The hepatitis B virus precore protein is retrotransported from endoplasmic reticulum (ER) to cytosol through the ER-associated degradation pathway*1

The hepatitis B virus precore protein is closely related to the nucleocapsid core protein but is processed distinctly in the cell and plays a different role in the viral cycle. Precore is addressed to the endoplasmic reticulum (ER) through a signal peptide, and the form present in the ER is the P22 protein. P22 is then cleaved in its C-terminal part to be secreted as HBe antigen. In addition, the form present in the ER is the P22 protein. P22 is then cleaved to the endoplasmic reticulum (ER) through a signal peptide, and plays a different role in the viral cycle. Precore is addressed to the ER form and retrotransported in the cytoplasm, allowing the idea that the hepatitis B virus develops a strategy to take advantage of the ER-associated degradation pathway, allowing distinct subcellular localization and probably distinct roles for precore and Grp78/BiP. Altogether, these data support the idea that the hepatitis B virus develops a strategy to take advantage of the ER-associated degradation pathway, allowing distinct subcellular localization and probably distinct roles for the viral precore protein.

Hepatitis B virus (HBV), a member of the Hepadnaviridae family, is a human hepatotropic virus responsible of chronic infections in 5–10% of infected patients. Persistence of HBV in the liver leads to development of cirrhosis and hepatocellular carcinoma. Although mechanisms involved in the establishment of viral persistence have not been yet elucidated, several studies have suggested that the viral precore protein plays an important role in this process (1–3).

HBV precore/core open reading frame contains two ATG initiation codons in-frame, which generate two proteins, the core protein (P21) and the precore protein (P25), that share almost their entire primary sequence with an extended N-terminal region for P25. The core protein is synthesized in the cytosol and self-assembles into a 180- or 240-subunit nucleocapsid structure (4). Biosynthesis of the precore protein uses another pathway as the P25 protein contains a 19-amino-acid signal peptide targeting the protein to the endoplasmic reticulum (ER) (5). During translocation of the nascent polypeptide into the lumen of the ER, the signal peptide is cleaved, leading to a 22-kDa precore protein (P22) that differs from P21 by 10 residues at its N-terminal end. P22 is further processed in the trans-Golgi network and cleaved by furin protease leading to HBe antigen secretion (6). Interestingly, a cytosolic form of precore has been observed in HBV transgenic mice and in HuH-7 cells expressing the precore gene (7, 8). This form, with an apparent molecular mass of 22 kDa, will be referred in this study as cytosolic P22 molecule. The cytosolic P22 molecules are assessed only in about 15% of P22 expressed proteins (8).

The biological function of precore in viral cycle could be played by HBe and/or P22 precore form, and until now, this role has not been clearly defined. Precore is an accessory protein, not required for viral replication or infection (9–11) but important for natural infection in vivo (1, 3, 12). It has been suggested that HBe could be involved in viral persistence by inducing immunologic tolerance (13, 14). Moreover, in HBV transfected cells, mutations leading to an abolition of precore gene expression result in a significant increase of HBV replication, suggesting that precore can negatively regulate this process (15, 16). This negative effect of precore was suggested to be due to cytosolic P22 molecules, which could form heterodimers with the core proteins (17). These hybrid nucleocapsids have been shown to be unstable and devoid of viral genome. In these studies, the authors presume that the cytosolic P22 protein is identical to the P22 protein present in the secretory pathway (here referred as ER P22). However, recently, some hybrid nucleocapsids containing a P22 molecule distinct from the ER P22 have been isolated in the serum of HBV chronically infected patients.
(18). In this case, the precore protein has conserved its signal peptide and has been cleaved at its C-terminal end. Therefore, various observations report the presence of a cytosolic P22 protein in infected cells, but its molecular identity and the mechanism explaining its presence in the cytosol remain controversial.

