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Hepatitis C Virus NS2 Protein Is Phosphorylated by the Protein Kinase CK2 and Targeted for Degradation to the Proteasome

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Hepatitis C virus (HCV) nonstructural 2 (NS2) protein is a hydrophobic transmembrane protein, described to be involved in different functions, such as apoptosis inhibition and gene transcription modulation. We investigated here NS2 protein turnover and found that NS2 was rapidly degraded by the proteasome in different cell lines, as in primary human hepatocytes. Since posttranslational modifications can influence protein turnover, we looked for potential phosphoacceptor sites in NS2. Computational sequence analysis in combination with screening of NS2 point mutants revealed that serine residue 168 was critical for degradation. In the quest of a protein kinase for NS2, we identified by sequence analysis that the serine residue 168 was part of a consensus casein kinase 2 (CK2) recognition site (S/TXXE). This motif was highly conserved since it could be found in the NS2 primary consensus sequences from all HCV genotypes. To verify whether CK2 is involved in NS2 phosphorylation, we showed by an in vitro kinase assay that CK2 phosphorylated NS2, as far as this CK2 motif was conserved. Interestingly, NS2 became resistant to protein degradation when the CK2 motif was modified by a single point mutation. Furthermore, inhibition of CK2 activity by curcumin decreased NS2 phosphorylation in vitro and stabilized NS2 expression in HepG2 cells. Finally, we showed in Huh-7.5 replicon cells that NS2, expressed in the context of the HCV polyprotein, was also sensitive to both proteasome-mediated degradation and CK2 inhibitor treatment. We suggest that NS2 is a short-lived protein whose degradation by the proteasome is regulated in a phosphorylation-dependent manner through the protein kinase CK2.

Hepatitis C virus (HCV) causes chronic hepatitis which frequently leads to cirrhosis and liver cancer (37). More than 170 million people worldwide are chronically infected with HCV (31). HCV is an enveloped virus which belongs to the *Flaviviridae* family. Its 9.6-kb positive-stranded RNA genome encodes a polyprotein of about 3,010 amino acids (37). Co- and posttranslational processing of this polyprotein by host and viral peptidases generates at least 10 polypeptides that include structural (core, E1, E2, and p7) and nonstructural (NS2 to NS5B) protein products (22). Since these proteins are not present at amounts detectable in the liver of many chronically HCV-infected patients (31), there are likely cellular mechanisms regulating this weak HCV protein level.

One major cellular mechanism known to function widely in selective intracellular protein turnover is the proteasome system. It plays a central role in the degradation of short-lived and regulatory proteins involved in cellular processes such as cell cycle and signal transduction (8). Most of the proteins that are degraded by the proteasome have to be tagged with multiple ubiquitin molecules through a cascade of enzymes, involving ubiquitin activating (E1), conjugating (E2), and ligase (E3) enzymes (28). However, recent evidence suggests that proteasomal degradation might occur in a ubiquitin-independent manner (18, 19). For both proteasome-mediated degradation pathways, the process specificity is determined by clearly defined motifs localized within the protein substrate, such as phosphorylation or dephosphorylation signals (9).

Many viruses encode proteins that take advantage of the degradation pathway (9). A particularly interesting example of a viral protein that exploits the proteasomal processing is the human immunodeficiency virus type 1 (HIV-1) protein Vpu. This viral protein mediates degradation of CD4, the primary receptor for HIV-1. This effect requires Vpu phosphorylation by the protein kinase casein kinase 2 (CK2) and the recognition of Vpu by the E3 ubiquitin ligase $SCF^{\beta TrCP}$ (for a review, see reference 30). As a consequence, downregulation of CD4 avoids superinfection of the host cell which is deleterious for the HIV and also facilitates maturation of the HIV envelope proteins (for a review, see reference 30). Another example is the E6 protein of the human papillomaviruses. The viral protein associates with p53 and targets it for rapid proteasome-mediated degradation. Consequently, p53-induced growth arrest and apoptosis-inducing activities are abolished, thus leading to virus survival (for a review, see reference 38).

