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Formation of Intracellular Particles by Hepatitis B Virus Large Surface Protein

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Hepatitis B virus small surface protein is synthesized as a transmembrane protein of the rough endoplasmic reticulum (RER) and then buds into the lumen in the form of subviral particles that are secreted. The closely related large surface protein is also targeted to the RER but is retained in a pre-Golgi compartment and cannot be secreted. It has been assumed that the large surface protein remains as a transmembrane RER protein and hence cannot form particles, possibly because of binding to a host factor on the cytosolic face of the RER membranes. We have reexamined this question and found the following results. (i) The retained large surface protein is associated not with RER but, rather, with a more distal compartment. (ii) Electron microscopy reveals intravesicular 20-nm particles, similar to those formed by the small surface protein. (iii) The large surface protein colocalizes with and binds to calnexin, an ER chaperone protein. Therefore, our results indicate that the large surface protein is capable of budding and forming particles, and hence its intracellular retention cannot be attributed to a cytosolic factor. We interpret the data as evidence that the large surface protein is retained by virtue of interacting with calnexin, a component of what is considered the quality control mechanism of the ER.

Hepatitis B virus (HBV) is a hepatotropic DNA virus that is distantly related to the retroviruses (reviewed in references 11 and 12). Three forms of the HBV surface protein are found in the virion envelope (reviewed in reference 10). The large surface protein is translated from the first ATG of the surface gene, while the middle and small forms are translated from in-frame ATG codons further downstream. All three forms are cotranslationally inserted into the rough endoplasmic reticulum (RER) as transmembrane proteins (8, 29). The middle and small forms, in the absence of any other viral proteins, can then bud into the lumen of a post-RER compartment to form spherical particles approximately 20 nm in diameter (18). The large form, in contrast, cannot be secreted by itself, although it is also targeted cotranslationally to the RER (5, 6, 20, 22, 24). If the large surface protein is coexpressed with the other forms, they form heteromultimers, whose behavior depends on the relative amounts of the various surface proteins: a small relative amount of large surface protein results in secretion, while a large amount results in retention (5, 6, 20). It has been suggested that the large surface protein is retained in the RER as a transmembrane protein because of binding to a cytosolic factor and hence cannot bud into the lumen (3, 18). This hypothesis is based on three observations: (i) large surface protein copurifies with microsomes in subcellular fractionation studies (6, 9); (ii) the N terminus of the large surface protein, in contrast to that of the small and middle forms, is cytosolically disposed upon completion of translation (1, 21, 26) and hence is uniquely in a position to interact with a cytosolic retention protein (Fig. 1); and (iii) modified large surface proteins with a luminal disposition of the N terminus is secretable (3, 9).

However, other data hint at the possibility that large surface protein actually buds into the lumen. Thus, Prange and Streeck (26) found that even though the N terminus of large surface protein is initially cytosolically disposed, after 4 h of chase the N terminus of most of the molecules is no longer susceptible to protease digestion from the cytosolic side. This result suggests that at least the N terminus of the large surface protein has translocated into the lumen, although a conformational change could also explain the lack of digestion. In addition, the hepatocytes of transgenic mice that overexpress the large surface protein contain numerous long filaments within distended vesicles (7). However, because of the concomitant expression of small and middle surface proteins in these studies, a definitive conclusion regarding the budding ability of large surface protein could not be reached. To resolve this important issue, we have done a detailed analysis of the intracellular localization and morphology of aggregates of the large surface protein, expressed in the absence of middle and small surface proteins. Our results indicate that, contrary to the prevailing hypothesis, this protein by itself can bud into the lumen to form 20-nm particles. However, the resulting particles are retained in a post-RER compartment. Furthermore, large surface protein colocalizes with and binds to calnexin, an integral ER membrane protein that binds to incompletely or incorrectly folded proteins within the lumen. Therefore, large surface protein is not retained as a membrane protein by interaction with a cytosolic protein. Rather, it is retained in the form of intraluminal particles, possibly by interacting with calnexin and other ER proteins with quality control functions.

MATERIALS AND METHODS

Plasmids. Plasmid pSVLM-S- (2) contains the large surface protein open reading frame of HBV subtype adw2 (31) under the control of the simian virus 40 enhancer/early promoter. However, the initiating ATG codons for middle and small surface proteins have been mutated to ACG codons, to prevent expression of these proteins from the endogenous HBV S promoter. Plasmid pSVsignalLM-S- is a derivative of pSVLM-S- with a fusion of the β-lactamase signal

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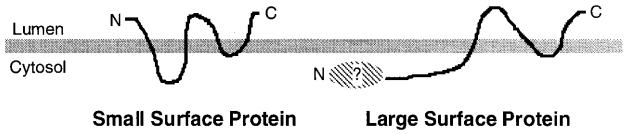


FIG. 1. Schematic diagram of the small and large surface proteins in the ER membrane, immediately after completion of translation. The detailed topology of the C-terminal portions of both proteins is as yet incompletely defined but has no bearing on our studies. The hatched ellipse with a question mark represents the putative cytosolic retention protein that is hypothesized to bind to the N-terminal portion of the large surface protein.

sequence to codon 7 of the large surface protein open reading frame (3). Plasmid pSVLM-S-ΔSau was derived from pSVLM-S- by digestion with the restriction enzyme *Sau*I and religation of the large fragment. This results in deletion of 114 bp from the large surface protein open reading frame, and thus a corresponding deletion of residues 81 to 118 of the 119-residue pre-S1 domain of the large surface protein expressed therefrom. Plasmid pCMVL contains the wild-type large surface open reading frame under the control of the cytomegalovirus intermediate-early promoter. Therefore, it is similar to pSVLM-S-, except that middle and small surface proteins are also expressed, at levels similar to the level of large surface protein, since the S promoter and the middle and small surface protein initiating ATG codons are all intact.