Newly synthesized proteins in the ER are subjected to a rigorous quality control system, and consequently, the misfolded proteins should be retrotransported back into the cytoplasm to be degraded by the ubiquitin-proteasome system. This multi-step process, called ER-associated degradation pathway (ERAD), has been characterized recently (for review, see Ref. 19). First, misfolded proteins are recognized by chaperone proteins, such as Grp78/BiP (20–22) or protein disulfide isomerase (PDI) and by ER resident chaperone-like lectins, such as the ER degradation enhancing α-mannosidase-like (EDEM) protein (23). Altogether, these chaperones lead to deglycosylation and disassembly of substrates and drive them to the translocon channel. The composition of this channel remains contentious and probably distinct in yeast or mammal cells. Indeed, it has been first proposed that Sec61 transmembrane protein could be the main transporter, but conflicting data have been reported (24, 25). More recently, the derlin-1 protein has been characterized to be part of the translocon channel (26–28). The emerging substrate is most likely ubiquitinated on lysyl residues at the cytosolic side of the ER membrane and recognized by a complex, which contains the ATPase P97 and its cofactors Ufd1-Npl4 (29, 30). The ATPase pulls the substrate out of the membrane, and the polyubiquitinated substrate is then addressed to the proteasome for degradation.

The aim of our study was to define and characterize the cytosolic form of the HBV precore protein and the mechanism allowing its accumulation in the cytoplasm. For this purpose, a cytosolic fractionation procedure was developed to distinguish the few cytoplasmic P22 molecules from the ER P22. Using this procedure, we isolated and characterized cytoplasmic P22 as a protein identical to the ER P22, and we demonstrated that i) P22 reaches the cytoplasm using the ERAD pathway and ii) P22 escapes to proteasomal degradation due to its low lysine content. We observed a Grp78/BiP redistribution from ER to cytoplasm that occurs along with the P22 retrotransport. Moreover, we demonstrated a specific interaction between P22 and Grp78/BiP, which could explain this Grp78/BiP relocation. All these data show a new mechanism by which P22 could be a candidate to explain viral persistence.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—pCEP4mp25 and pCEP4mp21 were previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. pCEP4mp25-M11Q plasmid was generated by site-directed mutagenesis of the second start codon of the PreC/C open reading frame on pCEP4mp25 plasmid. This avoids the expression of the core protein and allows the expression of a precore protein mutated in the 11th amino acid. Precore mutants encoding single or multiple arginine to lysine substitutions were generated by site-directed mutagenesis on pCEP4mp25 plasmid. Resulting plasmids were named as follows: pCEP4mp25-5K (R136K and R142K substitutions), pCEP4mp25-6K (R48K, R136K, and R142K), and pCEP4mp25-0K (K1R, K16R, and K91R), numbering of amino acids start at the first amino acid of P22. pCEP4mp25-His plasmid was constructed in two steps; i) a pCEP4m-His vector was created by the insertion of a His tag at HindIII and Xhol sites of pCEP4m, and ii) precore sequences were amplified by PCR and cloned into the Nhel and HindIII sites of pCEP4m-His. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under control of cytomegalovirus promoter. The pHPCAsp plasmid, which allows the expression of a precore protein deleted of its signal peptide (P22Δsp), was previously described in Ref. 31. In these constructions, precore and core genes were cloned under.
Retrotranslocation of P22 HBV via ERAD

Santa Cruz Biotechnology (SC-2006). Proteins were detected using the ECL SuperSignal West Pico kit (Pierce).

**Cytosolic Fractionation Procedure**—Cytosolic fractionation was performed 48 h after transfection. Cells were washed twice with PBS and incubated for 3 min on ice with 3 ml of a digitonin buffer (50 mM Tris, pH 8, 150 mM NaCl, 22.5 μg/ml digitonin (D1407), 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml apro- tinin, 1 μg/ml leupeptin, all products provided by Sigma). Digitonin buffer containing cytosolic proteins was withdrawn and centrifuged at 1,500 × g for 2 min at 4 °C. Pellet was dried and suspended in 60 μl of PBS, Nonidet P-40 1% (Sigma). Lysates were cleared of debris by centrifugation at 16,000 × g for 10 min at 4 °C. Beads were washed in PBS, Nonidet P-40 1% Nonidet P-40 buffer containing protease inhibitors (0.5 mM leupeptin, Sigma). Lysates were cleared of debris by centrifugation at 16,000 × g for 10 min at 4 °C. For each reaction, 2.5 μl of anti-BiP antibody (StressGen Biotechnologies, SPA827) were added to SDS-PAGE.

