Characterization of the Putative Membrane Fusion Peptides in the Envelope Proteins of Human Hepatitis B Virus

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Envelope proteins of virus contain a segment of hydrophobic amino acids, called as fusion peptide, which triggers membrane fusion by insertion into membrane and perturbation of lipid bilayer structure. Potential fusion peptide sequences have been identified in the middle of L or M proteins or at the N-terminus of S protein in the envelope of human hepatitis B virus (HBV). Two 16-mer peptides representing the N-terminal fusion peptide of the S protein and the internal fusion peptide in L protein were synthesized, and their membrane disrupting activities were characterized. The internal fusion peptide in L protein showed higher activity of liposome leakage and hemolysis of human red blood cells than the N-terminal fusion peptide of S protein. Also, the membrane disrupting activity of the extracellular domain of L protein significantly increased when the internal fusion peptide region was exposed to N-terminus by the treatment of V8 protease. These results indicate that the internal fusion peptide region of L protein could activate membrane fusion when it is exposed by proteolysis.

Key Words : Fusion peptide, HBV, Envelope protein, Infection, Viral fusion

Introduction

The fusion between virus and cellular membranes is an essential step for the infection of enveloped virus to target cells. The envelope proteins of viruses recognize receptors on the target cell membrane and mediate fusion between the viral and cellular membranes. The viral fusion proteins mediate this fusion process in a pH-independent manner at plasma membrane or induce low-pH triggered fusion at endosomal membrane. Most of the viral fusion proteins consist of the receptor recognition subunit and the membrane fusion subunit, which are processed from precursor proteins. The structure and biophysical characteristics of viral fusion proteins have been extensively studied using hemagglutinin subunit 2 (HA2) of influenza virus[1] or gp41 of human immunodeficiency virus (HIV).[2] HA2 activates membrane fusion at acidic pH in endosomes.[3,4] Whereas, gp41 induces membrane fusion at the surface of target cells in a pH-independent manner.[5] The fusion proteins contain a stretch of hydrophobic amino acids, called as "fusion peptide", which normally located at the N-terminus of the fusion proteins.[6,7] During membrane fusion, the fusion peptide region can insert directly into the target cell membrane and induce lipid mixing.[8,9] Synthetic peptides representing the fusion peptide region could insert into a target membrane[10] or induce the hemolysis of erythrocytes.[11]

Human hepatitis B virus (HBV) is a small DNA-containing envelope virus that belongs to *Hepadnaviridae* family. Despite representing a global health problem, the entry process of HBV life cycle is not well understood due to the lack of a convenient *in vitro* assay system of viral infection. Particularly, the fusion process induced by HBV envelope proteins has been poorly understood. HBV possesses three envelope proteins (S, M, and L proteins) that are translated from a single open reading frame at three different translational start codons.[12] L protein possesses an extension of 163 amino acids, termed as PreS region, which consists of PreS1 and PreS2 regions, at the N-terminus of S protein. M protein possesses an extension of 55 amino acids, termed as PreS2 region, at the N-terminus of S protein. The envelope proteins of HBV are assumed to have key roles in the binding and fusion with liver cells.[13] L protein has duel topology in which PreS region locates on the virus surface as well as inside the virus lumen.[14,15] The surface exposed PreS region of L protein involved in the recognition to receptors on target cells.[16,17] A potential fusion peptide sequence that consisted of a stretch of hydrophobic residues has been identified at the N-terminus S protein.[18] Since L and M proteins share the C-terminus region with S protein, the potential fusion peptide sequence of S protein also locates in the middle of L and M proteins. This sequence This sequence contains a core hydrophobic sequence (FLG-LL-AG) that observed in various viral fusion proteins,[19,20] and this sequence is conserved in hepadnavirus.[21,22] Synthetic peptide representing the N-terminus of S protein from HBV or woodchunk hepatitis virus (WHV) could bind to and destabilize lipid bilayers,[23-25] suggesting that this sequence could serve as fusion peptide. However, it is not clear whether the putative fusion peptide located either at the N-terminus of the S protein or internal region of L or M proteins are involved in membrane fusion.