Cell culture, transfection, and subcellular fractionation. Well-differentiated HuH-7 human hepatoma cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum at 37°C under an atmosphere of 7% CO_2 and 93% air. They were transiently transfected with plasmids by the calcium phosphate coprecipitation technique (15), and the cells and media were harvested 2 days after transfection. Cell extracts and secreted surface protein particles were prepared as described previously (34) and electrophoresed on 10 to 20% polyacrylamide–Tricine–sodium dodecyl sulfate (SDS) gels (Novex). For subcellular fractionation studies in the absence of calcium, the cells were washed three times with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS) plus 1 mM EGTA. The cells were placed in modified buffer B (10% [wt/vol] sucrose, 20 mM Tris HCl, 150 mM NaCl, 10 mM magnesium acetate, 1 mM EGTA [pH 7.6]) (29), and frozen-thawed eight times (1 min each) in dry ice and a 37°C water bath, respectively (25). After the nuclei and debris were cleared by a 5-s centrifugation at 14,000 rpm, the membranes were pelleted by centrifugation in a microcentrifuge at 14,000 rpm for 30 min (25) and analyzed by SDS-polyacrylamide gel electrophoresis, as described below. The resulting supernatant was precipitated with trichloroacetic acid and similarly electrophoresed. For fractionation studies in the presence of calcium, PBS with calcium and magnesium and without EGTA was used, as was buffer B without EGTA.

Electron microscopy and fluorescence microscopy. For electron microscopy, the cells were trypsinized, fixed in 2% glutaraldehyde in neutral phosphate buffer, postfixed in osmium tetroxide, and embedded in Epon. For conventional transmission electron microscopy, sections of ~80 nm were cut, stained with lead citrate and uranyl acetate, and examined under a Zeiss 10C microscope. For stereo electron microscopy, sections were cut at approximately double thickness, stained with lead citrate and uranyl acetate, and examined under the Zeiss microscope with a tilting stage.

For immunoelectron microscopy, transfected cells were washed with PBS and fixed in 10% neutral buffered formalin at room temperature for 30 min. The cells were then permeabilized with PBS plus 0.005% saponin and incubated in rabbit antibody against surface protein (Accurate Chemical; diluted 1:50 in PBS plus saponin) for 40 min at 37°C. After being washed, the cells were incubated in gold-conjugated goat anti-rabbit antibody (Nanoprobe; diluted 1:50 in PBS plus saponin) for 40 min at 37°C. After extensive washing in PBS and then in water (both containing saponin), the signal was enhanced with the LI Silver Enhancer Kit (Nanoprobe) for 6 min at room temperature in the dark. After additional washing, the cells were scraped off, postfixed in osmium tetroxide, and embedded in Epon. Thin sections were cut at ~80 nm and examined under the Zeiss 10C electron microscope without further staining.

For fluorescence microscopy, transfected cells grown in two-well chamber slides (Tissue Tek) were washed in PBS and fixed with methanol for 10 min. After further washing in PBS, the cells were incubated with various combinations of primary and secondary antibodies, as described previously (17, 34). For rab2 staining, the procedure of Chavier et al. (4) was followed. The primary antibodies used were as follows. Antibodies against surface protein were either a monoclonal murine antibody (1:50 dilution, clone F35-25; obtained from AMAC), or a polyclonal rabbit antibody (1:50 dilution; obtained from Accurate Chemical); monoclonal murine antibody (20 μg/ml) against ERGIC53 was obtained from H. P. Hauri (28); rabbit antibody against rab2 (1:50 dilution) was obtained from Santa Cruz Biotechnology; rabbit antibody against calnexin (1:200 dilution) was obtained from StressGen Biotechnologies; rabbit antibody against calreticulin (1:100 dilution) was obtained from Affinity Bioagents; monoclonal murine antibody against grp94 (1:200 dilution) was obtained from StressGen Biotechnologies. Fluorochrome-labeled secondary antibodies were either fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulins or Cy3-labeled goat anti-mouse immunoglobulins (Sigma Chemical Co.). The labeled lectins used were fluorescein isothiocyanate-labeled concanavalin A and lentil lectin (E-Y Laboratories).

Immunoprecipitation and Western blotting. For immunoprecipitation (19), transfected cells were transferred to methionine-free medium for 1 h and then labeled with 500 μCi of [^{35}S]methionine per ml (New England Nuclear; specific activity, >800 Ci/mmol) for 2 h. The cells were then transferred for an additional 2 h into medium containing 1.5 mg of unlabeled methionine per ml. The cells were lysed with buffer S (0.05 M Tris HCl, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40 [pH 7.4]) and precipitated with antibodies against calnexin or calreticulin (5 μl each) prebound to protein A-Sepharose beads (Sigma). After being washed with buffer S, half of the beads were boiled in SDS sample buffer, and the released proteins were electrophoresed on a 10 to 20% polyacrylamide–SDS–Tricine gel (Novex). The other half of the beads were placed in buffer S plus 1% SDS and incubated at 37°C for 40 min and then at room temperature overnight. After removal of the beads by brief centrifugation, additional buffer without SDS was added to make the final SDS concentration 0.1%. The supernatant was precipitated with 5 μl of monoclonal murine antibody against large surface protein (clone H166) prebound to rabbit anti-mouse immunoglobulin G-agarose beads (Sigma). After being washed with buffer S, the beads were boiled in sample buffer and the released proteins were electrophoresed on the same polyacrylamide gel.

Western blotting for large surface protein was performed as described previously (34). Western blotting for calnexin was performed with the rabbit antibody to calnexin diluted at 1:1,500 and the Amersham enhanced chemiluminescence detection kit.