Interestingly, the HCV also possesses several proteins whose turnover has been shown to be tightly regulated by the proteasome pathway. For example, binding of HCV RNA-dependent RNA polymerase (NS5B) to a ubiquitin-like protein was reported to decrease the NS5B quantity in the cell. This regulation of NS5B level was proposed to function as a mechanism to limit HCV RNA replication and thus to escape from host cell defenses (14). Moreover, the unglycosylated cytosolic form of HCV envelope protein 2 (E2) was described as sensitive to the proteasome pathway but seems to become resistant to degradation in cells expressing an activated protein kinase (i.e., protein kinase R [PKR], a double-stranded RNA-activated protein kinase). The resulting stabilized E2 was shown to interact with PKR as a device to prevent PKR-induced protein synthesis inhibition (27).

In the present study, we focused our investigation on the

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HCV nonstructural 2 (NS2) protein. NS2 in association with NS3 (NS2/NS3 protease) is the first activated viral protease within the HCV polypeptide responsible for the maturation of the remaining NS proteins (10). This NS2/NS3 autoprotease is essential for highly productive in vivo replication (20). Upon proteolytic processing of the HCV polypeptide (22), NS2 is a nonglycosylated transmembrane protein of 23-kDa anchored to the endoplasmic reticulum (ER) (32, 41). Until now, only a few properties have been attributed to the cleaved mature NS2 protein. Nevertheless, we recently reported that NS2 acts as an apoptosis inhibitor (12). It was also proposed that NS2 might interfere with cellular gene expression (11) and might be required for NS5A phosphorylation (21).

We investigated here the fate of HCV NS2. We found that NS2 is phosphorylated by the protein kinase CK2 and targeted for degradation to the proteasome.

MATERIALS AND METHODS

Plasmid constructions. The NS2 gene of the HCV-H strain was inserted into the KpnI and EcoRI sites of the pVM6 plasmid (Roche Molecular Biochemicals) in frame with the vesicular stomatitis virus G (VSV-G)-tagged sequence, yielding in the vector pVM6-NS2. The His_6 tag sequence located 3′ with respect to the multicloning site of the pVM6 vector was avoided by the introduction of a stop codon at the 5′ end of the NS2 sequence. The point mutant NS2S168A was constructed by site-directed mutagenesis by overlap extension with the PCR as previously described (17). The pVM6-lacZ vector was provided by Roche Molecular Biochemicals.

For colocalization studies, the NS2 gene was inserted either into the KpnI and BamHI sites of the pEGFP-C3 plasmid (Clontech) to express a N-terminal green fluorescent protein (GFP)-tagged NS2 protein or subcloned into the EcoRI and BamHI sites of the pEGFP-N3 vector, resulting in a C-terminal GFP-tagged NS2.

Antibodies, peptide, and chemicals. The following antibodies were used: VSV-G (Roche 1667–351 [5 μg/ml for immunoblotting]), phosphoserine (Zymed 61–8100 [1 μg/ml for immunoblotting]), Annexin V (kindly provided by F. Russo-Marie [1:1,000 for immunoblotting]), anti-α-tubulin (Sigma T5168 [1:10,000 for immunoblotting]), anti-E2 monoclonal antibody (H52; kindly provided by J. Dubuisson [1:1,000 for immunoblotting]), anti-NS2 polyclonal antiserum (WU107; kindly provided by C. M. Rice [1:5,000 for immunoblotting]), and horseradish peroxidase-labeled goat anti-rabbit and anti-mouse immunoglobulin (Dako Corp. P0448 and P0447 [1:2,000 to 5,000 for immunoblotting]). The Western blot detection kit was the SuperSignal kit (Pierce). The following chemicals were used: cycloheximide (CHX), curcumin (Sigma), MG132, Clasto-Lactacystin-β-Lactone (Biomol Research Laboratories), and hepatocyte growth factor (Sigma). For the in vitro kinase assay we used a CK2 peptide substrate (New England Biolobs P6011S; 500 μM).

Cell lines and transfection. HeLa cells were cultured and transfected by electroporation as described previously (13) or by using the Lipofectamine 2000 reagent kit (Invitrogen) according to the manufacturer's instructions.

