Intracellular Assembly and Packaging of Hepatitis B Surface Antigen Particles Occur in the Endoplasmic Reticulum

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Received 7 November 1985/Accepted 8 March 1986

Hepatitis B surface antigen (HBsAg) particles are secreted by Chinese hamster ovary cells that are stably transfected with the S gene of hepatitis B virus. The assembly of HBsAg into cylindrical and spherical particles occurred intracellularly within the endoplasmic reticulum. HBsAg particles accumulated within large dilated areas of the endoplasmic reticulum and remained within these structures for most of the time prior to secretion from the cells. Once the particles were formed, the HBsAg polypeptides did not appear to become associated with subsequent intracellular organelle membranes or the plasma membrane. HBsAg within the dilated structures did not bind wheat germ agglutinin, indicating that its oligosaccharide chains had not yet been processed to the complex form (containing terminal sialic acid-*N*-acetylglucosamine residues). The oligosaccharide chains of HBsAg are processed to the complex form and can be detected on the HBsAg after secretion, but this event was not detected within cells. In addition, HBsAg was not observed on the cell surface by indirect immunofluorescence or immunoprecipitation, although immunoelectron microscopy revealed some staining at or near the cell surface. These results suggested that HBsAg was either secreted from cells without being incorporated into the plasma membrane, or that the levels of HBsAg in the plasma membrane were below the limits of detection.

During a hepatitis B virus infection, two types of particles containing the hepatitis B surface antigen (HBsAg) are observed in the bloodstreams of patients (2). Infectious virions called Dane particles are composed of a nucleocapsid containing the hepatitis B core antigen surrounded by a lipoprotein envelope containing the HBsAg. Cylindrical and spherical forms of Australia antigen or 22-nm particles are composed of only the HBsAg lipoprotein envelope. Prior to recombinant DNA technology, study of the biosynthesis of these particles was limited to the use of liver explants from infected patients or chimpanzees. Several electron microscopic studies suggested that structures resembling cylindrical and spherical forms of HBsAg appeared to reside within the endoplasmic reticulum (ER) (8, 19, 37, 38). Mature Dane particles appeared to form when the nucleocapsid migrated from the nucleus and obtained its lipoprotein envelope by budding into the lumen of the ER. The mechanism for secretion of the cylindrical and spherical HBsAg and Dane particles was not known (1, 8, 19, 37, 38).

Mammalian cells transfected with only the S gene encoding HBsAg also secrete cylindrical and spherical particles of HBsAg (6, 7, 22, 24, 26). These particles, composed of both the glycosylated and unglycosylated HBsAg polypeptides characteristic of natural particles in infected plasma (29, 30), are secreted with a half-time of approximately 5 h from cells (28). This is considerably slower (2 to 15 times) than the rate of transport of other membrane and secretory proteins to the cell surface (9, 10, 17, 21, 23). In addition, the bulk of intracellular HBsAg contains only high-mannose oligosaccharide chains, whereas the secreted HBsAg contains only the complex form. Virtually all of the intracellular HBsAg,

therefore, has not been processed by the Golgi complex, but glycosylation presumably occurs immediately prior to secretion (28). In the present study we used a combination of indirect immunofluorescence, electron microscopy, and immunoelectron microscopy to define the cellular location of the HBsAg particle assembly.

MATERIALS AND METHODS

Cells and radiolabeling. The growth and radiolabeling of Chinese hamster ovary (CHO) DHFR⁻ and CHO 21.8 cells expressing HBsAg were described previously (28).

