Apical Secretion of Hepatitis B Surface Antigen from Transfected Madin-Darby Canine Kidney Cells*

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Hepatitis B surface antigen (HBsAg), the major envelope component of human hepatitis B virus, during infection drives the assembly and basolateral secretion from hepatocytes of both virions and subviral lipoprotein particles into the bloodstream. We studied the sorting behavior of HBsAg in the heterologous epithelial Madin-Darby canine kidney cells permanently transformed with the hepatitis B virus S gene. These cells, forming tightly packed monolayers in permeable supports, secreted HBsAg apically through a mechanism not involving transcytosis. This suggests that molecular features acting as apical addressing information, seemingly unfunctional or less efficiently used by the exocytic machinery of hepatocytes, could be contained in short hydrophilic regions of HBsAg. Lipids also could play a role in this asymmetric sorting because HBsAg is known to be secreted by forming macromolecular lipoprotein complexes rather than as a soluble protein. Together with available data, our results would imply not only the existence of tissue-specific variations in handling constitutively secreted proteins but also that these variations are strikingly dependent on the kind of protein examined. On the other hand, pulse-chase experiments with tunicamycin showed that the expression of apical information in HBsAg particles does not require N-linked glycosylation, contrasting with the known gp80 Madin-Darby canine kidney-endogenous apical secretory marker. This is the first experimental evidence that carbohydrate moieties in secretory proteins do not hold domain-specific sorting signals, a fact previously shown exclusively for membrane proteins. Thus, HBsAg provides a novel model system for the analysis of the molecular mechanisms of constitutive apical secretion.

Polarized epithelial cells form barriers between external milieu compartments and the internal environment of the organism, functionally connecting these compartments through the vectorial processes of transport, absorption, and secretion. To accomplish their physiological role, these cells exhibit a plasma membrane divided by tight junctions in apical outward facing and basolateral inward facing domains.

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Key points:
- HBsAg is a highly hydrophobic 226-amino acid protein synthesized as a transmembrane intermediate in hepatocytes and MDCK cells.
- HBsAg is secreted into the bloodstream as the hepatitis B surface antigen (HBsAg) encoded by the S gene of the hepatitis B virus (HBV).
- HBsAg is involved in the assembly and secretion of viral particles.
- The polarity of HBsAg secretion was assessed in permanent transfectants of MDCK cells.
- Pulse-chase experiments were performed to assess the effect of tunicamycin treatment on cell monolayers over nitrocellulose filters.
- The sorting behavior of HBsAg in MDCK cells was studied using a standard approach.

Materials and Methods:
Expression Plasmid Encoding HBsAg—Standard recombinant DNA techniques were followed (41). A Poull/BamHI, 3850-kilobase fragment carrying the HBsAg coding region (S gene) flanked by the early promoter and polyadenylation site from SV40 was isolated from plasmid LSV-HBsAg, kindly provided by Dr. P. Valenzuela (Chiron Co., Berkeley, CA). This fragment was inserted between the Poull/BamHI sites of a pSP64 vector and then removed with HindIII/BamHI for its final ligation into the corresponding sites of a pSV2-derived mammalian expression vector that contains the bacterial neomycin-resistance gene under control of the herpes simplex virus thymidine kinase promoter (kindly provided by Drs. D. Sabatini and T. Gottlieb, New York University Medical Center, New York).

Permanent Transformants of MDCK Cells—MDCK (strain II) cells were maintained in minimum Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Bioproducts for Science, Inc., IN), 200 units/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), and fungizone (Squibb). Permanent transformants were obtained after G418 (GIBCO) selection. The screening for HBsAg expression was made by incubating the cells for 12-24 h with 150 mg/ml sodium butyrate followed by metabolic labeling of the cell monolayers with 35S-methionine from the medium, and analysis by SDS-polyacrylamide gel electrophoresis and fluorography. Details of these procedures, including the G418 selection conditions, have been previously described (5, 42, 43).