The entry pathway of HBV and the mechanism of membrane fusion mediated by HBV envelope proteins have not been clearly resolved. Study of DHBV uptake in primary

Abbreviations: HBV, hepatitis B virus; LUV, large unilamellar vesicle; POPC, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-{1-glycerol}]; RBC, red blood cell

duck liver hepatocytes revealed that it entered via endocytosis,[26,27] and exposure of DHBV particle to low pH induced a conformational change in the large surface protein.[28] In addition, the fusion peptide at the N-terminus of S protein showed increased membrane-disrupting activity at acidic endosomal pH,[23] supporting that the fusion process takes place at endosome. In contrast, pretreatment of the HBV particle at low pH or inclusion of lysosomotropic agents, which increased the pH of the lysosomes, do not affect the infectivity of HBV toward primary human liver cells,[29] suggesting that membrane fusion mediated by HBV envelope proteins requires factors other than low pH. It should be noted that the infectivity of the HBV particle is significantly increased after treatment with proteases. Intact HBV hardly infect the HepG2 cell, a cell line derived from human hepatoblastoma. However, V8 protease-treated HBV particles efficiently infected and proliferated in HepG2 cells,[21] suggesting that proteolysis of HBV envelope proteins facilitates membrane fusion. One of the potential cleavage sites of the V8 protease locates at the N-terminus of potential fusion peptide sequence in L or M proteins, suggesting that the internal fusion peptide could be exposed at the N-terminus after proteolysis. The newly exposed fusion peptide sequence differs from the fusion peptide sequence at the N-terminus of S protein by lacking the two amino acids at N-terminus. Although the synthetic peptide representing the N-terminus of S protein showed lipid-mixing activity at low pH, the membrane-disrupting activity of the internal fusion peptide sequence of L protein, which is exposed by V8 proteolysis, has not been characterized.

In this study, we prepared two synthetic peptides representing the fusion peptide at the N-terminus of the S protein and inside of L or M proteins, and showed that the internal fusion peptide sequence had a higher membrane disrupting activity. In addition, we showed that the exposure of the internal fusion peptide sequence of L protein by proteolysis stimulated membrane-disrupting activity of L protein.

Materials and Methods

Reagents. POPC (1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPG (1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-{1-glycerol}]) were purchased by Avanti Polar Lipids (USA). Hypaque meglumine (60%) used for the purification of erythrocyte was obtained from the Winthrop Pharmaceuticals Division of Sterling Drug Inc. (USA). Calcein and Triton X-100 were purchased from Sigma (USA). All other reagents were reagent grade.

Peptide synthesis. The peptide corresponding to the N-terminus 16 residues of the S protein (FP1: H_2N-MENTTSGFLGPLLVLQ-$CONH_2$) and the peptide representing amino acids 166-181 of the L protein (FP2: H_2N-NTTSGFLGPLLVLQAG-$CONH_2$) were synthesized using a Synergy Peptide Synthesizer (Applied Biosystems, USA) by a solid phase synthesis method with Fmoc-amino acids on a Rink resin which produced an amide group at the C-terminus of peptide. The synthesized peptides were cleaved from the resin by the method of King *et al.*[30] and further purified on a Rainin HPLC apparatus (Rainin, USA) with a reverse-phase C_{18} column (Vydac, USA) using a linear gradient of 20% to 100% acetonitrile solution containing 0.1% trifluoroacetic acid. The peptide concentration was measured using fluorescamine.[31]

Preparation of expression vectors for recombinant proteins. A plasmid (pADRa) containing the HBV gene isolated from a male Korean hepatitis patient was used as a template DNA for the amplification of L protein.[32] The DNA fragment encoding the extracellular region of the L protein (L_{EC}; amino acid 1-220 of L protein) was amplified by the polymerase chain reaction (PCR) using the 5' primer (5'-CGGCGAATTCATGGGGACGAATCTTTCTGTTCCC -3') and the 3' primer (5'-GGCGAAGCTTTCAGGTTGG-GGACTGCGAATTTTG-3'). Likewise, a DNA fragment encoding the central region of the L protein (L_{CEN}; amino acid 166-220 of the L protein) was amplified using N-(5'-CGGCGAATTCAACACAACATCAGCTTCCTAGGA-3') and C-(5'-GGCGAAGCTTTCAGGTTGGGGACTGCGA-ATTTTG-3') primers. The amplified DNAs were digested with *Eco*RI and *Hin*dIII restriction enzymes and ligated to *Eco*RI/*Hin*dIII restriction sites of pTrx vector, which is a derivative of pET28a harboring thioredoxin gene.[32] The resulting plasmids, pTrx-L_{CEN} and pTrx-L_{EC}, were used for the expression of which producing chimera proteins of L_{CEN} or L_{EC} regions linked to the C-terminus of thioredoxin, respectively (Fig. 1). The amino acid sequences of recombinant L protein was the same as the previously reported sequence (gi11041688).[33]

