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Proteolytic Conversion of Hepatitis B Virus e Antigen Precursor to End Product Occurs in a Postendoplasmic Reticulum Compartment

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At least two proteolytic events are involved in the biogenesis of hepatitis B virus e antigen. The first proteolytic event removes the signal peptide and results in the translocation of the precursor protein, P22, into the lumen of the endoplasmic reticulum (ER). The second proteolytic event removes the carboxy-terminal arginine-rich sequence of P22 and converts it to the 16-kDa hepatitis B virus e antigen end product. In contrast to the first proteolytic event, the second proteolytic event is suppressed by brefeldin A, a chemical that inhibits the transport of protein from the ER to the Golgi apparatus. In subcellular fractionation experiments, P22 was detected in both the ER and the Golgi fractions, but P16 was detected only in the Golgi fraction. On the basis of these results, we conclude that the conversion of P22 to P16 occurs in a post-ER compartment, mostly likely the Golgi apparatus.

Hepatitis B virus (HBV) e antigen (HBeAg) is an important serological marker for HBV infection. Its presence is often associated with productive HBV replication (12, 20). This antigen is derived from a precursor protein named precore protein (2, 13, 17, 22). The precore protein is related to the core (capsid) protein of HBV, and both of them are products of the *C* gene of HBV (for reviews, see references 4 and 16).

At least two different proteolytic events are involved in the maturation of HBeAg. The first proteolytic event occurs cotranslationally (2, 3). This proteolytic event removes the signal peptide from the precore protein and is mediated by the signal peptidase located in the lumen of the endoplasmic reticulum (ER) (2, 3). After the removal of the signal peptide, the majority of the precore protein derivative, P22, is translocated across the ER membrane (3, 15). In the meantime, a small but significant portion of P22 is released back into the cytosol and transported into the nucleus (15).

Like other constitutively secreted eucaryotic proteins (7), P22 is transported via the ER and Golgi compartments to the cell surface and secreted. During this secretion process, P22 undergoes at least one additional proteolytic cleavage event. This second proteolytic event converts P22 into lower-molecular-mass species which range in size from 16 to 22 kDa and which are then secreted (6, 14, 22). Immunologically, these secreted molecules are known as HBeAg (13).

Although the first proteolytic event for the maturation of HBeAg is believed to occur in the ER lumen (2, 3), the subcellular location of the second proteolytic event is still unclear. In an effort to understand further the biogenesis of HBeAg, we have decided to localize the subcellular compartment in which this second proteolytic event occurs. A stable transformant, Alex-PC, was established from the Alexander hepatoma cell line (PLC/PRF/5) by cotransfecting cells with selection plasmid pSV2neo (19) and plasmid pRSV-PC (23). pRSV-PC contains the Rous sarcoma virus promoter and expresses the precore protein of HBV (23). Alex-PC was one of the stable transformants selected by the neomycin analog G-418 (23).

To determine whether the precore protein is expressed and processed to HBeAg in Alex-PC cells, we performed pulse-chase labeling and radioimmunoprecipitation experiments. As shown in Fig. 1A, precore protein derivative P22 was the predominant protein species in Alex-PC cells when there was no chase. The amount of P22 in the cells was reduced as the length of the chase increased (lanes 2 to 8). After 15 min of chase, a protein species with a molecular mass of 16 kDa (P16) appeared. The amount of P16 reached a peak after 30 min of chase and was gradually reduced thereafter. P16 was secreted into the medium after 30 min of chase and continued to accumulate in the medium during the chase (Fig. 1B). This result is consistent with those of previous reports (6, 14, 22) and indicates that P22 is proteolytically processed and secreted as P16 (HBeAg) in Alex-PC cells. In our previous studies, the HBeAg end product of P22 identified was heterogenous in size, with a molecular mass ranging from 16 to 22 kDa (14, 15). However, as shown in Fig. 1, HBeAg secreted from Alex-PC cells was homogeneous in size, with a molecular mass of 16 kDa (P16). Since in our previous experiments the host cells used were *Xenopus* oocytes (14) and COS cells (15), a transformed monkey kidney cell line, the discrepancy observed is likely due to the efficiency of proteolytic cleavage of P22 in different cell types.

