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Membrane Structure of the Hepatitis B Virus Surface Antigen Particle

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Expression of S protein, an envelope protein of hepatitis B virus, in the absence of other viral proteins, leads to the secretion of hepatitis B virus surface antigen (HBsAg) particles that are formed by budding from the endoplasmic reticulum membranes. The HBsAg particles produced by mouse fibroblast cells show a unique lipid composition, with 1,2-diacyl glycerophosphocholine being the dominant component. The lipid organization of the HBsAg particles was studied by measuring electron spin resonance (ESR) using various spin-labeled fatty acids, and the results were compared with a parallel study on HVJ (Sendai virus) and vesicles reconstituted with total lipids of the HBsAg particles (HBs-lipid vesicles). HVJ and the HBs-lipid vesicles showed typical ESR spectra of lipids arranged in a lipid bilayer structure. In contrast, the ESR spectra obtained with the HBsAg particles showed that the movement of lipids in the particle is severely restricted and a typical immobilized signal characteristic of tight lipid-protein interactions was also evident. Phosphatidylcholine (PC) in the HBsAg particles was not exchangeable by a PC-specific exchange protein purified from bovine liver, while phospholipase A_2 from *Naja naja* vemon was able to hydrolyze all the PC in the particles. These analyses suggest that the lipids in the HBsAg particles are not organized in a typical lipid bilayer structure, but are located at the surface of the particles and are in a highly immobilized state. Based on these observations we propose a unique lipid assembly and membrane structure model for HBsAg particles.

Key words: assembly, electron spin resonance, hepatitis B virus, membrane, phospholipid.

Hepatitis B virus (HBV) is an enveloped DNA virus of the hepadnavirus family and is one of the major causes of infectious liver diseases as well as primary hepatocellular carcinomas (*1*, *2*). During the chronic carrier state or the acute phase of HBV infection, the infected hepatocytes synthesize and secrete three types of virus-related particles, 42-nm infectious spherical virions (Dane particles) and 22-nm noninfectious spherical or tubular lipoprotein-like particles composed primarily of the envelope protein and host cell–derived lipids (*3*). Of these particles, the 22-nm spherical particles (HBsAg particles) are produced in large quantities, are strongly immunogenic, and can induce a protective immune response, so that they have been used to develop an effective vaccine against HBV (*4*).

The envelope proteins of HBV comprise three closely related transmembrane proteins, small (S, 226 amino acids), middle (M, 281 amino acids), and large (L, 400 amino acids) proteins (*5*). HBV encodes these three envelope proteins in one single open reading frame using three different in-frame translation start codons and a common stop codon. These envelope proteins can be independently secreted from cells as subviral particles lacking all other viral components (*6*). S protein is the dominant component of the 22-nm HbsAg particles and is unique among viral envelope proteins in that it can assemble into coreless, lipoprotein particles to be released from the cell. S protein expression, however, is not sufficient for the envelopment of HBV nucleocapsids, and the expression of L protein in

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Abbreviations: HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; PC, phosphatidylcholine; GPC, glycerophosphocholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine; CL, cardiolipin; L-PC, lysophosphatidylcholine; PL, phospholipid; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol; CH, cholesterol; CHE, cholesteryl ester; ER, endoplasmic reticulum; ESR, electron spin resonance; SLS, spin-labeled stearate; 5-SLS, 5-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivatives of 5-ketostearate); 7-SLS, 7-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivatives of 7-ketostearate); 12-SLS, 12-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivatives of 12-ketostearate); 16-SLS, 16-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivatives of 16-ketostearate); VET50, small unilamellar vesicle prepared by extrusion through 50 nm filter; TLC, thin-layer chromatography; DMEM, Dulbecco's-modified Eagle's medium.

addition to S protein is required for intact viral assembly (*5*, *7*).

In most viral systems, the envelope proteins are simple integral membrane proteins with a single transmembrane domain spanning a conventional lipid bilayer (*8*). The envelope proteins synthesized at the rough ER are transported *via* vesicles to a cellular target membrane where the virus particles are formed by the budding process. It is believed that the budding process does not disturb the integrity of the lipid membrane and that the composition of the viral lipids resembles the lipid composition of the host cell membrane where the virus is formed (*8*, *9*). In contrast, the HBV envelope proteins, including S protein, have multiple transmembrane domains and post-translational alterations in the transmembrane topology of HBV envelope proteins occur during virus formation (*10–12*). These results suggest that a dramatic reorganization of either the envelope proteins or the lipid bilayer (or both) must occur during the budding process (*13*, *14*).

