Proteolytic Processing of the Hepatitis B Virus e Antigen Precursor

CLEAVAGE AT TWO FURIN CONSENSUS SEQUENCES*

Received for publication, July 29, 2002, and in revised form, October 29, 2002
Published, JBC Papers in Press, November 1, 2002, DOI 10.1074/jbc.M207634200

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The hepatitis B virus P22 protein is a nonstructural protein that is the precursor of the 17-kDa secreted e antigen (HBeAg). The mature HBeAg is obtained after the removal of the C-terminal region of P22, a process which involves a proprotein convertase. Our studies show first that the protease could cleave P22 at the C-terminal side of Arg167 or Arg154 and second, that the maturation process can be either done in one step or in two steps with the generation of a processing intermediate (P20). Our data also demonstrate that the removal of the P22 C terminus, which occurs mainly in the trans-Golgi network, can also be achieved after exocytosis. Keeping in mind this characteristic and the amino acid sequence of the cleavage sites, we concluded that furin is involved in the maturation of the HBeAg. In addition, with the knowledge that in our experimental system, the HBeAg is a 164-amino acid protein and not a 159-amino acid protein as previously reported.

The hepatitis B virus (HBV) e antigen (HBeAg) is a 17-kDa protein secreted into the serum of patients infected with wild-type HBV. It derives from the precore protein, which is encoded by the PreC/C gene. The function of HBeAg and/or its precursors during the course of infection is still enigmatic, although conservation of a similar e antigen among all of the members of the hepadnavirus family argues for an important role of at least one of these proteins in the viral life cycle. Even though a role for infectivity or viral multiplication has been ruled out (1), at least one of these proteins in the viral life cycle. Even though a role for infectivity or viral multiplication has been ruled out (1), several reports have nevertheless brought circumstantial evidence that the PreC/C gene products could be involved in the establishment of persistent infection (2).

Most of the synthetic pathway of HBeAg is now clearly established (2). The precore protein (Fig. 1) is co-translationally directed to the secretory pathway by a 19-amino acid signal peptide. The latter is cleaved during translocation of the nascent chain into the lumen of the endoplasmic reticulum. The 22-kDa resulting protein (named P22) is 193 amino acids long and is further processed into mature HBeAg. The same biosynthetic pathway has been described for both the Duck Hepatitis B virus e antigen and the Woodchuck hepatitis virus e antigen (3, 4).

In 1983, Takahashi and co-workers (5) reported that the C-terminal extremity of the secreted HBeAg was located at position 149 (see Fig. 1), leading to the conclusion that the mature HBeAg was obtained by the cleavage of the last 34 amino acids from the P22 C terminus. Indeed, very little is known about the protease involved in this process, except that a proteolytic self-cleavage of P22 is excluded (6). More recently, it has been reported that a proprotein convertase is involved in the maturation of P22 (7). However, neither the exact nature of the protease nor the cleavage sites have been determined. This led us to study these points to get new insights in the mechanism of P22 processing and thus complete our understanding of the process of HBeAg synthesis.

Furin is a transmembrane proprotein convertase localized in the trans-Golgi network (TGN), transported to the plasma membrane and then retrieved back through the endocytic pathway (8). The consensus sequence for furin cleavage is RX/K/R, both arginine residues at positions P1 and P4 being absolutely required, giving the minimal furin cleavage site RX(R)(9). However in some cases, an alternate sequence 6 amino acids long with at least two basic residues of three at positions P6, P4, or P2, in addition to the P1, can also constitute a competent furin cleavage site (8, 9). Interestingly, six potential furin cleavage sites can be found in the 34-amino acid C-terminal domain of P22 (Fig. 1): two consensus cleavage sites (RXRR), two minimal cleavage sites (RXXXXR), and two alternate 6-amino acid sequences (RXRRXXR and RXRRXXR). These studies present how that a furin protease cleaves P22 at two positions within the C-terminal domain: one cleavage generating a processing intermediate (P20) and the other one generating the HBeAg. Our data also demonstrate that the removal of the P22 C terminus, which occurs mainly in the TGN, can also be achieved after exocytosis. In addition, we show that in our experimental system HBeAg is a 164-amino acid protein, in contradiction with the previous result published by Takahashi et al. (5).