Purification of recombinant proteins. The recombinant proteins were expressed in the *E. coli* strain BL21(DE3) harboring pTrx-L_{CEN} or pTrx-L_{EC}. The transformed *E. coli* cells were grown in LB liquid media containing 50 μg/mL kanamycin at 37°C until the OD_{600} reached 0.6-0.7. The expression of the recombinant proteins was induced by adding 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 3 h incubation, cells were collected by centrifugation at 5,000 × g for 10 min. About 10 g of cell paste was suspended in extraction buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, pH 7.5), and the cells were lysed by passing through a French Press Cell (SLM Instruments, USA). The recombinant proteins were expressed as inclusion bodies and recovered in the pellet fraction after centrifugation at 10,000 × g for 20 min. The inclusion body of Trx-L_{CEN} was washed 4 times with buffer A (10 mM Tris-HCl, pH 8.0) and dissolved in 8 M urea in the buffer A. The dissolved protein sample was loaded onto a Ni-NTA column (2 × 5 cm) equilibrated with buffer B (8 M urea in buffer A). After washing the resin with 20 mM imidazole in buffer B, the bound protein was eluted with 250 mM imidazole in the buffer B. The urea was removed from the eluted protein by serial dialysis against 4 M, 2 M and 0 M urea in 20 mM Tris-HCl buffer (pH 8.0). The refolded protein was applied to a Q-Sepharose column (Pharmacia, Sweden) and Trx-L_{CEN} was eluted by a linear gradient of 0.05 M-0.6 M NaCl in 20

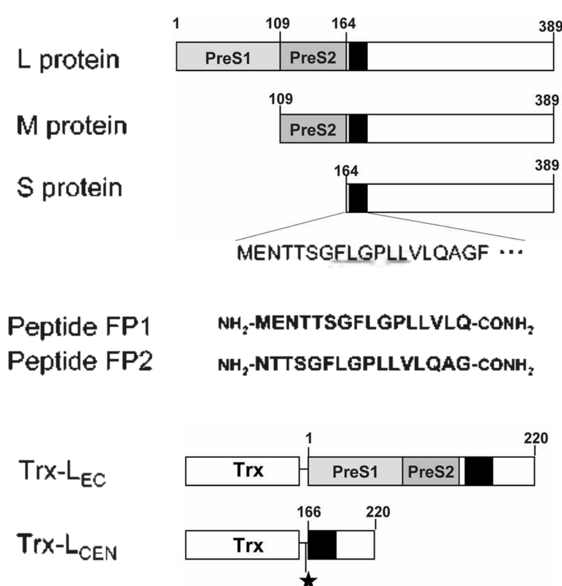

Figure 1. Schematic diagram of HBV envelope proteins, Trx-L_{CEN}, Trx-L_{EC} and the amino acid sequences of FP1 and FP2 peptides. PreS1 and PreS2 region of HBV M and L protein are indicated as grey boxes, and the 19 amino acid sequence from the N-terminus sequence of S protein is indicated. The putative fusion peptide sequence located at the N-terminus of the S protein or at the C-terminus of PreS2 region of M or L proteins is indicated as a black box. FP1 and FP2 peptides represent amino acid 164-179 or 166-181 of L protein, respectively. Trx-L_{EC} contains the extracellular region of the L protein (amino acid 1-220 of L protein) at the C-terminus of thioredoxin. Trx-L_{CEN} contains the fusion peptide region (amino acid 166-220 of L protein) at the C-terminus of thioredoxin. The thrombin recognition site in front of the putative fusion peptide is marked by asterisk.

mM Tris-HCl buffer (pH 8.0). Trx-L_{EC} was purified by the same procedure as for Trx-L_{EC} except that 8 M urea in 50 mM sodium phosphate buffer (pH 8.0) was used to dissolve the inclusion body.