To determine whether P22 is converted to P16 in the ER, we treated Alex-PC cells with brefeldin A (BFA), a chemical known to specifically inhibit protein transport from the ER to the Golgi apparatus (9, 10, 18). Because of its ability to inhibit protein transport, BFA will inhibit the proteolytic cleavage of P22 if the latter occurs in a post-ER compartment. As shown in Fig. 2, P16 was not detected in cells treated with BFA (lane 3) but was clearly detectable in cells not treated with BFA (lane 2). This result suggests that P22 is proteolytically converted to P16 in a post-ER compartment. The suppression of the processing of P22 to P16 by BFA is not likely to be a nonspecific artifact, because the first proteolytic event for the generation of P22, a step believed to take place in the ER lumen (2, 3), was unaffected

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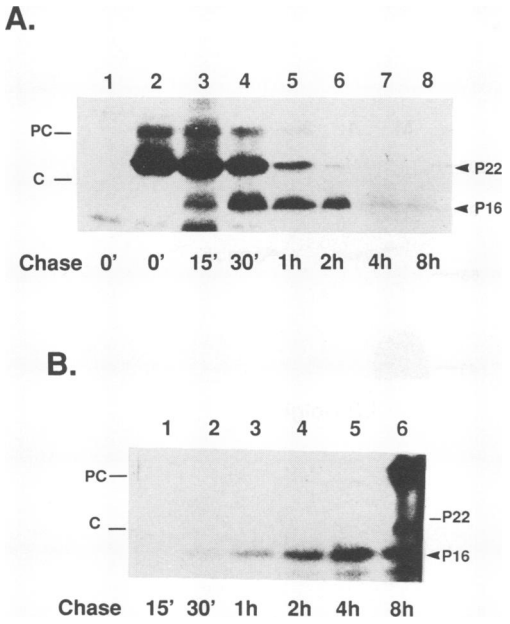

FIG. 1. Pulse-chase labeling analysis for precore protein. Alex-PC cells preincubated in methionine-free medium for 1 h were labeled with [^{35}S]methionine for 15 min. The cells were then incubated with fresh medium containing 5 μg of nonlabeled methionine per ml. At the indicated times, the medium was removed and the cells were washed twice with phosphate-buffered saline and lysed with 10 mM Tris-HCl (pH 7.0)–150 mM NaCl containing 0.5% Nonidet P-40. After brief centrifugation to pellet the nuclei, cell lysates were mixed with an equal volume of RIPA (10 mM Tris [pH 7.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) and immunoprecipitated with rabbit antiserum directed against the denatured core protein by our previously described procedures (15). The medium was mixed with an equal volume of RIPA and similarly immunoprecipitated. In all the lanes, samples derived from similar amounts of cells were loaded on the gel. (A) Immunoprecipitates of cell lysates. (B) Immunoprecipitates of the medium. Lane 1 of panel A is the parental Alexander cell control. PC and C indicate the locations of full-length precore (25-kDa) and core (21-kDa) proteins, respectively. These two protein markers were synthesized in vitro with rabbit reticulocyte lysates and run in parallel on the gel. The locations of P22 and P16 are indicated.

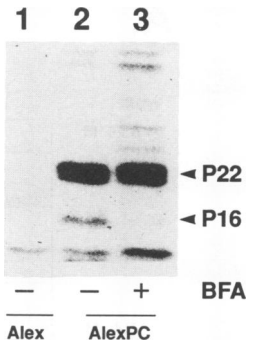

FIG. 2. Suppression by BFA of the proteolytic conversion of P22 to P16. Alex-PC cells were starved for methionine with methionine-free medium for 1 h and labeled with [^{35}S]methionine for 30 min. The cells were then lysed with 10 mM Tris-HCl (pH 7.0)–150 mM NaCl containing 0.5% Nonidet P-40 and radioimmunoprecipitated by the method described in the legend to Fig. 1. Lanes: 1, parental Alexander cell control; 2, Alex-PC cells without BFA treatment; 3, Alex-PC cells treated with BFA at a concentration of 5 μg/ml during starvation for methionine and labeling with [^{35}S]methionine. Arrowheads indicate the locations of P22 and P16. P22 appeared as a doublet band on the gel, possibly because of its phosphorylation (23).

by BFA. Furthermore, the processing of haptoglobin, a protein known to be processed in the ER, has also been shown not to be affected by BFA (10). Thus, the result shown in Fig. 2 suggests that the processing of P22 to P16 occurs in a post-ER compartment.