EXPERIMENTAL PROCEDURES

Plasmids—Plasmid pHPC (formerly named pMLP-PC), which encodes the HBeAg precursor, the precore protein, has been previously described (10). It contains the Pre-C sequence and C gene of the HBV subtype ayw genome, under the control of the adenovirus major late promoter. Substitution mutants were constructed by site-directed mu-
FIG. 1. Complete amino acid sequence of the precore protein, the HBeAg precursor. The precore protein is encoded by the entire HBV C open reading frame. This ORF contains two in-frame initiation codons, delimiting the PreC sequence (29 codons) and the C gene (183 codons). Conventionally, position number 1 is assigned to the first amino acid of the core protein encoded by the C gene. Consequently, the first amino acid of the precore protein is located at position −29. After the cleavage of the 19-amino acid signal peptide, the resulting P22 precursor begins at position −10. According to Takahashi et al. (5), the HBeAg derives from P22 by removal of its 34 C-terminal amino acids, and consequently ends at Val149. Positions of important amino acids are boxed and the two 6-amino acid putative furin cleavage sites present in the P22 C-terminal part are underlined.

FIG. 2. Existence of a processing intermediate in the HBeAg maturation process. A, COS-7 cells were transfected with plasmid pHPC, which encodes the HBeAg precursor. Forty-eight hours after transfection, proteins were radiolabeled for 3 h. Proteins from cell extracts or culture media were immunoprecipitated and separated on a 12.5% SDS-PAGE as indicated under “Experimental Procedures.” Results of three independent transfections are shown; the mock-transfected immunoprecipitated cell extract or medium is on the corresponding far left lane. On the right of the figure are indicated migration positions of 14C-labeled molecular mass standards (in kDa) and on the left are those of the HBeAg, its precursor P22, and P20. B, COS-7 cells were transfected with plasmid pHPC, and 48 h later, proteins were labeled for 3 h. Brefeldin A (BFA, 18 μM) was added (+) or not added (−) one hour before labeling and was present up to the end of labeling. Proteins from cell extracts or media were analyzed as in A. Migration positions of 14C-labeled molecular mass standards (in kDa), HBeAg, its precursor P22, and P20 are indicated.

RVKR-cmk during methionine/cysteine depletion and protein labeling. The inhibitor was also present in the lysis buffer (phosphate-buffered saline-Nonidet P-40).

RESULTS

Existence of a Processing Intermediate in the HBeAg Maturation Process—Given that six putative furin cleavage sites are present in the P22 C-terminal domain (see Fig. 1), the existence of other processing products in addition to the mature HBeAg can be envisaged. Transient expression of the precore protein was achieved by transfection of COS-7 cells with plasmid pHPC. Proteins from transfected cells were metabolically labeled with highly radioactive 35S-labeled amino acids, and the precore-derived proteins were immunoprecipitated from the cell lysate as described under “Experimental Procedures.” Fig. 2A shows the specifically immunoprecipitated proteins, with the typical occurrence of HBeAg alone in the medium and both P22 and HBeAg in the cell extract (12). Interestingly, an additional faint band with an apparent molecular mass of 20 kDa (named P20) was detected in the cell extract. P20 could either correspond to a processing intermediate, generated in exocytic compartments, or to the result of a degradation of some misfolded P22 molecules that occurs in the cytosol (13, 14). To determine whether P20 was formed in the secretory pathway, we used the fungal metabolite brefeldin A (BFA), which blocks the traffic of secreted proteins (15). Thus, pHPC-transfected cells were grown in the presence 18 μM BFA and proteins from cell extract or cell medium were analyzed (Fig. 2B). As expected, HBeAg was no longer secreted when BFA was present. Interestingly, in the cell extract, P22 was accumulated in higher amounts.
than in standard conditions, whereas neither P20 nor HBeAg was detected. From this data, we can exclude that P20 is a cytosolic degradation product of P22. Rather, as BFA blocks the traffic between the endoplasmic reticulum and the Golgi apparatus, we conclude that P20 is a processing intermediate generated from P22 in a post-endoplasmic reticulum compartment of the secretory pathway.

