Biochimica et Biophysica Acta 1848 (2015) 895–906

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Study of the putative fusion regions of the preS domain of hepatitis B virus

Carmen L. Delgado [a,1], Elena Núñez [a,2], Belén Yélamos [a], Julián Gómez-Gutiérrez [a], Darrell L. Peterson [b], Francisco Gavilanes [a,*]

[a] Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense, 28040 Madrid, Spain
[b] Department of Biochemistry and Molecular Biology, Medical College of Virginia, Virginia Commonwealth University, Richmond, 23298 VA, USA

ARTICLE INFO

Article history:
Received 15 October 2014
Received in revised form 1 December 2014
Accepted 22 December 2014
Available online 29 December 2014

Keywords:
Fusion protein
HBV
Hepatitis virus
Membrane fusion
Protein domain
Phospholipid vesicle

ABSTRACT

In a previous study, it was shown that purified preS domains of hepatitis B virus (HBV) could interact with acidic phospholipid vesicles and induce aggregation, lipid mixing and leakage of internal contents which could be indicative of their involvement in the fusion of the viral and cellular membranes (Núñez, E. et al. 2009. Interaction of preS domains of hepatitis B virus with phospholipid vesicles. Biochim. Biophys. Acta 17884:417–424). In order to locate the region responsible for the fusogenic properties of preS, five mutant proteins have been obtained from the preS1 domain of HBV, in which 40 amino acids have been deleted from the sequence, with the starting point of each deletion moving 20 residues along the sequence. These proteins have been characterized by fluorescence and circular dichroism spectroscopy, establishing that, in all cases, they retain their mostly non-ordered conformation with a high percentage of β structure typical of the full-length protein. All the mutants can insert into the lipid matrix of dimyristoylphosphatidylglycerol vesicles. Moreover, we have studied the interaction of the proteins with acidic phospholipid vesicles and each one produces, to a greater or lesser extent, the effects of destabilizing vesicles observed with the full-length preS domain. The ability of all mutants, which cover the complete sequence of preS1, to destabilize the phospholipid bilayers points to a three-dimensional structure and/or distribution of amino acids rather than to a particular amino acid sequence as being responsible for the membrane fusion process.

1. Introduction

Hepatitis B virus (HBV) is a small DNA virus which infects the liver. Although infection is usually followed by a complete recovery, in some cases the infection becomes chronic and may result in the development of cirrhosis and hepatocellular carcinoma. HBV infection is still a worldwide health problem despite the fact that an effective vaccine has been available for more than 25 years. About 240 million people are chronic carriers of HBV and about 1 million become infected yearly [1].

HBV envelope proteins are involved in the binding of the virus to the hepatocytes and in the cell entry mechanism [2]. There are three surface proteins designated as the small (S), medium (M) and large (L), that are the product of a single open reading frame. They share 226 amino acids (full-length S protein) at the C-terminus. The M protein has an extension of 55 amino acids, preS2, at the N-terminus of the S. The L protein is composed of the entire M protein and the preS1 region at the N-terminus which has 108–119 amino acids, depending on the HBV genotype. The regions preS1 and preS2 together are known as preS domains [3].

The preS domains are functional at different steps of the virus life cycle. Several studies have demonstrated that preS1, and not preS2, contains the main hepatocyte specific binding domain [4], specifically a region from 21 to 47 residues [5–9]. Other studies indicate that the full-length preS1 domain, with the exception of amino acids 78 to 87, is essential for the infectivity of HBV [10] and the species specificity has been attributed to the first 30 amino acids of preS1 (subtype ayw) [11]. It is also widely accepted that myristoylation at Gly2 residue of the preS1 domain plays an important role in specific binding to hepatocytes [3,12–14]. Several molecules have been proposed to play a role in binding of HBV to hepatocytes [3,15–17]. Very recently sodium

Abbreviations: HBV, Hepatitis B Virus; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine; Rh-PE, N-(lissamine rhodamine B sulfonyl)-diacylphosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; ANTS, 8-Aminonaphtalene-1,3,6-trisulfonic acid; DPX, p-xylenebis(pyridinium) bromide; NBD-F, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole, IPTG, isopropyl-D-thiogalactopiranosyde; CCA, Convex Constraint Analysis

* Corresponding author. Tel.: +34 91 3944266; fax: +34 91 3944159.
E-mail address: pacog@bbm1.ucm.es (F. Gavilanes).
[1] Present address: ASICI Pabellón Central, Recinto Ferial, 06300 Zafra, Badajoz, Spain.
[2] Present address: Janssen-Cilag, S.A., Paseo de las Doce Estrellas, 5-7, 28042 Madrid, Spain.

http://dx.doi.org/10.1016/j.bbamem.2014.12.020
0005-2736/

taurocholate cotransporting polypeptide has been identified as a HBV cellular receptor [18].