Antisera. Guinea pig antibodies to glutaraldehyde-fixed HBsAg (anti-gfHBs) were produced by incubation of 0.1 ml of HBsAg (4 mg/ml) with 0.015 ml of 16% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) and 0.006 ml of 1% glutaraldehyde (Electron Microscopy Sciences) for 1 h on ice. The mixture was subsequently diluted with 1 ml of phosphate-buffered saline (PBS; 0.14 M NaCl, 0.003 M KCl, 0.016 M phosphate [pH 7.2 to 7.4]) and dialyzed overnight against 500 ml of PBS at 4°C. The fixed HBsAg was emulsified with an equal volume of Freund adjuvant (GIBCO Laboratories, Grand Island, N.Y.), and two animals each received five 0.1-ml intradermal injections along their backs and one intramuscular injection of 0.25 ml in the quadriceps. The injections were given at 0, 2, and 4 weeks, with Freund complete adjuvant for the initial injection and Freund incomplete adjuvant for subsequent injections.

Anti-gfHBs was affinity purified on a column of HBsAg coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Inc., Piscataway, N.J.). Antiserum (8 ml) was passed through the column twice. The column was washed with PBS and then with two cycles of 0.5 M NaCl-0.2 M Tris

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(pH 8.5) and 0.5 M NaCl–0.2 M sodium acetate (pH 4.8). The anti-gfHBs was eluted with 3 M KSCN and dialyzed against PBS.

Immunoprecipitation. Immunoprecipitations were performed as described previously (28), except for immunoprecipitation of HBsAg on the cell surface. In this case, guinea pig anti-HBs was added to intact cells suspended in PBS, and the mixture was incubated for 1 h at 4°C. The cells were washed five times in PBS containing 1 mg of bovine serum albumin per ml (PBSA) and incubated for 1 h in PBSA at 4°C. A postnuclear supernatant containing plasma membranes (20) was prepared (28) and divided into two aliquots. One aliquot received additional anti-HBs, and the other received normal guinea pig serum. The samples were immunoprecipitated and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (28).

Immunofluorescence. Cells grown on cover slips were either (i) fixed and permeabilized for cytoplasmic immunofluorescence with methanol or (ii) fixed with paraformaldehyde and subsequently permeabilized with Nonidet P-40 (NP-40; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) or saponin (Eastman Kodak Co., Rochester, N.Y.). (i) After being washed three times with PBS, cells were incubated on ice with 50% methanol for 2 min, then with 100% methanol for 5 min, and then with 50% methanol for 2 min. The cells were washed three times with PBS and incubated for 30 min at 37°C with guinea pig anti-HBs antisera diluted in PBS containing 10% fetal bovine serum. Prior to incubation, the guinea pig antisera was centrifuged in an Airfuge (Beckman Instruments, Inc., Fullerton, Calif.) at 20 lb/in² for 15 min. The cells were washed three times with PBSA and incubated in PBSA for 1 h at room temperature. The cells were incubated for 30 min at 37°C with fluorescein-conjugated goat anti-guinea pig immunoglobulin G F(ab')₂ fragments (Cappel Laboratories, Cochranville, Pa.) diluted in PBS containing 10% fetal bovine serum. The cells were washed three times in PBSA and incubated overnight in PBSA prior to being mounted.

(ii) Cells permeabilized with NP-40 or saponin were first fixed with 3.7% paraformaldehyde for 30 min at room temperature. The cells were washed three times with PBS and permeabilized with 0.2% NP-40 or 0.05% saponin in PBS. NP-40-permeabilized cells were washed three times with PBSA, and saponin-permeabilized cells were washed three times with 0.02 M glycine in PBS. All subsequent incubations and washes were as described for the methanol-treated cells.

Cell surface immunofluorescence was performed as described for cytoplasmic immunofluorescence; however, the cells were fixed with paraformaldehyde and the methanol or detergent permeabilization steps were eliminated.

Tissue processing for plastic embedding. Confluent monolayers of 21.8 cells were fixed on petri dishes in Karnovsky fixative (3% glutaraldehyde, 1.6% paraformaldehyde, 0.1 M sodium cacodylate [pH 7.4], 0.05% CaCl₂) (M. J. Karnovsky, J. Cell Biol. 27:137A–138A, 1965) for 1 h at 4°C. Cells subsequently were scraped from the petri dishes and sedimented in an Eppendorf microfuge. Cell pellets were first treated with 1% osmium tetroxide (1 h) in veronal-acetate buffer (pH 6.0) and then dehydrated in ethanol and processed for plastic embedding (EMbed 812; Electron Microscopy Sciences). After being sectioned, the cell pellets were stained with 10% uranyl acetate and then by 1% lead citrate in distilled water.

