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Myristylation Is Involved in Intracellular Retention of Hepatitis B Virus Envelope Proteins

REINHILD PRANGE, ANNETTE CLEMEN, AND ROLF E. STREECK*

Institut für Medizinische Mikrobiologie, Johannes Gutenberg-Universität Mainz, Augustusplatz, D-6500 Mainz, Germany

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The envelope of hepatitis B virus contains three related proteins, one of which is myristylated. The nonmyristylated small and middle protein are assembled into empty envelope particles which are secreted from cells, whereas the myristylated large envelope protein is mainly found in complete virions and is not secreted in the absence of the nucleocapsid. The block to secretion can be partially overcome by mutation or deletion of the myristylation site. Creation of a myristyl attachment site in the small protein impairs the secretion of empty envelope particles but not their intracellular assembly. Myristylation may therefore play a crucial role in hepatitis B virus replication by channeling the envelope proteins into complete viral particles.

A variety of proteins, including the capsid precursor of retroviruses (34) and picornaviruses (13, 17), the VP2 protein of simian virus 40 and of polyomavirus (14, 31), the M35 and M25 proteins of vaccinia virus (6), the transforming tyrosine kinase $p60^{src}$ of Rous sarcoma virus (1), and several cellular enzymes, are modified cotranslationally by myristylation of N-terminal glycine residues (34). A recent addition to this group is the large envelope protein of the hepatitis B virus (HBV) (25).

The envelope of HBV carrying the hepatitis B surface antigen (HBsAg) contains three related viral proteins, the large, middle, and small envelope proteins. They are encoded in a single open reading frame of the HBV genome and initiate at three separate in-phase ATG codons spaced at intervals of 108 (or 119, depending on serotype) and 55 codons. The segments downstream of the three initiation codons are called the pre-S1 and pre-S2 regions and the S gene (33).

The small S protein is the predominant constituent of the envelope of complete virions and of noninfectious 20-nm empty envelope particles (7). Such particles are secreted from infected liver or transfected cell lines because of the unusual capacity of the small protein to self-assemble with host-derived lipids of the endoplasmic reticulum (ER) membrane (7, 28).

Unlike the two other envelope proteins, secretion of the myristylated large pre-S1 protein from transfected cells has not been observed (19, 20, 21, 24). Moreover, when pre-S1 and S proteins are synthesized together in the same cell, the secretion of 20-nm particles is blocked (19, 20, 21). Overexpression of the pre-S1 envelope gene in transgenic mice causes accumulation of filamentous envelope particles in hepatocytes, followed by cell damage and the development of liver cancer (2). During HBV replication, the pre-S1 envelope protein is preferentially enriched on virions (7, 33). These data indicate that the pre-S1 domain of the large envelope protein may have a pivotal function for the outcome of a viral infection and play an important role in virion assembly (7, 20, 21, 24). We have now determined whether these properties are due to the myristylation of the pre-S1 protein.

* Corresponding author.

Absence of myristylation causes partial secretion of pre-S1 mutant proteins. To study the function of N-terminal myristylation of the pre-S1 protein of HBV, we constructed mutants in which the myristyl attachment site was either changed or deleted. In one mutant, the N-terminal glycine codon was changed to an alanine codon (preS1.Ala2). In another mutant, the amino acid sequence 2 to 19 of the pre-S1 region was deleted (preS1Δ19) in order to remove the myristylation signal (34) and additional N-terminal sequences possibly involved in the intracellular retention of the pre-S1 protein (15). The entire pre-S1–pre-S2–S open reading frame encoding either the wild-type or the mutant sequences was inserted into the expression plasmid pNI2 carrying the human metallothionein IIa promoter (10). Synthesis of the pre-S1 proteins was thus controlled by the heterologous promoter, whereas the pre-S2 and S proteins remained under the control of their cognate promoter located in the pre-S1 region (30). For high-level synthesis of the S protein, the S gene lacking pre-S sequences was inserted into plasmid pNI2.