RESULTS

The ΔSau mutant of large surface protein is not secreted. It is now known that the N terminus of the large surface protein, unlike that of the small and middle surface proteins, is cytosolically disposed at the completion of translation (1, 21, 26). When the N terminus is forced to translocate into the ER lumen, by attachment of a heterologous signal sequence, the modified large surface protein becomes secretable (3, 9). These data support the hypothesis that the N-terminal region of the large surface protein binds to a host retention factor on the cytosolic surface of the ER that locks it in a transmembrane configuration and thus prevents its budding into the lumen (Fig. 1). Recently, Prange and Streeck (26) found that an internal deletion from residues 70 to 107 of the subtype ayw large surface protein (the so-called ΔSau deletion, which cor-

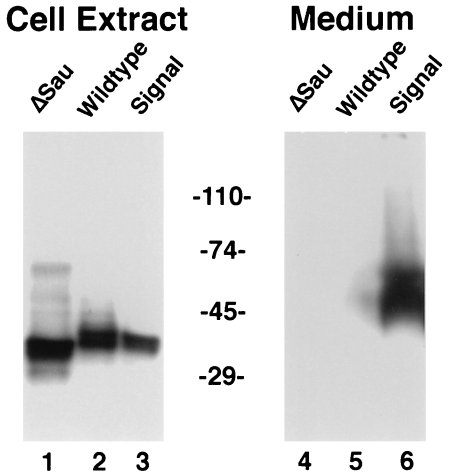
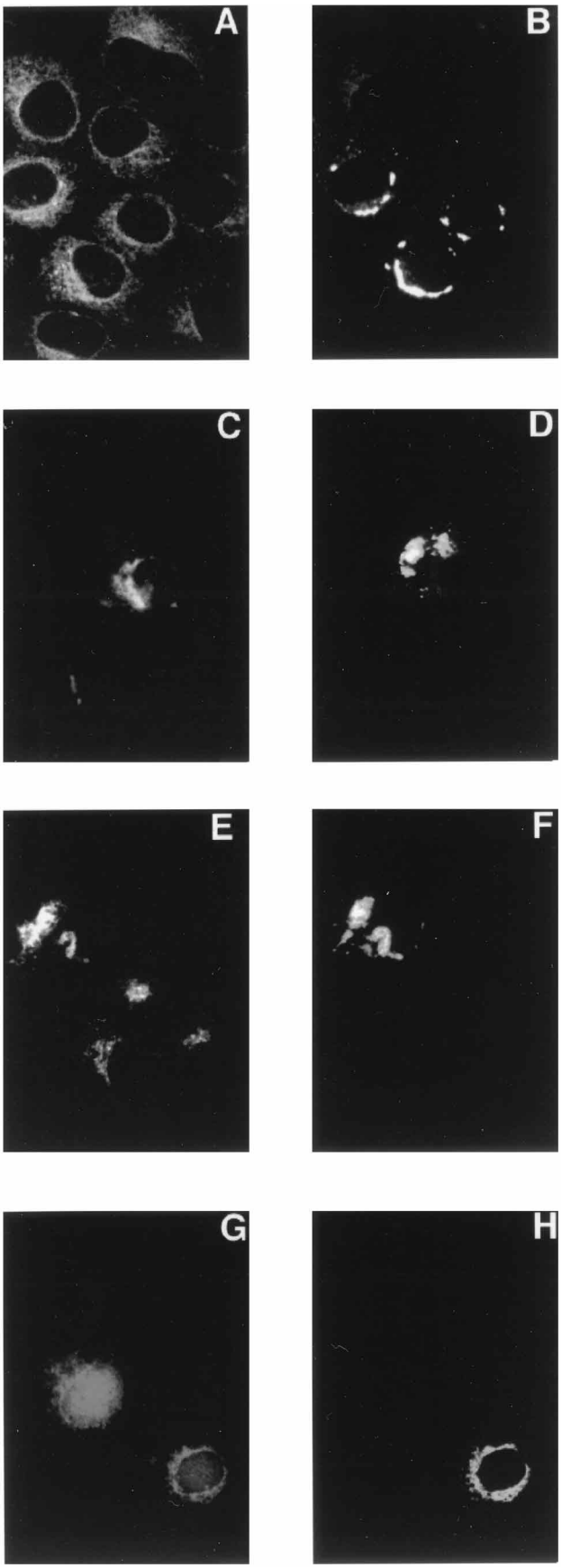


FIG. 2. Western blot of the large surface protein in cell extracts or medium from HuH-7 cells transfected with various plasmids. The wild-type plasmid is pSVLM-S-, which expresses large but not middle or small surface protein. The ΔSau plasmid is a mutant with a 114-bp deletion, which results in the synthesis of an internally deleted large surface protein with the N terminus on the luminal side of the ER membrane. The signal plasmid is pSVsignalLM-S-, which expresses a modified large surface protein with the β-lactamase signal peptide fused at its N terminus. This results in secretion of the large surface protein, which has a higher molecular weight than expected because of hyperglycosylation (3). The numbers represent the approximate molecular weight (in thousands) of prestained protein standards.



responds to residues 81 to 118 of subtype adw2 used by us), like attachment of the signal sequence, also causes the N terminus of the large surface protein to translocate cotranslationally into the ER lumen. Based on the above hypothesis, this mutant large surface protein should be secretable, since its N terminus would not be available for interaction with the cytosolic retention factor. However, when we transfected a plasmid expressing large surface protein with this deletion into HuH-7 cells, we could find no large surface protein secreted into the medium (Fig. 2, lane 4), despite high-level expression of the protein within the cells (lane 1). This result cannot be due to a secretion defect in the HuH-7 cells we used, since the large surface protein with a signal sequence was secreted (lane 6), and it raised the possibility that large surface protein was not retained by a cytosolic factor. It should be noted that the ATG start codons for the middle and surface proteins have been mutated in all of the plasmids we used for this study, so that the analysis would not be confounded by the coexpression of middle or small surface proteins, although similar results were obtained with a plasmid that expresses middle and small surface proteins in approximately equal amounts to that of large surface protein (35).