It is worth noting that P20 was detected only in the cell extract, suggesting it may not be secreted in the extracellular medium. However, we could not exclude that P20 had a short half-life after exocytosis and thus could not be detected when newly synthesized proteins were labeled for 3 h. To decide between these two possibilities, a pulse-chase protein labeling experiment was performed as described under “Experimental Procedures.” Fig. 3 shows that HBeAg and P20 were both immunoprecipitated from the cell media as well as from the cell extracts, demonstrating that P20 is actually excreted. This finding confirmed that P20 is generated in the secretory pathway. Moreover, these data strongly suggested that P20 could be further matured into HBeAg in the extracellular medium. All of these observations are in support of the conclusion that P22 is matured by a protease enzymatically active both in a post-endoplasmic reticulum compartment and at the cell surface, which is a well known characteristic of furin (8).

A Cellular Proprotein Convertase Is Involved in the Maturational Process of the HBeAg—To determine whether furin is the protease involved in P22 maturation, a specific inhibitor, the decanoyl-RVKR-chloromethylketone (dec-RVKR-cmk) (16) was used in the next experiment. The inhibitor, at a concentration of 20 μM, was added in medium from pHPC-transfected cells during depletion and was maintained through protein radiolabeling and cell lysis. As shown in Fig. 4, under these conditions, HBeAg was not present in the culture medium and P22 was the only pHPC-encoded protein detected in the cell extract. Thus, when the proteolytic activity of furin was inhibited, neither P20 nor HBeAg could be formed, indicating that this protease is involved in the biosynthetic pathway of HBeAg.

The C terminus of HBV P22 contains six putative furin cleavage sites (Fig. 1): two RXXR motifs (positions 151–154 and 154–157), two RXRR motifs (positions 164–167 and 172–175), one RXXXR motif (positions 152–157), and one RXXRRR motif (positions 154–159). Thus, we next determined among them which ones were effectively cleaved to generate P20 or HBeAg. To address this question, several independent substitution mutants were constructed to abolish each putative cleavage site (see Fig. 5). Introduction of different mutations in the 172RERRKR175 motif did not modify the P20 and HBeAg amounts in the cell extract or in the culture medium (data not shown), allowing us to exclude this sequence as a suitable cleavage site for the HBeAg maturation process. In contrast, abolishment of the cleavage motif 154RERR157 (mutants R164G and R167G; see Fig. 5) resulted in the disappearance of P20 in the cell extract (Fig. 6A, cells, lanes 164 and 167). Interestingly, the secretion of HBeAg was not modified (Fig. 6A, media, compare lane PC and lanes 164 and 167). From these results, it can be concluded first, that P20 results from the cleavage of P22 at the C-terminal side of Arg167 and second, that the formation of the HBeAg does not require the preliminary generation of P20.

We next focused on the cleavage that generated mature HBeAg. Replacement of Arg154 by a glycine (mutant R154G) had a drastic effect on P22 maturation. Indeed, formation of HBeAg was completely abolished (Fig. 6B, lane 154) whereas the amount of P20 was markedly increased in the cell extract. Concomitantly, P20 became detectable in the extracellular medium after a 3-h protein labeling which, as we showed above, was not the case for the wild-type protein (Fig. 6B, media, compare lanes PC and 154). From these data, we conclude that Arg154 is absolutely required to generate mature HBeAg. Interestingly, mutation R154G affects four putative furin cleavage sites: 151RRGR154, 154RGRSPR157, 154RSPR157, and 154RSPRR159. However, substitution of Arg157 or Arg159 by a glycine (mutants R157G and R159G, respectively) did not affect the synthesis of HBeAg (data not shown), allowing us to exclude the implication of the putative cleavage sites 154RGRSPR157, 154RGRSPR157, or 154RSPRR159 in the HBeAg maturation process. Furthermore, the results obtained with mutant R151G were identical to those obtained with mutant R154G (Fig. 6B, compare lanes 151 and 154), demonstrating that the presence of the cleavage site 151RRGR154 is critical for the formation of the HBeAg.