The plasmids were transfected into hepatoma HepG2 cells (16) by the calcium phosphate precipitation technique. Two days after transfection, cells were labeled with either 225 μCi of [^{35}S]methionine (New England Nuclear) or 1 mCi of [^{3}H]myristic acid (New England Nuclear) for 24 h. After the labeling periods, medium was collected and centrifuged briefly, and cells were lysed by using detergents as described previously (4, 5). For immunoprecipitation, clarified lysates and supernatants were precleared by using protein A-Sepharose (Pharmacia) and were incubated with rabbit antiserum to HBsAg particles from human serum (Behring-Calbiochem) in RIPA buffer (0.5% Nonidet P-40, 0.05% sodium deoxycholate, and 0.01% sodium dodecyl sulfate [SDS] with 2 mM phenylmethylsulfonyl fluoride and equal concentrations [2 μg/ml] of chymostatin, leupeptin, antipain, and pepstatin A). Immune complexes were isolated with protein A-Sepharose and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Lysates of cells transfected with the wild-type pre-S1 and the mutant preS1.Ala2 gene contained the 39-kDa nonglycosylated and the 42-kDa glycosylated forms of the large envelope (pre-S1) protein (8) (Fig. 1, lanes 1 and 3). The deletion mutant preS1Δ19 yielded shorter proteins of about 37 and 40 kDa, as expected (Fig. 1, lane 5). In addition, the

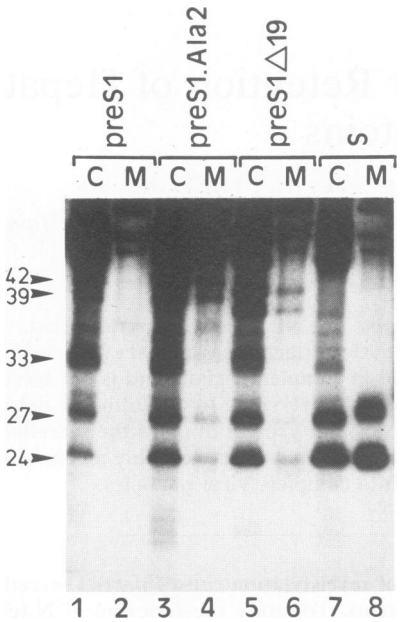

FIG. 1. Synthesis and secretion of wild-type and mutant pre-S1 proteins by using HepG2 cells. Cells (10^6) grown on a 6-cm petri dish were transfected with 20 μg of plasmids carrying the indicated genes and were labeled 2 days later with [^{35}S]methionine in methionine-depleted minimal essential medium supplemented with 6% dialyzed fetal calf serum. Equal amounts of cellular lysates (C) and cellular supernatants (M) were precipitated with rabbit antiserum against HBsAg from human serum and subjected to SDS-PAGE. Molecular masses (in kilodaltons), deduced from standards, are indicated on the left.

