Importance of the C terminus of the hepatitis B virus precore protein in secretion of HBe antigen

Damien Carlier,1 Olivier Jean-Jean,2 Nathalie Fouillot,1 Hans Will3 and Jean-Michel Rossignol1*

1 Laboratoire de Génétique des Virus, UPR 2431-CNRS, Avenue de la Terrasse, 91198 Gif sur Yvette cedex, 2 Département de Biologie, Ecole Normale Supérieure, 46 rue d’Ulm, 75230 Paris cedex 05, France and 3 Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, Hamburg, Germany

The hepatitis B virus (HBV) e antigen (HBeAg) is a 15 kDa soluble antigen derived from a precursor protein (precore protein) by two processing events, cleavage of the N-terminal signal peptide and cleavage of the C-terminal 34 amino acids. So far, the role of the C-terminal sequences in secretion has not been analysed in full. In this study deletion of the last 60 amino acids was found to abrogate HBeAg secretion whereas deletions of the last 10, 25 or 39 amino acids decreased its secretion rate. These data demonstrate that C-terminal precore protein sequences are crucial for HBe secretion and determine its secretion rate.

The hepatitis B virus (HBV) e antigen (HBeAg) is a 15 kDa non-particulate protein detectable in the serum of patients infected with wild-type HBV when active viral replication occurs. The function of this antigen in the life cycle of the virus is still enigmatic. While HBeAg is dispensable for viral viability (Tong et al., 1991), this antigen seems to be a major immunological target involved in virus elimination, because viruses unable to express HBeAg are selectively enriched during the natural course of infection and after interferon treatment (Günther et al., 1992). Additionally, a role in the induction of T cell tolerance in utero has been suggested (Milich et al., 1990).

Much of the mechanism of HBeAg biosynthesis is now well understood. HBeAg is derived from the precore protein encoded by the HBV C open reading frame (ORF). The C ORF contains two in-frame initiation codons delimiting the pre-C sequence (87 nucleotides) and the C gene (Fig. 1). Precore protein translation starts at the first ATG while translation of the core protein, which is the subunit of the viral nucleocapsid, starts at the second initiation codon of the C ORF. The precore protein of wild-type HBV isolates has a molecular mass of 25 kDa. It contains at its N terminus a 19 amino acid signal sequence and at its C terminus a strongly basic domain. In most HBV isolates, this domain contains 16 arginine residues arranged in clusters in a stretch of 34 amino acids (arginine-rich domain) while all HBV isolates of subtype adw have 17 arginine residues in a stretch of 36 amino acids. One HBV genome with only 15 arginine residues within 33 amino acids of the arginine-rich domain has also been described (Miska et al., 1993). The signal sequence directs the precore protein (P25) to the endoplasmic reticulum (ER). The signal peptide of most P25 proteins is cotranslationally cleaved and the resulting 22 kDa protein (P22) is translocated in the lumen of the ER (Junker et al., 1987; Garcia et al., 1988). The arginine-rich domain is then proteolytically removed (Garcia et al., 1988; Jean-Jean et al., 1989a; Wang et al., 1991), probably by a cellular aspartyl-like protease (Jean-Jean et al., 1989b; Nassal et al., 1989). Thus, the processed product (HBeAg) is secreted in a monomeric form into the blood (Wasenauer et al., 1992). It is worth noting that the mechanism which leads to the generation and the secretion of the e antigen is identical for two other members of the hepadnavirus family as established by Schlicht et al. (1987) for the duck hepatitis B virus (DHBV) and in our laboratory (Carlier et al., 1994) for the woodchuck hepatitis virus (WHV).

