Secreted Hepatitis B Surface Antigen Polypeptides Are Derived from a Transmembrane Precursor

Kay Simon,* Vishwanath R. Lingappa,‡§ and Don Ganem*§

Departments of *Microbiology, ‡Physiology, and §Medicine, University of California Medical Center, San Francisco, California 94143

Abstract. Hepatitis B surface antigen (HBsAg), the major coat protein of hepatitis B virus, is also independently secreted from infected cells as a lipoprotein particle. Secretion proceeds without signal sequence removal or cleavage of other segments of the polypeptide. We have examined the synthesis and transport of HBsAg in cultured cells expressing the cloned surface antigen gene. Our results show that HBsAg is initially synthesized as a integral membrane protein. This transmembrane form is slowly converted to a secreted lipoprotein complex in the lumen of the endoplasmic reticulum via a series of definable intermediates, after which it is secreted from the cell. This unusual export process shares many features with the assembly and budding reactions of conventional enveloped animal viruses. However, it differs importantly in its absence of a requirement for the participation of nucleocapsid or other viral proteins.

THE hepatitis B virus surface antigen (HBsAg)[1] is a 24-kD protein (also called p24s) that is encoded by the viral S gene. p24s and its glycosylated derivative (gp27s) are the major proteins in the virion outer coat. In the virus particle, this coat envelopes a nucleocapsid composed of a partially double-stranded DNA genome surrounded by core proteins. One of the remarkable features of HBsAg is that, unlike most viral envelope glycoproteins, both p24s and gp27s are also independently secreted from infected cells as subviral lipoprotein particles. Analysis of secreted HBsAg reveals that, unlike other secreted proteins, no amino acids are cleaved from the polypeptide during membrane translocation and export (4, 11, 13, 14, 18, 20). Notably, all the viral information required for particle formation and export resides within the S coding region: cultured cell lines which are stably transformed with the S gene alone can assemble and secrete HBsAg particles in the absence of other viral proteins (4, 11, 13, 14, 16, 17).

Though HBsAg has no cleaved signal sequence, analysis of its amino acid sequence reveals an uninterrupted stretch of hydrophobic residues at positions 80–98. Two other hydrophobic regions are also encoded, one at positions 3–22, which contains no charged residues, and the other at positions 170–226 in which 77% of the amino acids are hydrophobic. Recently, Eble et al. (5, 6) showed that when HBsAg mRNA is translated in vitro in the presence of microsomal vesicles, the HBsAg polypeptide is synthesized as an integral transmembrane protein that spans the bilayer at least twice. These results raise several important questions. Are transmembrane forms of HBsAg also synthesized in vivo? If so,

by what mechanism are they able to extricate themselves from cellular membranes and be secreted? In this study we examine the biogenesis of HBsAg particles with particular reference to these questions.

Materials and Methods

Materials

Cell Lines and Plasmids

Plasmid pSV24H was constructed by cloning the 1.9-kb Eco RI–Bg LII fragment of HBV (subtype adw) into the Eco RI- and Bam HI-cleaved plasmid pSV65; the latter is a derivative of pSP65 bearing the 343-bp SV-40 Pvu II–Hind III fragment containing the SV-40 early promoter and *ori* sequences. In the resulting recombinant, the HBV S gene and its polyadenylation signal are under the control of the SV-40 early promoter (cf. reference 19). The plasmid was introduced into mouse Ltk⁻ cells together with the HSV TK gene by calcium phosphate coprecipitation and tk⁺ cells selected in HAT medium as previously described (18).

Pulse-Chase Analysis

Cells were grown in DME-H16 medium containing 10% FCS. For labeling, 10-cm dishes of cells grown to subconfluence were preincubated for 30 min in 3 ml of medium without methionine and subsequently incubated for 1 h in 3 ml of medium containing 100 μCi/ml [35S]methionine. After the labeling period, the medium was removed and replaced with medium containing 200 mM unlabeled methionine, and cells were then chased for varying periods of time. At the end of each chase period, the medium was removed and diluted 1:1 into buffer A (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1 M EDTA, 2% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride [PMSF]); the cells were washed twice in PBS and then lysed in 2 ml of buffer A. The extract was spun at 10,000 *g* for 10 s to remove cellular debris.

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1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; HBsAg, hepatitis B virus surface antigen.