Large surface protein is retained as intraluminal particles in a post-RER compartment. To examine in more detail the question of the state of intracellularly retained large surface protein, we first sought to determine its intracellular localization by immunofluorescence analysis. HuH-7 cells were transfected with the plasmid that codes for the large surface protein, and the cells were probed with specific antibodies, followed by fluorochrome-conjugated secondary antibodies. As seen in Fig. 3B, the protein is present in a tight cluster near the nucleus, which is clearly different from the bulk of the intracellular membranes (chiefly the RER), as defined by costaining with concanavalin A (Fig. 3A). Based on previous studies showing that retained large surface protein contains endoglycosylase H-sensitive sugar groups (6, 22), this perinuclear region should not correspond to the Golgi apparatus; this expectation is confirmed by the finding that staining for large surface protein (Fig. 3D) does not colocalize with staining by lentil lectin, a marker for the Golgi apparatus (Fig. 3C). However, large portions of the large surface protein (Fig. 3F and H) do colocalize with two markers of an intermediate compartment, ERGIC-53 (28) and rab2 (4) (Fig. 3E and G, respectively). This colocalization was confirmed by confocal microscopy (data not shown). Therefore, it appears that large surface protein is not retained at its site of synthesis but, rather, travels to a region intermediate between the RER and Golgi apparatus.

We then performed transmission electron microscopy on HuH-7 cells transiently transfected with the plasmid encoding only large surface protein. Approximately 15% of the cells examined, commensurate with the percentage of transfected cells, contained smooth perinuclear vesicles with intraluminal particles approximately 20 nm in diameter (Fig. 4A), morphologically similar to small surface protein particles. Preembed-

FIG. 3. Two-color fluorescence analysis of HuH-7 cells transfected with pSVLM-S-, which expresses the large surface protein. In each row, the right-hand panel shows the immunostaining for large surface protein while the left-hand panel shows the same cells stained for various cellular markers, as follows. (A and B) Conconavalin A as a marker of intracellular membranes; (C and D) lentil lectin as a marker of the Golgi apparatus; (E and F) antibody to ERGIC-53, as a marker of a post-RER compartment; (G and H) antibody to rab2, another marker of a post-RER compartment. Note that not all of the cells in each field have been transfected. While the size and shape of the large-surface-protein staining area vary from cell to cell, the colocalization with ERGIC-53 and rab2 is consistent.

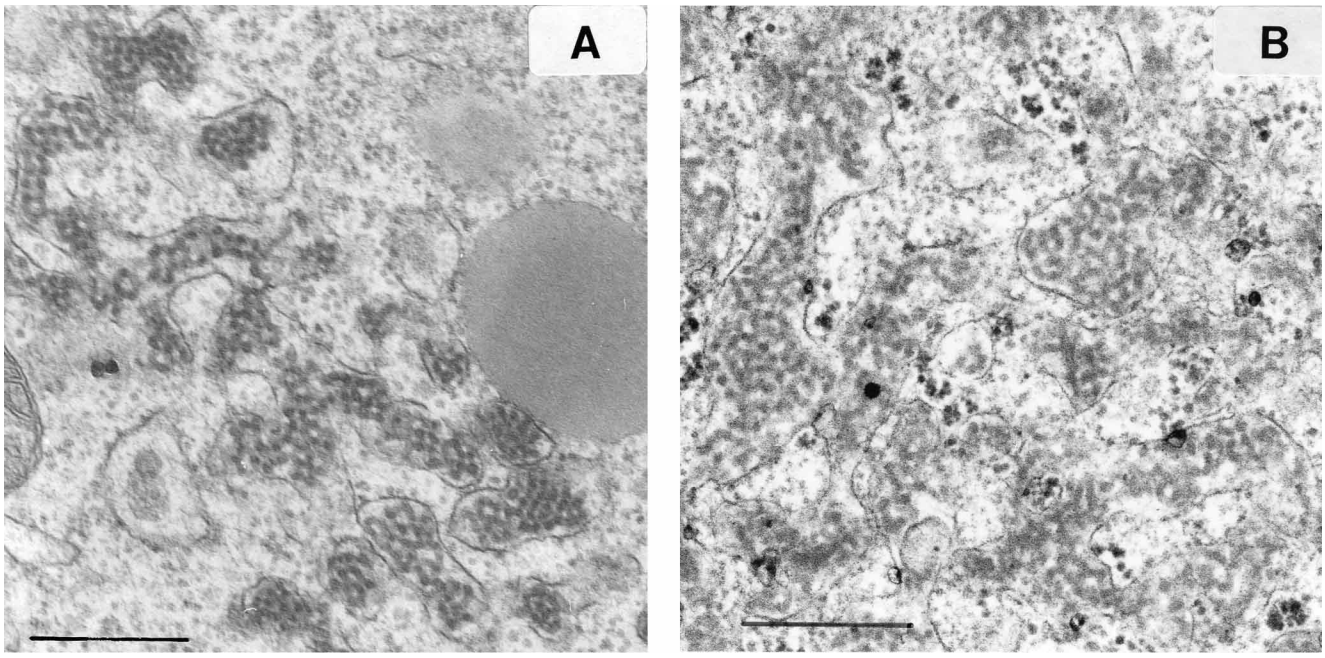


FIG. 4. (A) Transmission electron micrograph of a HuH-7 cell transfected with pSVLM-S-, which expresses only large surface protein. Note the presence of spherical and short filamentous particles within smooth vesicles. Bar, 400 nm. (B) Transmission electron micrograph of a HuH-7 cell transfected with pCMVL, which expresses middle and small surface proteins in an approximately equal amount to that of large surface protein. Note the similar particles in smooth vesicles. Bar, 400 nm.

ding immunogold staining with rabbit antibody against surface protein revealed that these particles were indeed large surface protein particles (Fig. 5A; compare with Fig. 5B, which shows staining with nonimmune serum). Similar particles were observed when the cells were transfected with a plasmid that expresses approximately equal amounts of large and of middle and small surface proteins (Fig. 4B). Therefore, mixed particles that contain an excess of large surface protein are retained similarly to pure large surface protein particles.