Hemolysis. The effect of peptides on the membrane integrity was examined by measuring their hemolysis activities on human red blood cells (RBC) as previously described.[34] Immediately before use, RBC were obtained from a healthy volunteer by vein puncture. Isolated RBC were washed four times with 20 mM Na-phosphate (pH 7.4) containing 100 mM NaCl, and then diluted with the same buffers. The prepared RBC were mixed with peptides and incubated for 1 h at 37°C with gentle shaking. After centrifugation for 10 min at 10,000 × g, the absorbance of the supernatant was measured at 540 nm. The value for 100% hemolysis was obtained from the supernatant treated with 0.2% Triton X-100. The RBC sample was obtained from a single individual and used within 4 days.

Preparation of lipid vesicles. Large unilamellar vesicles (LUV) were prepared from a solution of POPC and POPG in chloroform at a 7:3 molar ratio (POPC:POPG). The lipid was deposited as a film on the wall of a test tube by solvent evaporation with nitrogen. Final traces of solvent were removed for 2-3 h in a vacuum chamber attached to a liquid nitrogen trap. The lipid films were suspended in buffer C (20 mM MOPS, 100 mM NaCl, pH 7.4) yielding a final lipid concentration of 50 mM. The lipid suspensions were further processed with 5 cycles of freezing and thawing, followed by 10 passes through two stacked 0.1 μm polycarbonate filters (Nuclepore Corp., Pleasanton, CA) using the Mini extruder from Avanti Polar Lipids (USA).

Measurement of liposome leakage. Disruption of lipid vesicles was examined by measuring the increased fluorescence resulting from the leakage of calcein out of the vesicles, as described by Kendall and MacDonald.[35] The liposomes containing calein was prepared by resuspenstion of dry lipid with buffer C containing 20 mM calcein and 40 mM $CoCl_2$. After preparation of LUVs, the unentrapped calcein was removed by dialysis against buffer C. The calcein-covalt containing lipid vesicles (100 μM) were incubated in an appropriate buffer that contains 20 mM EDTA with the tested peptide or protein for 1 h or 16 h at 37°C, respectively. The excess EDTA in the extravesicular media would complex with the leaked cobalt and leave free calcein that generates fluorescence at 530 nm. The fluorescence intensity of calcein was measured using a Multi-frequency Cross Correlation Phase and Modulation Fluorometer (ISS, USA) with excitation and emission wavelength of 490 and 530 nm, respectively. The fluorescence intensity for 100% leakage was obtained after the addition of 0.2% Triton X-100.

Results

Preparation of potential fusion peptides and recombinant proteins containing HBV surface protein. Two 16-mer peptides representing putative fusion peptide from adr-type of HBV were prepared. Peptide FP1 represents amino acids 1-16 of S protein or 165-180 of L protein, and FP2 represent amino acids 3-18 of S protein or 167-182 of L protein. FP2 has 2 amino acid extensions at the N-terminus of FP1 peptide (Fig. 1). The synthesized peptides were purified by HPLC, and their molecular weights were confirmed by mass spectrometry (data not shown). In addition, the expression vectors for two chimera proteins of thioredoxin containing entire extracellular region of the L protein (Trx-L_{EC}) and C-terminus region with potential fusion peptide region (Trx-L_{CEN}) were prepared (Fig. 1). The recombinant proteins were expressed in *E. coli* as inclusion bodies and purified with >90% purity (data not shown).