Samples were immunoprecipitated with 4 μl of anti–HBsAg antiserum and 15 μl protein A–Sepharose.

Immunoprecipitation of sAg Translation Products

2–10 μl of antisera was added to each sample, and samples were incubated overnight at 4°C. After the incubation, 15 μl of a 50% slurry of protein A–Sepharose in buffer A was added. Samples were incubated for 1 h at 4°C, washed three times with buffer A, and twice subsequently with buffer containing 0.1 M Tris-HCl (pH 7.5) and 0.1 M NaCl to remove residual Triton X-100.

Crude Vesicle Preparation

Freshly labeled cells were washed twice with ice-cold PBS, and were then resuspended in 600 μl of an iso-osmotic buffer solution (buffer B) that contained 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM MgAc, and 10% sucrose (wt/vol). A crude vesicle preparation was obtained by homogenization with a ground-glass homogenizer, followed by a 10-s spin at 10,000 g to remove cellular debris.

Protease Protection

10-cm dishes of L cells were preincubated for 40 min in medium without methionine and then pulse-labeled in 3 ml medium containing 3 mCi/ml of [³⁵S]methionine. After the incubation period, crude vesicles were prepared as described above. Each sample was divided into three equal aliquots of 150 μl each, and these samples were then incubated at 4°C for 3 h either in the absence of proteinase K, in the presence of 3 mM proteinase K, or in the presence of proteinase K and 1% Triton X-100. Protease digestion was stopped by the addition of 2 mM PMSF followed by boiling in 2× volume 2% SDS in 0.1 M Tris-HCl (pH 8.9) for 10 min. For immunoprecipitation, all samples were diluted 10-fold in buffer A.

Carbonate Extraction

Vesicles were prepared as described. Each sample was divided into two aliquots of 50 μl each, and one aliquot was treated with 5 ml 0.1 M Na₂CO₃ (pH 11.0) for 30 min at 4°C. The extracted sample was then centrifuged for 45 min at 100,000 g in an ultracentrifuge. The top 4 ml was removed (supernatant), 900 μl of the remaining 1 ml of solution was discarded, and the

remaining 100 μl and pellet was resuspended in buffer A with 0.1% SDS. The supernatant was neutralized by addition of acetic acid and then added to 1 ml of 5× concentration buffer A including 0.5% SDS. All samples were immunoprecipitated as decribed above.

Results

We used a mouse L cell line stably transformed by a plasmid in which transcription of the S gene is driven by the SV-40 early promoter; construction of this plasmid and the transfection procedures are described in Materials and Methods (18, 19). To determine what percentage of the total HBsAg products were secreted by this cell line, we performed the pulse–chase experiment shown in Fig. 1. Cells were pulse labeled for 1 h in incubation medium containing [³⁵S]methionine and then chased in medium containing excess unlabeled methionine for varying time intervals. At the end of each incubation period, medium and cells were harvested and analyzed by immunoprecipitation with anti–HBsAg antisera, followed by gel electrophoresis. The results of this experiment show that >95% of the HBsAg synthesized is secreted within a 10 h time period, and that the half-time for secretion is ~3 h. Both p24s and gp27s are released into the medium with similar kinetics. In previous studies, we (18) and others (4, 11, 13, 14) have shown that the material secreted from such transformed cell lines is particulate, as judged by electron microscopy and by equilibrium density centrifugation in CsCl.