Electron microscope immunocytochemistry. Confluent monolayers of 21.8 cells were fixed in a mixture of 2%

paraformaldehyde and 0.2% glutaraldehyde in PBS for 1 h at 4°C. Cells were then washed in cold PBS, scraped from tissue culture plates, sedimented in Eppendorf tubes, and suspended in 10% gelatin in PBS (37°C). After repeated pelleting at 37°C, the gelatin was allowed to harden on ice. The gelatin-embedded cells were postfixed (overnight) with 2% paraformaldehyde–0.2% glutaraldehyde in PBS to allow gelatin fixation. The cell-containing pellet was cut into 1-mm³ blocks and stored in 8% paraformaldehyde–PBS containing 1 M sucrose. Prior to being cryosectioned at –90°C, blocks were immersed in 2.3 M sucrose in PBS. Cryosectioning and immunoincubation procedures were carried out as described previously (5, 16, 31). Cryosections were immunoincubated for single labeling with anti-gfHBs (50 µg/ml) and then with protein A–gold conjugates (8 nm diameter). For double labeling, protein A–gold conjugates (5 nm diameter) were used to label HBsAg. Subsequent incubations for double labeling were performed in the following sequence: protein A (50 µg/ml; Pharmacia Fine Chemicals) mucin (0.5 mg/ml; Sigma Chem. Co., St. Louis, Mo.) (16), lectin (wheat germ agglutinin [WGA], 40 µg/ml or *Ricinus communis* agglutinin, 40 µg/ml; Vector Laboratories, Burlingame, Calif.), anti-lectin antibodies (α-WGA, α-RCA; Vector Labs), and 8- to 12-nm protein A–gold conjugates. Between all steps, the sections were extensively washed in PBS–0.02 M glycine buffer. The immunoincubated sections were next stained with uranyl acetate (neutral and acidic) and embedded in 0.75% methyl cellulose in distilled water (Tylose M-H 300; Fluka Chemical Corp., Hauppauge, N.Y.).

RESULTS

Construction of cell lines. We constructed a plasmid capable of expressing high levels of HBsAg in stable mammalian cell lines to study the biosynthesis of HBsAg. This was accomplished by modifying plasmid p342E (6) so that a dihydrofolate reductase (DHFR) cDNA was joined to hepatitis B virus sequences at a *TaqI* site situated 100 base pairs downstream of the polyadenylation site of the HBsAg gene (34), creating plasmid pEHD22 (29). It should be noted that the DHFR cDNA is not directly preceded by a promoter element. Expression of the DHFR gene is dependent upon transcripts extending through the HBsAg gene, which appears to maximize the amount of HBsAg produced.

Plasmid pEHD22 was introduced into CHO cells deficient in DHFR (36) by the calcium phosphate coprecipitation technique (14) as described in reference 35. A total of 1 µg of plasmid DNA was precipitated by the calcium phosphate coprecipitation technique and added to 5×10^5 DHFR[–] cells. At 3 h later, the cells were treated with 20% glycerol (11) and fed with nonselective media. At 2 days later, the cells were passaged into several 10-cm petri dishes and fed with selective media (Ham F12 lacking glycine, hypoxanthine, and thymidine). Colonies arose 2 to 3 weeks later. Individual clones were isolated from one set of plates by using glass cloning cylinders. Plates containing 80% of the colonies were trypsinized to provide a representative pool of each transfection. These pools were split into 60-mm dishes, and the amount of HBsAg was determined after 2 days by a radioimmunoassay (Ausria II; Abbott Laboratories, North Chicago, Ill.). DHFR⁺ colonies arose at a significantly lower frequency with pEHD22 than with either pEHED22, a control monocistronic plasmid having a second simian virus 40 promoter situated between the HBsAg gene and the DHFR cDNA, or pED22, a DHFR expression plasmid