We have previously shown that the lipid composition of HBsAg particles shows clear differences from those of host cells and the endoplasmic reticulum (ER) membranes where the particles bud (*15*). More than 80% of the total membrane lipid of the HbsAg particles produced by human hepatoma cells is PC, and 1,2-diacyl GPC is preferentially incorporated into HbsAg particles, although the host hepatoma cells contain extremely high concentrations of ether-linked phospholipids. To obtain further insight into the mechanisms of HBsAg particle assembly, we analyzed the lipid composition of the HBsAg particles produced by mouse fibroblast cells (*16*). We also studied the organization of the membrane phospholipids of HBsAg particles by measuring electron spin resonance (ESR) spectra using various spin-labeled fatty acids, and the accessibility of the membrane phospholipids to phospholipase A_2 and PC-specific phospholipid exchange protein. A unique lipid assembly system and new membrane structure model of HBsAg particles will be discussed.

MATERIALS AND METHODS

Cell Line and Culture Conditions—MS128 cells (*16*) were cultured in Dulbecco's-modified Eagle's medium (DMEM, Flow Laboratories) supplemented with 5% fetal calf serum. Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. For the production of HBsAg particles from MS128 cells, the culture medium of the confluent cell layer was replaced with DMEM supplemented with 1% fetal calf serum. With a medium change every 3 days, MS128 cells continuously produce HBsAg particles.

Preparation of HBsAg Particles—HBsAg particles were purified from the culture medium of MS128 cells as described previously (*15*). Briefly, the culture medium was concentrated by press filtration with a membrane filter (molecular weight 100,000 cut-off), and the particles were purified by affinity column chromatography using a monoclonal antibody. In a typical experiment, 1 mg HBsAg particles as protein were obtained from 1.5 liters of culture medium. Western blotting and SDS–polyacrylamide gel electrophoresis of the HBsAg particles preparations from MS128 cells showed them to contain only the polypeptides encoded by the S-gene and preS-2 gene, and no other protein bands were detected (data not shown).

Lipid Analysis—Total lipids in HBsAg particles corresponding to 5 mg protein were extracted by the method of Bligh and Dyer (*17*). 10^8 cells were harvested and washed three times with saline, and total lipids were extracted as described above. The total lipid contents were determined gravimetrically. Dried lipid extracts were dissolved at approximately 20 mg of lipids/ml in chloroform–methanol (2:1, v/v) and stored under nitrogen at –20°C until used for further analysis. Phospholipid, diacylglycerol, triacylglycerol, cholesterol, and cholesteryl ester were separated by thin-layer chromatography (TLC) (DC-Fertigplatten Kieselgel 60, Merk) using *n*-hexane–diethyl ether–acetic acid (70:30:1, v/v) as a developing solvent. Monoacylglycerol was separated by TLC developed with petroleum ether–diethyl ether–acetic acid (50:50:1). After development, the plates were sprayed with a 0.002% primuline solution in acetone–water (5:1, v/v), and the lipid bands were visualized under UV light. The amounts of triacylglycerol, diacylglycerol, and monoacylglycerol were determined from the quantity of fatty acyl moieties. Fatty acids were analyzed as methyl esters by gas chromatography using a glass column packed with 20% EGSS-X on chromosorb WAW (*18*). The methyl ester of 15:0 fatty acid was used as an internal standard. The amounts of cholesterol and cholesteryl ester were determined by the method of Heider and Boyett (*19*).

Subclass Analysis of Phospholipids—Phospholipid subclasses were analyzed as described previously (*15*). Briefly, individual phospholipids were separated by two-dimensional TLC developed in the first direction with chloroform–methanol–acetic acid (65:25:13, v/v) and in the second direction with chloroform–methanol–88% formic acid (65:25:10, v/v). The phospholipids were extracted by the method of Bligh and Dyer and 1-alkenyl-2-acyl, 1-alkyl-2-acyl, and 1,2-diacyl GPC were separated. Briefly, phosphatidylcholine was hydrolyzed with phospholipase C (*B. cereus*) and the resultant diradyl glycerol was extracted and acetylated. Three types of 1,2-diradyl-3-acetyl-glycerols were separated by TLC developed with petroleum ether–diethyl ether–acetic acid (90:10:1, v/v) and then with toluene, and extracted from the plates. The amounts of 1-alkenyl-2-acyl, 1-alkyl-2-acyl, and 1,2-diacyl compounds were estimated from the amount of fatty acids as described previously (*20*).