Many of the large surface protein particles had a circular profile, but some were slightly elongated or irregular (Fig. 4A). Therefore, these particles seemed to be largely spherical, with some short filaments mixed in. The presence of filaments raised the possibility, albeit remote, that the apparent spherical

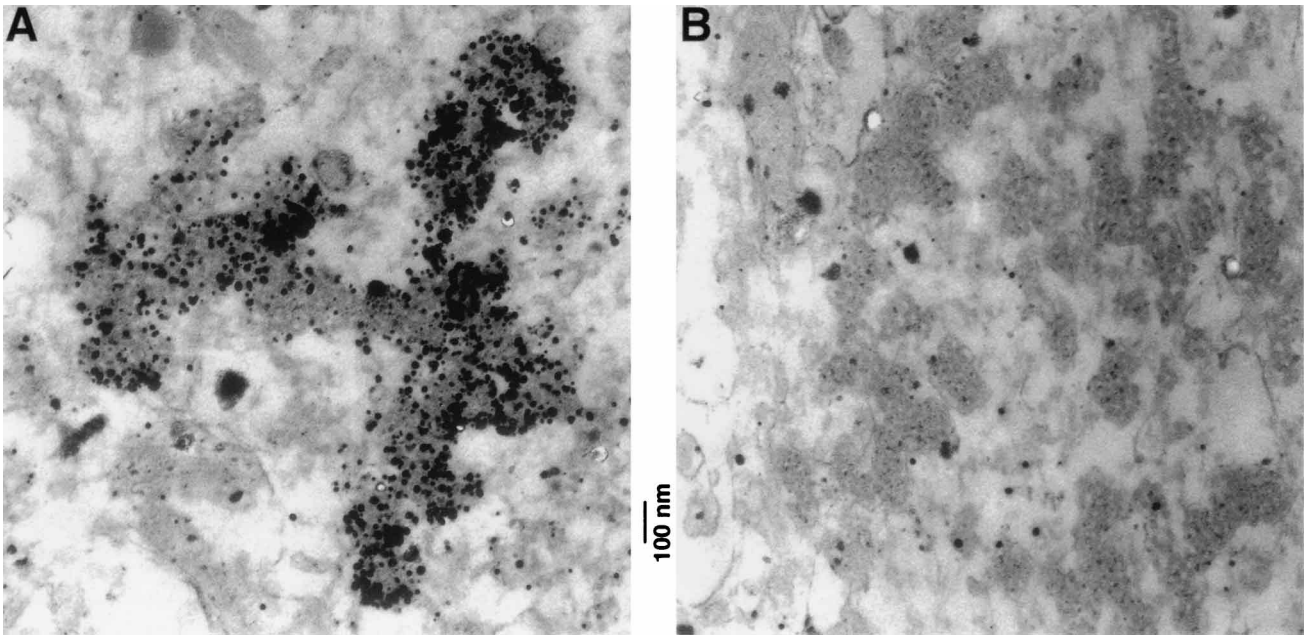


FIG. 5. Immunoelectron microscopy of intraluminal particles in HuH-7 cells transfected with pSVLM-S-. (A) The cells were exposed to rabbit antibody to surface protein; (B) the cells were exposed to nonimmune serum. The antibodies were then visualized by using nanogold-labeled anti-rabbit antibodies with silver enhancement.

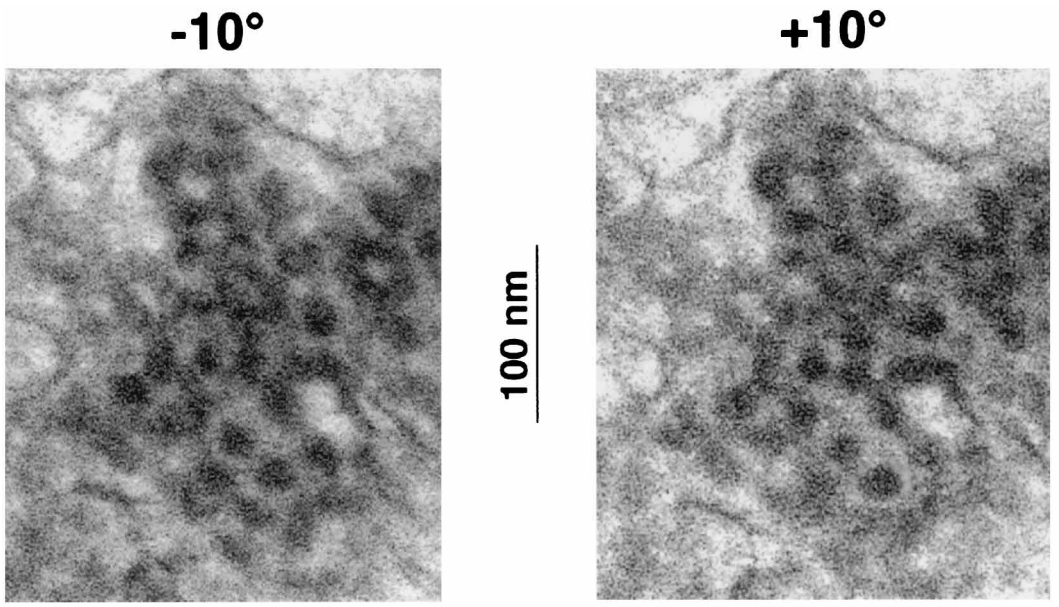


FIG. 6. Stereo electron microscopy of intraluminal particles in HuH-7 cells transfected with pSVLM-S-. The panel on the left was taken with the stage tilted at −10°, while the panel on the right was taken with it tilted at +10°.