FIG. 1. Immunofluorescence localization of HBsAg in methanol-fixed and permeabilized 21.8 (A) and DHFR⁻ (B) cells or intact 21.8 (C) and DHFR⁻ (D) cells. Note that the staining pattern in permeabilized cells (A) is largely perinuclear but not typical of the Golgi complex in flattened cells (compare Fig. 8 of reference 31, RCA label). Note the intense staining of circular and elongated structures. N, Nucleus. Original magnification, $\times 630$ (panel A), $\times 250$ (panels B through D).

lacking the HBsAg cistron (Table 1). When the amount of HBsAg produced from each culture was determined, however, significantly higher levels of HBsAg were produced from cells transfected with pEHD22. One clone was picked for further analysis. After continued passing for several

weeks, this line was subcloned and expanded. Southern analysis demonstrated that subclone 21.8 contains approximately 40 copies of pEHD22 integrated as a linear concatamer into the cellular DNA (data not shown).

Localization of HBsAg within cells. To localize HBsAg within 21.8 cells, we used a combination of immunofluorescence, electron microscopy, and immunoelectron microscopy. A guinea pig antibody to HBsAg (anti-HBs) that was previously shown to react specifically with HBsAg (28) was used to observe punctate areas of cytoplasmic immunofluorescence (Fig. 1A). Preimmune serum resulted in background levels of fluorescence (Fig. 1B). In flattened cells the fluorescence was localized to a perinuclear region (Fig. 1A), which could be distinguished from Golgi staining described previously (31). The punctate fluorescence in most cells appeared as spheres or cylindrical structures (Fig. 1A), which were observed whether the cells were permeabilized

TABLE 1. Number of DHFR⁺ colonies and level of HBsAg produced in plasmid-treated CHO cells

Plasmid	No. of DHFR ⁺ colonies	Amt of HBsAg produced (ng/10 ⁶ cells/day)
pED22	935	0
p342E	0	—
pED22 + p342E	340	12
pEHED22	510	110
pEHD22	20	980

FIG. 2. Determination of HBsAg in the plasma membrane of intact 21.8 cells by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Intact 21.8 cells labeled with [^{35} S]methionine (16 to 18 h) were incubated with anti-HBs to react with HBsAg on the cell surface. Unbound anti-HBs was removed by washing the cells, and a postnuclear supernatant fraction (containing plasma membrane) was isolated. Cell surface HBsAg was immunoprecipitated from the postnuclear supernatant by addition of protein A-sepharose (lane 1). Total HBsAg was immunoprecipitated by addition of more anti-HBs to the postnuclear supernatant and incubation for 2 h at room temperature before addition of protein A-sepharose (lane 2). Secreted HBsAg was immunoprecipitated from the medium as a control (lane 3). Molecular weight standards (lane 4) were a ^{14} C-methylated protein mixture from Amersham (14,300 to 200,000 molecular weight).

with methanol, NP-40, or saponin. A reticular fluorescence pattern was observed much less frequently than were spheres or cylindrical structures (data not shown). No staining of the periphery of permeabilized cells was detected, and cell surface immunofluorescence of nonpermeabilized 21.8 cells (Fig. 1C) was no greater than the staining of the parental CHO DHFR⁻ cells not synthesizing HBsAg (Fig. 1D).

Additional experiments were attempted to determine whether HBsAg could be detected at the cell surface. Anti-HBs was incubated with intact 21.8 cells to immunoprecipitate any HBsAg from the cell surface (Fig. 2, lane 1). HBsAg was quantitatively immunoprecipitated only when anti-HBs was added to lysed 21.8 cells (lane 2). On the basis of these experiments, none of the HBsAg synthesized by 21.8 cells could be detected at the cell surface, but rather appeared to be cytoplasmically located.