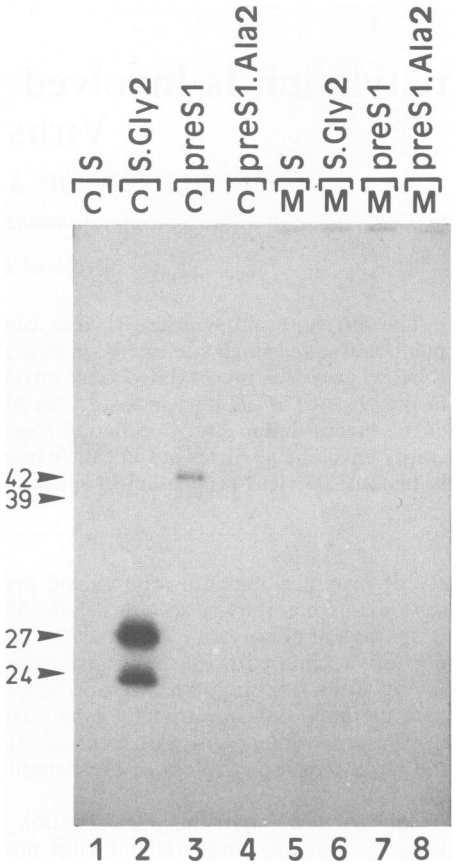

FIG. 2. Myristylation of wild-type and mutant envelope proteins. HepG2 cells were transfected with the genes indicated and labeled with [^{3}H]myristic acid in Dulbecco's modified Eagle's medium–1% fetal calf serum in the presence of 1% dimethyl sulfoxide. Labeled proteins were immunoprecipitated and analyzed by SDS-PAGE. C and M refer to cellular lysates and cellular supernatants, respectively. Molecular masses (in kilodaltons) are indicated on the left.

24-kDa nonglycosylated and the 27-kDa glycosylated forms of the small envelope (S) protein were present (Fig. 1, lanes 1, 3, and 5), as confirmed by transfection with the S gene alone (Fig. 1, lane 7). Finally, the band observed at 33 to 36 kDa should correspond, at least in part, to the middle envelope (pre-S2) protein (Fig. 1, lanes 1, 3, and 5).

The envelope proteins were not secreted from cells transfected with the wild-type pre-S1 gene (Fig. 1, lane 2). However, pre-S1 proteins as well as minor amounts of the S protein were clearly identified in cellular supernatants after transfection with the pre-S1 mutants (Fig. 1, lanes 4 and 6). It should be noted, however, that secretion of the pre-S1 mutants was only partial and that secretion of the S protein was more efficient in the absence of any pre-S1 protein (Fig. 1, lane 8).

Additional evidence for the secretory phenotype of the pre-S1 mutants was obtained by using a pre-S1-specific immunoassay (18). Three days after transfection, envelope proteins carrying the wild-type pre-S1 sequence could only be detected in the cellular lysate, whereas 20 to 30% of the mutant proteins were secreted under these conditions (data not shown).

By using metabolic labeling, incorporation of [^{3}H]myristate was detected in the wild-type pre-S1 protein (Fig. 2, lane 3) but not in the mutant preS1.Ala2 (Fig. 2, lane 4) or preS1Δ19 (data not shown). Consistent with the results of the methionine labeling studies (Fig. 1), the myristylated wild-type pre-S1 protein could only be identified in cellular lysates, not in the supernatants of transfected cells (Fig. 2, lane 7). Taken together, these data demonstrate a strong but not absolute correlation between the absence of myristylation and the release of the block to secretion of the pre-S1 protein.

A single point mutation causes myristylation and intracellular retention of the S protein. To provide further evidence that myristylation affects the secretion of the HBV envelope, we introduced an artificial myristylation site into the S protein. Besides the glycine residue in position 2, the known N-myristyl proteins carry a serine, threonine, or alanine residue in position 6 (34). Since the S protein of HBV already contains a serine in position 6, we converted the glutamic acid in position 2 into glycine by a single point mutation (S.Gly2). Expression of the mutant S.Gly2 in HepG2 cells in the presence of labeled myristate yielded myristylation of the S protein. Both the glycosylated (GP27) and the nonglycosylated (P24) forms of the S.Gly2 protein were efficiently myristylated (Fig. 2, lane 2). No myristate incorporation could be detected in the wild-type S protein (Fig. 2, lane 1), thus demonstrating that no fatty acid label was converted to amino acids under the labeling conditions used. Most importantly, secretion of the mutant S.Gly2 was almost completely blocked (Fig. 2, lane 6). Only after very long exposure of the autoradiogram could small quantities of the