Determination of the Distribution of Radioactivity in Phospholipids—MS128 cells were maintained for 3 days in DMEM supplemented with 1% fetal calf serum, and then labeled for 13, 24, or 72 h with ^{32}Phosphorus (Amersham; 20 μCi/1 × 10^7 cells). The cells were washed three times with ice-cold Tris-HCl buffered saline (TBS) (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) and scraped into 1 ml/dish of the same buffer. HBsAg particles were purified from the culture medium by affinity column chromatography as described above. Total lipids in cells and HBsAg particles were extracted by the method of Bligh and Dyer (*17*), and the phospholipids were purified by two-dimensional TLC as described above. Spots were located by staining with iodine vapor, scraped into liquid scintillation vials, and the radioactivity was counted (LS5000TD counter; Beckman Instruments, Fullerton, CA).

ESR Measurement of HBsAg Particles, HVJ (Sendai Virus), and Lipid Vesicles Composed of Total Lipids of HBsAg Particles—HVJ was purified as described previously (*21*). Total lipids from HBsAg particles in chloroform

were placed in a test tube and the solvent was removed in a rotary evaporator under reduced pressure. The dried lipid film was dispersed with a vortex mixture in TBS and sonicated for 30 min in a bath type sonicator. Samples of either the purified HBsAg particles (1.5 mg protein/ml), HVJ (1.5 mg protein/ml), or lipid vesicle composed of the total lipids of HBsAg particles in TBS were incubated with 0.1 μg of spin-labeled fatty acids (5-, 7-, 12-, and 16-SLS) for 1 h at room temperature. ESR spectra were recorded with a JEOL JES-RE3X spectometor (X-band, 100 kHz field modulation, 0.2 mT modulation width) equipped with a temperature controller (*22*).

Exchange of PC between HBsAg Particles and Liposomes—A PC-specific exchange protein was purified from bovine liver by sequential column chromatographies through DEAE-cellulose, CM-cellulose, and Sephadex G-50 columns as described by Kamp *et al.* (*23*). The phospholipids in HBsAg particles were radio-labeled by culturing MS128 cells in medium containing the ^{3}H-choline (Amersham; 15 μCi/1 × 10^7 cells) for 3 days, and the HBsAg particles were purified from the culture medium by affinity column chromatography as described above. With this labeling procedure, 93% of the ^{3}H-choline was incorporated into PC and 7% into SM (data not shown). Small unilamellar vesicles (VET50) containing ^{3}H-choline–labeled PC (Amersham) were prepared by the extrusion technique according to the manufacturer's protocol. Briefly, multilamellar vesicles (1 mM in phospholipid concentration) containing PC from HBsAg particles and ^{3}H-choline–labeled PC were prepared and passed three times through a Lipex Extruder (Lipex Biomembranes, Vancouver, BC, Canada) equipped with a 100 nm filter, then filtered through 50 nm filters. Multilamellar vesicles composed of egg yolk PC were prepared in TBS (*24*) and used as acceptor vesicles. The reaction mixture contained 0.16 nmol of phospholipid P from HBsAg particles or VET50, 10 nmol PC from the acceptor vesicle, and various amounts of the exchange protein in 1 ml of TBS. The exchange reactions were carried out at 37°C for various periods. The mixture was chilled on ice and centrifuged (22,000 ×*g*) at 0°C for 20 min to precipitate the acceptor vesicles. An aliquot of the supernatant was prepared for liquid scintillation counting.

Phospholipase A2 Treatment of HBsAg Particles—The HBsAg particles labeled with ^{3}H-choline and VET50 containing ^{3}H-choline labeled PC were prepared as described above. The phospholipid concentration in the stock preparations of both the HBsAg particles and VET50 was 1 μM. Either the HBsAg particles or VET50 (0.4 ml) was mixed with various amounts of phospholipase A_2 from *Naja naja* venom (Sigma) and digested at 37°C for 1 h in TBS containing 5 mM $CaCl_2$. The reaction was stopped by adding 30 μl of 0.1 M EDTA and the lipids were extracted, followed by the separation of PC and lysoPC by TLC. Each spot was scraped into a liquid scintillation vial in order to count the radioactivity as described above.