**Co-immunoprecipitation**—Two cell dishes (100 mm) were washed twice with PBS, and cells were lysed in 1 ml of PBS, 1% Nonidet P-40 buffer containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml apro tinin, 1 μg/ml leupeptin, Sigma). Lysates were cleared of debris by centrifugation at 16,000 × g for 10 min at 4 °C. For each reaction, 2.5 μl of anti-BiP antibody (StressGen Biotechnologies, SPA827) were mixed overnight to 50 μl of protein A-coupled Sepharose (Sepharose CL-4B, GE Healthcare, 17-0780–01) at 4 °C in PBS, 1% Nonidet P-40 buffer. Then, lysates were incubated with the anti-BiP antibody and the protein A-coupled Sepharose overnight at 4 °C. Beads were washed in PBS, Nonidet P-40 1% buffer five times and boiled in Laemmli’s sample buffer. Half of the immunoprecipitates and 20% of the crude lysate were subjected to SDS-PAGE.

**RNA Extraction and RT-PCR**—Total RNA was extracted using RNeasy Plus Qiagen kit according to the manufacturer’s protocol, and then 1 μg of total RNA were treated with Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR amplification was realized using Taq polymerase from Invitrogen. For the glyceraldehyde-3-phosphate dehydrogenase transcript (sense primer, 5’-ATGGCATGGACTGTTGAGAACCAGG-3’, anti-sense, primer 5’-GATG-3’), cycling conditions were as follows: 95 °C for 3 min, 20 cycles (95 °C for 45 s, 55 °C for 30 s, and 72 °C for 30 s). For unspliced and spliced Xbp-1 transcripts (sense primer, 5’-CTTGTAGTTGAGAACCAGG-3’, anti-sense primer, 5’-GGGCGTTGGATATATATGTTG-3’), 95 °C for 3 min, 32 cycles (95 °C for 45 s, 60 °C for 30 s, and 72 °C for 30 s). RT-PCR products were separated by 8% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.

**RESULTS**

The Cytosolic P22 Is Identical to the ER P22—Precore has been reported to be present in the cytoplasm; however, the mechanism allowing this localization remains unknown. To characterize this point, we developed a cytosolic fractionation protocol to isolate and specifically detect cytoplasmic precore proteins, discriminating them from ER forms. Since the cytoplasmic precore forms are not abundant (around 15% of all precore derivatives), classical methods to permeabilize cells, which often slightly damage ER membrane, were not convenient. For this reason, we devised plasma membrane permeabilization conditions using mild non-ionic detergent digitonin, which selectively perforates the plasma membrane without ER membrane damage. The soluble cytosolic protein components, released out of the cells, are further precipitated by methanol. The insoluble remnant “ghost” cells are lysed in Laemmli buffer, and this extract will be referred as the non-cytosolic fraction. We did such an experiment on HEK293T cells transfected with a plasmid encoding precore (p25). Cytosolic fractionation assay was performed, and the presence of proteins in both fractions was analyzed by Western blot using antibodies directed against either precore protein or PDI, an endogenous luminal ER protein. As shown in Fig. 1A, distinct forms of the precore protein were detected in the non-cytosolic fraction. Additional experiments, described in the legend of supplemental Fig. S1, allowed us to characterize the P22 ER form (Fig. 1A, arrow) and the P21 protein, slightly expressed with this vector (lower band, triangle). The middle band, indicated by a star, is most likely due to an aberrant site of signal peptide cleavage. Cytosolic fraction analysis revealed the presence of precore protein in the cytosol (Fig. 1A, arrow). Indeed, the absence of PDI from the cytosolic fraction demonstrates that the fraction is not contaminated by luminal ER components. As the precore form detected in the cytosol displayed the same electrophoretic migration than the P22 ER form, we assumed that it is a form of 22 kDa that will be referred as the P22 cytosolic form. In an additional control, we overexpressed the HA-tagged secreted alkaline phosphatase protein (SEAP-HA), the subcellular localization of which is restricted to the secretory pathway. We performed the same experiment as in Fig. 1A, and we showed that SEAP-HA is not detected in the cytosol fraction (Fig. 1B). Altogether, these data rule out the possibility that the presence of P22 in the cytosol may be an artifact due to overexpression.