To confirm this observation, we preformed a subcellular fractionation experiment with a discontinuous sucrose gradient prepared with 2.0, 1.3, 1.0, 0.6, and 0.25 M sucrose solutions. The profile of this sucrose gradient is shown in Fig. 3A. Bole et al. (1), using this method, reported that the ER membrane can be enriched in the interface of 2.0 and 1.3 M sucrose solutions and that the Golgi membrane can be enriched in the interface of 1.0 and 0.6 M solutions. To determine the efficiency of this separation method, we performed two control experiments. In the first control experiment, we used GRP78, a resident protein of the ER (5, 8, 11), as a marker for the ER fraction. As shown in Fig. 3B, GRP78 was detected only in the ER interface and not in the Golgi interface, indicating that the Golgi fraction was not significantly contaminated by ER proteins. In the second control experiment, horseradish peroxidase-conjugated wheat germ agglutinin (WGA) was used as a marker for *trans*-Golgi proteins. WGA is a lectin that binds specifically to *trans*-Golgi proteins containing clustered terminal *N*-acetylneuraminic acid residues as well as *N*-acetylglucosamine-containing oligosaccharide chains (21). As shown in Fig. 3C, protein samples prepared from the Golgi interface reacted efficiently with WGA, whereas protein samples prepared from the ER interface did not show significant reactivity with WGA. Since protein samples prepared from the ER interface at a concentration of 125 μg/ml had about the same reactivity with WGA as did the protein samples prepared from the Golgi interface at a concentration of 1 μg/ml, less than 1% of the proteins in the ER interface were contaminating Golgi proteins. These two control experiments demonstrated that this discontinuous gradient is an efficient method for separating ER and Golgi membranes. Thus, this method was used to study the subcellular localization of P22 and P16.

As shown in Fig. 4, although P22 was detected in both the ER and the Golgi fractions (lanes 3 and 4), P16 was detected only in the Golgi fraction (lane 4). This result confirms the result shown in Fig. 2, which indicated that the conversion of P22 to P16 takes place in a post-ER compartment. Because of the association of P16 with the Golgi fraction, this result also suggests that this post-ER compartment is the Golgi apparatus. As a control experiment, Alex-PC cells were also treated with BFA and fractionated on the gradient. As shown in Fig. 4, P22 was detected in both the ER and the Golgi fractions (lanes 1 and 2), but P16 was no longer detected in either fraction. The detection of a small amount of P22 in the Golgi fraction (lane 2) could have been due to the incomplete blockage of protein transport by BFA or to a change in a biochemical property of a fraction of the ER membrane which was subsequently fractionated into the Golgi interface.

In summary, our results demonstrate that the production of P22 is unaffected by BFA but that the conversion of P22 to the P16 HBeAg end product is suppressed by BFA (Fig.