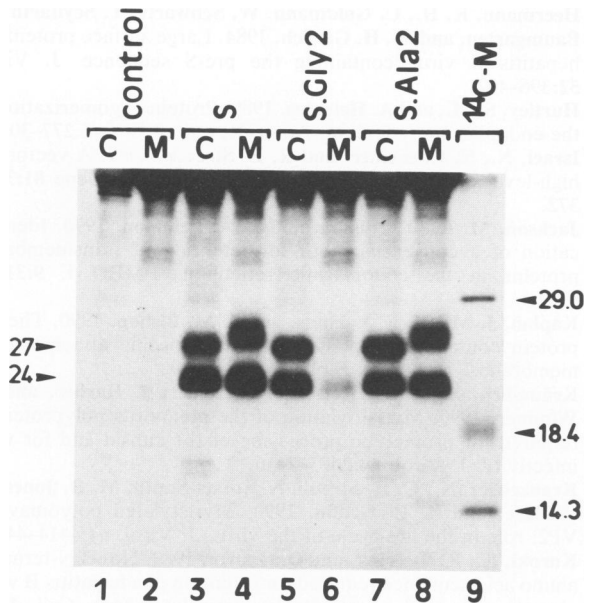

FIG. 3. Intracellular retention of the mutant S.Gly2 protein. Nontransfected HepG2 cells (lanes 1 and 2) or cells transfected with the indicated genes (lanes 3 to 8) were labeled with [^{35}S]methionine and [^{35}S]cysteine (New England Nuclear). Proteins were immunoprecipitated from cellular lysates (C) and cellular supernatants (M) and were analyzed by SDS-PAGE. Molecular masses of ^{14}C-labeled marker proteins (GIBCO-Bethesda Research Laboratories) (lane 9) as well as deduced molecular masses (in kilodaltons) are indicated.

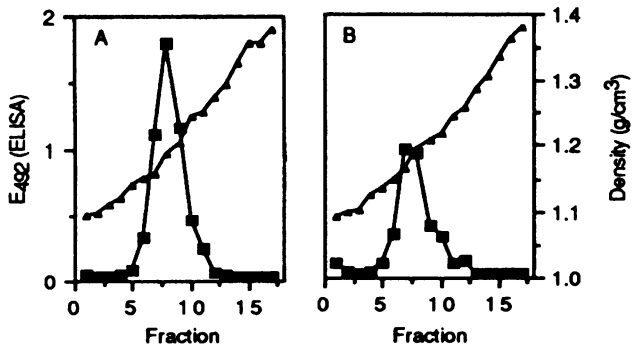

FIG. 4. Assembly of intracellular wild-type S and mutant S.Gly2 proteins into lipoprotein particles. HepG2 cells transfected with the wild-type S (A) or mutant S.Gly2 (B) gene were harvested 72 h after transfection. Equal amounts of the soluble cellular fractions were layered on a CsCl step gradient (10 to 50% [wt/wt]) and centrifuged in a SW 60Ti rotor (Beckman). Gradients were fractionated from the top, and each fraction was assayed for HBsAg by enzyme-linked immunosorbent assay (ELISA) (Auszyme, Abbott). ■, extinction at 492 nm (E_{492}); ▲, density.

myristylated S.Gly2 protein be detected in the supernatant of transfected cells.

To determine whether the S protein tolerates an amino acid substitution in position 2, glutamic acid was replaced by alanine in another mutant (S.Ala2). After transfection and metabolic labeling with [^{35}S]methionine, the wild-type S protein, the mutant S.Ala2, and the myristylated mutant S.Gly2 were immunoprecipitated from whole-cell lysates by using antiserum to HBsAg particles. As shown in Fig. 3 (lanes 3, 5, and 7), similar amounts of protein were found, indicating that the replacement of glutamic acid in position 2 did not affect the overall stability of the S protein or the conformation of the surface antigen. However, whereas secretion of the myristylated S.Gly2 protein was dramatically decreased (lane 6), the mutant S.Ala2 was secreted into the cellular medium as efficiently as the wild-type S protein (lanes 4 and 8). This demonstrates that the amino acid exchange per se cannot account for the block to secretion of the mutant S.Gly2.

The mutant S.Gly2 was glycosylated to the same extent as the wild-type S protein (Fig. 3, lanes 3 and 5), indicating translocation into the ER. As judged by electrophoretic mobility (Fig. 3, compare C and M lanes), the mutant S.Gly2 retained intracellularly did not undergo oligosaccharide processing (Fig. 3, lane 5). This suggests that the block to secretion occurs prior to transport to the medial cisternae of the Golgi apparatus.