particles merely represented cross sections of cylindrical infoldings of the limiting membrane in regions enriched for large surface protein. In other words, perhaps these were abortive forms frustes of budding and were really short filaments attached to the surrounding membrane at some point. To rule out this possibility, we performed stereo electron microscopy on semithick sections of transfected cells. As shown in Fig. 6, some of the particles can be clearly seen to be spheres that are well separated from the limiting membrane and hence must be truly intraluminal. Therefore, these particles appear to be a mixture of spheres and short filaments.

Large surface protein binds to calnexin. The finding that large surface protein is retained within the lumen of a membranous compartment is incompatible with the hypothesis that it is bound by a cytosolic retention factor but raises the possibility that a luminal protein is involved. It is known that there are several ER proteins which bind to secreted proteins in transit within the secretory pathway and are involved in the cellular quality control mechanism (14). To determine if any of these bind to large surface protein, we performed double-label immunofluorescence studies on cells transfected with the plasmid that expresses the large surface protein. Interestingly, in all of the transfected cells, large surface protein (Fig. 7B) colocalized with a significant portion of calnexin (Fig. 7A), an integral membrane protein that binds transiently to folding intermediates of secreted proteins and also more stably to misfolded proteins (16, 19, 23, 27). In contrast, no colocalization was observed with either calreticulin or grp94 (Fig. 7C and E, respectively), two other ER quality control proteins. This result is consistent with specific binding of calnexin to large surface protein. To confirm this inference, we performed sequential immunoprecipitations. Transfected HuH-7 cells were pulse-labeled with [^{35}S]methionine and lysed under gentle conditions after 2 h of chase with unlabeled methionine. The lysates were then precipitated with antibodies against calnexin under gentle conditions and analyzed by SDS-polyacrylamide gel electrophoresis. As seen in Fig. 8A, lane 1, in addition to the calnexin band, another labeled band with a mobility similar to that of large surface protein was present. This protein band was confirmed to be large surface protein, since it was brought down after disruption of the immune complexes with SDS and reprecipitation with antibodies to large surface protein (lane 2). As a negative control, we performed a similar experiment with antibodies against calreticulin, an ER quality control protein that does not colocalize with large surface protein. As seen in Fig. 8B, no large surface protein coimmunoprecipitated with calreticulin. We also attempted to determine if calnexin was coprecipitated by antibodies to large surface protein. However, no distinct radiolabeled band corresponding to calnexin was observed (Fig. 8C). This failure probably resulted from the fact that only ~15% of the cells were transfected and from the presence of preexisting unlabeled calnexin in the cell, which would effectively dilute out the specific activity of the [^{35}S]methionine used for labeling. It should be noted that a significant portion of the large surface protein appeared to be bound to calnexin, as determined by comparing the intensity of the large surface protein band in Fig. 8A and C. An even larger portion may be bound in vivo, since the extensive washings during immunoprecipitation may dissociate part of the complex.

Large surface protein cofractionates with membranes only in the presence of calcium. Our results demonstrating that large surface protein is actually present within the lumen seem to be in conflict with previous data showing that large surface protein behaved as an integral membrane protein in subcellular fractionation studies (9). However, since calnexin is an integral membrane protein, the binding of calnexin to large surface protein could explain why the latter protein would cofractionate with membranes. To confirm this inference, we took advantage of the fact that calnexin is known to require calcium ions for binding to other proteins (19, 23). Therefore, in the absence of calcium, large surface protein should detach from calnexin and hence behave as an intraluminal rather than membrane-bound protein, if our data were correct. Indeed, when we performed cell fractionation studies on HuH-7 cells in the absence of calcium and the presence of EGTA, a calcium-chelating compound, most of the large surface protein did

