Biochemical and Biophysical Research Communications 408 (2011) 447–453

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Real time monitoring of antigenicity development of HBsAg virus-like particles (VLPs) during heat- and redox-treatment

Qinjian Zhao [a,*], Yang Wang [a,1], Dicky Abraham [b], Victoria Towne [b], Ronald Kennedy [c], Robert D. Sitrin [b]

[a] Merck Research Laboratories, West Point, PA 19486, USA
[b] Merck Manufacturing Division, West Point, PA 19486, USA
[c] Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

ARTICLE INFO

Article history:
Received 29 March 2011
Available online 19 April 2011

Keywords:
Hepatitis B virus
Surface antigen
Virus-like particles
Disulfide bond isomerization
Neutralizing antibody
Epitope evolution

ABSTRACT

The Hepatitis B virus major surface antigen (HBsAg) is a cysteine-rich, membrane-bound protein which self-assembles into 22-nm spherical virus-like particles (VLPs). While this VLP based human vaccine has been demonstrated to be safe and efficacious since 1986, the structural and exact molecular basis for its antigenic determinants has not been elucidated. Maturation of the yeast-derived purified VLPs was characterized for the changes in 37 their biophysical properties [7]. Using rapid and label-free surface plasmon resonance technique with a neutralizing monoclonal antibody – A1.2, the epitope evolution kinetics of purified VLPs was monitored in real time. Evidence supporting the mechanism that the correct disulfide bond pairing is the molecular basis for shaping up the native virion-like epitopes was obtained. At least 10-fold enhancement in antigenicity probed by A1.2 of the VLPs was achieved by heat-treatment ($t_{1/2} \sim 6$–10 h), and another 2- to 3-fold enhancement was obtained when they were treated with redox buffer. This antigenicity development, presumably via disulfide formation/isomerization, was shown to be inhibited by a free radical scavenger and facilitated in the presence of light. Relative antigenicity determination with surface plasmon resonance was shown to be a valuable tool for process characterization in the kinetic monitoring mode or for final VLP product assessment in the end point antigenicity testing mode.

1. Introduction

Hepatitis B virus (HBV), the prototype member of the taxonomic family *hepadnaviridiae*, still poses a major health threat for causing hepatitis, liver cancer, and liver cirrhosis with 350 million carriers worldwide [1,2]. The 42-nm infectious virions of HBV, or Dane particles, are composed of three structural protein components: the lipid-associated major surface antigen (HBsAg); Pre-S protein that is continuously expressed with HBsAg; and the core antigen surrounding the DNA in the core of the particle. In addition to the intact 42-nm virions, abundant 22-nm non-infectious subviral spherical particles and tubules containing only HBsAg and lipids also exist in the plasma of chronically-infected individuals. These non-infectious, plasma-derived 22-nm virus-like particles (VLPs) had been used in prophylactic vaccines against HBV infection (Heptavax B, Licensed to Merck, 1981) before the introduction of a yeast-derived recombinant HBsAg vaccine [1]. The HBsAg is a 25-kDa protein with 226 amino acids, which self-assembles into immunogenic spherical particles containing host cell-derived lipids with ~100 copies of the HBsAg molecules per particle. Due to the comparable protective efficacy against HBV infection, yeast-derived HBsAg has replaced plasma-derived HBsAg as the preferred vaccine, making it the first recombinant vaccine (RECOMBIVAX HB®, Merck) licensed for human use in 1986 [1–7].

The HBsAg, expressed in yeast as lipid containing VLPs, forms disulfide-linked dimers and higher-order oligomers spontaneously during expression, downstream purification and storage [6,7]. Work from early years of HBsAg VLP characterization showed that cross-linking is essential for both antigenicity and immunogenicity of these *in vitro* assembled VLPs [7–9]. Antigenicity development is coupled with the cross-linking of HBsAg. Although RECOMBIVAX HB® has been used as a successful prophylactic vaccine for ~25 years, the detailed molecular structure for the antigenic determinants has not been fully elucidated. We previously reported the changes in conformational flexibility and dynamics of this lipid-associated oligomeric protein and the particle compaction during

Abbreviations: Cys, cysteine; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; HBsAg, major surface antigen of Hepatitis B virus; HBV, Hepatitis B virus; PBS, phosphate buffered saline; RAMFc, rabbit anti-mouse IgG Fc; SPR, surface plasmon resonance; VLP, virus-like particle.

* Corresponding author. Fax: +1 215 993 3348.
E-mail addresses: qinjian_zhao@merck.com (Q. Zhao), yang_wang@sinobiological.com (Y. Wang).
[1] Present address: Sino Biological, Inc., Beijing, China.

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.04.048

spontaneous maturation of the yeast derived HBsAg VLPs [7]. Here we report the immunochemical analysis using a conformation-sensitive monoclonal antibody (mAb) to probe the epitope evolution/transformation during maturation of the purified VLPs. Most importantly, the subtle structural changes were tracked in real time with a conformation-sensitive monoclonal antibody (mAb) A1.2 through the use of label-free, rapid, and biosensor-based surface plasmon resonance (SPR) method. Comparing to freshly purified VLPs, the heat-induced maturation was shown to enhance the specific antigenicity by up to 10-fold. Redox facilitated refolding was shown to yield another ~2- to 3-fold additional enhancement in antigenicity as compared to heat-induced maturation. It has been demonstrated that the formation of the neutralizing epitope(s) can be further improved by utilization of redox buffer at elevated temperatures, thus opening up the possibility of forming more virion-like epitopes on the recombinant lipid containing VLPs. The antigenicity of the redox-treated VLPs is comparable to that of human plasma-derived HBsAg subviral particles. The technique of tracking the antigenicity development in real time should facilitate the characterization and development of other VLP-based vaccines, which are gaining more popularity [10,11], where disulfides can be critical for proper presentation of the epitopes on VLP surface or necessary for VLP stability.