Two hypotheses could explain the presence of P22 molecules in the cytoplasm. First, cytosolic P22 could be the result of a retrotransport from ER to cytoplasm and consequently would be devoid of signal peptide. On the other hand, cytosolic P22 could correspond to a form, which still contains the signal peptide but has been subjected to a proteolytic cleavage in its C-terminal end (18). In all cases, these two forms will display the same electrophoretic migration and will not be distinguishable. To resolve this problem, we designed a new precore construction, named precore-His (P25-His), allowing the expression of a precore protein fused to a histidine tag at its C-terminal end. As shown in the diagram of the Fig. 2A, the first hypothesis should lead to a P22-His cytosolic form identical to the size of the ER form and still containing the histidine tag (23K). According to the second hypothesis, the cytosolic form would be shorter than the ER form and devoid of histidine tag (22K). Thus, HEK293T cells were transfected with a plasmid encoding either P25 or P25-His. A cytosolic fractionation assay was performed, and proteins expression was analyzed by Western blot using antibodies directed against precore, His tag, or PDI. PDI protein was detectable only in the non-cytosolic fractions, indicating that cytosolic fractions are not contaminated by luminal ER components (Fig. 2B). Precore and precore-His were equally
expressed in the non-cytosolic and cytosolic fractions. More importantly, precore-His cytosolic form showed the same electrophoretic migration as ER P22-His and clearly an apparent molecular mass greater than the wild type P22 cytosolic form (Fig. 2, compare lanes 5 and 6). Furthermore, the precore-His cytosolic form was recognized by the antibody directed against His tag. Taken together, these data demonstrate that a cytosolic precore derivative is the result of a retrotransport from ER to cytoplasm of the ER P22 protein.

**P22 Retrotransport Utilizes the ERAD Pathway**—To better understand and validate the retrotransport mechanism, we investigated the pathway involved in the translocation of P22 from luminal ER to cytosol. We assume that P22 could be retrotransported through the ERAD pathway, which is the main characterized pathway involved in translocation from ER to cytoplasm. However, as ERAD substrates are targeted to the ubiquitin-proteasome system, the question that arises is how precore could remain intact into the cytosol. The extremely low lysine content of precore (3 lysine residues on 193 amino acids) may favor the escape to ubiquitin conjugation. As a consequence, P22 will accumulate in the cytoplasm because it is not a suitable substrate for proteasome degradation. To test this hypothesis, distinct arginyl residues in positions 136, 142, and 48 were successively mutated into lysyl residues. The resulting mutant proteins, designated as P22-5K and P22-6K, were expressed in HEK293T cells. To assess the proteasome involve-
ment, 40 h after transfection, cells were treated or not with the MG132 proteasome inhibitor and then subjected to the fractionation assay. A Western blot analysis showed that wild type and mutant precore proteins were identically expressed in the non-cytosolic fractions (Fig. 3A, lanes 2–4). Interestingly, although precore wild type protein was easily detectable in the cytosolic fractions, few P22-5K proteins and none of the P22-6K mutant proteins were observed (lanes 9–11). In cells treated with MG132, P22-5K and P22-6K expression levels increased in non-cytosolic fractions (lanes 6 and 7), and more importantly, became detectable in the cytosol (lanes 13 and 14). This experiment strongly supports our hypothesis that wild type P22 protein is detectable in the cytosol due to its capacity to escape the proteasome degradation because of its low lysine content. Moreover, a mutant devoid of lysine (P22-0K) was constructed and tested in the same experiment. As shown in Fig. 3B, precore wild type and mutant proteins were easily and equally detectable in the cytosol in the absence of MG132 (lanes 5 and 6) as well as in the presence of the proteasome inhibitor (lanes 7 and 8). This P22-0K mutant behaved exactly like the wild type protein, demonstrating that the escape of P22 from the proteasome is efficient and complete.