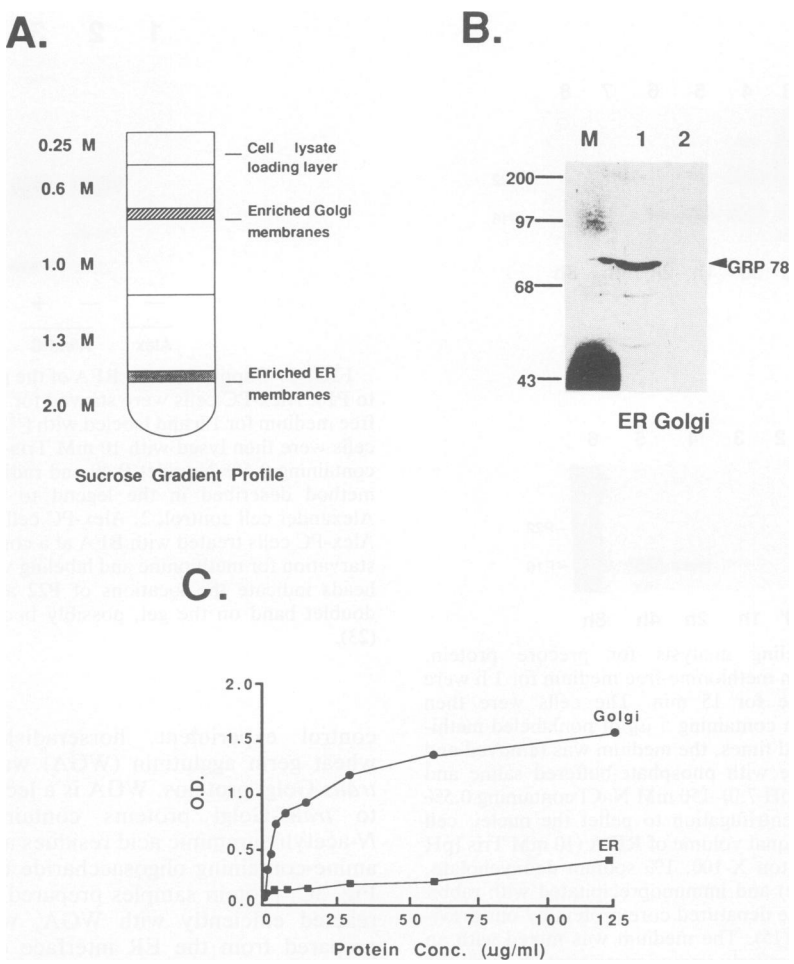

FIG. 3. Analysis of subcellular fractionation. Subcellular fractionation was performed by the procedure of Bole et al. (1), with a slight modification. In brief, cells grown on a 10-cm plate were washed once with ice-cold phosphate-buffered saline and twice with a 0.2 M sucrose solution in 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.2) and scraped off in 2.0 ml of a 0.2 M sucrose solution in 25 mM HEPES (pH 7.2) containing 1 μg of aprotinin per ml. The cells were homogenized by 10 strokes with a tightly fitting B pestal in a precooled Dounce homogenizer. After centrifugation at 200 × *g* and 4°C for 5 min to remove cell debris, the cell lysate was layered on top of a discontinuous sucrose gradient containing 1.5 ml of 2.0 M, 3.4 ml of 1.3 M, 3.4 ml of 1.0 M, and 2.2 ml of 0.6 M sucrose solutions in 25 mM HEPES (pH 7.2) containing 1 μg of aprotinin per ml. After centrifugation for 2 h in a Beckman SW41 rotor at 40,000 rpm and 4°C, the interface of the 2.0 and 1.3 M sucrose solutions (ER fraction) and that of the 1.0 and 0.6 M sucrose solutions (Golgi fraction) were isolated with needled syringes. The samples prepared from five plates of cells were pooled and concentrated about 50-fold with a Centricon-3 concentrator (Amicon). The protein concentrations of the samples were measured with a Bio-Rad protein assay kit. (A) Discontinuous sucrose gradient profile. (B) Western blot (immunoblot) of GRP78. Approximately 200-μg protein samples from the ER (lane 1) or Golgi (lane 2) fractions were loaded on the gel for analysis. Lane M contains prestained protein molecular mass markers (in kilodaltons) (Bethesda Research Laboratories). Parts of the markers came off during hybridization. The primary antibody used was rabbit anti-GRP78 (5, 8, 11), and the secondary antibody used was alkaline phosphatase-conjugated goat anti-rabbit. The color development reaction was carried out with a Bio-Rad alkaline phosphatase substrate kit. (C) Enzyme-linked immunosorbent assay of Golgi proteins. Protein samples diluted in 0.1 M $NaCO_3$ (pH 9.6) to various concentrations (Conc.) were used to coat wells of microtiter plates. After being coated with 0.25-ml protein samples at 4°C overnight, the wells were washed three times with TN solution (20 mM Tris-HCl [pH 7.4] containing 0.05% Nonidet P-40) and blocked with 1% bovine serum albumin in 0.1 M $NaCO_3$ (pH 9.6) for 3 h at 40°C. After three washes with TN solution, 0.24 ml of a 1:1,000 dilution of horseradish peroxidase-conjugated WGA (ICN) was added to each well. The plates were further incubated at 40°C for 2 h and washed four times with TN solution. Finally, the samples were treated with an Abbott OPD kit, and color development was measured with a microtiter plate reader (Dynatech). O.D., optical density.

2). This result confirms the fact that the first proteolytic event for the biogenesis of HBeAg occurs in the ER compartment and suggests that the second proteolytic event, the maturation of P16, takes place in a post-ER compartment. The latter is confirmed by the subcellular fractionation results shown in Fig. 4, which demonstrated that P16 could not be detected in the ER fraction. Although the exact compartment in which the conversion of P22 to P16 takes place remains to be determined, the association of P16 with the Golgi fraction suggests that this proteolytic cleavage event takes place in the Golgi apparatus.

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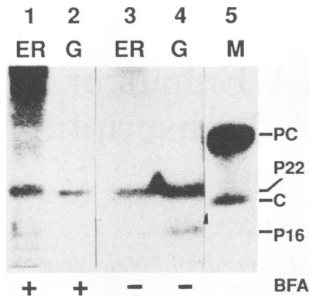

FIG. 4. Immunoprecipitation analysis of P22 and P16 fractionated on the sucrose gradient. Alex-PC cells labeled with [^{35}S]methionine were homogenized and fractionated on a discontinuous sucrose gradient by the method described in the legend to Fig. 3. Lanes: 1 and 2, cells treated with 5 μg of BFA per ml; 3 and 4, cells not treated with BFA; 5, full-length precore (PC) and core (C) protein markers. The locations of P22 and P16 are also indicated. The background signal in lane 1 is higher than that in lane 3, possibly because of the accumulation of nonspecific protein signals in the ER fraction because of BFA treatment. ER, ER fraction; G, Golgi fraction.