Polarity Assessment of HBsAg Secretion—Cells grown to confluence in 75-cm² flasks were plated at a density of about 10⁶ cells either over nitrocellulose membranes (0.45-μm pore size) in 30-mm Millicell-HA chambers (Millipore Corp., Bedford, MA) or over Nucleopore polycarbonate membranes (0.4-μm pore size) in 24.5-mm Transwell chambers (Costar Corp., Cambridge, MA) after being incubated overnight in 20% horse serum to block the luminal surface. Unattached cells were removed after 8 h, and the released medium was supplemented with 30% fetal bovine serum. Sodium butyrate (10 mM) was added after a transepithelial electrical resistance higher than 100 ohms/cm² was achieved, usually 2-3 days after plating, as measured with an EVOM electrometer (World Precision Instruments, UK). Sodium butyrate was maintained for 16 h before and during the metabolic labeling of the cells, which was carried out by adding 1.3 ml of methionine-free Medium Eagle's medium containing 1% methionine to the basolateral chamber and 1 ml of unlabeled medium to the apical chamber, both supplemented with 30% fetal bovine serum. After metabolic labeling, apical and basolateral compartments were collected separately, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (5, 42, 43) using radioactive molecular mass standards (Bethesda Research Laboratories). Fluorograms were developed on preflashed Kodak AR X-Omat films (Eastman), and scanning densitometry was performed in a Shimadzu model CS-9000 (Shimadzu Scientific Instruments, Inc., Japan) densitometer.

Transient Expression of rGH—The previously obtained neomycin-resistant MDCK cells were additionally transfected in 75-cm² flasks with an expression plasmid bearing the rGH under the control of the SV40 early promoter using previously described procedures (5). One day after transfection, 10⁶ cells were plated into Millicell chambers, treated with sodium butyrate for 12 h, and 48 h after plating, the polarity of HBsAg and rGH secretion was assessed as described in the previous section.

Pulse-Chase Experiments—Pulse-chase experiments were made in 35-mm plastic dishes by preincubating the cells for 1 h in methionine-free medium and then for 30 min with 10 μCi/ml [35S]methionine (Du Pont-New England Nuclear). After each chasing period, in the presence of 100-fold excess methionine (3 mg/ml), HBsAg was immunoprecipitated from cell extracts and medium using a rabbit polyclonal antibody raised against HBsAg particles produced in recombinant yeast (44).

Discussion:
To study the sorting behavior of the HBsAg in MDCK cells, we followed a standard approach (5, 42). The S gene of the HBV was first inserted into an expression vector (depicted in Fig. 1), transfected into MDCK cells, and then permanent transformants that express and secrete HBsAg were obtained by selection in G418. Incubation with sodium butyrate was needed to achieve detectable levels of expression, as already
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FIG. 1. HBsAg expression vector. The HBsAg coding region (S gene) flanked by the early promoter and polyadenylation site of SV40 was cloned into a pSV2-based plasmid containing the bacterial neomycin-resistance gene under control of the herpes virus thymidine kinase (TK) promoter as illustrated. For the convenience of the cloning strategy, two SV40 promoters were placed in tandem.

FIG. 2. Expression and secretion of HBsAg in permanent transformants of MDCK cells. MDCK cells stably transformed with the pSV2-HBsAg-TKNeo plasmid and grown in plastic dishes were labeled for 8 h with 125 gCi/ml [35S]methionine in methionine-free medium, either untreated (lane 1) or pretreated (lane 2) for 12 h with 10 mM sodium butyrate to enhance the expression. HBsAg was immunoprecipitated from the medium and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Two bands representing unglycosylated (p24) and glycosylated (gp28) HBsAg were detectable only after pretreating the cells with sodium butyrate.

observed for other cDNAs expressed under the control of the SV40 early promoter in these cells (42, 43). Immunoprecipitation from the medium of metabolically labeled cells pretreated with butyrate showed secretion of the characteristic unglycosylated (p24) and glycosylated (gp28) forms of HBsAg (Fig. 2). Pulse-chase experiments revealed that about 90–95% of newly synthesized HBsAg is secreted in 6–8 h (Fig. 3), showing efficient secretion, although at a lower rate than that reported for the 81-kDa endogenous protein (5).

To assess the polarity of HBsAg secretion, the cells were grown over permeable filter supports in Millicell chambers that allow a separate analysis of products released either apically or basolaterally. To our surprise, a predominant apical release of HBsAg was found (Fig. 4) with no more than 10–15%, and most frequently less than 5%, of the HBsAg being basolaterally detected in different experiments. The incubation period of 8 h was long enough for HBsAg to cross the protein-saturated filter, as judged by the relatively rapid equilibrium observed between both chamber compartments after opening the tight junctions by EDTA during the last hour of labeling (Fig. 4, lanes 3 and 4). Similar results were obtained in different colonies of transformant MDCK cells as well as attaching the monolayers to polycarbonate filters of Transwell Costar chambers (not shown). The possibilities that either basolateral secretion followed by transcytosis or a preferential degradation in the basolateral compartment could account for the apical appearance of HBsAg were discarded. In fact, previously labeled HBsAg placed in the basolateral compartment was almost entirely recovered by immunoprecipitation after 8 h of incubation (lanes 5 and 6). Thus, there was no detectable transcytosis, and significant degradation of the protein was not observed as judged by the recovery.