The role of the arginine-rich domain in the generation of e antigen is still unclear. HBe-like proteins from which arginine-rich domain but not the peptide signal have been removed have been found in the sera of HBV carriers (Takahashi et al., 1992), rendering it conceivable that the signal sequence is not the only requirement for secretion of precore derived proteins. Furthermore, earlier work based on in vitro translation experiments suggested that the arginine-rich domain of the precore protein could act as a translocation stop signal and thus must be removed in the ER to allow translocation of the HBeAg (Bruss & Gerlich, 1988). Recent in vivo experiments have not supported this conclusion as it was shown that the removal of the arginine-rich domain takes place in a
Fig. 1. Schematic representation of the HBV C ORF and primary sequence of the C terminus of the core and precore proteins. The HBV C ORF contains two in-frame AUGs which delimit the pre-C sequence and the C gene. Numbers above the C ORF indicate the positions of the first methionine of the precore protein (−29), the C terminus of HBeAg (149) and the C terminus of the precore and core proteins (183). The C-terminal sequence of HBV precore and core proteins is shown in the single-letter code. The arrows indicate the position of the C terminus of each precore protein mutant. The large arrowhead indicates the C terminus of HBeAg.

post-ER compartment (Schlicht, 1991; Wang et al., 1991; Carlier et al., 1994). Moreover, the presence of the C-terminal domain seems crucial for HBeAg production in that cells infected with a recombinant vaccinia virus expressing a precore protein truncated by 34 amino acids at the HBeAg C terminus secreted about 20 times less HBeAg than those infected with a recombinant expressing the entire precore protein (Schlicht & Wase-nauer, 1991). In addition, DHBV precore proteins lacking the last 89 amino acids are blocked in their intracellular transport while the secretion of those lacking the last 36 amino acids is not modified (Schlicht, 1991). In an attempt to define the role of the different parts of the C-terminal sequence of the HBV precore protein in intracellular transport, we have studied the secretion of precore proteins C-terminally truncated at different positions.

To this end, nonsense mutations were introduced in the 3′ end of gene C in plasmid pHPC (formerly pMLPPC) which expresses the precore protein of HBV subtype ayw (Jean-Jean et al., 1987). Stop codons were introduced in pHPC by site-directed mutagenesis (Zoller & Smith, 1984) at codons 173, 158, 144 and 123 of the C gene (see Fig. 1). Thus, the corresponding plasmids pHPC-10, pHPC-25, pHPC-39 and pHPC-60 express truncated precore proteins respectively deleted by 10, 25, 39 or 60 C-terminal amino acids. Variant proteins are named according to the number of deleted amino acids: for example, P25-10 is the precore protein with the last 10 amino acids deleted, P22-10 and HBe-10 are the corresponding HBeAg precursor and secreted e antigen respectively. The C-terminal sequence of these proteins is shown on Fig. 1.

Adenovirus-transformed human embryo cells (line 293-31) (Harrisson et al., 1977) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Cells at 50% confluency were transfected by the calcium phosphate method (Graham & Van der Eb, 1973) with 20 μg of DNA of the appropriate plasmid per 100 mm dish. Forty-eight hours post-transfection cells were grown in 10 ml of methio-
Short communication

1 3 5 7

pHPC

1 3 5 7

pHPC-10

1 3 5 7

pHPC-25

1 3 5 7

pHPC-39

1 3 5 7

pHPC-60

Fig. 3. Secretion rates of the HBe-like proteins encoded by the deleted plasmids. Cells (line 293) were transfected by either pHPC or plasmids encoding precore proteins lacking the last 10 (pHPC-10), 25 (pHPC-25), 39 (pHPC-39) or 60 amino acids (pHPC-60). Forty-eight hours after transfection, cells were grown for 1 h in methionine-free DMEM then for 1 to 7 h in 4 ml of methionine-free DMEM containing 500 μCi EXPRESS35S35S protein labelling mixture. At the time indicated above each lane, the cell culture medium was collected and the immunoprecipitated proteins were analysed by SDS-12-5% PAGE.

nine-free cysteine-free DMEM for 1 h, then for 3 h in 4 ml of methionine-free cysteine-free DMEM containing 500 μCi EXPRESS35S35S protein labelling mixture (New England Nuclear). The medium was clarified by centrifugation at 650 g for 10 min and concentrated to 200 μl using a Centricon 10 microconcentrator (Amicon). Cells were lysed in 1 ml phosphate-buffered saline (PBS)-2% Nonidet P40. Cell debris and nuclei were removed by centrifugation at 12000 g for 5 min and the resulting supernatant (cell extract) was collected. Two hundred microlitres of concentrated medium or an aliquot fraction of cell extract (107 c.p.m.) were immunoprecipitated according to Weimer et al. (1987) using 5 μl of anti-hepatitis B core antigen rabbit antiserum (Dako). Immunoprecipitated proteins were separated by 0.15% SDS-12.5% PAGE as described by Laemmli (1970). Gels were prepared for fluorography by treatment with Amplify (Amersham), dried and autoradiographed with Fuji RX films.