Having established that this cell line secreted HBsAg with high efficiency, we then characterized the translocation properties of newly synthesized surface antigen polypeptides. First, we used proteolytic digestion of vesicle preparations to determine whether the transmembrane form previously observed in cell-free systems is also synthesized in vivo.
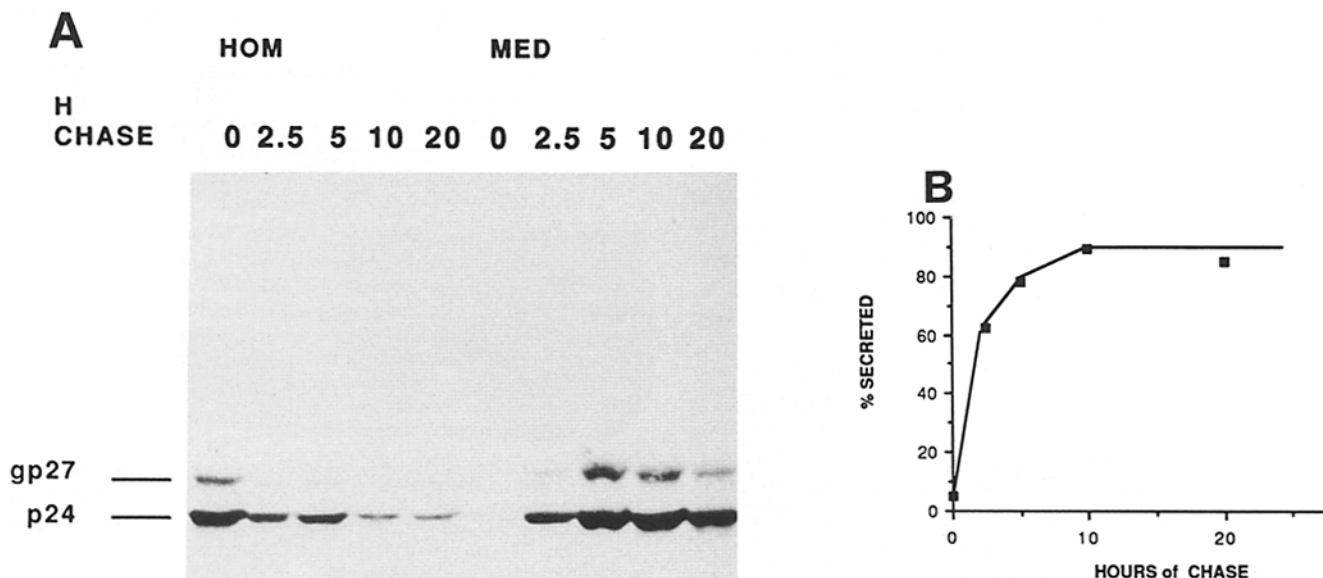

Figure 1. HBsAg is efficiently secreted from murine L cells. (A) Cells were pulse labeled for 1 h in medium containing [³⁵S]methionine and then chased for the times indicated (in hours). At each time point, medium was collected and cell homogenates were prepared by detergent extraction. Labeled products were immunoprecipitated with anti–HBsAg antiserum and visualized by gel electrophoresis. HOM refers to the homogenate prepared from lysed cells, and MED refers to the medium. P24 and gp27 refer to the unglycosylated and glycosylated forms of HBsAg, respectively. (B) Products visualized in A were quantitated by scanning densitometry and were plotted as percent of total product secreted vs. time. Total product is calculated as the sum of homogenate and medium at each individual time point.

Cells were pulse labeled with [^{35}S]methionine for 8 min, and a crude vesicle extract was prepared by homogenization in iso-osmolar buffer. Vesicles were incubated in the presence of proteinase K under conditions that had previously been shown to digest the transmembrane form produced in vitro (5). The results of this experiment are shown in Fig. 2 A. Lane 1 shows the 24-kD (p24S) form of HBsAg seen in the absence of proteinase K. In the presence of proteinase K (Fig. 2 A, lane 2), all of the detectable HBsAg peptide chains have shifted down to a molecular mass of ~17,000 D (labeled F). This shift down in molecular mass is identical to the change previously observed (5) when proteolysis was performed on the products generated in cell-free translation/translocation experiments. A control experiment in which vesicles were incubated with proteinase K in the presence of Triton X-100 (Fig. 2 A, lane 3) demonstrates that the polypeptides are extensively degraded under the same conditions when the vesicles are not intact. Because all of the HBsAg

detected at this early time point is susceptible to protease, we conclude that HBsAg in these cells is initially synthesized as a transmembrane protein.