Enhancement of membrane-disrupting activity of FP2 that lacks two amino acids at the N-terminus of FP1. The membrane disrupting activity of FP1 and FP2 was examined by measuring their hemolytic activity on RBC and the leakage activity on liposomes. FP1 at a concentration of 100 μM did not significantly induce hemolysis of RBC, and less than 10% of RBC were disrupted. In contrast, the same concentration of FP2 effectively stimulated hemolysis of RBC. More than 80% of RBC were disrupted in the presence of 100 μM of FP2 (Fig. 2). The membrane disruption by these peptides was further examined using vesicles of synthetic lipids. Leakage of vesicles induced by FP1 or FP2

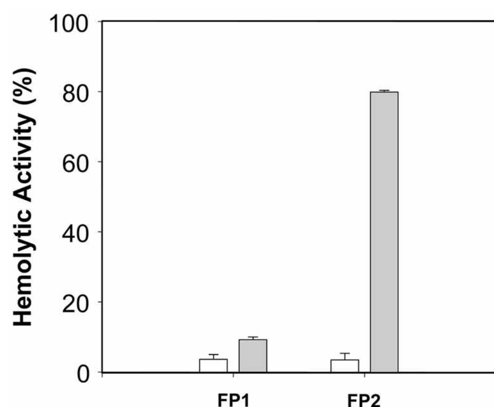

Figure 2. Hemolysis of RBCs by FP1 and FP2 peptides. Hemolysis of RBC is monitored as the increase of absorbance at 540 nm. Samples are incubated with 100 μM of FP1 and FP2 peptides (gray bars) for 1 h at 37 °C with constant shaking in 20 mM Na-phosphate, 100 mM NaCl pH 7.4. The value for 100% hemolysis is obtained from the RBC in the presence of 0.2% Triton-X 100. The value of negative control is obtained from RBC without peptides (white bars).

was examined by measuring the amount of calcein released during one hour of incubation at 37 °C. As shown in Figure 3A, FP1 could not perturb the integrity of the vesicles at a concentration up to 20 μM. When the concentration of FP1 was increased to 30 μM, less than 30% of the vesicles were disrupted. In contrast, FP2 initiated leakage of the vesicles at 5 μM, and more than 70% of the liposomal contents had leaked out at 30 μM peptide. These results indicated that FP2 from the internal N-terminus region of the S protein had stronger activity of membrane disruption than FP1. The pH dependence of these peptides on the leakage of liposome was also examined. As shown in Figure 3B, the liposome-leakage activity of FP2 was not changed at the tested pH range. In contrast, the hemolysis activity of FP1 was slightly increased at pH 4.5, although its membrane-disrupting activity is much less than FP2.

Stimulation of membrane disrupting activity of L protein by the treatment of V8 protease. Membrane fusion peptides of viral envelope proteins need to be exposed by proteolysis in order to initiate membrane fusion process, and the treatment of V8 protease enhanced infectivity of HBV particle. One of the recognition sites of V8 protease is located at the N-terminus of FP2 sequence, and FP2 sequence is exposed by the treatment of V8 protease.[21] To examine the activation mechanism of FP2 sequence in L protein, the membrane-disrupting activity of the extracellular region of L protein in the presence or absence of V8 protease-treatment was examined. When Trx-L_{EC} which contained the entire extracellular region of L protein including FP2 sequence, was treated with V8 protease, it processed to several fragments of 23, 20, 14, or 6 kDa (Fig. 4A, lane 3). The 6 kDa fragment (Fig. 4A, arrow) corresponds to the size of putative fragment containing FP2 sequence. When Trx-L_{EC} was incubated with lipid vesicles, it failed to disrupt liposomes up to 100 μM and slightly induced leakage of