It has been shown that rGH is secreted in a nonpolarized fashion from transfected MDCK cells during transient expression experiments (5). To estimate the fluid phase transit of the apical and basolateral pathways and to determine whether our permanent transformants of MDCK cells still maintain the capability of basolateral secretion, we performed similar experiments. The described vector bearing rGH cDNA (5) was overtransfected into HBsAg transformant MDCK cells, and the simultaneous secretion of both rGH and HBsAg was assayed by immunoprecipitating each protein in tandem. A clear difference in the secretion pattern of these proteins was observed with a relatively nonpolarized secretion of rGH. As seen in Fig. 5, less than 5% of HBsAg versus approximately 40% of the rGH was found basolaterally.
were overtransfected with a rGH expression plasmid (5), excluding the possibility that our transformant cells had lost the ability for basolateral secretion.

MDCK cells have two co-existing secretory pathways, one directed to the apical and the other to the basolateral plasmalemma (5, 11, 17). A number of exogenous secretory proteins, including rGH, expressed after transfecting these cells with appropriate plasmids (5, 12), are secreted in roughly equal amounts through both cell surface domains. This should occur with proteins that either do not express any sorting information for polarity or possess tissue-specific addressing information nonfunctional in MDCK cells, thus being secreted through both cell surfaces at random, in amounts proportional to their default partition (not signal-mediated) within the bulk phase of each pathway. Instead, the apical secretion of HBsAg most probably reflects that specific sorting information, remaining to be identified, is being intracellularly deciphered, presumably at the trans-Golgi network (3), and actively conducts this protein into a selected secretory pathway through a receptor(s)-mediated process, as has been suggested for the endogenous MDCK apical secretory marker (5).

Previous examples of exogenous secretory products apically released in transfected MDCK cells had been restricted to the ectoplasmic domains of several plasma membrane proteins. In fact, soluble anchor-minus versions of the transmembrane proteins aminopeptidase (38), polymeric immunoglobulin receptor (37), and neutral endopeptidase (36) and of the glycosyl phosphatidylinositol-linked proteins Thy-1 (39) and placental alkaline phosphatase (40) are all secreted preferentially apical, though in some cases with less than 80% polarity (37, 40) when expressed in MDCK cells. This, together with observations made using chimeric proteins (46, 47), suggests that the ectodomain of apical membrane proteins expresses targeting information, and, therefore, secretory and plasma membrane proteins destined to the apical cell surface could share sorting events taking place within a luminal compartment of the exocytic route, probably at the trans-Golgi network and maybe involving the same receptor. For transmembrane proteins, though, sorting events can also be directed from the cytosolic compartment as suggested by the influence of cytosolic-specific sequences in basolateral sorting of several plasma membrane proteins (48–52). Some transmembrane apical proteins could also require, in some yet unclear way, their anchoring and/or cytosolic segments for proper sorting. For instance, a truncated soluble form of the apical marker, influenza hemagglutinin, is secreted unpolarized in transfected MDCK cells (42), although this observation disagrees with that of other investigators (46). There is usually uncertainty about the characteristics that could be acquired by secretory products derived through genetically engineering the cDNA of plasma membrane proteins. Also, it is still unclear whether plasma membrane and secretory proteins follow the same exocytic pathways (4, 53). The biogenesis of HBsAg particles includes a transmembrane precursor in the endoplasmic reticulum (29, 30); however, this is a natural process that more likely should lead to a product conserving the native characteristics of endogenous substrates for constitutive secretory pathways, especially those forming lipoprotein particles.

The opposite polarity by which HBsAg was secreted from transfected MDCK cells compared with the basolateral release known to occur from infected hepatocytes to the bloodstream (25) was not foreseen. Former evidence led to the belief that a direct apical exocytic pathway does not exist in hepatocytes and that bile-secreted proteins would be mainly...
transported by transcytosis after bulk phase or receptor-mediated basolateral endocytosis from the plasma or through a paracellular route (4, 19, 20). In challenging this view, Sassano and Palade (18) have recently presented evidence suggesting that the major source of bile albumin is newly synthesized albumin directly secreted from hepatocytes. A direct apical secretory pathway seemingly of considerable transporting capacity, as judged by the biliary albumin concentration, would, therefore, be functional in these cells. However, only very small amounts of HBsAg have been detected by radioimmunoassay in bile during acute HBV infection and in chronic carriers (54).