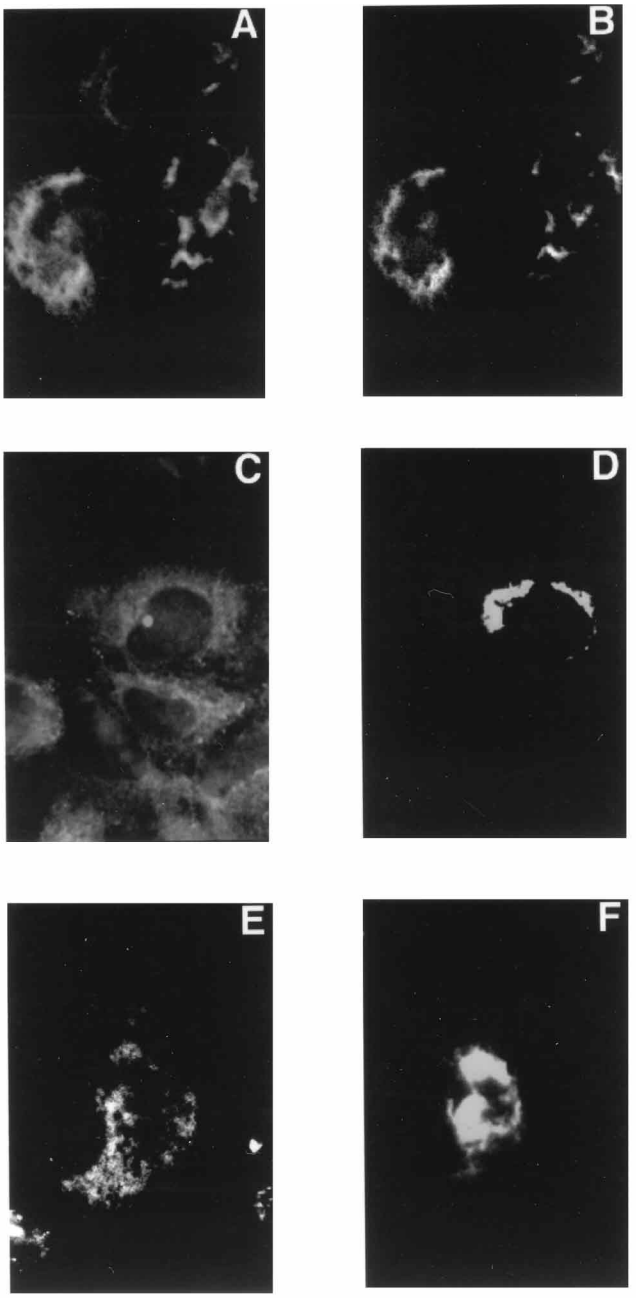


FIG. 7. Two-color immunofluorescence analysis of HuH-7 cells transfected with pSVLM-S-. In each row, the right-hand panel shows the staining for large surface protein while the left-hand panel shows the same cells stained for various ER proteins, as follows. (A) Calnexin; (C) calreticulin; (E) grp94. While the size and shape of the large-surface-protein staining area vary from cell to cell, the colocalization with calnexin is consistent.

not sediment with the membrane pellet but stayed in the supernatant fraction following low-speed centrifugation (Fig. 9A, lane 2). In contrast, calnexin was found in the membrane fraction (lane 3), as expected for this transmembrane protein. We could further show that the large surface protein was in a particulate form, since it was sedimented by high-speed centrifugation (data not shown). On the other hand, when the identical experiment was performed in the presence of calcium, large surface protein was found in the membrane fraction (i.e., the pellet from the low-speed centrifugation) (Fig. 9B, lane 1). It should be noted that in previous studies demonstrating an association between large surface protein and membranes, calcium chelators were not used (6, 9).

DISCUSSION

The HBV small surface protein has the unique property among viral envelope proteins of being secretable by itself as subviral particles. The closely related large surface protein, in contrast, is not secreted. Previous analyses have shown that the large surface protein is also synthesized as a transmembrane RER protein, but, unlike the small surface protein, its N terminus is not carried cotranslationally into the ER lumen by a downstream translocation signal (1, 21, 26). In contrast, modified large surface proteins, with the N terminus forced to translocate into the ER lumen by heterologous signal sequences, are secreted as particles (3, 9). Together, these data suggested that the large surface protein is locked in a transmembrane configuration at its site of synthesis (i.e., the RER) by a cytosolic factor that interacts with its N terminus. However, we have made a series of observations that contradict this scenario. (i) An internally deleted large surface protein (ΔSau mutant), which has been previously shown to cotranslationally translocate its N terminus into the ER lumen (18), is nevertheless not secreted. (ii) Nonsecreted large surface protein accumulates not in the RER but in a more distal pre-Golgi compartment. (iii) Electron microscopy and cell fractionation in the absence of calcium ions both reveal that retained large surface protein forms intraluminal particles with morphology similar to small surface protein particles. Therefore, large surface protein actually can bud into the lumen of form particles, presumably by a mechanism similar to that for small surface protein particles.

Our results rule out the possibility that direct binding to a cytosolic factor is responsible for the intracellular retention of large surface protein, since the retained protein is entirely

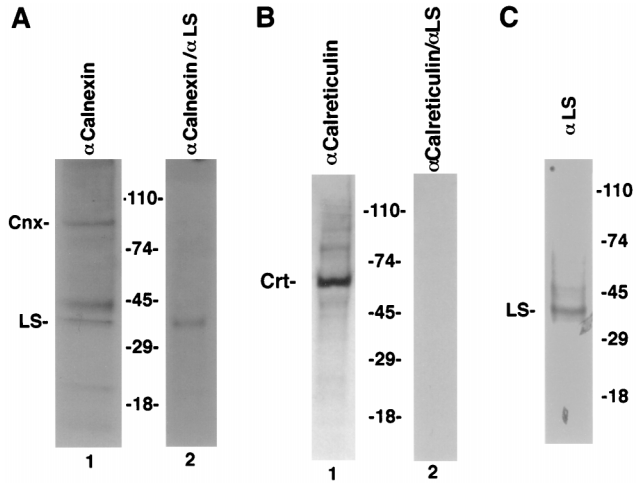


FIG. 8. Two-stage immunoprecipitation of HuH-7 cells transfected with pSVLM-S-, after pulse-labeling with [^{35}S]methionine and a 2-h chase with unlabeled methionine. (A and B) The cells were first precipitated under gentle conditions with antibodies against calnexin (A) or calreticulin (B). A portion of the precipitated material was electrophoresed (lane 1 of each panel), while the remainder was dissolved in SDS-containing buffer and reprecipitated with antibodies against large surface protein. This second precipitate was electrophoresed in lane 2 of each panel. Precipitated proteins were visualized by autoradiography. The numbers represent the approximate molecular weights (in thousands) of prestained protein standards. Crt, calreticulin; Cnx, calnexin; LS, large surface protein. (C) Autoradiogram of proteins directly precipitated by antibodies to large surface protein.