RESULTS AND DISCUSSION

Lipid Composition of HBsAg Particles—Our previous analysis of the lipid compositions of HBsAg particles and the particle-producing human hepatoma cell lines demonstrated that 1,2-diacyl GPC is the dominant lipid component of HBsAg particles, showing a markedly different phospholipid composition from that of the host cell membranes (*15*). In order to clarify whether this preferential incorporation of phospholipid into HBsAg particles is due to the specific nature of the host hepatoma cells or to the S protein that may direct the assembly of the phospholipids, we analyzed the lipid compositions of HBsAg particles and particle-producing mouse fibroblast cells (MS128 cells) stably transfected with the cloned HVB DNA encoding S protein (*16*). The HBsAg particles from the culture medium of MS128 cells consisted of 22–27 nm particles with a density of 1.21–1.22 g/ml in CsCl and an average lipid to protein ratio of 0.346 by weight (data not shown). These values are consistent with those observed for HBsAg particles produced by the hepatoma cell lines huGK-14 and PLC/PRF/5 (*15*). Table I shows the lipid compositions of HBsAg particles and host cell line MS128 cells. More than 90% of the envelope lipids of the HBsAg particles are phospholipids. Cholesterol accounts for 16% of the lipids of the host cell membrane, but only 3% of lipids in the HBsAg particles. The phospholipid compositions of the HBsAg particles and the host cell line are shown in Table II. In the HBsAg particles, PC is the dominant phospholipid, accounting for more than 80% of all phospholipids, followed by small amounts of PE, SM, and PI. Neither PS nor CL was detected in the preparations. In contrast, the host cells contained largely PC and PE, about 45 and 25%, respectively, along with small amounts of SM, PI, PS, PA, PG, and CL.

Table III shows the subclass compositions of PC from HBsAg particles and the host cell line. In the HBsAg parti-

TABLE I. **Lipid compositions of HBsAg particles and their host cells.**

	MS128		huGK-14*
	HBs particle	Cell	HBs particle
PL	94.5±2.0	73.1±1.3	90.6±4.0
MG	ND	1.5±0.1	ND
DG	0.4±0.1	3.2±1.5	ND
TG	1.6±1.5	6.2±0.3	4.4±2.1
CH	3.1±1.2	16.4±0.7	3.7±1.8
CHE	1.6±0.6	0.3±0.1	1.2±0.4

Total lipids from HBsAg particles corresponding to 5 mg of protein or 10^9 host cells were extracted. Each class of lipids was separated and quantified as described in "MATERIALS AND METHODS." Data are presented as mole % of total lipids and each value represents the mean ± SD of three defferent preparations; ND, not detected. *Results from the previous study (*15*) are shown.

TABLE II. **Phospholipid compositions of HBsAg particles and their host cells.**

	MS128		huGK-14*
	HBs particle	Cell	HBs particle
PC	84.0±1.9	45.2±0.7	83.6±2.4
PE	7.3±1.0	25.3±0.9	7.2±0.6
SM	4.4±1.1	10.0±0.5	2.9±1.9
PI	4.2±0.2	6.8±0.3	5.3±2.5
PS	ND	5.8±0.0	ND
PA	ND	2.3±0.1	ND
PG	ND	0.9±0.5	ND
CL	ND	1.0±0.1	ND
L-PC	ND	ND	ND

Data are presented as mole % of total phospholipids and each value represents mean ± SD of three different preparations; ND, not detected. *Results from the previous study (*15*) are shown.

cles, more than 85% of the PC is 1,2-diacyl GPC, whereas the host cells contain large amounts of ether-linked phospholipids; 33.4 and 3.8% of PC are 1-alkyl-2-acyl GPC and 1-alkenyl-2-acyl GPC. The fatty acid composition of the 1,2-diacyl GPC obtained from the HBsAg particles from MS128 cells is almost identical to that of the host cells; the major components are palmitic (16:0), palmitoleic (16:1), stearic (18:0), and oleic (18:1) acids (data not shown).

As shown in Tables I–III, the lipid compositions of the HBsAg particles from MS128 cells are identical to those of HBsAg particles from huGK-14 and PLC/PRF/5 cells (*15*). These lipid compositions are quite different from those of patient plasma-derived HBsAg particles and the particles produced by Chinese hamster ovary cells and yeast cells (*25*). It is likely that during circulation or the cell culture period, the exchange of lipids between the HBsAg particles and lipoproteins present in either human plasma or the culture medium may cause the discrepancy.

Metabolic Labeling of PC—To confirm further that the PC in HBsAg particles was not incorporated into the particles by lipid exchange or transfer during the purification procedures of the particles, MS128 cells were metabolically labeled with ^{32}P, and the distribution of radioactivity in the phospholipids of the HBsAg particles and host cells was compared. As shown in Table IV, the distributions of radioactivity in the phospholipids of host cells and HBsAg particles correlates well with the phospholipid compositions shown in Table II; 58% of radioactivity of the host cells and 84% that of HBsAg particles was incorporated into PC. The results clearly demonstrate that PC synthesized by the

TABLE III. **Subclass compositions of choline-containing glycerophospholipids of HBsAg particles and their host cells.**

	MS128		huGK-14*
	HBs particle	Cell	HBs particle
1-Alkenyl-2-acyl	1.6±1.0	3.8±0.7	5.1±1.5
1-Alkyl-2-acyl	10.4±1.5	33.4±2.0	1.4±0.4
1,2-Diacyl	85.0±5.3	62.9±2.0	93.6±11.3

The results, expressed as percentage of total PC, are mean±SD of three defferent preparations. *Results from the previous study (*15*) are shown.