The HepG2 human hepatoma cell line was cultured in a mixture of minimal essential medium (75%) and M199 (25%) (Eurobio) supplemented with 10% fetal calf serum and 100 IU of penicillin/ml, 50 μg of streptomycin sulfate/ml, 5 μg of bovine insulin/ml, 5×10^{-7} M hydrocortisone hemisuccinate, and 1% L-glutamine. These cells were transfected by electroporation as previously described (7).

To establish NS2 and NS2S168A stable transfectants (HepG2-NS2 and HepG2-NS2S168A), HepG2 cells were transfected with the pVM6-NS2 and pVM6-NS2S168A plasmids by using the Lipofectamine Plus reagent kit (Invitrogen) according to the manufacturer's instructions. After 48 h, the transfected cells were selected in HepG2 medium lacking penicillin and streptomycin sulfate, supplemented with 0.4 mg of G418 antibiotic (Invitrogen)/ml for 2 weeks, and were then maintained in culture medium containing 0.5 mg of G418/ml.

The Huh-7.5 cell line was cultured in Dulbecco modified Eagle medium (Eurobio) with 10% heat-inactivated fetal bovine serum (HyClone), 0.1 mM nonessential amino acids, 100 IU of penicillin/ml, and 50 μg of streptomycin sulfate/ml and then transfected by using a Lipofectamine 2000 reagent kit.

The Huh-7.5 replicon cell line containing the full-length HCV genome, kindly provided by C. M. Rice, was cultured in Huh-7.5 medium supplemented with 0.5 mg of G418/ml.

Primary cell culture and transfection. Fragments of normal adult human liver were obtained from patients undergoing hepatic resection for liver metastases (the fragments were taken at a distance from the metastasis in macroscopically normal liver tissue). Access to this biopsy material was in agreement with French laws and satisfied the requirements of the French National Ethics Committee. Hepatocytes were isolated by the procedure of Guguen-Guillouzo and Guillouzo as described previously (16). To transfect human hepatocytes, cells were seeded in six-well plates (5×10^5 cells per well) and cultured in HepG2 medium (see above) supplemented with 1.4×10^{-6} M hydrocortisone hemisuccinate and 1 g of albumin/liter. After 24 h, 10 ng of hepatocyte growth factor/ml was added to the culture medium. After 48 h, cells were transfected with 1 μg of pVM6-lacZ or pVM6-NS2 plasmid by using the Lipofectamine 2000 reagent kit (Invitrogen) according to the manufacturer's instructions. Cells were harvested 24 h after transfection and lysed, and 20 μg of proteins was subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting analysis.

Cell culture treatment with chemicals. Transfected cells were incubated with 10 μM MG132, 5 μM Clasto-Lactacystin-β-Lactone, or 100 μM curcumin (dissolved in 0.25% dimethyl sulfoxide) for 16 to 24 h.

For the time course assay, stable transfectants HepG2-NS2 and HepG2-NS2S168A were treated with 10 μM MG132 for 16 h, washed two times in culture medium without proteasome inhibitor, and incubated for different times with 5 μg of CHX/ml. For dose-dependent inhibition by curcumin, the HepG2-NS2 cell line was pretreated with MG132 and washed as described for the time course assay prior to simultaneous exposure to 5 μg of CHX/ml and various concentrations of curcumin (1, 10, and 100 μM) for 6 h. The cells were then harvested and lysed, and the protein concentration was determined by the Bradford method (Bio-Rad). A total of 50 μg of proteins was subjected to SDS–12.5% PAGE, followed by Western blot analysis as described previously (12). To ensure comparable loading of the samples, blots were incubated with anti-Annexin V antibody (kindly provided by F. Russo-Marie). Specific bands were quantified by scanning the autoradiographs, and the band density was measured with the assistance of DensyLab Software (Microvision Instruments, Evry, France).

Fluorescence staining. HeLa cells (5×10^5) were transfected with 2 μg of pEGFP-C3 or pEGFP-C3-NS2 by using the Lipofectamine 2000 reagent kit and were left untreated or were exposed to 10 μM MG132. For the GFP expression study, nuclei were stained for 15 min at room temperature with 0.5 μg of Hoechst 33342 (Sigma)/ml, and the cells were washed, fixed, and analyzed by fluorescence microscopy using an Olympus AX60 microscope. For colocalization studies, cells were transfected with the indicated GFP expression vectors and incubated with 1 μM ER-Tracker Blue White DPX (Molecular Probes) for 30 min under the appropriate cell growth condition. The cells were then washed and fixed in 4% paraformaldehyde. Images were acquired with a Leica DMRXA2 microscope by using charge-coupled device cameras and subsequently processed by using Metamorph software.

