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Mutational analysis of hepatitis B virus pre-S1 (9–24) fusogenic peptide

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ABSTRACT

A hollow nanoparticle known as a bio-nanocapsule (BNC) consisting of hepatitis B virus (HBV) envelope L protein and liposome (LP) can encapsulate drugs and genes and thereby deliver them *in vitro* and *in vivo* to human hepatic tissues, specifically by utilizing the HBV-derived infection machinery. Recently, we identified a low pH-dependent fusogenic domain at the N-terminal part of the pre-S1 region of the HBV L protein (amino acid residues 9 to 24; NPLGFFPDHQLDPAFG), which shows membrane destabilizing activity (*i.e.*, membrane fusion, membrane disruption, and payload release) upon interaction with target LPs. In this study, instead of BNC and HBV, we generated LPs displaying a mutated form of the pre-S1 (9–24) peptide, and performed a membrane disruption assay using target LPs containing pyranine (fluorophore) and *p*-xylene-bis (*N*-pyridinium bromide) (DPX) as a quencher. The membrane disruption activity was found to correlate with the hydrophobicity of the whole structure, while the peptide retained a random-coil structure even under low pH condition. One large hydrophobic cluster (I) and one small hydrophobic cluster (II) residing in the peptide would be connected by the protonation of residues D16 and D20, and thereby exhibit strong membrane disruption activity in a low pH-dependent manner. Furthermore, the introduction of a positively charged residue enhanced the activity significantly, suggesting that a sole positively charged residue (H17) may be important for the interaction with target LPs by electrostatic interaction. Collectively, these results suggest that the pre-S1 (9–24) peptide may be involved in the endosomal escape of the BNC's payloads, as well as in the HBV uncoating process.

1. Introduction

Enveloped viruses require fusion with the plasma membrane or endosomal membrane for their uncoating process, thereby allowing them to release the internal components (*e.g.*, genome, capsids, and polymerase) to the cytoplasm [1,2]. In general, fusion protein, a part of the envelope proteins, possesses fusion peptides or fusion loops forming a hydrophobic membrane at proximal regions at the junction between the ectodomain and membrane anchor. When the fusion of two membranes proceeds by the formation of a dehydration interface, and a hemifusion stalk is formed followed by a fusion pore, either the fusion peptides or fusion loops could be deposited onto fusion proteins by priming events (*e.g.*, proteolytic cleavage, receptor attachment, low pH environment), leading to a change in the secondary structure caused by the acidic conditions in endosomes. After insertion into the endosomal membrane, a dehydration interface can be formed, which then enhances the membrane perturbation to form the hemifusion stalk [1]. For example, the fusion peptide of influenza virus hemagglutinin protein was shown to slightly increase α-helicity by the protonation onto residues E15 and D19 in endosomes, leading to the fusion between the viral membrane and endosomal membrane [3]. Hepatitis B virus (HBV) is also an enveloped virus, consisting of small [S, 226 amino acid residues (aa)], middle (M; pre-S2+S, 281 aa), and large [L; pre-S1+pre-S2+S, 389 aa (serotype, ayw) or 400 aa (serotype adr)] envelope proteins [4]. HBV is considered to attach specifically to human hepatic cells, enter the cells by receptor-mediated endocytosis, and then execute subsequent membrane

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fusion for the transfer of its genome and accessory proteins to the cytoplasm (*i.e.*, uncoating process) [5]. The following fusogenic domains were independently identified in L protein: the C-terminal half of the pre-S2 region [pH-independent, from 149 to 160 aa of L protein (ayw)] [6], the N-terminal part of the S region [low pH-dependent, 164–186 aa of L protein (ayw)] [7], and the whole pre-S1 region (low pH-dependent) [8]; however, it has thus far remained controversial as to which domains in the L envelope protein are responsible for the uncoating process of HBV in endosomes. In 2003, HBV envelope L protein particles synthesized in *Saccharomyces cerevisiae* were found to infect human hepatic cells *in vitro* and *in vivo* by HBV-derived infection machinery [9–11]. We therefore designated an HBV bio-mimicking nanoparticle as a bionanocapsule (BNC). Recently, using a lipid mixing assay with liposomes (LPs) labeled with fluorescence resonance energy transfer (FRET) pairs, the membrane portion of the BNC was found to strongly fuse with LPs at low pH, indicating that L protein harbors functional low pH-dependent fusogenic activity. When the BNC was digested by trypsin to remove the whole pre-S1 region and N-terminal half of the pre-S2 region, but to retain the former two fusogenic domains (see above), the trypsinized BNC was found to lose its fusogenic activity completely [12], strongly suggesting that the pre-S1+pre-S2 regions are necessary for HBV membrane fusion. Finally, by using LPs displaying the pre-S1-derived peptides (20 aa each), we identified that a low pH-dependent fusogenic domain resides in the N-terminal part of the pre-S1 region (NPLGFFPDHQLDPAFG, aa 9–24 of the L protein). Moreover, it was demonstrated that the BNC could not only fuse with target LPs but also disrupt its own membrane (payload release) and target LPs (membrane disruption) [12]. Since HBV displays the pre-S1+pre-S2 region outwardly as in the BNC, this suggests that the pre-S1 (9–24) peptide may play a pivotal role in the HBV uncoating process, as well as in the endosomal escape of the BNC's payload. In this study, we examined whether the pre-S1 (9–24) peptide itself possesses sufficient activity not only for membrane fusion but also for membrane disruption by using target LPs (a model of the endosomal membrane) containing a fluorophore and its quencher. By delineating the crucial aa for these activities by using mutated pre-S1 (9–24) peptides, we propose the mechanism for how the fusogenic peptide interacts with the membrane.