Greater than 95% of the synthesized HBsAg polypeptides are eventually secreted as a lipoprotein complex; therefore, a mechanism for the dissociation of these peptides from the ER membrane must exist. To further characterize the molecular events underlying this process, we examined in detail the events subsequent to the initial synthesis of the transmembrane form. To detect changes in the membrane association of HBsAg polypeptides that might occur subsequent to initial synthesis and translocation, cells were incubated in the presence of [^{35}S]methionine for longer time periods before preparing vesicles and subjecting them to protease digestion. The results of experiments in which cells were pulse labeled for either 20 or 40 min are shown in Fig. 2 A. Quantitation of these results by scanning densitometry reveals that after a 20-min incubation, 50% of the chains have been converted
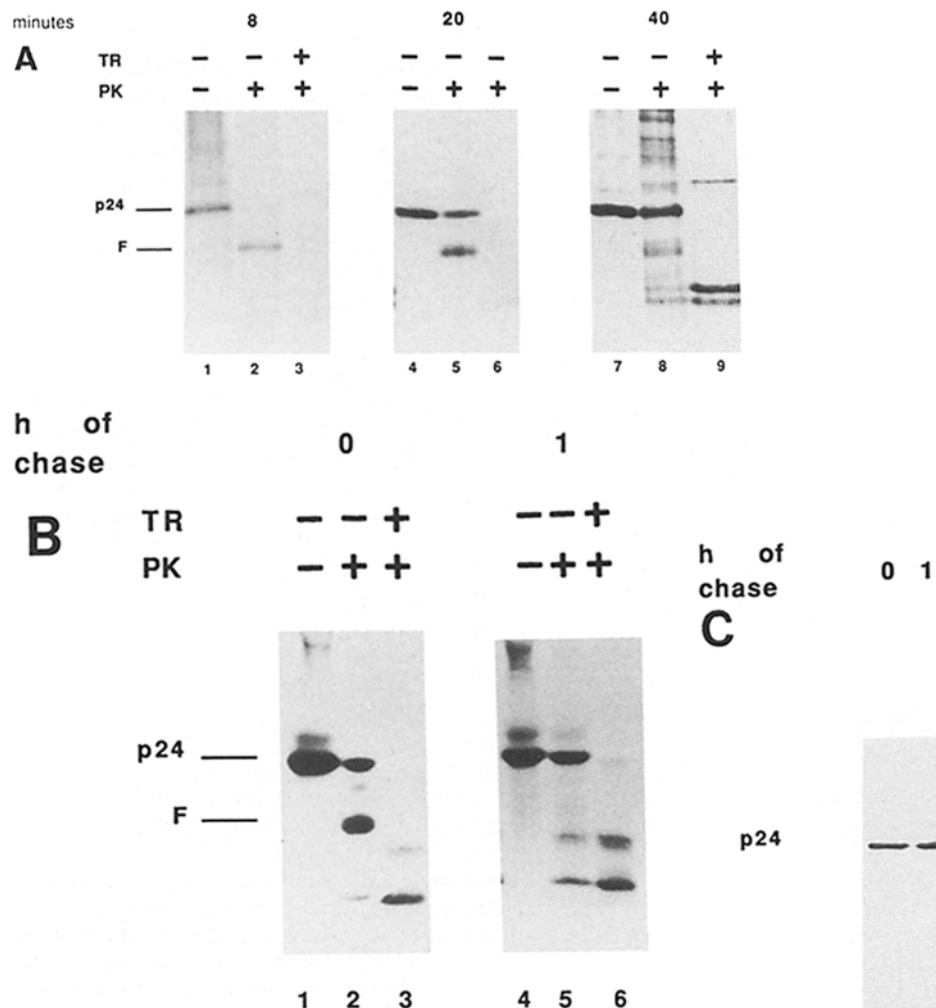
Figure 2. Digestion of vesicle preparations with proteinase K shows that HBsAg is converted from a protease-sensitive to a protease-resistant form. (A) Cells were pulse labeled for 8 (lanes 1–3), 20 (lanes 4–6), or 40 (lanes 7–9) min with [^{35}S]methionine. At each time point, vesicles were prepared and incubated either in the absence of proteinase K (lanes 1, 4, and 7), in the presence of proteinase K (lanes 2, 5, and 8), or in the presence of proteinase K and 1% Triton X-100 (lanes 3, 6, and 9). F denotes the fragment generated from protease digestion of the exposed region of HBsAg. Products were visualized by immunoprecipitation with anti-HBsAg antisera followed by SDS-PAGE. (B) Cells were either pulse labeled for 20 min (lanes 1–3) in medium containing [^{35}S]methionine or pulse labeled for 20 min followed by a 1-h chase (lanes 4–6) in medium containing excess methionine. After the incubation period, cells were harvested and vesicles were prepared and subsequently incubated with proteinase K in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of Triton X-100. Lanes 1 and 4 are control samples that were incubated in the absence of proteinase K. Products were visualized by immunoprecipitation with anti-HBsAg antisera followed by SDS-PAGE. (C) Cells were either pulse labeled for 20 min with [^{35}S]methionine and then harvested (0 h of chase) or chased for 1 h in medium containing excess unlabeled methionine (1 h of chase). Samples were immunoprecipitated with anti-HBsAg antiserum and analyzed by SDS-PAGE.

