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Comprehensive proteomic analysis of host cell lipid rafts modified by HBV infection

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ABSTRACT

Lipid rafts are cholesterol- and sphingolipid-rich membrane microdomains that have been shown to participate in the entry, assembly and budding of various viruses. However, their involvement in HBV replication remains poorly characterized. In a preliminary study, we observed that HBV release could be markedly impaired by methyl-β-cyclodextrin mediated depletion of cholesterol in lipid rafts, and that this effect could be reversed by replenishment of exogenous cholesterol, suggesting that lipid rafts play an important role in the HBV life cycle. To further understanding how HBV exploited host cell lipid rafts to benefit replication, comprehensive proteomic approaches were used to profile the proteome changes of host cell lipid rafts in response to HBV infection using 2DE-MS/MS, in combination with SILAC-based quantitative proteomics. Using these approaches, a total of 97 differentially expressed proteins were identified. Bioinformatics analysis suggested that multiple host cell pathways were involved in the HBV infection processes including signal transduction, metabolism, immune response, transport, vesicle trafficking, cell adhesion and cellular ion homeostasis. These data will provide valuable clues for further investigation of HBV pathogenesis.

1. Introduction

The human hepatitis B virus (HBV), a prototype member of the *Hepadnaviridae* family, is a highly infectious enveloped DNA virus [1]. The infectious HBV virion, or Dane particle, is a spherical particle consisting of an icosahedral nucleocapsid and a lipid bilayer envelope in which the three envelope proteins (S, M, L) are anchored [2]. Empty subviral spherical or filamen-

Abbreviations: 2DE, 2 dimensional polyacrylamide gel electrophoresis; CAV1, caveolin 1; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; DRMs, detergent-resistant membranes; ER, endoplasmic reticulum; ESI-Q-TOF, electrospray ionization quadrupole time-of-flight; GOEAST, Gene Ontology Enrichment Analysis Software Toolkit; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IPA, Ingenuity Pathways Analysis; MLV, murine leukemia virus; MS, mass spectrometry; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; MβCD, methyl-β-cyclodextrin; NDV, Newcastle disease virus; PID, Pathway Interaction Database; RSV, respiratory syncytial virus; SILAC, stable isotope labeling with amino acids in cell culture; SREBP1c, nuclear sterol regulatory element-binding protein 1c; SV40, simian virus 40; SVPs, subviral particles; TEM, transmission electron microscope; TfR1, transferrin receptor 1.

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tous envelope particles (SVPs) which lack the nucleocapsid are also secreted in great excess compared with the infectious HBV virion [2]. In spite of the availability of an effective vaccine against HBV, about 2 billion people worldwide have been infected with HBV and about 350 million live with chronic infection [3]. With no improved effective treatment for those people already infected with HBV, an estimated 600,000 persons die each year due to the acute or chronic liver diseases caused by this virus [3]. Thus, a thorough understanding of HBV infection and morphogenesis is urgently required for the development of innovative antiviral treatments [4].

Recent studies have brought new insights into the role of lipid rafts in various virus life cycles [5–8]. Lipid rafts or caveolae can be defined as detergent-resistant membrane domains (DRMs) by their cholesterol- and sphingomyelin-rich nature, enrichment in glycosylphosphatidylinositol (GPI)-anchored proteins, cytoskeletal association and resistance to detergent extraction [9,10]. These microdomains can organize signaling molecules at the membrane to modulate many signal transduction cascades and cellular responses [11,12]. They also serve as docking sites for vehicles involved in protein sorting and membrane trafficking [13,14]. It is well documented that several viruses, (e.g. HIV, HCV, HSV, SV40, RSV, influenza virus, MLV and NDV), can hijack lipid rafts during some stages of their life cycle such as internalization or fusion, cell entry, assembly, replication, and budding [15,16]. Additionally they can perturb cell signaling pathways by interfering with proteins present in lipid rafts [17]. More recently, a dependence on the caveolin-1 (a marker for caveolea/lipid raft compartments) mediated endocytosis pathway for HBV cellular entry has been established [18]. Cholesterol in the HBV envelope has been reported to be a key determinant in this process, implying that the HBV envelope contains highly ordered lipids, e.g. raft-like structures that are important for infectivity [19].