Protein kinase CK2 assay. Recombinant glutathione *S*-transferase (GST), GST-NS2, and GST-NS2S168A proteins were produced and purified as already described (12). Equal amounts of immobilized GST fusion proteins were incubated for 2 h at 30°C with 1 μl of recombinant CK2 (500 U; New England Biolabs) in a CK2 buffer with 200 μM ATP. In the case of GST-NS2, kinase reaction was left untreated or was supplemented either with 500 μM concentrations of CK2 peptide substrate (New England Biolabs) or 100 μM curcumin. Immobilized GST proteins were spun down and resuspended in sample buffer (Sigma) prior to loading on an SDS-polyacrylamide gel and Western blotting analysis.

RESULTS

NS2 is an unstable protein degraded by the proteasome in different mammalian cell types. The objective of the present study was to investigate the NS2 protein turnover. Since NS2 is a protein of a hepatotropic virus, we first examined NS2 protein expression in a liver physiological context, e.g., HepG2 cells, a human hepatoma cell line, or human hepatocytes in primary cell culture. To do this, NS2 or β-galactosidase proteins were tagged with VSV-G at their N termini and expressed in cells. After 24 h, protein levels were examined by Western blotting. As expected, Western blot analyses showed expres-

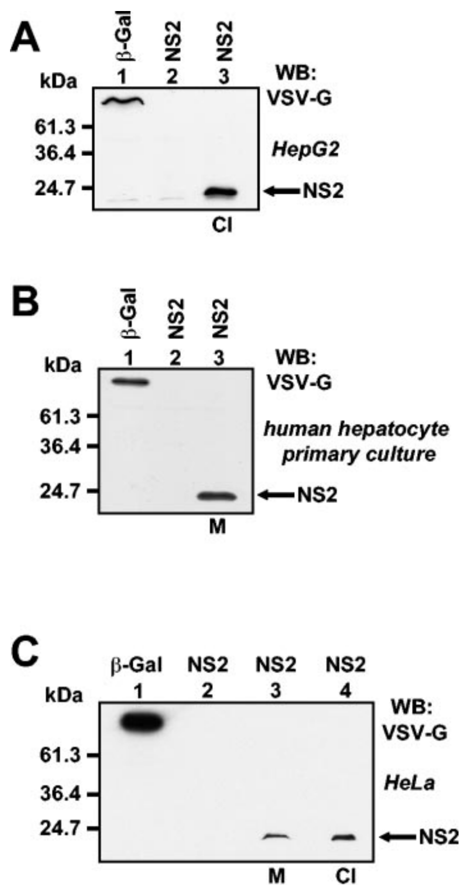

FIG. 1. Degradation of the HCV NS2 protein by the proteasome. (A) The HepG2 human hepatoma cell line was transfected with 24 μg of pVM6 plasmids expressing either VSV-G-tagged β-galactosidase (lane 1) or VSV-G-tagged NS2 (lanes 2 and 3). Cells, transfected to express the NS2 protein, were left untreated for 16 h (lane 2) or exposed to a 5 μM concentration of proteasome inhibitor Clasto-Lactacystin-β-Lactone (lane 3). Cell lysates were analyzed by Western blotting (WB) with anti-VSV-G antibody. (B) Hepatocytes were isolated from human liver biopsies, seeded, and prepared as described in Materials and Methods. Cells were then transfected with 2 μg of pVM6-lacZ (lane 1) or pVM6-NS2 (lanes 2 and 3) plasmids and exposed to 10 μM concentrations of proteasome inhibitor MG132. Total protein extracts were probed with anti-VSV-G antibody. (C) HeLa cells were transfected with 24 μg of pVM6 plasmids expressing either VSV-G-tagged β-galactosidase (lane 1) or VSV-G-tagged NS2 (lanes 2 to 4). Cells were exposed to proteasome inhibitors (Clasto-Lactacystin-β-Lactone and MG132) for 16 h (lanes 3 and 4, respectively) or left untreated (lane 2). Proteins from total cell lysates were analyzed with anti-VSV-G antibody. Cl, Clasto-Lactacystin-β-Lactone; M, MG132.

