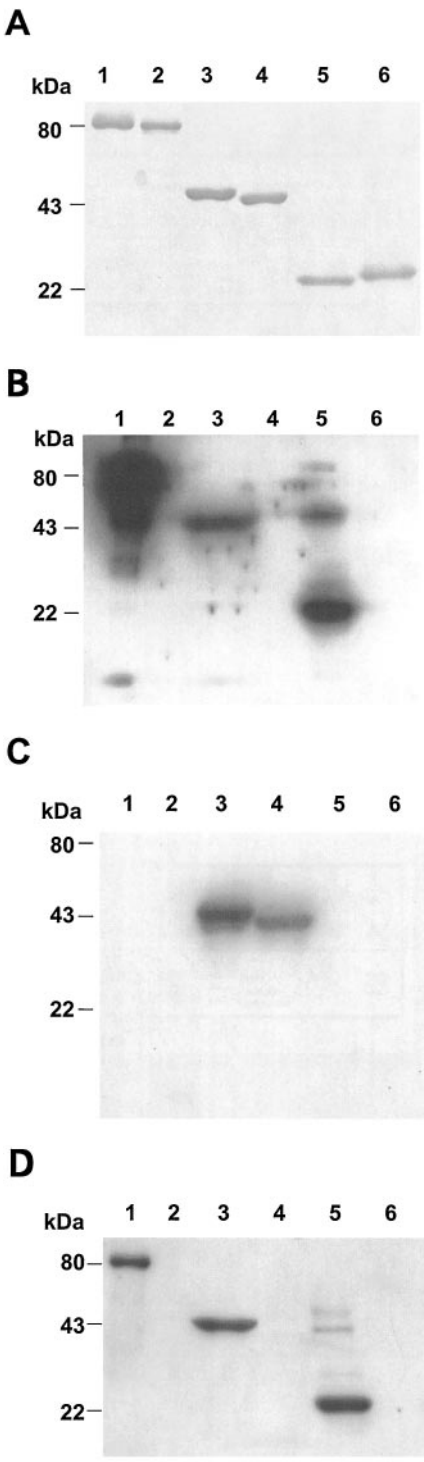

FIG. 8. **E2 protein binding specificity of the C-s3 fragment (aa 600–632).** The E2 protein binding activity of the MBP-fused C-s3 fragment (aa 600–632) was examined by Far-Western blot analysis as indicated in Fig. 1. *Lane 1*, human LF; *lane 2*, human TF; *lane 3*, MBP-fused C-s3 fragment (aa 600–632); *lane 4*, MBP2 without LF; *lane 5*, TRX-fused C-s3 fragment (aa 600–632); *lane 6*, TRX without LF. *A*, Coomassie Brilliant Blue staining after 12% SDS-PAGE. *B*, Western blot analysis using rabbit anti-human LF polyclonal antibody. *C*, Western blot analysis using rabbit anti-MBP polyclonal antibody. *D*, Far-Western blot analysis using the E2 protein as a probe.