Analysis of the cell culture supernatant by immunoprecipitation revealed that in the medium of pHPC-25 transfected cells not even trace amounts of HBe-related protein could be detected (Fig. 2a, lane 5) despite the fact that significant amounts of the corresponding precursor P22-25 could be immunoprecipitated from the cell extract (Fig. 2b, lane 5). When only the last 10 amino acids of the distal part of the C terminus of the HBV precore protein were deleted, the amount of HBe-related protein in the medium was similar to that of the wild-type HBeAg (lane 2) while it was clearly reduced for deletions of 25 amino acids (lane 3) or of 39 amino acids (lane 4). It is also worth noting that, in most cases, the size of the secreted HBe protein was dependent on the position of the stop codon relative to codon 149 which encodes the last residue of HBeAg (Takahashi et al., 1983). HBe-10 had the same electrophoretic mobility as the wild-type HBeAg (Fig. 2a, compare lane 2 to lane 1) while HBe-29 (Fig. 2a, lane 3) had an increased mobility as expected. HBe-25 (Fig. 2a, lane 4) migrated at a position indicating that its shortened arginine-rich domain was not cleaved, suggesting that the deletion of the last 25 amino acids of the P25 C-terminus could affect the removal of the arginine-rich domain.

Within cells (Fig. 2b), the increased amounts of P22-25 and P22-39 compared to P22 (compare lanes 3 and 4 to lane 1) correlated negatively with the low levels of the corresponding fully processed products in the medium (Fig. 2a). This result suggests that the more C-terminal sequence is deleted, the less efficient the processing and secretion of HBeAg. P22-60 did not accumulate intracellularly as much as P22-25 and P22-39 (Fig. 2b, lane 5) despite the fact that this mutant was the most deficient in secretion. One plausible explanation is that P22-60 may be unstable.

To verify these hypotheses, the time courses of secretion of the C-termnially truncated precore protein cells were analysed. Human 293 cells were transfected with the different plasmids. Forty-eight hours post-transfection cells were grown in 10 ml of methionine-free cysteine-free DMEM for 1 h; the medium was then replaced by 4 ml of methionine-free cysteine-free DMEM containing 500 μCi EXPRESS35S35S protein labelling mixture (New England Nuclear) and cells were grown for 1, 3, 5 or 7 h. At the indicated time, cells and media were collected and treated as previously described. Results are shown in Figs 3 (media) and 4 (cells). Fig. 3 illustrates that HBe-25, HBe-29 and surprisingly HBe-10 showed kinetic delays in secretion when compared to HBeAg. After labelling for 1 h, the amount of newly synthesized wild-type HBeAg (panel pHPC) was greater than that of
In this paper, we show that deletions in the C terminus of the HBV precore protein can result either in a decrease of the secretion rate or in the abrogation of the secretion of HBeAg. These results demonstrate that the HBV precore protein has two sequences (the amino-terminal signal sequence and the carboxy-terminal sequence) which both harbour secretion-competence signals for HBeAg and heterologous proteins.