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REFERENCES

1. **Bole, D. G., L. M. Hendershot, and J. F. Kearney.** 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. **102**:1558–1566.
2. **Bruss, V., and W. H. Gerlich.** 1988. Formation of transmembrane hepatitis B virus e antigen by cotranslational in vitro processing of the viral precore protein. Virology **163**:268–275.
3. **Garcia, P. D., J.-H. Ou, W. J. Rutter, and P. Walter.** 1988. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translation can be aborted and the product released into the cytoplasm. J. Cell Biol. **106**:1093–1104.
4. **Genam, D., and H. E. Varmus.** 1987. The molecular biology of hepatitis B virus. Annu. Rev. Biochem. **56**:651–693.
5. **Hendershot, L. M., J. Ting, and A. S. Lee.** 1988. Identity of the immunoglobulin heavy-chain-binding protein with the 78,000-dalton glucose-regulated protein and the role of posttranslational modifications in its binding function. Mol. Cell. Biol. **8**:4250–4256.
6. **Junker, M., P. Galle, and H. Schaller.** 1987. Expression and replication of the hepatitis B virus genome under foreign promoter control. Nucleic Acids Res. **15**:10117–10132.
7. **Kelly, R.** 1985. Pathways of protein secretion in eukaryotes. Science **230**:25–32.
8. **Li, X., D. W. Warren, J. Gregoire, R. C. Pederson, and A. S. Lee.** 1989. The rat 78,000-dalton glucose-regulated protein (GRP78) as a precursor for the rat steroido-genesis-activator polypeptide (SAP): the SAP coding sequence is homologous with the terminal end of GRP78. Mol. Endocrinol. **3**:1944–1952.
9. **Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner.** 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell **56**:801–813.
10. **Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara.** 1986. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J. Biol. Chem. **261**:11398–11404.
11. **Nakaki, T., R. J. Deans, and A. S. Lee.** 1989. Enhanced transcription of the 78,000-dalton glucose-regulated protein (GRP78) gene and association of GRP78 with immunoglobulin light chains in a nonsecreting B-cell myeloma line (NS-1). Mol. Cell. Biol. **9**:2233–2238.
12. **Nielsen, J. O., O. Dietrichson, and E. Juhl.** 1974. Incidence and meaning of the e determinant among hepatitis B antigen positive patients with acute and chronic liver disease. Lancet **ii**:913–915.
13. **Ou, J.-H., O. Laub, and W. J. Rutter.** 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc. Natl. Acad. Sci. USA **83**:1578–1582.
14. **Ou, J.-H., D. N. Standring, F. R. Masiarz, and W. J. Rutter.** 1988. A signal peptide encoded within the precore region of hepatitis B virus directs the secretion of a heterogeneous population of e antigen in Xenopus oocytes. Proc. Natl. Acad. Sci. USA **85**:8405–8409.
15. **Ou, J.-H., C.-T. Yeh, and T. S. B. Yen.** 1989. Transport of hepatitis B virus precore protein into the nucleus after cleavage of its signal peptide. J. Virol. **63**:5238–5243.
16. **Robinson, W. S.** 1990. Hepadnaviridae and their replication, p. 2137–2169. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
17. **Roossnick, M. J., S. Jameel, S. H. Loukin, and A. Siddiqui.** 1986. Expression of hepatitis B viral core regions in mammalian cells. Mol. Cell. Biol. **6**:1391–1400.
18. **Serafini, T., G. Stenbeck, A. Brecht, F. Lottspeich, L. Orci, J. E. Rothman, and F. T. Wieland.** 1991. A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein beta-adaptin. Nature (London) **349**:215–220.
19. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
20. **Takahashi, K., M. Imai, F. Tsuda, T. Takashi, Y. Miyakawa, and M. Mayumi.** 1976. Association of Dane particles with e antigen in the serum of asymptomatic carriers of hepatitis B surface antigen. J. Immunol. **117**:102–105.
21. **Tartakoff, A., and P. Vassalli.** 1983. Lectin-binding sites as markers of Golgi subcompartment: proximal-to-distal maturation of oligosaccharides. J. Cell Biol. **97**:1243–1248.
22. **Weimer, T., J. Salfeld, and H. Will.** 1987. Expression of the hepatitis B viral core gene in vitro and in vivo. J. Virol. **61**:3109–3113.
23. **Yeh, C.-T., and J.-H. Ou.** 1991. Phosphorylation of hepatitis B virus precore and core proteins. J. Virol. **65**:2327–2331.