to a protease-resistant form (Fig. 2 *A*, lanes *4–6*), and that after a 40-min pulse, >80% of the antigen has become protease resistant (Fig. 2 *A*, lanes *7–9*). A small amount of the protease-sensitive transmembrane form (labeled *F*) is seen after a 40-min labeling period (Fig. 2 *A*, lane *8*); this is generated from cleavage of chains synthesized at the end of the labeling period before sufficient time has elapsed for conversion to the protease-resistant form. These studies suggest that conversion of p24s from a protease-sensitive to a protease-resistant form is almost complete in <1 h.

To demonstrate more clearly that the changes we observed were in fact due to a conversion of the transmembrane form to a protease-resistant form, we also performed a pulse–chase analysis in which cells were pulse labeled for 20 min (Fig. 2 *B*, lanes *1–3*), and then chased for 1 h (Fig. 2 *B*, lanes *4–6*) before preparing vesicles and digesting with protease. As before, about half of the antigen is in a transmembrane form (labeled *F*) after a 20-min pulse; after a 1-h chase, virtually all of the transmembrane HBsAg form has disappeared and has been converted to a protease-resistant form. A control experiment in which cells were labeled for 20 min (Fig. 2 *C*, lane *1*) and then chased for 1 h (Fig. 2 *C*, lane *2*) demonstrates that there has been no incorporation of isotope or degradation of the protein during the chase period. We conclude that by 1 h subsequent to the initial synthesis of HBsAg, there has been a quantitative conversion of the HBsAg polypeptide chains from a protease-sensitive to a protease-resistant form.

What is the nature of the protease-resistant material? One interpretation is that these HBsAg chains have been transferred into the endoplasmic reticulum (ER) lumen. Alternatively, protease resistance could be due to a change in polypeptide conformation and/or to aggregation into complexes which, though still in the ER membrane, are no longer accessible to protease.

To determine whether the acquisition of protease resistance represents an actual transfer of polypeptide chains into the ER lumen, we next performed a pulse–chase analysis coupled with extraction of vesicles in Na_2CO_3 (pH 11.0). This procedure distinguishes between integral membrane proteins (which are embedded in the lipid bilayer and cannot be extracted in the absence of nonionic detergents) and proteins that are either soluble or associated with the membrane

by electrostatic interactions that are disrupted by the low salt concentration and high pH of the carbonate buffer. After incubation with alkali, the treated vesicles are centrifuged to separate extracted proteins (supernatant) from proteins remaining associated with vesicle membranes (pellet); products from each fraction were immunoprecipitated and visualized by SDS-PAGE. In previous studies, we have established that secreted HBsAg particles, like more conventional secreted proteins, remain in the supernatant after pH 11 extraction and sedimentation (22).

The HBsAg chains visualized after a 15-min pulse were not extracted with alkali; all remained associated with the vesicle pellet (Fig. 3, lanes *1* and *2*). To our surprise, we found that even when cells were pulse labeled for 15 min and subsequently chased for as long as 1 h (Fig. 3, lanes *3* and *4*), none of the detectable HBsAg had become carbonate extractable, even though by this time >80% of the polypeptide chains are protease resistant. Similarly, after a 2-h chase (Fig. 3, lanes *5* and *6*), only 31% of the HBsAg was carbonate extractable, as judged by densitometric analysis of the data. However, after a 4-h chase (Fig. 3, lanes *7* and *8*), 70% of the HBsAg proteins were extractable with carbonate, indicating that intracellular assembly of the particulate form does eventually result in the appearance of HBsAg polypeptides that no longer display the behavior of typical transmembrane proteins.