Taken together, our results clearly demonstrate that first, furin is involved in the HBeAg maturation process and second, the removal of the P22 C-terminal domain implies specific cleavages at the C-terminal side of Arg167 and Arg154. Alterna-
Furin Maturates the HBe Antigen

Fig. 5. Substitution mutants of the precore protein. The wild-type sequence of the P22 C-terminal region is shown on the upper line, with the six putative furin cleavage sites underlined and numbered. Names of all of the substitution mutants referred to in this paper are on the left. In each case the corresponding sequence is shown, with the mutation indicated in bold and the number of the cleavage sites abolished.

A

| mutant | putative cleavage site(s) abolished |
|--------|-----------------------------------|
| R151G  | GVRG                              |
| R154G  | GRRG or GRRR                       |
| R157G  | GRRG or GRRR                       |
| R159G  | GRRG or GRRR                       |
| R164G  | GRRR                              |
| R167G  | GRRR                              |

B

The Secreted HBeAg C Terminus Is Located at Arg154—A cleavage of P20 or/and P22 at position 154 is not in agreement with the C terminus of the HBeAg previously reported at position 149 for subtype adr (5). Because subtypes adr and ayw share the same nucleotide sequence for the PreC/C gene, this may indicate that a further step of maturation is required to eliminate the last 5 amino acids. Alternatively, the size of the HBeAg produced by ex vivo cell transfection could be different from that of the HBeAg purified from the sera of HBV infected patients by Takahashi et al. (5). We thus decided to determine accurately the length of the HBeAg produced in our experimental system. For this purpose, cells were transfected with plasmids encoding wild-type (pHPC) or truncated HBeAg precursors ending exactly at Val149 or Arg154 (C149 or C154 proteins respectively). Analysis of immunoprecipitated proteins by SDS-PAGE showed that the HBeAg encoded by pHPC migrated at the same position as the C154 protein (Fig. 7). This result demonstrates that in our experimental system, the accurate HBeAg C terminus is located at Arg154 rather than at Val149. It is important to note that the same result was obtained in a human epithelial kidney cell line (HEK 293, data not shown).

Discussion

In this report, we present new data on the last unresolved steps of HBeAg biosynthesis. Our studies established that two endoproteolytic cleavages occur during P22 maturation, one cleavage giving rise to a 177-amino acid intermediate (P20) and the other generating the 164-amino acid mature HBeAg. It should be noted that these two proteolytic events are independent. In other words, the maturation of the HBeAg does not require the prior formation of the P20 intermediate. This is clearly demonstrated by our experiments in which mutated precursors that cannot produce P20 are not impaired in the HBeAg secretion. Using a specific inhibitor and P22 molecules bearing mutated cleavage motifs, we also provide evidence that these endoproteolytic cleavages are due to a member of the proprotein convertase family, most likely the furin.

Seven proteases belonging to the mammalian proprotein convertase (PC) family have been so far identified: furin/PACE, PC1/PC3, PC2, PC4, PACE 4, PC5/PC6, and LPC/PC7/PC8/SPC7 (see Refs. 9 and 17 for reviews). All PCs typically cleave their substrates on the C-terminal side of arginine residues. In contrast, they have different tissue distribution. Expression of PC2 and PC1/PC3 is limited to neuroendocrine tissues, whereas that of PC4 is highly restricted to testicular spermatogenic cells. In contrast, furin, PACE4, PC5/PC6, and LPC/PC7/PC8/SPC7 are expressed in a broad range of tissues and cell lines (9). Thus, with the exception of PC5/PC6, which is not present in the TGN (9), we assume that these three ubiquitous proteases could be responsible for the proteolytic processing of P22 into HBeAg.