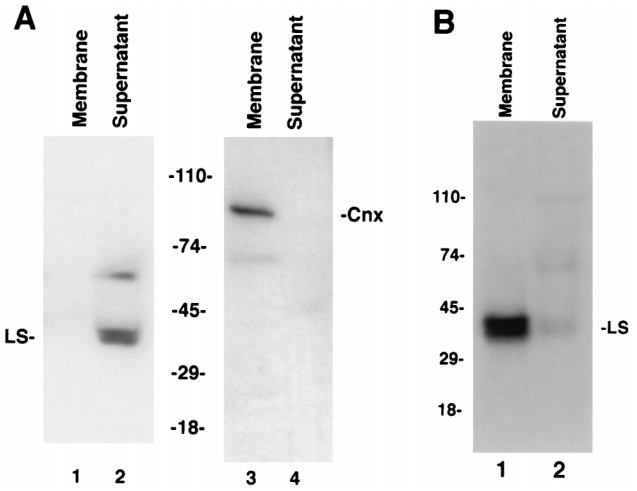


FIG. 9. Western blot of large surface protein in subcellular fractions of HuH-7 cells transfected with pSVLM-S-. (A) The fractionation was performed in the absence of added calcium ions and in the presence of EGTA; (B) the fractionation was performed in the presence of calcium ions. The right-hand portion of panel A represents the same blot reprobed for calnexin (Cnx) to confirm that this integral ER membrane protein was not released from the membrane fraction. The numbers represent the approximate molecular weights (in thousands) of prestained protein standards. LS, large surface protein.

intraluminal. Instead, large surface protein particles either lack a signal for secretion or are retained by a factor within the secretory pathway. The latter possibility is much more likely, since there is no evidence that a positive signal is needed for constitutive secretion (33). Calnexin, a transmembrane protein of the ER, is a strong candidate for the retention factor, since it not only colocalizes with intracellular large surface protein by immunofluorescence but also coimmunoprecipitates with the latter. Furthermore, large surface protein particles are bound to membranes in the presence of calcium but not in its absence, correlating with the known calcium dependence of calnexin binding to other proteins (19, 23). Such a role for calnexin would not be surprising, since it is one of the ER quality control proteins implicated in the proper folding of secreted proteins and the retention of abnormal proteins (16, 19, 23, 27).

Additional factors besides calnexin are almost certainly also involved in retention of large surface protein. Our electron micrographs clearly show particles in the center of the lumen, well separated from the membrane. Calnexin, as an integral membrane protein, obviously cannot bind directly to those particles. Therefore, particles which are not immediately adjacent to the membrane must be bound by other proteins. One possibility is that large surface protein particles bind directly to each other and thus form large paracrystalline arrays of particles. Another possibility is that there are soluble host proteins in the lumen that bridge these particles. We have ruled out two ER luminal proteins, calreticulin and grp94, as candidates, since they do not colocalize with large surface protein, but other ER proteins remain to be tested.

Our results also render untenable the attractive scenario that the reason for the intracellular retention of large surface protein is to increase the chance of its binding to cytosolic nucleocapsid particles, thereby facilitating virion morphogenesis (24). Indeed, the question is raised of how large surface protein can efficiently envelop the nucleocapsid, now that we have shown that there is a competing pathway for the newly synthesized protein (budding as subviral particles into the lumen). However, it is known that the small surface protein by itself can efficiently envelop the cytosolic ribonucleoprotein particle of hepatitis D virus (32). Since the small surface protein clearly can efficiently bud into the lumen, the few hours that the small (and presumably the large) surface protein spends in the membrane must be sufficient for HBV virion morphogenesis as well.

In turn, our results raise the question of why large surface protein particles are not secreted. Since preliminary immunoprecipitation analysis reveals that the N-terminal region of the large surface protein is exposed on the surface of these particles (35), and since the receptor-binding domain of large surface protein (and hence of virion particles) is thought to reside in this region (reviewed in references 10 and 11), the logical explanation is that large surface protein, if allowed to be secreted, would compete with virions for receptor binding. In other words, large surface protein particles, unlike small surface protein, would interfere with HBV infection. Confirmation of this conjecture must await identification of the HBV receptor.

Another question raised by our studies is why our cells that express large surface protein contained relatively few filaments, whereas numerous long filaments were seen in both infected people and transgenic mice (7, 13). One possibility is that particles composed purely of large surface protein are mainly spherical whereas mixed particles can be long filaments. However, transfected cells expressing all three forms of surface protein (but with overexpression of the large form) also contain only spheres and short filaments (Fig. 4B). Another possibility, which we cannot rule out at present, is that HuH-7 cells, unlike normal hepatocytes, do not have all of the cellular factors necessary for the formation of long filaments. However, the possibility we favor is that the long filaments are formed in situ from spheres and short filaments over extended periods. Because we used a transient-transfection system, the particles we studied are all recently formed. In contrast, the filaments in the intact liver may be many days old, since the half-life of intracellular large surface protein is >1 day (35). This interpretation is favored by two facts. First, low levels of nonionic detergents can apparently convert filaments into spheres (30). Second, many of the filaments found in transgenic mice (7) and infected humans (13, 35) are so long (up to 800 nm) that it is difficult to imagine that they can bud in this form into the lumen. Unfortunately, we have not been able to obtain cells that stably express large surface protein to confirm this hypothesis (35). As an alternative, we are attempting to generate cells by inducible expression, which may resolve this issue.

In summary, we have shown that the place and mechanism of large surface protein retention are not what we and others had expected. Further experiments will be needed to dissect the amino acid residues and cellular factors involved in the intracellular retention of large surface protein. The information gathered may provide further insights into the biology of HBV and the molecular and cellular biology of the mammalian secretory process.

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