sion of the VSV-G-tagged control protein, β-galactosidase (Fig. 1A and B, lanes 1). In contrast, the NS2 protein was undetectable (lanes 2) and could only be visualized when transfected cells were treated with a 5 or 10 μM concentration of proteasome inhibitors, such as Clasto-Lactacystin-β-Lactone (Fig. 1A, lane 3) or MG132 (Fig. 1B, lane 3). Furthermore, we examined NS2 protein expression in HeLa cells in order to verify whether NS2 is also degraded in a nonhepatic context. Once again, as expected, β-galactosidase expression was detected 24 h posttransfection (Fig. 1C, lane 1). However, even in this cell line, Western blot analysis showed that NS2 expression could only be detected in the presence of proteasome inhibitors (Fig. 1C, lanes 3 and 4). Indeed, in the absence of any proteasome inhibitor, a very weak signal could only be visualized after long exposure (data not shown). Although we could note that MG132 treatment slightly increased the protein level of the stably expressed β-galactosidase protein (data not shown), suggesting a probable overall protein stabilizing effect of this drug, our results clearly provide evidence that NS2 is an unstable protein which is degraded by the proteasome through a liver-nonspecific mechanism.

Protein stability and subcellular localization of GFP-tagged NS2. Since we could not exclude that the VSV-G tag had an effect on NS2 stability or its membrane association, we decided to replace this protein tag by generating NS2 as a GFP-tagged fusion protein. This GFP tag allowed us to analyze by fluorescence microscopy both NS2 expression and subcellular localization at the same time, particularly in the absence of an efficient anti-NS2 antibody to perform immunofluorescence studies. To do this, HeLa cells were transfected with plasmids expressing either GFP or GFP-NS2. After 24 h, GFPs were observed by direct fluorescence, and the numbers of GFP-positive cells were evaluated after nuclear Hoechst staining. As expected, fluorescence analyses showed that the GFP control protein (Fig. 2Aa and b) could be detected in cells untreated or exposed to proteasome inhibitor MG132. In contrast, the GFP-NS2 was nearly undetectable (Fig. 2Ac) and could mainly be visualized when cells were treated with MG132 (Fig. 2Ad). Interestingly, similar results were obtained when the GFP tag was fused to the C terminus of NS2 (data not shown), indicating that the protein tag position had no effect on NS2 degradation.

Since NS2 is a transmembrane protein that associates with ER membranes (32), we verified that the GFP tag did not alter NS2 subcellular localization. HeLa cells transfected to express GFP or GFP-NS2 were exposed to 10 μM MG132 and stained with ER-Tracker. As shown in Fig. 2B, GFP-NS2 displayed punctate structures in the perinuclear space (Fig. 2Bd). By costaining these cells with ER-Tracker (Fig. 2Be), we observed almost completely overlapping staining signals (Fig. 2Bf), indicating that GFP-NS2 colocalized with ER membranes. This specific localization was not due to overexpression of GFP, since GFP alone showed a diffused cellular distribution (Fig. 2Ba, b, and c).

These results indicate that protein tagging had no effect on the ER-membrane association of NS2, and regardless of the used protein-tag and of its position within the protein sequence, NS2 remained sensitive to proteasomal degradation. Therefore, we hypothesize that NS2 by itself harbors proteins domains which are responsible for targeting NS2 as a substrate to the proteasome.