To test whether precore is retrotransported through the ERAD pathway, two dominant negative mutants of this pathway were used. The P97 QQ mutant is a dominant negative mutant of the P97 ATPase, which does not affect the binding of substrates but significantly reduces the ATPase activity (30). Derlin-1GFP, corresponding to the derlin-1 protein fused to the GFP protein at its C-terminal end, plays a dominant negative role by disturbing retrotranslocon channel (33). HEK293T cells were transfected with plasmid encoding precore protein, and 24 h later, subjected to a second transfection with plasmids encoding either P97 wild type protein or dominant negative mutants. Then, a cytosolic fractionation assay was performed, and fractions were analyzed as already described. Co-expression of wild type P97 or dominant negative mutants did not affect ER precore protein expression (Fig. 4, lanes 2–6). In contrast, a strong reduction of P22 amount in the cytosol was observed when precore was co-expressed with dominant negative mutant (Fig. 4, compare lanes 11 and 12 with lanes 9 and 10). Tubulin expression level was not affected under the same conditions, showing cell integrity. These data clearly demonstrate that precore proteins use the ERAD pathway to drive a pool of P22 molecules from ER to cytoplasm and escape the proteasome degradation due to its low lysine content.

BiP Chaperone Protein Is Relocated to the Cytoplasm in the Presence of P22 and Interacts Specifically with the Precore Protein—Interestingly, among the several ER luminal proteins we used to check the purity of the cytosolic fraction, we noticed that the BiP chaperone protein behaves distinctly. Indeed, when cells expressing precore protein were subjected to the cytosolic fractionation assay, BiP was clearly detectable in the corresponding cytosolic fractions (Fig. 5). This observation is specific as SEAP-HA overexpression did not induce the same BiP relocation. Moreover, as precore protein is closely related to P21 core protein, we also analyzed whether P21 could induce the same effect (Fig. 5). It turned out that, in the presence of
none of the BiP molecules were found in the cytosolic fraction, showing that in our experiments, BiP relocation is restricted to precore protein.

To test whether this relocation is the consequence of an interaction between precore and BiP, we performed immunoprecipitation experiments. HEK293T cells were transfected with plasmids encoding precore, core, or SEAP-HA protein. Cellular extracts were immunoprecipitated with an antibody directed against BiP protein, and the co-immunoprecipitated proteins were analyzed by Western blot using antibody directed against precore/P21 proteins. As shown in Fig. 6A, only the precore protein is detected (lanes 5 and 6). To analyze whether an unrelated protein overexpressed in the ER could also be co-immunoprecipitated by BiP, we analyzed the co-immunoprecipitated proteins by Western blot using antibody directed against HA tag (Fig. 6B). It turned out that SEAP-HA protein was not co-immunoprecipitated with BiP (Fig. 6B, lane 4), demonstrating that precore protein is specifically associated with BiP in vivo. Altogether, these results show that the relocation of BiP in the cytosol could be the consequence of an interaction between these two proteins.