Few data on the function(s) of the C-terminal domain of the precore protein in the secretion process have hitherto been reported. Schlicht & Wasenauer (1991) have shown that 20 times less HBe protein is secreted when the last 34 amino acids of P22 (the arginine-rich domain) are missing. Our results extend the conclusion of this study by demonstrating that the low secretion efficiencies of C-terminally truncated precore proteins are due to a slow down in intracellular transport of the HBeAg precursor. This is reminiscent of observations reported for the precursor of the Saccharomyces cerevisiae protease A (PrA), a vacuolar hydrolase which contains a prodomain proteolytically removed when PrA is about to reach its final destination. PrA deleted in the prodomain shows a two- to fourfold delay in delivery to the vacuole (Klionsky et al., 1988). Thus, it is tempting to speculate that the arginine-rich domain could function as a prodomain and may contribute to the overall conformation of P22 which may be required for its efficient intracellular transit. Interestingly, Schlicht & Wasenauer (1991) have observed that efficient HBeAg secretion is not dependent on the transient presence of the arginine-rich domain when the pre-C sequence is replaced by the signal sequence of influenza virus hemagglutinin. Moreover, Schlicht & Wasenauer demonstrated that in this case the secreted HBe-like antigen has a dimeric structure. This finding is consistent with recent reports showing that the monomeric structure of HBeAg is determined by an intramolecular disulphide bond between a cysteine encoded by the pre-C sequence (Cys7; counting starts at the first methionine of the core protein) and Cys61 (Wasenauer et al., 1992, 1993; Nassal & Rieger, 1993). Thus, it appears that the C-terminal domain of P22 is required for successful transport when Cys7 is present and consequently when the protein has a monomeric structure. The C-terminal domain of P22 could have an important role in disulphide bond formation in the mature HBeAg similar to the prodomain of the transforming growth factor β-1 (Sha et al., 1991).

Alternatively, some sequences of the C terminus of P22 might interact with a cellular protein acting as a chaperone in the secretion process. Deletions in the C terminal of P22 would result in a reduction in these interactions, explaining the slow intracellular transport of the truncated forms of the precore protein. One of

![Fig. 4. Time course of synthesis of the precore proteins encoded by the deleted plasmids. Cells (line 293) were transfected by either pHPC or plasmids encoding precore proteins lacking the last 10 (pHPC-10), 25 (pHPC-25), 39 (pHPC-39) or 60 amino acids (pHPC-60). Forty-eight hours after transfection, cells were grown for 1 h in methionine-free DMEM and for 1 to 7 h in 4 ml of methionine-free DMEM containing 500 µCi [35S] methionine-labelling mixture. At the time indicated above each lane, cell extracts were prepared and the immunoprecipitated proteins were analysed by SDS-12.5% PAGE. Positions of molecular mass markers (kDa) are indicated.

HBe-10 (panel pHPC-10) while HBe-25 and HBe-39 antigens were barely detectable (panels pHPC-25 and pHPC-39). In cell extracts corresponding to a labelling period of 3 h, HBeAg and HBe-10 were present in similar amounts, greater than the amounts of HBe-25 and HBe-39 antigens, as previously observed in Fig. 2(a). This indicates that the delays observed in secretion cannot be attributed to a difference in the transfection efficiencies and confirms that HBe-25 and HBe-39 antigens are secreted more slowly than the wild-type antigen.

When cells were transfected with pHPC-60, no e antigen-related protein was detectable even after synthesis labelling for 7 h (panel pHPC-60). In the corresponding cell extracts (Fig. 4, panel pHPC-60), the amount of P22-60 decreased after 3 h labelling, suggesting that P22-60 is less stable than the other precore proteins. From these results, we cannot conclude that the lack of secretion of HBe-60 is due to the relative instability of P22-60. Alternatively, the lack of secretion of HBe-60 could lead to degradation of its intracellular precursor. These data suggest that the region of P22 between the residue Val124 and the C terminus (Fig. 1) contains a sequence which could positively modify the rate of intracellular transport of HBV P22.

![Image]
these sequences could be the region Val^{124}–Arg^{133} (missing in P22–69) which was recently reported by Sällberg et al. (1993) to be at the surface of the precore protein.

It is worth noting that some HBV carriers with high viral replication have no HBeAg in their serum, despite the fact that the pre-C sequence contains no mutation which could prevent HBeAg synthesis. According to our finding, it is conceivable that the corresponding HBV genomes have mutations in the C-terminal end of the precore protein which prevent secretion of HBeAg. Such mutations have already been described for some genomes (Miska et al., 1993), but their consequences for HBeAg secretion have so far not been determined.

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References


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