Since misfolded and unassembled polypeptides are generally retained in the ER (9), we investigated whether myristylation of the mutant S.Gly2 affected its folding or its incorporation into 20-nm particles. Transfected cells were lysed by freezing and thawing in hypotonic buffer as described previously (4). After clarification at 15,000 × *g* for 30 min, the soluble cell fraction was analyzed by isopycnic cesium chloride gradient centrifugation. As shown in Fig. 4, both the intracellular wild-type S protein (panel A) and the mutant S.Gly2 protein (panel B) banded at a buoyant density of about 1.19 g/cm^3, which is typical of serum-derived HBsAg lipoprotein particles (29).

Biological significance of myristylation of HBV envelope proteins. Our data demonstrate that the introduction of an artificial myristylation site into the HBV S envelope protein was sufficient to impair the secretion of empty envelope subviral particles. Neither improper assembly nor the amino acid exchange in position 2 of the protein was responsible for the intracellular retention. Thus, myristylation alone can account for the block to secretion. Conversely, prevention of myristylation of the pre-S1 protein by elimination of the myristic acid attachment site led to a release, although only partial, of the block to secretion. This indicates that other features of the protein are also involved in the intracellular retention. We suggest that the myristic acid residue is not responsible per se for the retention but acts by introducing conformational changes facilitating or enhancing intra- or intermolecular contacts and interactions with other cellular components. The lack of myristylation of the pre-S1 protein would then impair but not totally prevent these as yet unknown interactions, thereby leading to the partial secretion of the envelope protein. Related findings have been made for other myristylated proteins; e.g., myristylation of the *src* protein acts in conjunction with multiple domains of the protein to facilitate attachment to a receptor of the plasma membrane (12, 26).

Analysis of deletion and point mutations in the pre-S1 region of the adw2 serotype of HBV led Kuroki et al. to postulate a novel ER retention signal located in the pre-S1 amino acid sequence 6 to 19 (15). Unlike the ayw serotype used in our work, the adw2 pre-S1 region carries an N-terminal extension of 11 residues, including an additional myristylation signal. Single and double mutation of the myristic acid attachment sites, Gly-2 and Gly-12, did not lead to secretion of the pre-S1 protein (15). This contrasts with our finding, described in this work, that mutation of Gly-2 released the block to secretion of the pre-S1 protein, although only partially. The retention signal predicted by

Kuroki et al. (15) is not operative in the ayw sequence, since deletion of the pre-S1 amino acid sequence 2 to 19 (corresponding to the sequence 12 to 30 of the adw2 serotype) did not lead to a significant increase of secretion compared with that of the mutant preS1.Ala2. Interestingly, the N-terminal extension of the adw2 pre-S1 protein carries a cluster of basic residues (KPRK) at positions 7 to 10 which might contribute to retention in the ER (11, 23). It is also conceivable that pre-S2 sequences may be involved in the partial retention of the pre-S1 mutants described by Kuroki et al. (15) and in this work.

Myristylation of viral proteins has been found to be essential for their assembly into infectious particles for a variety of viruses. These include picornaviruses (13, 17), simian virus 40 and polyomavirus (14, 31), and vaccinia virus (6). In retrovirus assembly, targeting to the membrane of the *gag* precursor by myristylation appears to be the driving force for the budding process (3).

Interaction between nucleocapsid and envelope proteins seems to be a critical step in the assembly of retroviruses and HBV particles. For retroviruses, this interaction is achieved through myristylation of the capsid. For HBV, retention of the envelope by myristylation of the pre-S1 protein may be an important step for creating the local environment facilitating the contacts between capsid and envelope critical to the budding of complete virions, which is thought to take place at the ER (22). Concomitant with virion assembly, the myristate moiety of the pre-S1 protein may be buried within a cleft or a pocket in the structure of the virion, thereby allowing transit out of the cell. Since infectious HBV particles can be produced in vitro by transfection of cell lines (27, 32), the function of myristylation in the viral replicative cycle can now be established.

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