Little is known about the involvement of the envelope proteins in the fusion between the viral and the host cell membrane. A peptide comprising the 16 amino acids at the N-terminal end of S protein have been shown to interact with model membranes, causing liposome destabilization in a pH-dependent manner and adopting an extended conformation during the process [19,20]. Evidence for the role of the N-terminal S peptide in woodchuck hepatitis B virus (WHV) or duck hepatitis B virus (DHBV) infectivity has also been obtained by others researchers [21,22]. On the other hand, we have previously shown that isolated preS domains (subtypes adw and ayw) are able to interact with acidic phospholipids vesicles and, as a result of this interaction, cause the destabilization of the bilayer, both at neutral and acidic pH [23]. Furthermore, the addition of this type of phospholipid vesicles led to a conformational change in the preS domain, increasing its helical content [23]. Similar results were obtained with the DHBV preS domain, despite the difference in the amino acid sequence [24]. Both domains share a similar hydrophobic profile, indicating that a three-dimensional conformation rather than a particular amino acid sequence would be responsible for the properties observed [24]. These results point to the possibility that both S and preS regions could contribute to the fusion of the viral and cellular membranes. Moreover, it has been shown that the preS2 domain plays an important role in virus assembly but it is dispensable in virus entry [25].

With the aim to locate the region of the preS domains responsible for the fusogenic properties, five deletion mutants along the preS1 sequence (subtype ayw) have been prepared. In each mutant, 40 amino acids were deleted, with the starting point of each deletion moving 20 residues along the sequence. Thus, the mutants are designated $preS_{\Delta 1-40}$, $preS_{\Delta 20-60}$, $preS_{\Delta 40-80}$, $preS_{\Delta 60-100}$ and $preS_{\Delta 80-120}$, indicating the deleted region in each case. All the proteins were characterized spectroscopically, and their interaction with phospholipids was studied.

2. Materials and methods

2.1. Enzymes and reagents

Restriction enzymes, ligases, DNA polymerase and other molecular biology reagents were obtained from New England Biolabs, Promega, Invitrogen, Novagen or BRL. N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine (NBD-PE), N-(lissamine rhodamine B sulfonyl)-diacylphosphatidylethanolamine (Rh-PE) and dimyristoylphosphatidylglycerol (DMPG) were provided by Avanti Polar Lipids. 8-Aminonaphtalene-1,3,6-trisulfonic acid (ANTS), p-xylenebis(pyridinium) bromide (DPX) and 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) were purchased from Molecular Probes. Triton X-100 was obtained from Boehringer Mannheim. Sepharose CL-6B Ni-nitrilotriacetic acid (NTA) was purchased from Qiagen. All other reagents were obtained from Merck and Sigma. All solvents were of HPLC grade.

2.2. Cloning of preS-his and $preS_{\Delta 1-40}$

To clone the preS domain (subtype ayw) and the $preS_{\Delta 1-40}$ deletion mutant in the plasmid pT7-7 (Gibco BRL), the plasmid pET3d-preS-his-ayw [26], which contains the full-length preS domain (subtype ayw) followed by a region encoding a six-histidines tag at the carboxy-terminal end, was used as template. The forward primers used were preS-*Nde*I(+): 5-′GGA GAT ATA CAT ATG GGG CAG AAT C and Δ1-40-*Nde*I(+): 5′C AAC AAG CAT ATG TGG CCA GAC GC; the reverse primer for both cloning was preS-*Hind*III(-): 5′-GCA GCC AAG CTT CTA CTA ATG GTG. Underlined are the *Nde*I and *Hind*III restriction sites included in the primers.