Precore Protein Does Not Induce the IRE-1/XBP-1 Unfolded Protein Response—BiP is a chaperone protein playing multiple activities in the cell. Among them, BiP regulates the unfolded protein response (UPR), which is activated during ER stress. It has been previously shown that the presence of BiP in ER negatively regulates the UPR. Consequently, the cellular redistribution of BiP observed in our experiments could activate UPR. The UPR is characterized by three pathways (34). One of them, the inositol-requiring enzyme 1 (IRE-1) pathway, leads to the production of a distinct spliced form of the xbp-1 transcript, which encodes the active transcription factor XBP-1 (35). XBP-1 induces a subset of genes encoding molecules involved in ER-associated protein degradation. As one HBV promoter is thought to be sensitive to the IRE-1 pathway, we examined the effect of precore expression on IRE-1 UPR. HEK293T cells were transfected with plasmids encoding precore, core, and SEAP-HA proteins. RNAs were extracted 48 h after transfection, and an RT-PCR was performed to detect spliced or unspliced xbp-1 transcripts. As a positive control, we treated cells with tunicamycin that is known to induce UPR and the xbp-1 splicing (36). As expected, the spliced form of xbp-1 is clearly observed after tunicamycin treatment (Fig. 7, lane 1), whereas in non-stressed cells, transfected with the empty vector, or in cells expressing SEAP-HA, only the unspliced form is detected (lanes 2 and 3). Interestingly, cells expressing core or precore proteins behave as non-stressed cells as the unspliced form is the main transcript observed (lanes 4 and 5). To confirm these results, the same experiments were performed in a human hepatoma cell line, HuH-7 cells. Results shown in Fig. 7 were identical to those obtained in the previous experiment, the
The unspliced form of xbp-1 is observed in all conditions, except in cells treated with tunicamycin (lanes 6–10). These results demonstrate that BiP relocation observed under precore expression does not induce the xbp-1 transcript splicing and hence the IRE-1 UPR.

**DISCUSSION**

Hepatitis B precore protein is a non-classical protein, which has several derivatives, each of them having most likely distinct roles in the infected cells. In this study, we focused on the precore cytoplasmic form. First, we set up a fractionation experiment, allowing specific detection of the few cytoplasmic precore molecules present in the cytoplasm when compared with the large amount of P22 molecules present in the ER lumen. Thus, the use of a digitonin extraction buffer allowed us to differentiate cytoplasmic P22 from ER P22, which is a significant progress to further characterize the role of precore. Furthermore, our results provide new information on the identity of the precore form present in the cytoplasm. Indeed, Kimura et al. reported recently the existence of HBV DNA-negative nucleocapsids containing precore protein with an uncleaved signal sequence but lacking the C-terminal arginine-rich domain (18). They hypothesized an inefficient translocation of precore, leading to particle formation in the cytosol. Here we show, using a strategy with a C-terminally tagged version of precore, that the precore cytoplasmic form is identical to the P22 ER form. Our result supports the idea that P22 is a consequence of a retrograde transport rather than an abortive translocation. They are in agreement with the hypothesis that intracellular hybrid nucleocapsids observed in vitro (17) are composed of P22 without signal peptide. The discrepancy observed with Kimura et al. results could be explained by the fact that they studied extracellular particles present in the serum of patients with persistent HBV infection.

We also demonstrate that precore takes advantage of the ERAD system to leave the ER, as shown by the use of well characterized dominant negative mutants of this pathway. The ERAD system retrotranslocates misfolded ER luminal or membrane proteins into the cytosol for degradation by the 26 S proteasome (19). It implicates that (i) precore should be either recognized as an unfolded protein or implicated in a specific interaction with a component of the ERAD pathway and (ii) precore should remain intact in the cytosol. We responded clearly to the last point by demonstrating that due to its low lysine content, precore escapes proteasome degradation. Indeed, mutants with increased lysine content were degraded in the cytosol, whereas treatment with proteasome inhibitor allowed their detection. This strategy has already been

**FIGURE 6.** Precore specifically interacts with BiP in vivo. A and B, HEK293T cells were transfected with vectors expressing SEAP-HA, P21, or P25 proteins. Cells were lysed in a PBS, 1% Nonidet P-40 buffer. Cellular extracts were subjected to a specific BiP immunoprecipitation (IP) using an anti-BiP antibody. Immunoprecipitates and crude extracts were subjected to an electrophoresis on a 12.5% SDS-PAGE and probed with the antibodies indicated on the left.