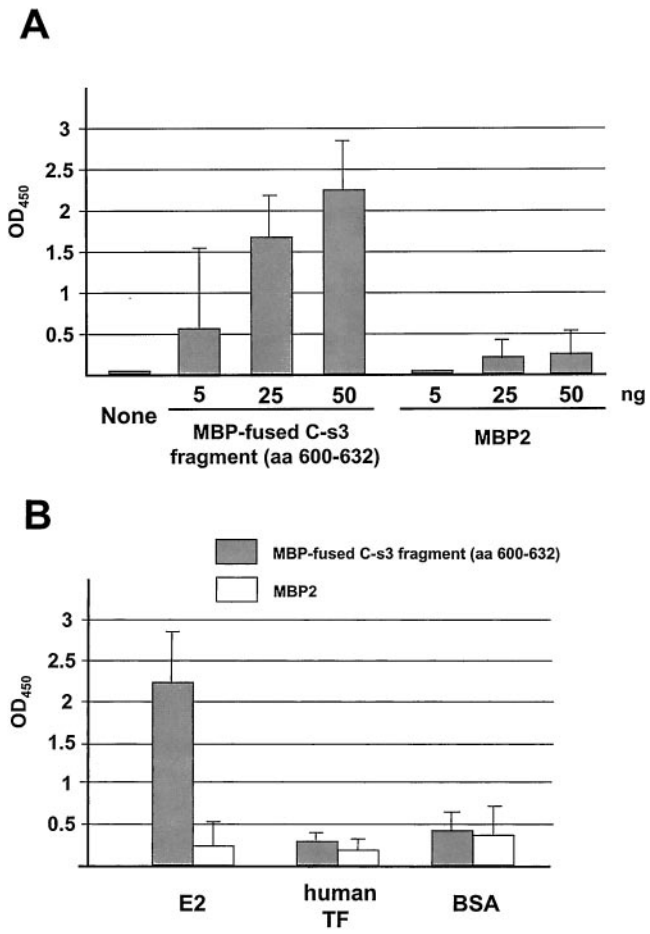

FIG. 9. **ELISA-based E2 protein binding activity of the C-s3 fragment (aa 600–632).** *A*, the MBP-fused C-s3 fragment (aa 600–632) bound to the E2 protein in a dose-dependent manner. An ELISA-based binding assay was performed to examine the E2 protein binding activity of the MBP-fused C-s3 fragment (aa 600–632), as described under "Experimental Procedures." MBP2 was used as a control protein without LF. 5, 25, and 50 ng of the MBP-fused C-s3 fragment (aa 600–632) or MBP2 were used for binding to the E2 protein. *B*, binding specificity of the MBP-fused C-s3 fragment (aa 600–632). To examine the E2 protein binding specificity of the MBP-fused C-s3 fragment (aa 600–632), human TF and BSA were also coated to immunoplates and were used as negative controls in the ELISA-based binding assay. MBP2 was used as a control protein without LF. 50 ng of the MBP-fused C-s3 fragment (aa 600–632) or MBP2 were used for binding to the E2 protein, human TF, or BSA.

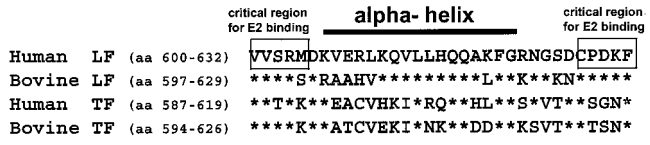

FIG. 10. **Amino acid sequence alignment of C-s3 and C-s3-relevant fragments of bovine LF, human TF, and bovine TF.** *Capital letters* indicate amino acids that differed from those in the sequences of aa 600–632 of human LF. The identical amino acids are indicated with *asterisks*. The α-helix structure (aa 606–622) identified in human LF (22) is depicted by a *thick bar*. Both the N-terminal five aa and C-terminal five aa, which are considered to be critical for E2 protein binding, are shown in *boxes*.

600–632). However, we cannot exclude the possibility that Cys at aa 628 paired with other Cys residues in TRX during the refolding process of the Far-Western blot analysis; this could have provided E2 protein binding activity. To exclude this possibility, we constructed the MBP-fused C-s3 fragment (aa 600–632), because Cys was not present in the MBP portion including the linker region. Although the MBP-fused C-s3 fragment (aa 600–632) possesses only one Cys, this fusion protein showed similar E2 protein binding activity with that of the TRX-fused C-s3 fragment (aa 600–632). Therefore, the present results suggest that a disulfide bond is not required for binding to the E2 protein, in contrast to the

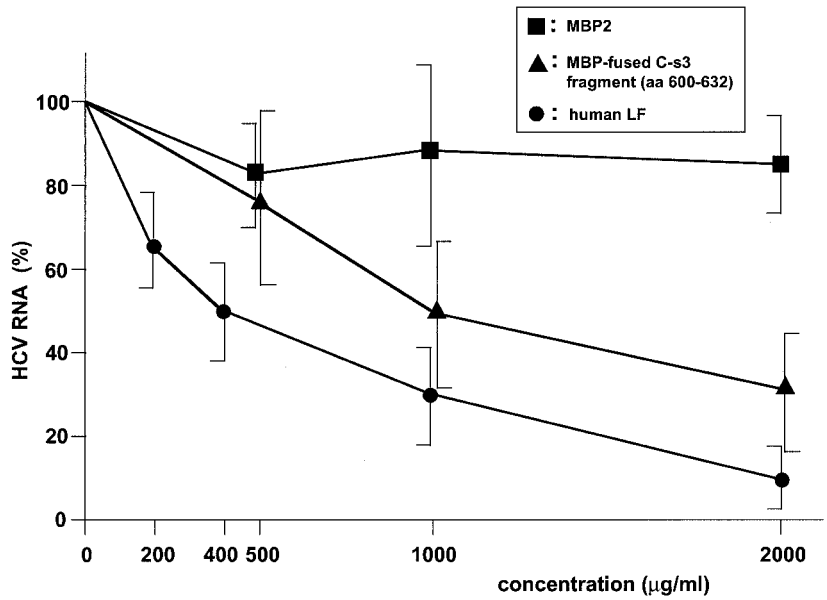

FIG. 11. **Anti-HCV activity of the MBP-fused C-s3 fragment (aa 600–632) in PH5CH8 cells.** PH5CH8 cells and inoculum 1B-2 were used for the HCV-inhibiting assay, as described under "Experimental Procedures." The *number* in the *axis* of the *ordinate* indicates the percent of the amount of HCV RNA determined by quantitative RT-PCR using LightCycler PCR (49). Approximately 2,000 copies of HCV RNA per μg of cellular RNA was reproducibly obtained using this HCV infection system (36, 49). In addition to the MBP-fused C-s3 fragment (aa 600–632), human LF and MBP2 were also used for the assay as control materials.