Discussion

The above results outline a number of biochemically assayable steps in the biogenesis of secreted surface antigen particles and suggest a model for the mechanism of particle formation (cf. Fig. 4). HBsAg is initially synthesized as a transmembrane protein, even in cells that efficiently secrete this protein. Within 1 h, this material is converted to a form that in our assays is protease resistant, but not extractable with carbonate. We suggest that this second form represents polypeptide chains that have undergone a conformational change and/or aggregation in the lipid bilayer in such a way as to make them no longer susceptible to protease; this partially assembled form must be composed of proteins that are still embedded in the lipid bilayer, since they are resistant to carbonate extraction. In the third step, the assembled HBsAg
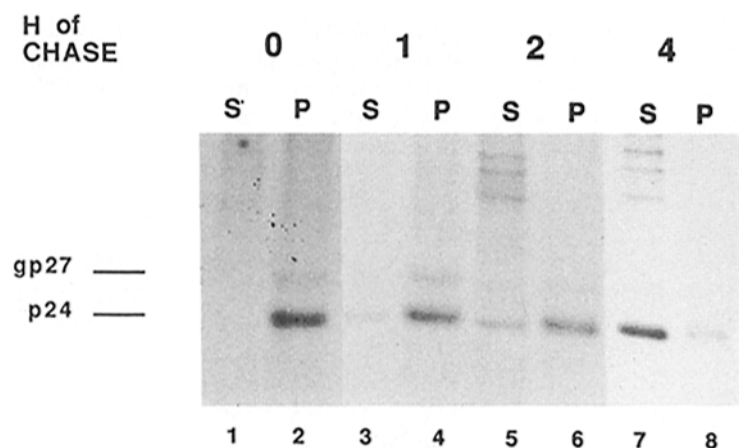
Figure 3. Newly synthesized HBsAg becomes carbonate extractable within a period of hours. Cells were pulse labeled with [^{35}S]methionine for 15 min then chased for the indicated periods. At each time point, homogenates were prepared and exposed to Na_2CO_3 (pH 11), then centrifuged into supernatant (*S*) and pellet (*P*) fractions as detailed in Materials and Methods. Each fraction was then precipitated with anti-HBsAg and the precipitated products visualized by SDS-PAGE and autoradiography.

complexes are completely transferred into the ER lumen, at which time they become carbonate extractable. Since morphologically mature 22-nm particles have been observed by electron microscopy in the ER lumen of infected and stably transformed cells (9, 16), we assume that the particles extruded into the ER lumen represent the fully assembled lipoprotein complex. However, we cannot exclude the possibility that further associations with lipids occur en route to the cell
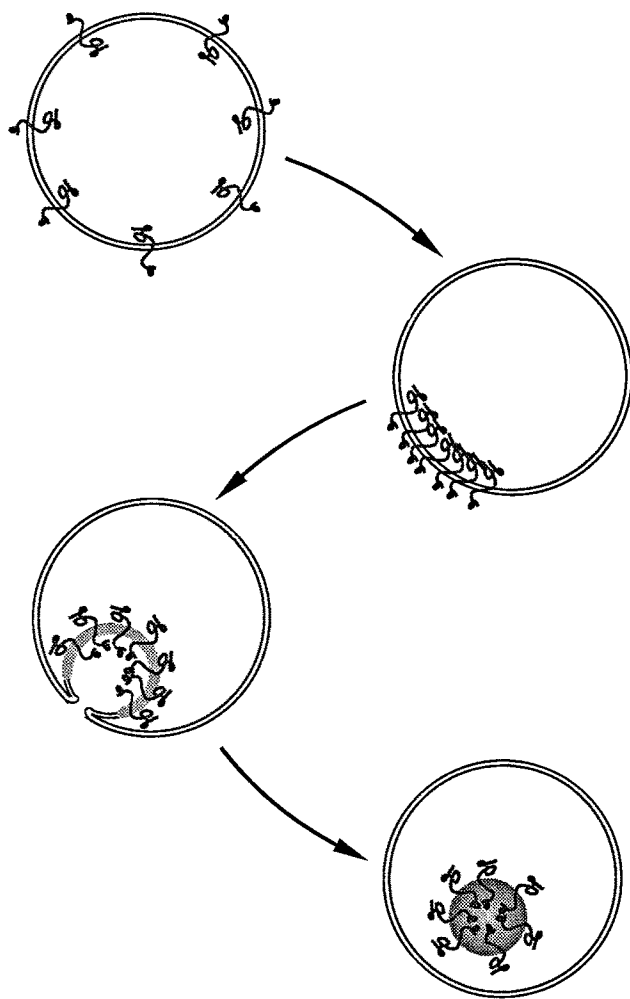
Figure 4. A model for the biogenesis of HBsAg particles. Particle formation is schematized as occurring in several steps. The process begins with the synthesis of transmembrane p24s in the ER membrane (step 1). For simplicity, the monomers are depicted as having a single transmembrane domain, although in reality each subunit is known to span the bilayer at least twice (6). Within 40 min, all of this material has become protease resistant, which may represent simply a conformational change (not shown) or aggregation in the membrane (as depicted in step 2), or both. At this stage, the antigen is still integrally associated with the bilayer. Over the ensuing 3 h, the bulk of this material is transferred to the ER lumen (steps 3 and 4) as a subviral particle no longer integrally associated with the membrane. Lipid is retained in the extruded particle; the structure of the lipid in the mature particle is poorly understood but may no longer be in a bilayer (see text). The reorganized lipid is depicted schematically with stipple. Once formed, the complex is rapidly exported to the cell exterior via the normal vesicular pathway (not shown).