In our experimental system, HBeAg can be generated in one step, by a cleavage at the C-terminal side of Arg154. That this cleavage is caused by the furin is supported by two complementary results. First, we have shown that HBeAg can be generated in the TGN or in the extracellular medium, implying that the processing enzyme is present in the TGN as well as at the cell surface or secreted. This is one of the features of furin, which cycles between the TGN and the plasmic membrane and
which is also secreted in an active form (8, 17, 18). Second, furin is the only member of the PC family that does not have a strong requirement for a basic residue in position P2 (9, 19) and consequently is able to recognize a motif as RRGR164. The situation is less clear for the generation of P20 that arises in the sera of infected patients. For instance, in the case of pro-hormone processing, a carboxypeptidase removes one or two basic residues after the cleavage by the proprotein-convertase (18). Howev-
er, the involvement of LPC/PC7/PC8/SPC7 or PACE 4 cannot be totally excluded. To overcome this problem, LoVo cells unable to produce functional furin were used (21). Unfortunately, the low efficiency of transfection obtained with this cell line did not allow us to draw a clear conclusion. It is worth noting that four of six putative furin sites were not cleaved. This cannot be explained by the nature of P1 and P2 residues, which are neither a lysine nor a hydrophobic residue, a characteristic reported to impede proper cleavage by furin (22, 23). A possible explanation could be the nonaccessibility of some putative cleavage sites to the protease that could either be caused by the folding of P22 or to the presence of cellular proteins that interact with its C-terminal domain. We have previously reported two such proteins which interact specifically with the C-terminal domain of P22 (12, 24).

The next question is: what could be the function of P20 in the life cycle of HBV? One hypothesis derives directly from our previously reported data (10, 24). We have shown that the P22 C-terminal domain plays an important role during the intra-
cellular transport of P22 through the secretory pathway, because a sequence located between amino acids 144 and 165 is required for an efficient HBeAg secretion via an interaction with a cellular tunicamycin-sensitive protein named TSP (24). Consequently, it could be envisaged that TSP would interact with a precursor of the HBeAg shorter than P22, like P20, which ends at Arg167. Other experiments will be required to conclude about this point.

In our experimental system, our results strongly indicate that the HBeAg ends at Arg164 instead of Val149, as previously reported by Takahashi et al. (5). However, it is worth noting that these authors have determined the C terminus of the HBeAg purified from patient's sera whereas we have studied the generation of HBeAg in cell culture. Therefore, it could be speculated that another proteolytic event arises in the sera of infected patients. For instance, in the case of pro-hormone processing, a carboxypeptidase removes one or two basic residues after the cleavage by the proprotein-convertase (18). How-
ever, this second proteolytic event always occurs in the secretory pathway, implying that only the mature hormone is secreted. Although we cannot exclude that another proteolytic event occurs in the patient's sera during the HBeAg processing, our hypothesis is supported by previous results of Salfeld and co-workers (25). These authors have studied the HBeAg isolated from patients infected by the adw subtype of HBV that encode the sequence TTTV149 instead of the classical 146TTVVRRGR154 sequence. Interestingly, they reported that the adw HBeAg has a slower migration than the ayw HBeAg. This observation is not consistent with TTVV149 as the C-terminal extremity of the HBeAg but rather favors of our hypothesis. Because our results have been reproduced in two independent cell lines (COS-7 and HEK 293), demonstrating that they was not due to the specificity of COS-7 cells, we suggest that HBeAg actually ends at position 154 in vivo.

Acknowledgments—We thank for their technical assistance Marie-Thérèse Bidoyen and Anne Thouard.

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J. Biol. Chem. 2003, 278:891-895.
doi: 10.1074/jbc.M207634200 originally published online November 1, 2002

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