2. Materials and methods

2.1. Probe LPs for the lipid mixing assay

Dipalmitoylphosphatidylcholine (DPPC; NOF, Tokyo, Japan), dipalmitoylphosphatidylethanolamine (DPPE, NOF), dipalmitoylphosphatidylglycerol sodium salt (DPPG-Na, NOF), cholesterol (Nacalai Tesque, Kyoto, Japan), *N*-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE; Life, Carlsbad, CA), and lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero- 3-phosphoethanolamine, triethylammonium salt (Rh-DHPE; Life) were mixed at 15:9:30:40:4:2 (mol), dissolved in chloroform/methanol (2:1, v/v), and then evaporated at 37 °C to form a thin lipid film. After hydrating the film with phosphate-buffered saline (PBS) at 60 °C, the mixture was sonicated for 15 min to obtain probe LPs of ~100 nm, which was determined by Zetasizer Nano ZS (Malvern, Worcestershire, UK). Lipid concentration was estimated from the cholesterol content determined by a Cholesterol E-Test Wako kit (Wako, Osaka, Japan).

2.2. Peptide-displaying LPs

DPPC, cholesterol, and *N*-(3-maleimide-1-oxopropyl)-L-α-phosphatidylethanolamine, dioleoyl (NOF) were dissolved in ethanol at 55:40:5 (mol), instilled into Tris–HCl (pH 8.0) buffer with gentle stirring at room temperature, and then subjected to a Sephadex G-25 gel filtration column (GE Healthcare, Buckinghamshire, UK) equilibrated with PBS. The LPs were incubated with synthetic peptides (purity, >95%; with a C-/N-terminal Cys modification; SCRUM, Tokyo, Japan; Table 1) at room temperature for 2 h, maintained at 4 °C for 24 h, and then subjected to a Sephadex G-25 gel filtration column.

2.3. Lipid mixing assay

The probe LPs (NBD- and Rh-labeled, 10 μg) were pre-warmed in citrate buffer (pH 7.4, 9 mM citric acid, 91 mM Na_2HPO_4) or low-pH citrate buffer (pH 4.5, 56 mM citric acid, 44 mM Na_2HPO_4) at 37 °C for 30 min. After the incubation with 20 μM free peptides or 60 μg peptide-displaying LPs (20 μM as peptide) at 37 °C for 30 min, the NBD-derived fluorescence (excitation at 470 nm, emission at 540 nm) was measured on a Varioskan multiplate spectrophotometer (Thermo, Waltham, MA). Triton X-100 was added at 1.0% (v/v) to quench the FRET effect. The percentage of NBD-derived fluorescence was calculated as follows: $\%F = (F_{NBD}-F_0)/(F_{100}-F_0)$, where F_0 is the initial NBD-derived fluorescence before the addition of samples, F_{NBD} is the NBD-derived fluorescence after 30-min incubation with each sample, and F_{100} is the NBD-derived fluorescence after addition of Triton X-100.

2.4. Pyranine leakage assay

As described in Section 2.1, the mixture of DPPC, DPPE, DPPG-Na, and cholesterol at 15:15:30:40 (mol) was allowed to form a thin lipid film. By hydrating with PBS containing 35 mM pyranine (fluorophore; Sigma–Aldrich, St. Louis, MO) and 50 mM *p*-xylene-bis (*N*-pyridinium bromide) (DPX, quencher; Sigma–Aldrich) at 60 °C, the mixture was sonicated to obtain ~100-nm LPs, and then subjected to a Sephadex G-25 gel filtration column. The LPs (4 μg) were pre-warmed in citric acid buffer at pH 7.4 or pH 4.5 at 37 °C for 30 min. After incubation with 20 μM free peptides or 60 μg peptide-displaying LPs (20 μM as peptide) at 37 °C for 30 min, the amount of leaked pyranine was determined by the pyranine-derived fluorescence (excitation at 416 nm, emission at 512 nm). Triton X-100 was added at 1.0% (v/v) to release pyranine from the LPs. The percentage of leaked pyranine was calculated as follows: pyranine leakage (%) $= (F_{pyranine}- F_0)/(F_{100} - F_0)$, where F_0 is the initial pyranine-derived fluorescence, $F_{pyranine}$ is the pyranine-derived fluorescence after 30-min incubation with each sample, and F_{100} is the pyranine-derived fluorescence after the addition of Triton X-100.

2.5. Circular dichroism (CD)

The CD spectra were obtained with 50 μM peptide in phosphate buffer (pH 7.4; 2 mM KH_2PO_4, 8 mM Na_2HPO_4) or low-pH phosphate buffer (pH 5.3; 97.5 mM KH_2PO_4, 2.5 mM Na_2HPO_4) at 25 °C on a JASCO J-725 CD spectropolarimeter (Tokyo, Japan) using a 10-mm cell.

3. Results

3.1. Fusogenic and membrane disruption activity of the pre-S1 (9–24) peptide

The BNC showed membrane fusion, membrane disruption, and payload release activity in a low pH-dependent manner. Furthermore, low pH-dependent fusogenic activity was identified in the pre-S1 (9–24) region of the BNC by a lipid mixing assay [12]. When pre-S1 (9–24) peptide [wild type (WT) sequence]-displaying LPs