The PCR reaction conditions were: 1 min at 94 °C, followed by five cycles at 94, 60 and 72 °C, each for 1 min, by 30 cycles at 94, 55 and 72 °C, each for 1 min, and a final "filling in" step at 72 °C for 7 min. The fragment of amplified DNA was purified by Wizard PCR Prep system (Promega) and was subcloned into the linear plasmid pCR2.1 (Invitrogen), with 3′T ends and ampicillin resistance gene. pCR2.1-preS and pT7-7 plasmids were subsequently digested with restriction enzymes *Nde*I (Boehringer Mannheim, 10 U/μL) and *Hind*III (New England Biolabs, 20 U/μL), in a final volume of 10 μL at room temperature for 16 h. After digestion, a 1% agarose gel was run. Once the suitable fragments had been copurified, using the QIAEX system (QIAGEN), they were ligated with bacteriophage T4 DNA ligase (Gibco BRL, 1 U/μL), at room temperature for 1-2 h, and *Escherichia coli* DH5αF′ cells were transformed with half of the reaction mixture. The positive colonies were identified by digestion of purified plasmid DNA with the restriction enzymes *Nde*I and *Hind*III. The individual cDNA sequences were confirmed by automated DNA sequencing. The resulting recombinant plasmids were called pT7-7-preS and pT7-7-Δ1-40.

2.3. Cloning of $preS_{\Delta 20-60}$, $preS_{\Delta 40-80}$, $preS_{\Delta 60-100}$ and $preS_{\Delta 80-120}$

The rest of the mutant cDNAs were obtained by the method described by Pogulis [27]. Briefly, the regions on both sides of the deletion were amplified separately by PCR, using the plasmid pT7-7-preS as template. Internal primers of each mutant had a complementary region of 18 nucleotides, corresponding to the 9 final nucleotides and the first 9 nucleotides located before and after the deletion. The conditions of this first PCR reaction were the same as described above for preS and $preS_{\Delta 1-40}$. After running the PCR product on a 1.75% agarose gel and copurifying both regions using the QIAEX system (QIAGEN), a second elongation PCR using Taq Gold polymerase was carried out without primers in order to expand the area. The PCR conditions were: 15 cycles at 94, 52 and 72 °C, each for 1 min, followed by 7 min at 72 °C.

Finally, a third PCR using the amplified sequences as template, and the oligonucleotides preS-*Nde*I(+) and preS-*Hind*III(-) as primers, resulted in a DNA fragment with a region of 120 nucleotides deleted and 3′A ends. The PCR conditions were: 32 cycles at 94, 55 and 72 °C, each for 1 min, followed by a final step of 7 min at 72 °C. Then, they were cloned into the pCR2.1 plasmid, digested with restriction enzymes *Nde*I and *Hind*III and subsequently cloned into the plasmid pT7-7 digested with the same enzymes. The sequences of the different genes were confirmed by automated DNA sequencing.

2.4. Expression and purification of preS-his and deletion mutants

pT7-7 recombinant plasmids with the preS-his domain or the different deletion mutants were used to transform *E. coli* HMS174 (DE3) cells. In all cases, the expression of the recombinant protein was under the control of the T-7 promoter, inducible by isopropyl-D-thiogalactopiranosyde (IPTG).

A single colony was used to inoculate 50 mL of M9 medium supplemented with 0.17% glucose, 1.06 mM $MgSO_4$, 0.053 mM $CaCl_2$ and 100 μg/mL ampicillin. Following overnight incubation at 37 °C, the culture was used to inoculate 950 mL of the same medium. This culture was grown to an optical density at 600 nm of 0.6 and the IPTG was added to a final concentration of 0.5 mM, and incubated at 37 °C for 4 h to induce protein expression. Cells were harvested by centrifugation at 7400 g for 10 min in a GS-3 rotor (Sorvall). The cell pellet was resuspended in ice cold 10 mM MOPS pH 8.0, 10 mM imidazole, 0.3 M NaCl, 6 M Urea to avoid proteolytic degradation of proteins. Cells were lysed by sonication and centrifuged at 89500 g for 30 min in a Beckman SW-28 rotor.

Recombinant proteins were purified using a single affinity chromatography step in Sepharose CL-6B Ni-nitrilotriacetic acid (NTA)-agarose column (Qiagen) equilibrated with 10 mM MOPS pH 8.0, 10 mM imidazole, 0.3 M NaCl, 6 M Urea. After washing the non-specifically bound proteins with 10 mM MOPS pH 8.0, 30 mM imidazole, 6 M Urea, elution of the proteins was performed with 10 mM MOPS pH 8.0, 200 mM