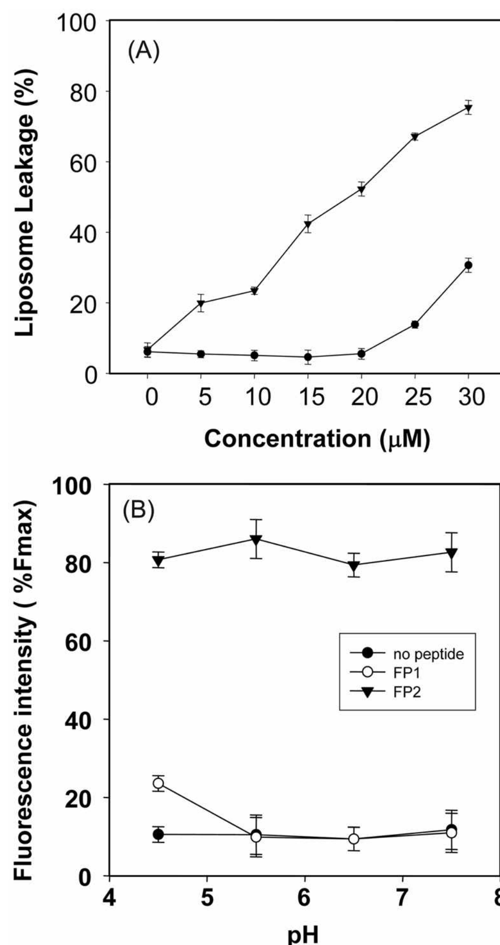

Figure 3. Disruption of liposomes by FP1 and FP2 peptides. (A) Fluorescence intensity of the POPC:POPG (7:3) liposomes containing calcein-cobalt complex are measured in the presence of different concentration of FP1 (●) or FP2 (▼) peptides. Samples are incubated for 1 h at 37 °C with constant shaking in 20 mM MOPS pH 7.5, 100 mM NaCl, and then 10 mM EDTA is added for the dequenching of calcein. (B) The leakage of calcein from liposomes in the presence of FP1 (○) and FP2 (▼). Basal level of liposome-leakage at different pHs is indicated as filled circle (●). The concentration of lipid vesicle was 100 μM. *Fmax* is the intensity of fluorescence of calcein from liposomes disrupted by 0.2% Triton X-100.

liposomes at 200 or 500 μM (Fig. 4B, open circles). In contrast, V8 protease-treated Trx-L_{EC} significantly increased the leakage of liposomes at 100 μM or higher concentrations. At 200 μM of V8 protease-treated Trx-L_{EC}, more than 50% of liposomes were disrupted (Fig. 4B, filled circles). These results indicate that the membrane-disrupting activity of internal FP2 sequence in L protein is activated by the treatment of V8 protease that cleaves in front of FP2 sequence.

Stimulation of membrane disrupting activity of fusion peptide by proteolysis. Activation of FP2 sequence of L protein by V8 protease-treatment suggested that the FP2 sequence would exposed at the N-terminus of processed protein, and the newly exposed FP2 sequence serve as fusion peptide during membrane fusion process. To examine

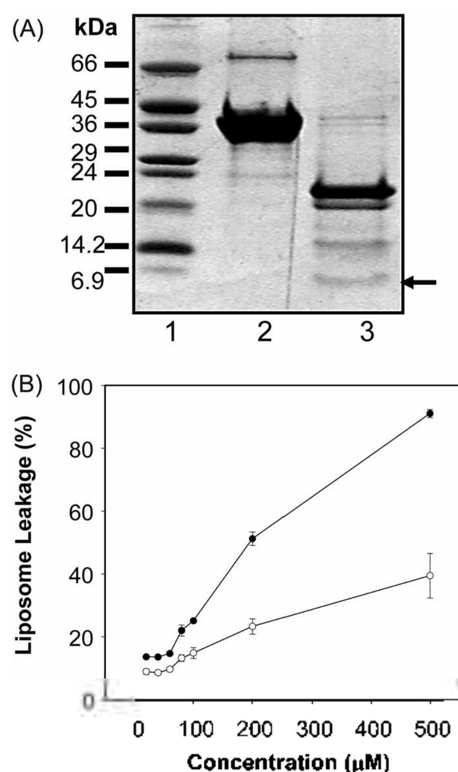

Figure 4. Activation of the liposome-disrupting activity of Trx-L_{EC} by the treatment of V8 protease. (A) The peptide fragments of Trx-L_{EC} after the treatment with V8 protease are analyzed using a 16% Tricine-SDS PAGE. Lane 1; molecular weight standards (66, 45, 36, 29, 24, 14.2, and 6.9 kDa). Lane 2; Trx-L_{EC}, lane.3; Trx-L_{EC} after 6 h incubation with V8 protease. (B) Lipid vesicles are incubated with Trx-L_{EC} in the presence (●) or absent (○) of V8 protease. The reaction mixture of lipid vesicle (100 μM) and different concentration of Trx-L_{EC} or V8 protease-treated Trx-L_{EC} is incubated for 6 h at 37 °C with constant shaking in 20 mM MOPS, 100 mM NaCl, pH 7.4. Fluorescence of the reaction mixture is measured at 530 nm after addition of 10 mM of EDTA.