Plastic-embedded thin sections of 21.8 cells viewed under the electron microscope revealed large dilated structures (Fig. 3B) occasionally decorated with ribosomes, giving them a dilated rough ER appearance (Fig. 3A). The dilated structures varied considerably in size and were filled with apparently spherical and cylindrical particles of approxi-

mately 15 nm in diameter (Fig. 3). These large, dilated structures were never observed in the parental DHFR⁻ cells (data not shown), nor in other CHO cell lines (16) or baby hamster kidney cells (31) when similar techniques were used. The spherical and cylindrical structures were similar in appearance to HBsAg particles secreted by these same 21.8 cells, although the diameter (15 nm) was somewhat smaller than the diameter of secreted particles (22 nm) visualized by negative stain. The cylindrical tubules were oriented in long parallel rows of repeating units (Fig. 3A). The tubules appeared to be of variable length, with several tubules at least 150 to 200 nm long. In some cases the spherical structures also appeared to be closely apposed to one another in long chains (Fig. 3A). The spherical and cylindrical structures were also detected in nondilated ER (Fig. 3A, inset) and in partially dilated perinuclear spaces (Fig. 3B; see Fig. 5B, inset). Large arrays of spherical or cylindrical structures were not observed in the Golgi complex.

Electron microscope immunocytochemistry was used to determine whether the spherical and long cylindrical structures inside the dilated ER contained HBsAg. Initial experiments with the guinea pig anti-HBs used for immunofluorescence (Fig. 1) and immunoprecipitation (28) experiments were unsuccessful owing to poor reactivity with glutaraldehyde-fixed cells. Consequently, we used guinea pigs in an attempt to elicit antibodies against glutaraldehyde-fixed HBsAg (anti-gfHBs). The anti-gfHBs were specific for HBsAg, as evidenced by immunoprecipitation of radiolabeled polypeptides from 21.8 cells (Fig. 4). The only polypeptides specifically immunoprecipitated with anti-gfHBs (lanes 4 through 7) were constituents of HBsAg (P20, GP23, and GP25) and were the same polypeptides immunoprecipitated with anti-HBs against native HBsAg (lanes 8 and 9). Higher-molecular-weight polypeptides were nonspecific contaminants (compare preimmune to immune lanes in Fig. 4).

Electron microscope immunocytochemistry experiments with anti-gfHBs and protein A-gold conjugates on 21.8 cells revealed uniform labeling of large dilated vesicular structures (Fig. 5A) within the cytoplasm as well as nondilated ER (Fig. 5). Labeling of the dilated structures was not due to nonspecific trapping of antibody in these structures, since anti-HBs against native HBsAg labeled these structures only weakly. The dilated structures were similar in size and frequency of occurrence to the structures seen in plastic-embedded sections (Fig. 3) and appeared to contain similar tubules and spheres (Fig. 5). The label was specific for the dilated and nondilated regions of the ER. Only background levels of label were observed in the cytoplasm, plasma membrane, or mitochondria (Fig. 5A). The majority of HBsAg within 21.8 cells therefore appeared to be confined to the large, dilated structures. These dilated structures were never observed in wild-type CHO cells viewed either in plastic-embedded sections or by immunoelectron microscopy (data not shown).

Origin of dilated structures containing HBsAg. Some of the dilated structures containing HBsAg appeared to be studded with ribosomes (Fig. 3A). Occasionally, the dilated structures were constricted at one end (Fig. 3A), and connections were observed at these constricted sites with rough ER (Fig. 3A, inset). Dilated structures studded with ribosomes could also be observed lining the nuclear membrane (Fig. 3B; Fig. 5B inset), and these were densely labeled with anti-gfHBs (Fig. 5B). Oblique sections through the nucleus gave the appearance that dilated areas were engulfed in the nucleus (Fig. 5B). This indicates that the dilated structures are ER