surface. The rate-limiting step in this export process would appear to be extrusion into the ER, since the half-time for carbonate extractability is very similar to the half-time for secretion.

From the ER lumen the particles are rapidly exported from the cell via the constitutive pathway of vesicular transport. Particles traverse the Golgi cisterns, since secreted HBsAg glycoproteins are endoglycosidase H resistant, whereas all intracellular gp27s chains are susceptible to this enzyme (17, 22). (The absence of intracellular endoglycosidase H–resistant HBsAg is also consistent with our postulate that the rate-limiting step in export is assembly in the ER.) The fact that export of HBsAg is inhibited by monensin (17) is also indicative of trans-Golgi passage of this protein complex.

These experiments clearly demonstrate that a transmembrane form of HBsAg serves as precursor to the secreted form, and indicate that the molecular events leading to the export of this complex differ from other known processes for protein secretion. Most secreted proteins contain a cleaved signal sequence at their amino terminus (1, 2); they are completely translocated into the ER lumen cotranslationally, and they are not derived from a transmembrane intermediate. By contrast, there have been a number of reports of secreted polypeptides which, like HBsAg, are derived from a transmembrane precursor; proteins sharing this feature include the secretory component of immunoglobulin A (15), transforming growth factors α and β (3, 12), and EGF (10, 21). However, in all of these cases, the secreted product is generated by proteolytic cleavage of the ectodomain of the larger transmembrane precursor. No such cleavage events are required to liberate HBsAg.

The biogenesis of HBsAg particles can also be instructively compared to the assembly and budding of enveloped viruses. In this case, the analogous transmembrane proteins are the envelope glycoproteins that, by interacting with the nucleocapsid, participate in a budding event that results in virion formation (7). During this process, the envelope glycoproteins undergo lateral interactions in the membrane and selectively self-assemble while at the same time excluding host cellular proteins (7). Maturation of HBsAg particles also includes a process of self-association and selective exclusion of host proteins; however, HBsAg biogenesis is distinctive in that participation of other viral components is not required for the self-aggregate to be released from the original membrane in which it was embedded. In addition, although 22-nm particles contain lipid, the lipid/protein ratio of these structures is much lower than that of conventional cellular membranes (8), and the behavior of mature HBsAg particles at pH 11 also suggests that the proteins are not simply embedded in a conventional vesicle-like structure (22). If indeed the lipid within the complexes no longer exists as a bilayer, then the extrusion process that delivers HBsAg complexes to the ER lumen may well involve a substantial reorganization of lipid as well as proteins within the membrane environment, a feature that would further distinguish this process from conventional viral budding events.

Inspection of the HBsAg primary amino acid sequence reveals several structural features that might be essential for directing the assembly process. Studies performed with cell-free systems established that the hydrophobic sequence at positions 80–98 is a membrane-spanning region which directs the translocation of the carboxy terminus (5, 6). This trans-

membrane region as well as the two other hydrophobic regions (amino acids 3–22 and 170–226) are highly conserved between the hepadnaviruses of different species. Any or all of these regions might be capable of interacting with the membrane lipid bilayer or of promoting lateral interactions between individual p24s subunits. We are currently examining surface antigen mutants constructed in vitro to define the molecular determinants that direct the assembly and export process. Analysis of these mutants with the biochemical assays for assembly intermediates reported here should aid in the further elucidation of the mechanism of this unusual export process.

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