A serine residue at position 168 of NS2 is critical for its degradation. The specificity of the proteasome-mediated degradation process is characterized by recognition of degradation signals within the protein substrate (9). Since protein phosphorylation was described as one of the posttranslational modifications that can constitute a degradation motif and thus modify protein stability (8, 9, 15), we wondered whether NS2 might be degraded in a phosphorylation-dependent manner. In this case, mutations introduced in potential phosphorylation sites of NS2 would modify the NS2 protein turnover. To ex-

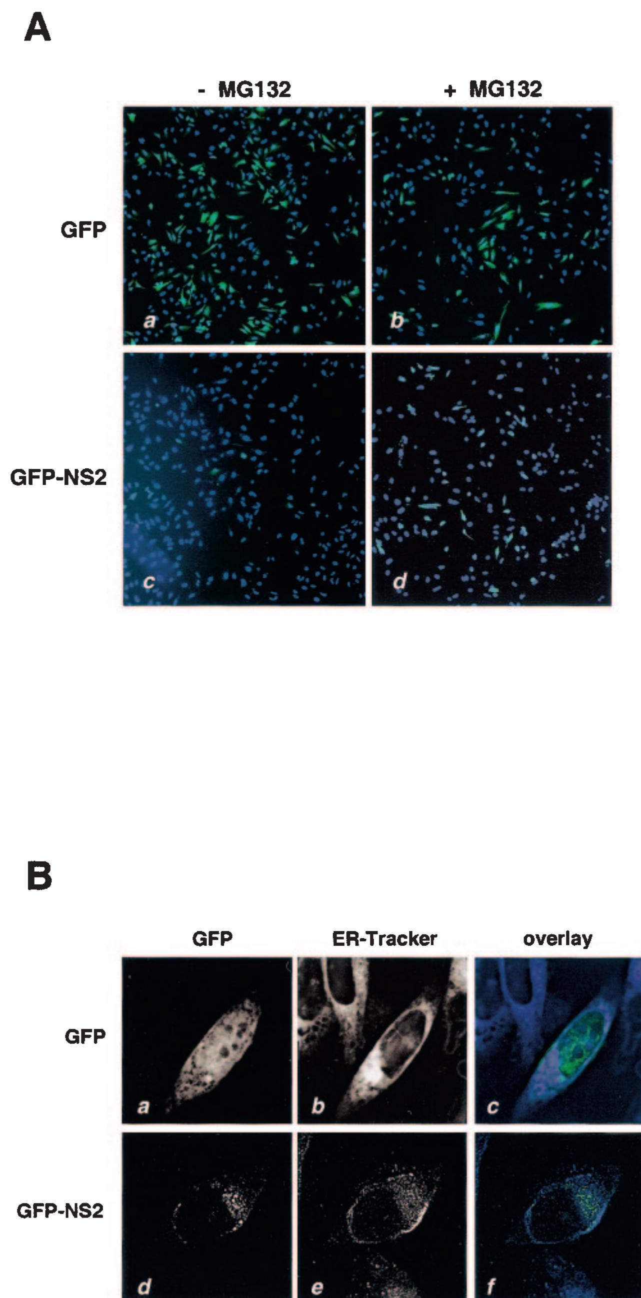

FIG. 2. Protein stability and subcellular localization of GFP-NS2. (A) Protein expression analysis of GFP-NS2. HeLa cells were transfected with 2 μg of the control vector pEGFP-C3 (subpanels a and b) or the plasmid pEGFP-C3-NS2 that expresses the GFP-tagged NS2 (subpanels c and d). Cells were exposed to a 10 μM concentration of proteasome inhibitor MG132 for 16 h (subpanels b and d) or left untreated (subpanels a and c). After 24 h, cells were fixed, stained with Hoechst 33342, and observed under a fluorescence microscope. Magnification, × 20. (B) Cytochemical staining. HeLa cells were transfected with 2 μg of plasmids expressing GFP or GFP-NS2 (subpanels a and d) and treated with 10 μM MG132. At 24 h posttransfection, the ER was stained with 1 μM ER-Tracker (subpanels b and e), and cells were fixed. Colocalization was visualized by overlay (subpanels c and f). Images were obtained by using a ×40 oil immersion objective lens.