FIG. 4. Characterization of antibodies to HBsAg by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of radiolabeled polypeptides from 21.8 cells. Immunoprecipitates with antibodies to glutaraldehyde-fixed HBsAg either as guinea pig serum (α_g -gfHBs) (lanes 4 and 5) or affinity-purified antibodies (α_p -gfHBs) (lanes 6 and 7) were compared with immunoprecipitates by using antiserum to native HBsAg (α -HBs) (lanes 8 and 9) or preimmune serum (lane 2 and 3). Intracellular HBsAg consists of P20, an unglycosylated polypeptide, and GP23, a glycosylated polypeptide containing only high-mannose oligosaccharide chains. Secreted HBsAg, in addition to P20, contains GP25, a glycosylated polypeptide bearing complex oligosaccharide chains. Molecular weight standards were a ^{14} C-methylated protein mixture from Amersham (14,300 to 200,000 molecular weight).

from the perinuclear space, which follows the indentations of the nucleus.

On the basis of morphology and location of the dilated structures within cells, HBsAg particles appear to be assembled and packaged into dilated ER cisternae. Double-label immunoelectron microscopy with the lectin WGA, were performed to determine whether any of the HBsAg within the dilated ER contained complex oligosaccharide chains. WGA binds terminal *N*-acetyl glucosamine and sialic acid residues that are added to oligosaccharides in the medial and *trans* cisternae of the Golgi complex, respectively (16; A. M. Tartakoff, Int. Cell Biol. Abstr., Tokyo). The absence of labeling with WGA would confirm that none of the HBsAg within the dilated ER had been processed by Golgi enzymes.

The staining of 21.8 cells double labeled with anti-gfHBs (5-nm particles) and WGA (8-nm particles) is illustrated in Fig. 6. All of the anti-gfHBs labeling within the cell was confined to large, dilated structures and ER (Fig. 6). There was also occasional anti-gfHBs labeling of the plasma membrane, although some of the label was at a considerable distance from the cell surface. This appears to indicate that

some HBsAg may be at or near the cell surface. The WGA labeling within the cell was equivalent to background levels. The primary region of WGA labeling was the plasma membrane, which contains membrane glycoproteins with complex oligosaccharide chains that have been modified in the Golgi complex (18). This labeling pattern indicates that HBsAg within the dilated ER does not contain complex-type oligosaccharide residues and therefore has not been processed by Golgi enzymes.

DISCUSSION

Pulse-chase experiments have established that the half-time for secretion of HBsAg from the cell is 5 h and that high-mannose oligosaccharide chains added to HBsAg in the ER are not converted to the complex form (by enzymes within the Golgi complex [16]) presumably until just before secretion (28). These experiments indicated that HBsAg was in some pre-Golgi or early Golgi compartment for virtually all of its intracellular lifetime (28), but a more definite location could not be assigned. The present study involved the use of a combination of techniques to more accurately define the intracellular location of HBsAg. Electron microscopy revealed that HBsAg was assembled into cylindrical and spherical particles within cells. These intracellular HBsAg particles were packaged inside large dilated structures that resided in the cytoplasm of cells. The dilated structures were part of the ER, as evidenced by their location within the cell and the connections between the dilated areas and either cytoplasmic ER or the ER lining the nucleus (perinuclear space). In addition, immunoelectron microscopy revealed that the oligosaccharide chains of HBsAg in the dilated areas did not contain *N*-acetyl glucosamine-sialic acid residues indicative of conversion to complex oligosaccharide chains that occur in the Golgi complex (16).