A prominent basolateral exocytic pathway would not require special addressing information to produce asymmetric secretion as has been shown experimentally by expressing rGH (6) and the ε light chain (55) in the polarized cell line, Caco-2. Similarly, in hepatocytes, as a current assumption sustains (4), a default mechanism of protein segregation could determine the predominant sinusoidal secretion of newly synthesized secretory proteins that do not hold specific sorting information. This notion is compatible with the unpolarized secretion of the hepatic form of α-galactosidase previously observed in MDCK cells (5). However, the apical secretion of HBsAg in MDCK cells suggests that distinctly secreted proteins, while sharing similar polarity in hepatocytes, can otherwise be handled in completely different fashions by other epithelial cells and that some of the endogenous hepatic proteins could hold addressing information by becoming functional in the heterologous cellular systems. Strikingly, HBsAg seems to mimic the sorting behavior of the kidney bile and hepatic forms of the gp80 protein (21), all of them bearing special molecular features to interact with lipids. In fact, the gp80 apical secretory protein of MDCK cells has as homologous proteins SP-40,40 and apolipoprotein-J of human plasma, produced and basolaterally secreted by hepatocytes (21–23). Apolipoprotein-J is a component of a subclass of high density lipoproteins bearing apoA-I, and the SP-40,40 protein was identified forming complexes with complement components, presumably through hydrophobic interactions (22, 23). It could be that elements featuring apical information in this kind of protein are not functional in hepatocytes, or in these cells, are less efficiently recognized or overridden by interactions with other basolaterally addressed proteins. Perhaps, in hepatocytes, intracellular associations with apolipoproteins, such as apoA-I, might command the basolateral release of apol and HBsAg. It would be of great interest to test the secretion of HBsAg in Caco-2 cells because these cells produce lipoproteins and are thought to display similar sorting characteristics with hepatocytes, as none of their endogenous secretory proteins have been found to be secreted preferentially to the apical pole (6).

The functional meaning of the HBsAg sorting characteristics for the HBV life cycle remains to be elucidated. Envelope glycoproteins of several viruses emulate endogenous substrates of constitutive (nonregulated) polarized exocytic routes as part of the viral strategy for exiting from infected epithelial cells toward external or internal body fluids (43, 56, 57). Hepadna viruses are not strictly hepatotropic. Evidence exists of epithelial cell infection by these viruses in pancreas and kidney (58–60), and bile duct epithelium (61). In human carriers, low amounts of HBsAg and/or viral DNA have been detected in apically derived secretions such as milk (62), pancreatic juice (54), and semen and saliva (63), the last two seemingly important in non-parenteral HBV transmission. The presence of HBsAg in these fluids has been generally attributed to either transepithelial transport from the blood (64) or blood leakage from the intravascular space (63). However, the structural features driving HBsAg into specific exocytic pathways also may play a role in extraphepatic tissues as part of the mechanism of viral exit from the organism.

The only moieties known so far as functional as apical targeting information are glycosylphosphatidylinositol anchored, a special class of membrane proteins to the bilayer (65). The presence of such a signal in HBsAg can be practically excluded because glycolipids were not detected in a detailed analysis of highly purified subviral particles (28). We found that inhibition of N-glycosylation by tunicamycin does not affect the HBsAg-polarized secretion. Because tunicamycin treatment produces a loss of polarity in the previously known apical constitutive secretory marker, the gp80 protein (12), this result constitutes the first formal evidence that oligosaccharides do not hold sorting information in secretory proteins, a fact previously shown for plasma membrane proteins only (2, 66, 67). This observation would also argue against the possibility that HBsAg was apically addressed by interacting with gp80 in MDCK cells.

The apical sorting determinants of HBsAg and the gp80 could differ in their N-linked carbohydrate dependence for functional expression. For instance, the active information could reside in a particular peptide conformation stabilized upon glycosylation in the gp80 protein but not in the HBsAg. It seems less likely that the two different apical signals could exist in these two proteins and both be recognized by the same cell. It is possible that lipid association properties, for instance prompting the interaction with the membrane of apical exocytic vesicles that are likely to be enriched in glycosphinogilipids (3), could be the important sorting determinant in both gp80 and HBsAg proteins. On the other hand, if the recognition would occur in an aqueous environment, a small hydrophilic region of 30–40 amino acids between two hydrophobic regions (68) presumably facing the surface of subviral HBsAg particles (28, 69) could be suitable for holding a sorting signal. Mutants of this protein should enable a test of this possibility. Finally, because HBsAg particles are biotechnologically produced in large amounts (44), this viral glycoprotein could also provide a convenient ligand system for attempting the detection of hypothetical intracellular receptors mediating apical protein segregation.

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