amine this hypothesis, we first looked for potential phosphorylation sites in NS2. By computational analysis using the NetPhos 2.0 Server (3) located at the ExPASy Proteomics tools home page (http://us.expasy.org/tools/), we found that the serine residue at position 168 of NS2 had the highest probability to be phosphorylated. Since this approach has to be taken with caution, we introduced single point mutations at different predicted phosphorylation sites of NS2 (a serine at position 168 or 211, a threonine at position 172) in order to study whether they might stabilize NS2. HepG2 cells were transfected with plasmids encoding either VSV-G-tagged NS2 or its single point mutants (NS2S168A, NS2T172A, or NS2S211A), and protein expression was analyzed by Western blotting 24 h later. As expected, a very weak signal could only be revealed after long exposure for the wild-type form of NS2 (Fig. 3A, lane 2, lower panel). In contrast, the NS2S168A mutant was easily detected (lane 3), and its expression level was comparable to that detected with the NS2 wild-type protein in the presence of a proteasome inhibitor (lane 4). In addition, neither NS2T172A nor NS2S211A could be visualized by Western blot analysis (data not shown). Finally, we determined the protein turnover rate of both NS2 and NS2S168A, in a time course experiment. For this investigation, we established HepG2 cell lines continuously expressing VSV-G-tagged NS2 (HepG2-NS2) or VSV-G-tagged NS2S168A (HepG2-NS2S168A) and pretreated them with the reversible proteasome inhibitor MG132. After 16 h, cells were washed and incubated in medium containing the protein synthesis inhibitor CHX for different times (0, 30, 60, and 90 min). Then, cells were harvested and cell lysates were analyzed by immunoblotting. Figure 3B and C show that 60% of the NS2 protein was already degraded within 30 min of treatment and was only weakly detected after 90 min. Compared to the short-lived wild-type NS2 protein, the point mutant NS2S168A remained stable during the time course assay. Taken together, these results indicate that the point mutation S168A of the predicted phosphorylation site confers stability to NS2 protein and thus favor the idea that NS2 might probably be degraded by a phosphorylation-dependent mechanism.

Serine residue 168 belongs to a consensus CK2 phosphorylation sequence motif. In the quest of a protein kinase, which might phosphorylate NS2, we performed an amino acid sequence analysis of the residues surrounding serine residue 168 (Fig. 4, asterisk) and looked for known motifs recognized by kinases. By this approach, we identified that serine residue 168 was part of a consensus phosphorylation site (S/TXXE, where X is any amino acid) for CK2 (24) (Fig. 4, black box). This observation was of particular interest, since we have shown in Fig. 3 that the mutation of serine 168 to alanine confers stability to NS2 protein and thus correlates directly with a modified CK2 recognition motif.

Since the NS2 protein from different HCV genotypes might differ in their amino acid sequence, we wondered whether this CK2 recognition motif might be conserved. To investigate this possibility, we first determined the primary consensus sequence of NS2 for each HCV genotype (Fig. 4). To do so, we identified and aligned all HCV isolates recorded for each HCV genotype in the HCV database (http://hcvpub.icbp.fr). The NS2 consensus sequences obtained for the different genotypes