TABLE IV. **Incorporation of ^{32}P into PL in HBs particles of MS128 cells.**

	HBs particle	MS128 cell
PC	83.5±2.0	57.2±0.7
PE	9.7±0.8	22.1±0.2
PS	0.9±0.6	4.8±0.9
PI	3.8±0.8	9.5±0.8
SM	2.0±1.2	4.0±1.0
PA	0.1±0.5	2.4±0.2

1×10^7 MS128 cells were cultured with 20 μCi ^{32}P for 72 h. The HBsAg particles released into the culture medium were collected and purified. Phospholipids were separated by two-dimensional TLC and the redioactivity of each spot was determined.

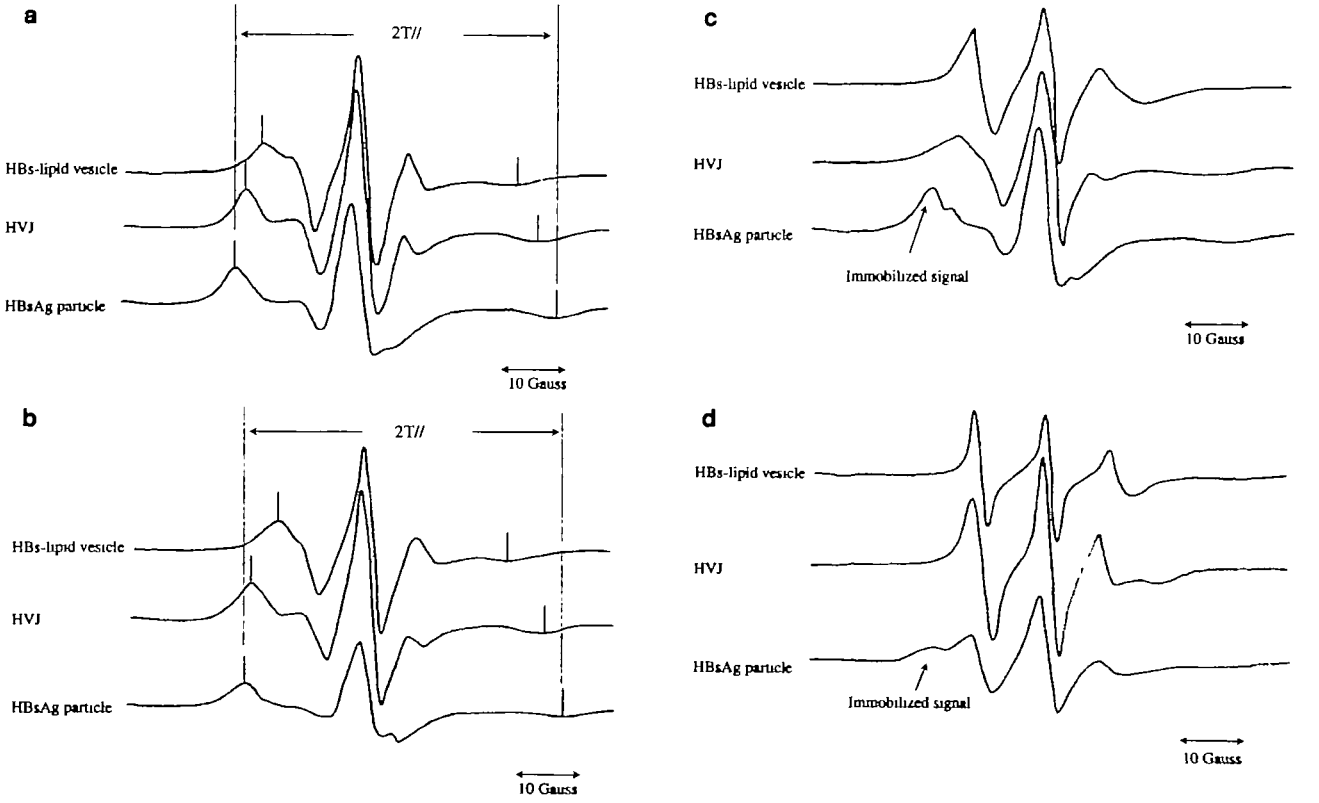

Fig. 1. **Comparison of the ESR spectra of HBsAg particles, HVJ virion, and HBs-lipid vesicles using various spin-labeled fatty acids.** HBs-lipid vesicles were prepared from total lipids of HBsAg particles as described in "MATERIALS AND METHODS." Purified HBsAg particles, HVJ virion, and HBs-lipid vesicles were labeled with either 5-SLS (a), 7-SLS (b), 12-SLS (c), or 16-SLS (d), and ESR spectra were measured at 23°C. For each spectrum, the outer vertical hatch marks indicate 2T//. Arrows in (c) and (d) indicate a typical immobilized signal. Horizontal bar, 10 gauss.

host cells is preferentially incorporated into the HBsAg particles.