**FIGURE 7.** Precore does not induce the IRE-1/XBP-1 unfolded protein response pathway. HEK293T and HuH-7 cells were transfected either with the empty pCEP4m vector or with vector expressing P25, P21, or SEAP-HA proteins. Forty-eight h after transfection, RNAs were extracted, and RT-PCR experiments were performed with oligonucleotides specifically amplifying the xbp-1 or the glyceraldehyde-3-phosphate dehydrogenase (gapdh) transcripts. Both unspliced (u.s.) and spliced (s.) forms of xbp-1 transcript are indicated on the right. TM, tunicamycin-treated cells.
reported for some bacterial and plant toxins. Toxins use different endocytic mechanisms to enter cells and then are transported to the cytosol from distinct organelles (37). Several toxins are subject to retrograde transport via the Golgi apparatus to the ER. Ricin A, a plant toxin, and more recently the Pertussis bacterial toxin, have been shown to reach the cytoplasm using the ERAD pathway and to escape the proteasome due to few sites being available for ubiquitination (38, 39). In addition, some viruses are able to hijack the ERAD pathway. For example, US11 human cytomegalovirus protein and K3 Kaposi’s sarcoma-associated herpesvirus protein mediate dislocation and degradation of major histocompatibility complex class I molecules through the ERAD pathway (33, 40, 41). In contrast to precore protein, US11 and K3 are transmembrane glycoproteins, which are not further present in the cytosol. To our knowledge, it is the first time that a viral non-transmembrane protein is shown to use this pathway to be addressed into the cytosol. Hence, our results could open perspectives to study the luminal ERAD pathway using precore as a new substrate model.

Interestingly, we showed a specific redistribution of Grp78/ BiP protein, a multifunctional chaperone, from ER to cytoplasm in the presence of precore. This non-classical localization of BiP is not observed in control cells nor in cells overexpressing the SEAP protein. The co-immunoprecipitation experiment demonstrates an interaction between BiP and precore. Furthermore, this interaction is specific as an overexpressed unrelated protein SEAP-HA is not observed in association with BiP. Therefore, the relocation of BiP is most likely the consequence of this interaction.

To explain the consequence of BiP redistribution under precore expression, we can address some hypotheses. BiP is known to be involved in the UPR (42). The UPR is a complex signal transduction cascade activated following ER stress. Three distinct pathways have been characterized, involving three ER transmembrane proteins: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and IRE-1 (34). BiP is known as the sensor of these three pathways since under normal conditions, BiP is bound to the ER luminal domain of these three proteins. Under stress conditions, BiP is released from these proteins, causing activation of UPR. According to Rao et al., this activation is coupled to a redistribution of BiP in the cytosol (43). In HBV infection, the IRE-1 activation pathway has been shown to have a role in activating a viral promoter (44), and recently, it has been disclosed that the hepatitis B X protein could activate IRE-1 and ATF6 pathways (45). Thus, we could also suspect a role of precore in activation of UPR through BiP redistribution. In this regard, we addressed the question of the induction of the IRE-1 pathway. As we did not observe any induction of this pathway by precore, BiP redistribution is probably not associated to the UPR. Additional experiments, especially analysis of ATF6 and PKR-like ER kinase pathways, are going to be performed to confirm this point.

Years ago, reports suggested that BiP may protect against ER stress-induced cell death through the inhibition of caspase-3 (46, 47). As one study links this activity to BiP redistribution from lumen to cytosol (43), precore could indirectly behave like an anti-apoptotic protein, which would participate in the establishment of viral persistence. Recent works support the idea that BiP interacts with the large HBV envelope protein by enhancing its specific folding (48–50). We could hypothesize that precore could disturb this folding, leading to a decrease of infectious particles budding. The direct consequence of this decrease would be an accumulation of the viral DNA in the nucleus, which is responsible for the viral persistence. In this regard, precore would participate in viral persistence enhancement. Finally, we could not exclude that precore could also promote a redistribution or degradation of other cellular proteins, like major histocompatibility complex-I molecules, known to be down-regulated in HBV infection (51). All these points are under investigation.