In a preliminary study we found that depletion of cholesterol in host cell lipid rafts by methyl-β-cyclodextrin resulted in a remarkable reduction in HBV release. To better understand the functional role of host raft microdomains on the HBV life cycle, a combination of 2DE-MS/MS and SILAC-based quantitative proteomics was applied to globally profile changes in the lipid raft proteome using the *in vitro* cell model HepG2.2.15 a cell line stably producing HBV virus and the corresponding parental HepG2 cells. As a result, 97 nonredundant proteins were identified by 2DE-MS/MS, of which 34 proteins were identified by both proteomic approaches. Bioinformatics analysis of these derived protein datasets was performed to identify functional signaling networks and further understand the diverse host cell biological processes that may be involved in the HBV life cycle.

2. Experimental procedures

2.1. Cell culture

HepG2.2.15 and HepG2 cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% dialyzed FBS (Hyclone, USA), penicillin (10^7 U/L) and streptomycin (10 mg/L) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. ELISA and cell cytotoxicity assay

Cells were pretreated with varying concentrations of methyl-β-cyclodextrin (MβCD, Sigma C-4555) at 37 °C for 1 h, and subsequently treated with 400 μg/mL water-soluble cholesterol (Sigma) for another 1 hour. Supernatant samples were taken at intervals for ELISA analysis using HBsAg ELISA kits (Lantu Biotech Co., Ltd) according to the manufacturer's instructions.

Cell viability was measured using the MTT cytotoxicity assay. Briefly, cells were incubated with 0.5 mg/mL MTT at 37 °C for 4 h. 150 μL of dimethyl sulfoxide (DMSO) was added to solubilize formazan crystals, and the absorbance was detected at 570 nm with a 630 nm reference filter using a spectra max MS (MDC, USA). Background absorbance readings from reagent and media alone were deducted. All samples were examined in triplicate and results were expressed as fold changes relative to the negative controls.

2.3. Isolation of lipid rafts

Low-density lipid rafts were isolated as described previously with some modifications [20]. Briefly, cells were lysed with 1% Triton X-100 buffer (25 mM HEPES, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1%Triton X-100) containing protease inhibitor (Sigma, P8340). Cell lysates were homogenized using a Dounce homogenizer, and incubated on ice for 30 min. Lysates were mixed with an equal volume of 80% sucrose cushion, placed in the bottom of an ultracentrifuge tube, and overlaid with 30% and 5% sucrose respectively. The gradient mixture was centrifuged at 180,000 ×*g* for 18 h at 4 °C in a SW41 rotor (Beckman). After centrifugation, twelve fractions, 1 mL for each, were collected for subsequent western blot analysis. The buoyant band at the interface between 5% and 30% sucrose gradients was diluted with 25 mM HEPES (pH6.5, 150 mM NaCl, 1 mM EDTA and 1 mM PMSF), and centrifuged at 20,000 rpm for 30 min at 4 °C to obtain a pellet of detergent-resistant lipid rafts for further 2DE analysis and transmission electron microscopy [21].

2.4. 2DE and image analysis

2DE was performed as previously reported with minor modifications [22]. Briefly, the lipid rafts extracted as described earlier were dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.2% pH3–10 ampholyte, Bio-Rad, USA) containing protease inhibitor (Sigma, P8340). Protein concentration was determined using the DC protein assay kit (Bio-Rad). Each sample containing equal protein contents (1.6 mg) was loaded into an IPG strip (17 cm, pH3–10NL, Bio-Rad) using a passive rehydration method. Separation in the second dimension was performed using 12% SDS-PAGE at 30 mA constant current per gel after isoelectric focusing (IEF) and equilibration. The gels were stained with CBB R-250 (Bio-Rad, 161–0438) and scanned with a Bio-Rad GS-800 scanner. Quantitation of each spot in a gel was determined (OD) using PDQuest software 7.1 (Bio-Rad). Differentially expressed proteins were defined as statistically meaningful ($p<0.05$) based on both of the following criteria: (1) abundance alterations >2.0-fold and (2) observed more than three times in the total of four