These results clearly demonstrate that 1,2-diacyl GPC is preferentially incorporated into HBsAg particles, regardless of the host cells employed to produce the particles. Since the expression of S protein in the absence of viral proteins leads to the formation and secretion of HBsAg particles, it is likely that the assembly process of S protein directs the preferential incorporation of the lipid into the particles. S protein may preferentially interact with PC, or, alternatively, the budding process of the particles may involve a restricted area or domain on the ER membrane (*26*), such as the lumenal leaflet of the ER membrane bilayer where PC has been reported to be enriched (*27, 28*).

ESR Study of the Lipid Organization in HBsAg Particles—To study the molecular motion and organization of the lipids in HBsAg particles, the ESR spectra of various spin-labeled fatty acid probes incorporated into the particles were compared with those of HVJ (Sendai virus) and reconstituted lipid vesicles composed of total lipids from HBsAg particles (HBs-lipid vesicles). Upon incubation of the spin-labeled fatty acids, 5-, 7-, 12-, and 16-spin-labeled stearate (5-SLS, 7-SLS, 12-SLS, and 16-SLS; *N*-oxy-4',4'-dimethyloxazolidine derivatives of either 5-, 7-, 12-, or 16-ketostearate) with each sample at 24°C, the spin-labeled fatty acids readily inserted into the membranes, because no spectrum for probes partitioning into the aqueous buffer was observed (Fig. 1, a–d). The overall splitting values (2T//) of 5- and 7-SLSs observed with the HBsAg particles are significantly higher than those observed with HVJ or HBs-lipid vesicles, suggesting that the mobility of the fatty acyl chains of the HBsAg particles is highly restricted (*22, 29*). For each sample, the peak height ratios were used to assess the mobility of each SLS (Fig. 2a) (*30*). The peak height ratios of the SLSs observed with both HVJ and HBs-lipid particles increased with the increase in the reporter group from the C-terminal of the SLSs, clearly indicating that the fatty acyl chains are more mobile in the interior of the membranes (Fig. 2b). In contrast, no significant increase in the peak height ratio was observed with HBsAg particles, indicating that the movement of the nitroxide that lies in the membrane interior is severely restricted. As indicated by the arrows in Fig. 1, c and d, typical immobilized signals (*31*) were observed for the spectra of 12- and 16-SLS in the HBsAg particles. This immobilized signal was not observed with HVJ or HBs-lipid vesicles. An ESR spectrum showing an immobilized signal is not a ubiquitous feature of lipid bilayers such as liposomes, biomembranes, and other enveloped viruses (*32–34*), but is found when the alkyl chain is bound firmly to proteins such as bovine serum albumin (*35*) and PC-specific exchange protein (*31*).

The present ESR analyses demonstrate the striking difference in the ESR spectra of HBsAg particles and HVJ. Enveloped viruses such as HVJ and influenza virus have been shown to possess membranes acquired during the process of assembly by budding at the cell surface (*8, 36*). The lipid compositions of these viruses reflect the composition of the host cell plasma membranes. ESR studies of these viruses have shown that the viral lipids are arranged in a bilayer structure and that the organization of the membrane lipids is not affected by the presence of the envelope proteins, suggesting that the majority of the lipids are in a fluid state, not associated with the envelope proteins (*32–34*). In contrast, ESR spectra obtained for the HBsAg particles show that the mobility of lipids in the particles is extraordinarily restricted, and this restricted movement is more evident using probes that lie in the membrane interior. Judging from the fatty acid composition of the lipids of HBsAg particles, this immobilization of the methyl terminus of the fatty acids is likely to be caused by interaction