whether the exposed FP2 sequence of L protein could induce membrane disruption, a chimera protein (Trx-L_{CEN}) that contains amino acids 168 to 220 of L protein at the C-terminus of thioredoxin and thrombin cleavage site was prepared. Treatment of Trx-L_{CEN} with thrombin generated a 6 kDa processed protein (Fig. 5A, lane 3) whose N-terminus amino acid sequence is identified as "NH_2-Gly-Ser-Asn-Thr-Thr-"by N-terminus sequencing. The newly exposed sequence of 6 kDa fragment has two thrombin recognition residues (Gly-Ala) followed by FP2 sequence. When Trx-L_{CEN} was incubated with lipid vesicles, it hardly disrupted the vesicles up to 100 μM, indicating that the P2 sequence internally located in Trx-L_{CEN} could not disrupt the lipid vesicle as in the case of Trx-L_{CEN}. Incubation of the vesicle with thrombin treated Trx-NS, however, significantly induced vesicle leakage. At 40 μM or higher concentration of thrombin-treated Trx-NS, most of the vesicles were disrupted (Fig. 5B). These results indicated that FP2 sequence of L protein showed membrane disrupting activity only when it exposed to N-terminus after proteolysis.

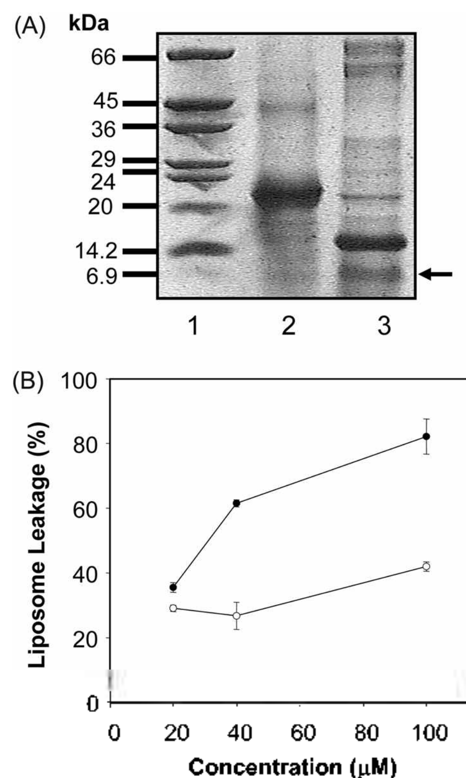

Figure 5. Activation of the liposome-disrupting activity of Trx-L_{CEN} by the treatment of thrombin. (A) The peptide fragments of Trx-L_{CEN} after the treatment with thrombin are analyzed using a 16% Tricine-SDS PAGE. Lane 1; molecular weight standards (66, 45, 36, 29, 24, 14.2, and 6.9 kDa). Lane 2; Trx-L_{CEN}, lane.3; Trx-L_{CEN} after 16 h incubation with thrombin. The 6kDa band indicated by an arrow had the same N-terminus sequence as the FP2 peptide. (B) Lipid vesicles are incubated with Trx-L_{CEN} in the presence (●) or absent (○) of thrombin which cleave in front of the FP2 sequence of Trx-L_{CEN}. The reaction mixture containing 100 μM of lipid vesicles and different concentration of Trx-L_{CEN} is incubated for 16 h at 37 °C with constant shaking in 20 mM MOPS, 100 mM NaCl, pH 7.4. After addition of 10 mM EDTA, the intensity of fluorescence is measured at 530 nm.