2. Materials and methods

2.1. Materials

A1.2, a murine mAb IgG1 with viral neutralizing activities, was prepared and characterized as previously reported [12]. The spin-trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO), potassium thiocyanate (KSCN), reduced form and oxidized form of glutathione, and other buffer salts of highest purity were purchased from Sigma (St. Louis, MO). Biacore CM5 chip, amine coupling kits, and HEPES buffered saline and other related buffers for cleaning and maintenance were purchased from Biacore or GE Health care (Piscataway, NJ).

2.3. Recombinant HBsAg VLPs

HBsAg was over-expressed in yeast (*Saccharomyces cerevisae*) and purified according to previously published procedures [6,7]. The purified HBsAg was used at concentrations in the range 40–200 μg/mL and was stored or diluted in phosphate-buffered saline (PBS) at 4 °C. Protein concentrations were determined by a Lowry method with commercially available reagents (Pierce). HBsAg maturation by heat-treatment and redox treatment were performed at various scales, from 0.7 mL (7 mm polypropylene vials), 1.0 mL (10 mm glass vials), 4.0 mL in glass vials on the Biacore 2000 sample block or up to 300 mL bench scale in blue glass and stainless steel containers. At small scales, aliquots were drawn from the vial at programmed time, whereas for bench scale maturation, samples were taken at various time intervals and frozen at −70 °C until analysis.

2.4. Surface plasmon resonance (SPR) instrumentation

The antigen–antibody biomolecular interaction analyses were performed with a BIAcore 2000 or 3000 instrument equipped with four flow cells and sample racks with temperature control (Uppsala, Sweden). The temperature of the sensor chip was maintained at 20 °C for all experiments. The running buffer contained 10 mM HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.005% (w/v) polysorbate 20 and the flow rate was maintained at 5 μL/min during and between sample injections (except during acid regeneration where the flow rate was 20 μL/min).

2.5. Analysis of HBsAg antigenicity by SPR

Affinity-purified polyclonal rabbit anti-mouse IgG Fcγ antibody (RAMFc) was chemically immobilized to the CM5 sensor chip surface using the vendor-supplied amine coupling kit. The HBsAg-specific mAb A1.2 was captured by RAMFc, and the antigenicity of the HBsAg sample was determined by injecting 10 μg/mL HBsAg onto the chip with mAb A1.2 captured on the sensor chip. The antigenicity of HBsAg was defined as $\Delta RU_{HBsAg}/\Delta RU_{A1.2}$. The sensor chip surface with covalently-coupled RAMFc was regenerated after binding analysis with acid after each cycle. Non-specific binding was negligible when RAMFc or A1.2 was missing from the chip.

For the kinetic studies at 10 μg/mL HBsAg in PBS, the $\Delta RU_{HBsAg}/\Delta RU_{A1.2}$ ratios at different time intervals were measured repeatedly after each regeneration cycle, then normalized a fully "aged" VLP preparation. Aliquots were automatically drawn from the sample rack on the Biacore platform with a 23-min time interval by injecting the VLPs onto the sensor chip. Temperature of the sample rack was controlled at 25 °C or 37 °C by an external circulating water bath for monitoring antigenicity over time.

2.6. Antigenicity development with light exposure

For the experiments of light exposure effect on epitope development, two identical 9 mm glass vials (from Biacore) with 10 μg/mL HBsAg in PBS were exposed to strong light approximately 30 cm from a 100 W tungsten lamp in a well-ventilated chemical hood. The control vial was wrapped tightly with aluminum foil to prevent light exposure, while simulating the heat radiation with the vial with light exposure. Aliquots were drawn at different time intervals for the antigenicity analysis and were compared to a standard with constant antigenicity.

2.7. Data analysis

The report points were made within Biacore control software for the RU values. Thus the changes in RU values were calculated from two report points. The ratio between the HBsAg binding over A1.2 binding was calculated as a measure of antigenicity – HBsAg binding for a given amount of captured A1.2. Subsequently, this ratio for a sample was normalized to that of a reference. Non-linear least square fits for the first-order kinetics of epitope development were performed to derived the apparent rate constant for a given maturation condition using Grafit [13]. With these apparent first order rate constants, the analysis of the pH dependence of the rate constants was carried out using Grafit [13]. Eq. (1) was used for deriving the pK_a for the ionizable group by fitting the data to a single ionization curve, assuming that the k_{obs} or y axis runs from 0 to a data-defined upper limit [13]:

$$y = \frac{\text{Limit}.10^{(\text{pH}-\text{p}K_a)}}{10^{(\text{pH}-\text{p}K_a)}+1} \quad (1)$$

3. Results

Mouse mAb A1.2 is a structurally sensitive probe for native virion-like conformation and it has neutralizing activity against the virus [12,14]. Thus, the binding to A1.2 of VLPs is a surrogate of its efficacy to elicit neutralizing antibodies *in vivo* with virion-like epitopes. Here, we demonstrated that A1.2 functions as an exquisite conformational probe as A1.2 is highly sensitive to small structural changes in the antigenic loop on the surface of the VLPs. SPR analysis is the method of choice for tracking the epitope development for HBsAg VLPs in nearly real time because (a) it is simple and quick with about 20 min turn-around time; (b) it is a label-free