Acknowledgments—We thank Dr. Yihong Ye for providing us the P97 dominant negative mutant and Dr. Hidde L. Ploegh for the Derlin-1 dominant negative mutant. We are grateful to members of the Molecular Virology team in Laboratoire de Génétique et Biologie Cellulaire for helpful comments and discussions. We thank S. Larmory for careful reading of this manuscript.
Retrotranslocation of P22 HBV via ERAD

scovics, A., and Nagata, K. (2001) EMBO Rep. 2, 415–422
24. Schmitz, A., Herrgen, H., Winkeler, A., and Herzog, V. (2000) J. Cell Biol. 148, 1203–1212
25. Wahlman, J., DeMartino, G. N., Skach, W. R., Bulleid, N. J., Brodsky, J. L., and Johnson, A. E. (2007) Cell 129, 943–955
26. Knop, M., Finger, A., Braun, T., Hellmuth, K., and Wolf, D. H. (1996) EMBO J. 15, 753–763
27. Lilley, B. N., and Ploegh, H. L. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 14296–14301
28. Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T. A. (2004) Nature 429, 841–847
29. Halawani, D., and Latterich, M. (2006) Mol. Cell 22, 713–717
30. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001) Nature 414, 652–656
31. Laine, S., Thouard, A., Derancourt, J., Kress, M., Sitterlin, D., and Rossignol, J. M. (2003) J. Virol. 77, 12875–12880
32. Kothe, M., Ye, Y., Wagner, J. S., De Luca, H. E., Kern, E., Rapoport, T. A., and Lencer, W. I. (2005) J. Biol. Chem. 280, 28127–28132
33. Lilley, B. N., and Ploegh, H. L. (2004) Nature 429, 834–840
34. Schroder, M. (2008) CMLS Cell Mol. Life Sci. 65, 862–894
35. Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002) Nature 415, 92–96
36. Yoshida, H., Oku, M., Suzuki, M., and Mori, K. (2006) J. Cell. Biol. 172, 565–575
37. Lord, J. M., Roberts, L. M., and Lencer, W. I. (2005) Curr. Top. Microbiol. Immunol. 300, 149–168
38. Deeks, E. D., Cook, J. P., Day, P. J., Smith, D. C., Roberts, L. M., and Lord, J. M. (2002) Biochemistry 41, 3405–3413
39. Worthington, Z. E., and Carbonetti, N. H. (2007) Infect. Immun. 75, 2946–2953
40. Loureiro, J., and Ploegh, H. L. (2006) Adv. Immunol. 92, 225–305
41. Wang, X., Ye, Y., Lencer, W., and Hansen, T. H. (2006) J. Biol. Chem. 281, 8636–8644
42. Lee, A. S. (2005) Methods (San Diego) 35, 373–381
43. Rao, R. V., Peel, A., Logvinova, A., del Rio, G., Hermel, E., Yokota, T., Goldsmith, P. C., Ellerby, L. M., Ellerby, H. M., and Bredesen, D. E. (2002) FEBS Lett. 514, 122–128
44. Huang, Z. M., Tan, T., Yoshida, H., Mori, K., Ma, Y., and Yen, T. S. (2005) Mol. Cell. Biol. 25, 7522–7533
45. Li, B., Gao, B., Ye, L., Han, X., Wang, W., Kong, L., Fang, X., Zeng, Y., Zheng, H., Li, S., Wu, Z., and Ye, L. (2007) Virus Res. 124, 44–49
46. Liu, H., Miller, E., van de Water, B., and Stevens, J. L. (1998) J. Biol. Chem. 273, 12858–12862
47. Yu, Z., Luo, H., Fu, W., and Mattson, M. P. (1999) Exp. Neurol. 155, 302–314
48. Awe, K., Lambert, C., and Prange, R. (2008) FEBS Lett. 582, 3179–3184
49. Cho, D. Y., Yang, G. H., Ryu, C. J., and Hong, H. J. (2003) J. Virol. 77, 2784–2788
50. Lambert, C., and Prange, R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5199–5204
51. Chen, Y., Cheng, M., and Tian, Z. (2006) Cell Mol. Immunol. 3, 373–378