Discussion

Fusion peptide sequences of the envelope proteins of HBV have been characterized in this study. Due to the utilization of the same open reading frame, the fusion peptide sequence locates at the N-terminus of S protein as well as at the middle of extracellular region of L protein. It is noticeable that the N-terminus 16-mer peptide of the S protein from the ayw-type HBV could insert into membranes and efficiently destabilize acidic phospholipid bilayers at pH 5.0.[23-25] The membrane disrupting activity of this peptide increased 2-3 fold at pH 5.0, and 30-55% of RBCs or liposomes were disrupted at the concentration of 40-70 μM.[23] In contrast, disruption of RBCs or liposomes was not observed in the presence of 100 μM of FP1, which differs from the 16-mer peptide from the S protein of ayw-type HBV by single amino acid substitution of isoleucine to threonine at the forth residue (Fig. 1). The presence of hydrophilic threonine in the

4th amino acid of FP1 would increase polarity of FP1 and reduce its membrane disrupting activity. Compare to FP1, the internal fusion peptide (FP2) of HBV envelope proteins has much stronger membrane-disrupting activity. About 80% of RBCs or liposomes were lysed in the presence of 100 or 30 μM of FP2, respectively. This membrane-disrupting activity of FP2 is comparable or higher than the reported activity of the 16-mer peptide from the S protein of ayw-type HBV, although direct comparison of these values may inaccurate due to the difference in the composition and concentration of lipid used in the experiments. Compared to FP1, FP2 lacks methionine and glutamate at N-terminus and gains alanine and glycine at the C-terminus. The pH-independence of FP2 was explained by lack of negative charged amino acids, whose charge effect would be disappeared at acidic pH condition. Also the absence of charged residue in FP2 would contribute to the increased membrane disrupting activity of FP2 compare to FP1. The lipsome-disrupting activity of protease-treated Trx-L_{CEN} and Trx-L_{EC} appears lower than FP2. More than 200 μM of Trx-L_{EC} or 40 μM of Trx-L_{CEN} were required for disrupting liposomes (Fig. 4B and Fig. 5B). Two possible explanations are possible for the lower liposome disrupting activity. First, the concentration of 6 kDa fragment resulted from the proteolysis of Trx-L_{CEN} or Trx-L_{EC} may be lower than the initial concentration of Trx-L_{CEN} or Trx-L_{EC} used for proteolysis due to incompleteness of proteolysis reaction. Secondly, the affinity of the 6 kDa fragment to lipid bilayer may be lower than that of FP2 peptide. The 6 kDa fragment has FP2 sequence and extra 39 residues that consists of hydrophilic residues. The presence of hydrophilic residues in 6 kDa fragments may reduce the affinity of 6 kDa fragment to lipid bilayer and its liposome-disrupting activity.

Induction of membrane-disrupting activity of recombinant L protein by cleavage in front of FP2 sequence indicated that a proteolysis-dependent exposure of FP2 sequence is required for the initiation of membrane fusion. Processing of precursor membrane fusion protein are frequently observed in viral envelop proteins. Hemagglutinin of influenza virus is synthesized as a precursor protein, HA0, which is cleaved at residue 329, generating the fusion peptide at the amino terminal residues of the newly formed HA2 transmembrane subunit. Prevention of the processing of HA0 impairs the fusion activity of HA2.[36] Similar to hemagglutinin, a precursor form of the envelope protein of HIV (gp160) was processed into two subunits (gp120 and gp41) in the ER, and the fusion peptide was located at the N-terminus of gp41.[37] Processing any of the envelope proteins of HBV during the translocation, assembly process or infection process into the liver cell has not been reported. However, the induced infectivity of HBV to HepG2 cells by treatment with V8 protease[38] suggested that the proteolysis of the envelope proteins might induce the infectivity of HBV. Particularly, a serine protease inhibitor Kazal (SPIK) was highly expressed in HBV-insusceptible hepatoma cell lines such as HepG2 or Huh7 cells compare to the basal level expression in HBV susceptible human liver cells.[39] High level serine protease inhibitor in HBV-insusceptible cells supports the requirement protease activity for the membrane fusion activity of HBV envelope proteins to target cells.

In this study, we have shown that internal fusion peptide sequence of HBV surface proteins such as L protein has characteristics of fusion peptide, and proteolysis-dependent exposure of the fusion peptide sequence could activate its membrane disrupting activity. Identification of protease that responsible for cleavage at FP2 sequence of HBV envelope protein and effect of pH on this process would elucidate molecular mechanism of HBV infection.

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