HBsAg particles accumulate in characteristically dilated areas of the ER, and the extended half-time for secretion appears to reflect the residence time in the ER. The rate-limiting step in secretion therefore seems to be the transfer of HBsAg from the dilated ER to the Golgi complex. This has also been observed for several membrane proteins and secretory proteins that are transported to the cell surface at different characteristic rates (9, 10, 17, 23). In each case, the rate of transport to the cell surface was a function of the rate of transport from the ER to the Golgi complex (9, 10, 17, 23). The differential rates of transport from the ER to the Golgi complex were postulated to be dependent on the interaction of each protein with specific receptors in the ER (9, 10, 23). Proteins with a slow rate of transport would interact only weakly with their specific receptor or may not interact with any receptor at all, but rather would be carried along by bulk fluid-phase transport (23). Since HBsAg is transported to the cell surface very slowly, it may be an example of a protein which interacts very weakly with a transport receptor protein or may be transported via bulk fluid transfer of the lumen contents. Alternatively, the elongated particles of HBsAg that are assembled in the ER may be difficult to transport to the Golgi complex owing to size constraints. Many studies indicate that protein transport is directed from

FIG. 3. A and B. Plastic-embedded thin sections revealing ultrastructure of 21.8 cells containing HBsAg in dilated structures often partially decorated with ribosomes. Panel A inset. RER connection to dilated region. Symbols: M, mitochondria; N, nucleus; pm, plasma membrane; G, Golgi complex; rer, rough endoplasmic reticulum; *, dilated structures; $\rightarrow$, ribosomes on dilated structures; $\rightarrow$, spherical and cylindrical particles; $\blacktriangleright$, A, chains of spherical structures, B, perinuclear spaces; $\blacktriangle\blacktriangle\blacktriangle$, parallel rows of cylindrical structures. Panel A: magnification $\times 55,000$; scale bar, 0.25 μ m. Inset: magnification, $\times 48,000$; scale bar, 0.25 μ m. Panel B: magnification $\times 37,000$; scale bar, 0.5 μ m.

FIG. 6. Double immunolabeling of HBsAg (5-nm gold) and WGA (8- to 12-nm gold) in 21.8 cells. Dilated structures (*) similar to those seen in Fig. 3 and 5 and ER are uniformly labeled for HBsAg with affinity-purified anti-gfHBs, but no labeling was detected for WGA. The plasma membrane (pm), on the other hand, shows intense labeling with WGA. Magnification, $\times 62,000$; scale bar, $0.25 \mu\text{m}$.

the ER to the Golgi complex (4, 5, 13, 15, 16, 32, 33). If this transport is mediated by transport vesicles, there may be a limit to the size of particles that can be accommodated inside the vesicles. The rate of transport then would be determined by the rate of interconversion from a more elongated particle to a more spherical particle. There is evidence that purified spherical particles can spontaneously form elongated particles *in vitro* (data not shown).

The pathway of intracellular transport and maturation has been delineated by electron microscopy for several viral membrane glycoproteins, the most widely studied being the G protein of vesicular stomatitis virus and the p62-E1 complex of Semliki Forest virus (3, 4, 15, 32). These proteins are synthesized in the rough ER, transported to the Golgi complex, and ultimately incorporated into the plasma membrane. At each stage they are integrally associated with each organelle membrane. Although HBsAg is an extremely hydrophobic integral membrane protein (12), it appears to be transported through the cell like a secretory protein, i.e., once the HBsAg particles form in the lumen of the ER, the HBsAg polypeptides no longer become integrally associated with each subsequent organelle membrane.

In previous electron microscopic studies, mature virions (Dane particles) were also detected in the lumen of the ER and apparently had obtained the viral envelope containing HBsAg by budding through the ER membrane (8, 19, 37, 38). Thus, both virions and HBsAg particles appear to follow a similar assembly pathway. The present study also revealed that HBsAg particles are assembled at a time when the HBsAg polypeptides contain immature high-mannose oligosaccharide chains. Oligosaccharide processing to the complex form occurs after assembly. A similar postassembly carbohydrate processing has been described for a coronavirus (27) and may occur with LaCrosse virus as well (25). Although assembly of mature virions in each of these instances occurs in the ER, oligosaccharide processing of glycoproteins on the surface of virions or particles can occur after assembly, provided the virions and particles are transported through the Golgi complex before secretion from the cell.

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