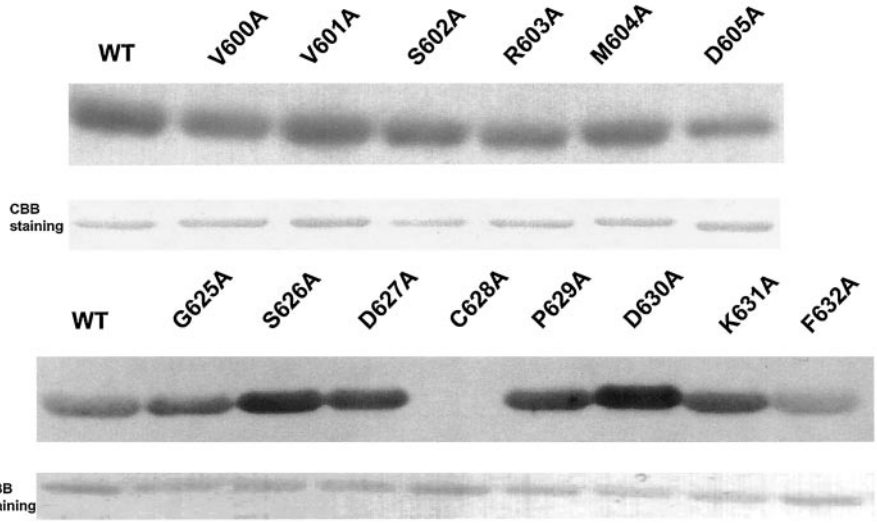

FIG. 12. **Site-directed mutagenesis of the TRX-fused C-s3 fragment (aa 600–632).** Site-directed mutagenesis to Ala was carried out in aa 600–605 and 625–632, respectively. *Lane WT*, TRX-fused C-s3 fragment (aa 600–632). Far-Western blot analysis was performed using the E2 protein as a probe (*upper panel*), as indicated in Fig. 1. The purified TRX-fused proteins were stained with Coomassie Brilliant Blue (*CBB*) after 12% SDS-PAGE to confirm their purities (*lower panel*).

case involving CD81 LEL (55). However, it is of note that site-directed mutagenesis to Ala in both terminal regions of the C-s3 fragment (aa 600–632) revealed that Cys at aa 628 is the most critical residue for binding to the E2 protein. To clarify whether only the Cys residue at this position is necessary for binding to the E2 protein, further experiments (*e.g.* substitution of amino acids other than Ala) will be needed.

Because it is well known that E1 and E2 proteins form a non-covalently linked heterodimer, which probably represents the surface of infectious virus particles (59), it is important to clarify whether the C-s3 fragment (aa 600–632) identified in this study binds to the heterodimer of E1 and E2 proteins. To date, aa 441–500 of E2 protein has been identified as the E1 protein heterodimeric binding region (60). Although our preliminary results estimated that the C-s3 fragment (aa 600–632) binds to aa 411–500 of the E2 protein, the E2 protein binding activity of the C-s3 fragment (aa 600–632) may not be affected by heteromeric complex formation between E1 and E2 proteins, because LF prevents HCV infection by direct interaction between LF and HCV (36).

We demonstrated the anti-HCV activity of the MBP-fused C-s3 fragment (aa 600–632) in our HCV infection system using PH5CH8 cells (36, 49). Although this result suggests that the E2 protein binding activity contributes to the prevention of HCV infection, our results revealed that the anti-HCV activity of the MBP-fused C-s3 fragment (aa 600–632) was severalfold weaker than that of human LF. However, site-directed mutagenesis to an Ala residue within aa 600–605 and aa 625–632 of human LF revealed that several positions strengthened the E2 protein binding activity. This result suggests that some peptides possess stronger binding activity than that of the C-s3 fragment (aa 600–632) and that these peptides could be obtained by screening peptide libraries, *e.g.* phage libraries. The antiviral activities of such peptides will be evaluated using our cell culture assay system (36, 49). Furthermore, such peptides may be useful for the removal of circulating HCV. In any case, the present study broadens the possibilities for developing anti-HCV peptides in the future.

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