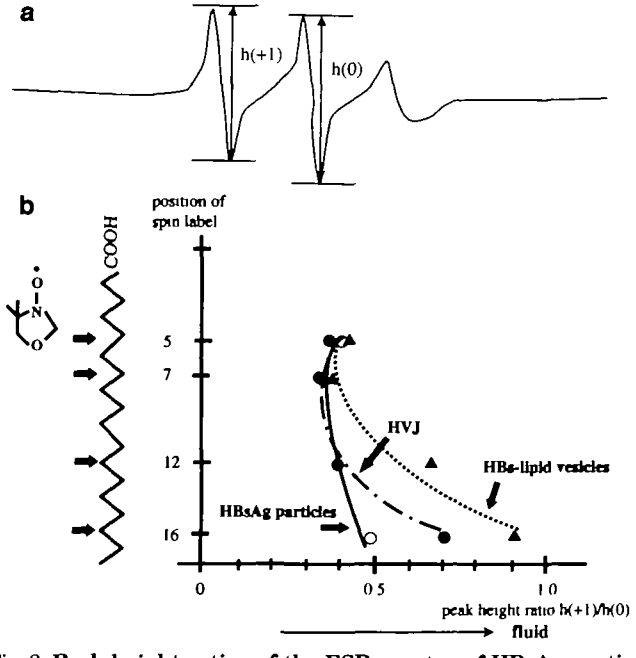

Fig. 2. **Peak height ratios of the ESR spectra of HBsAg particles, HVJ virion, and HBs-lipid vesicles labeled with various spin-labeled fatty acids.** Peak height ratios indicate the values of h(+1)/h(0) (a). The peak height ratios are sensitive to membrane fluidity and lie between 0 and 1 (*30*). From the ESR spectra of HBsAg particles (●), HVJ virion (▲), and HBs-lipid vesicles (○) labeled with various spin-labeled fatty acids, h(+1)/h(0) values were plotted against the positions of the spin label in stearate (b).

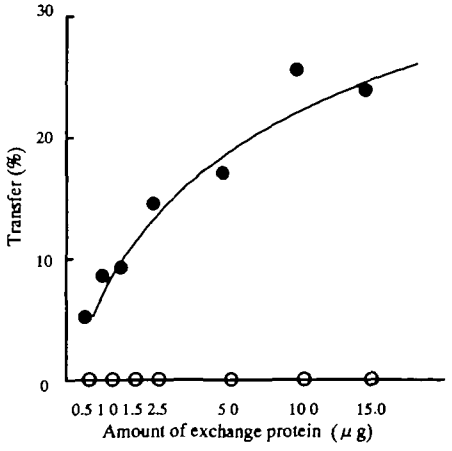

Fig. 3. **Accessibility of PC in HBsAg particles to the PC exchange protein.** The exchange of PC between HBsAg particles and multilamellar vesicles (○), VET50 and multilamellar vesicle (●) in the presence of various amounts of purified PC exchange protein was measured after incubation at 37°C for 60 min.

with S protein, not by lipid-lipid interactions, suggesting that the lipids in the HBsAg particles are not organized in a typical lipid bilayer such as those of other enveloped viruses.

Accessibility of Phospholipids to PC-Specific Exchange Protein and Phospholipase A2—The spatial arrangements of lipids in biomembranes, lipoproteins, and enveloped viruses have been studied by measuring their sensitivities to phospholipases and exchange proteins (*37–39*). To obtain further insight into the lipid organization of HBsAg particles, we examined the sensitivities of PC in HBsAg particles to PC-specific exchange protein and phospholipase A_2. In these experiments, the PC in HBsAg particles was metabolically labeled with ^{3}H-choline, and small unilamellar vesicles prepared by extruding the vesicles through filter papers with a pore size of 50 nm (VET50) were used as a control bilayer membrane. In HBsAg particles labeled with ^{3}H-choline, 93 and 7% of the ^{3}H-choline was incorporated into PC and SM, respectively (data not shown). The HBsAg particles and VET50 were used as donor membranes, and multilamellar vesicles composed of PC was used as an acceptor membrane. As shown in Fig. 3, the PC-specific exchange protein purified from bovine liver exchanged more than 30% of the PC in VET50 during the assay period, while no PC in the HBsAg particles was exchanged even at higher concentrations of the exchange protein. No exchange of PC in the HBsAg particles was observed even after prolonged incubation at higher concentrations of exchange protein (data not shown). The results indicate that the PC in the HBsAg particles is not accessible to the PC-specific exchange protein.

A family of phospholipid exchange proteins exchanges lipids between various membranes including biological membranes, artificial lipid vesicles, and serum lipoproteins (*37*). The phospholipid exchange protein purified from bovine liver exchanges PC present on the outer leaflet of bilayer membranes. The PC-specific exchange protein was also used to study the transbilayer distribution of PC in the envelope of influenza virus (*38*, *39*). The results showed that PC exists in two pools in the membrane: about half the PC is readily exchangeable, while the remaining half is nonexchangeable or only very slowly exchangeable. The authors suggested that the exchangeable pool corresponds to the PC present in the outer surface of the membrane bilayer. In contrast, our results show that PC in the HBsAg particles is inaccessible to the PC-specific exchange protein. The PC-specific exchange protein did not accelerate the exchange of phospholipid between intact red blood cells and liposomes, while it readily exchanged PC in sealed ghosts, suggesting that some structural change in the membrane may make PC accessible to the exchange protein (*40*). Since the present ESR spectral analyses of HBsAg particles indicate that the lipids are highly immobilized in the particles, it is likely that the lack of PC exchange by the HBsAg particles is due to the immobilization of the PC molecules in the particles, which may not allow complex formation with the exchange protein.

As shown in Fig. 4, up to 55% of the PC in VET50 is degraded by phospholipase A_2, while almost all the PC in the HBsAg particles is degraded by the enzyme. Aggerbeck *et al.* showed that phospholipase A_2 is able to hydrolyze all the PC, PS, and PE in human serum low density lipoproteins (LDL) (*41*). Based on the precise kinetic analyses of lipid hydrolysis the authors concluded that the phospholipase A_2–sensitive phospholipids are located on the surface of the LDL particles. Although further detailed and precise kinetic analyses, as well as the effect of lipid hydrolysis on the structural integrity of HBsAg particles, is needed, the present results suggest that the majority of PC is located at the surface of the HBsAg particles and that the lipids of the

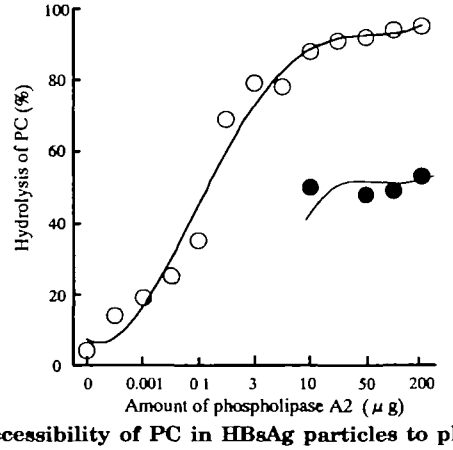

Fig. 4. **Accessibility of PC in HBsAg particles to phospholipase A_2.** Hydrolysis of PC in HBsAg particles (○) and VET50 (●) by various amounts of phospholipase A_2 was measured after incubation at 37°C for 60 min.

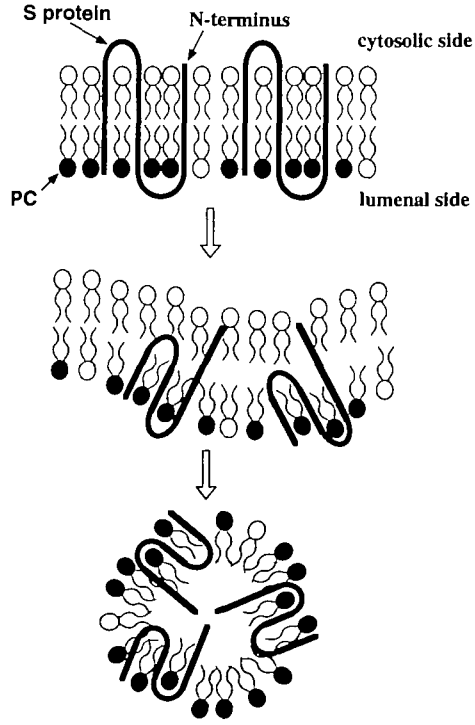

Fig. 5. **Schematic model of HBsAg particle formation.** The lumenal and cytoplasmic sides of the membrane are indicated. S protein has three transmembrane domains and the C-terminal region locates in the lumenal side of the membrane (*13*, *14*). PC is suggested to be enriched in the lumenal half of the bilayer membrane (*27*, *28*). During the budding process of HBsAg particles into the lumenal side of the membrane, PC molecules in the lumenal side are preferentially incorporated into the particle.

HBsAg particles may not be organized in a typical bilayer structure.

In this study we demonstrated that PC is the dominant component of HBsAg particles, and that the lipid organization HBsAg particles differs significantly from those of other enveloped viruses such as HVJ and influenza virus. We propose that PC in the particles is not aligned in a lipid bilayer configuration, but is located on the surface of the particles in tight association with S protein. Electron microscopic studies have shown that the site of HBsAg formation is the ER membrane where particles bud into the lumenal side of ER (*26*). Since PC is suggested to be enriched in the lumenal half of the bilayer of the ER membrane (*27, 28*), it is intriguing to speculate that the formation of HBsAg particles may involve the lumenal leaflet of the ER membrane. Based on recent analyses of the transmembrane topology of S protein (*13, 14*), a model for the formation of HBsAg particles is shown in Fig. 5.

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