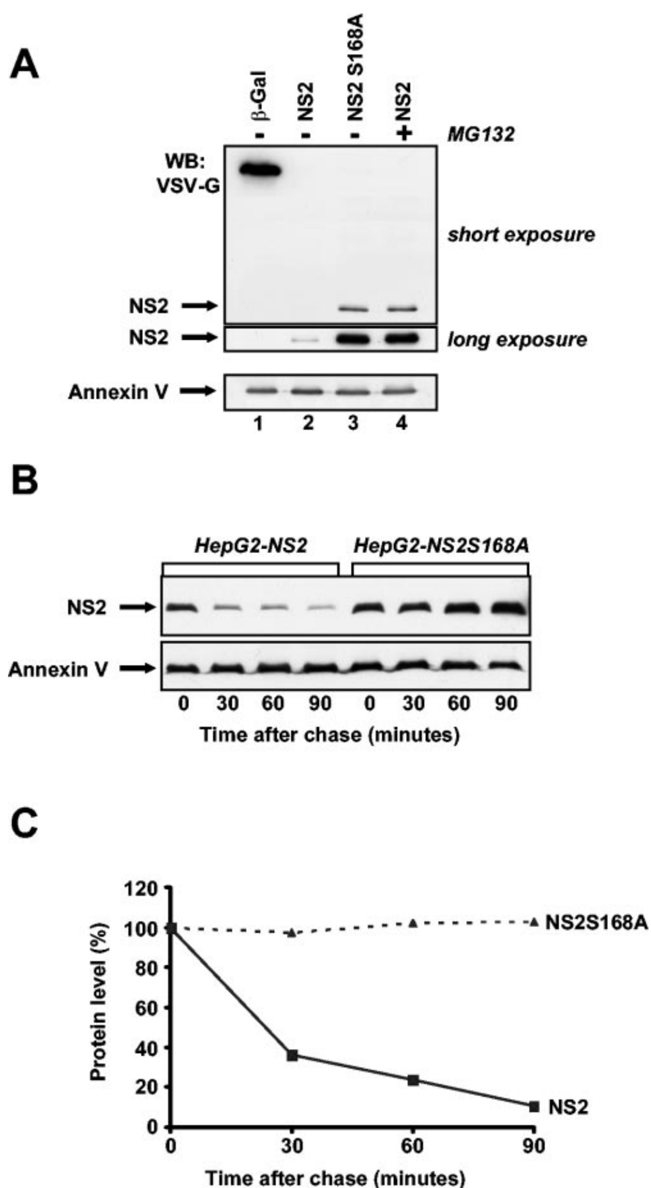

FIG. 3. The NS2S168A mutant is resistant to proteasome-mediated degradation. (A) The serine residue at position 168 is critical for NS2 degradation. HepG2 cells were transfected with 24 μg of different pVM6 plasmids to express either VSV-G–β-galacotosidase (lane 1), VSV-G–NS2 (lanes 2 and 4), or VSV-G–NS2S168A (lane 3). A plus sign indicates treatment of the transfected cells with MG132 for 16 h. Total cell lysates were analyzed by immunoblotting with anti-VSV-G antibody. To ensure comparable loading of the samples, the blot was also probed with anti-Annexin V antibody (lower panel). (B) Time course assay of NS2 and NS2S168A. HepG2-NS2 and HepG2-NS2S168A cell pools, continuously expressing VSV-G–NS2 or VSV-G–NS2S168A, were pretreated with 10 μM MG132 for 16 h, followed by exposure to CHX over a 90-min time course. Total cell lysates were analyzed by immunoblotting with anti-VSV-G antibody. The numbers indicated under the panels refer to the different CHX exposure times. (C) Quantification of NS2 and NS2S168A degradation in the time course assay. The results obtained in panel B were quantified by using a densitometer, and the NS2 and NS2S168A values were plotted after normalization with those obtained for Annexin V.

were then aligned and compared to the NS2 protein sequence of the HCV-H strain used in the present study.

As shown in Fig. 4 (arrows), the two predicted phosphorylation sites, a threonine residue at position 172 and a serine residue at position 211, were not found in all HCV consensus sequences. In contrast, the serine-168 residue (asterisk) belongs to the identified CK2 recognition motif (S/TXXE) that is highly conserved between NS2 proteins from all HCV genotypes.

Protein kinase CK2 phosphorylates NS2 in vitro. To verify whether NS2 is a substrate of CK2, we performed an in vitro kinase assay. To this aim, purified GST or GST-NS2 proteins were immobilized on glutathione-agarose beads and exposed to recombinant protein kinase CK2 with or without CK2 peptide substrate. Then, phosphorylated serine residues of recombinant proteins were detected by Western blotting with anti-phosphoserine antibody. As shown in Fig. 5A, NS2 is phosphorylated by CK2 (lane 2), whereas no serine phosphorylation signal could be observed for GST (lane 1). In addition, phosphorylation of NS2 could be inhibited in the presence of a CK2 peptide substrate (lane 3), indicating that NS2 seems to be a substrate of CK2.

We further examined the capacity of both curcumin, a recently identified CK2 inhibitor (39), and the NS2S168A mutation to affect CK2-directed serine phosphorylation (Fig. 5B). As expected, serine phosphorylation on recombinant NS2 was significantly decreased in the presence of curcumin (Fig. 5B, lane 3). However, a weak phosphorylation signal could still be detected. This result indicates that curcumin, which was described to inhibit CK2 activity (39), does not completely abolish CK2 activity. Interestingly, the GST-NS2S168A mutant was not phosphorylated by CK2 (lane 4). We suggest that the serine 168 seems to be the only CK2 serine phosphorylation site in NS2, although we cannot exclude the possibility that the serine residue mutation might inhibit phosphorylation on another site by indirect effects on, e.g., protein structure and phosphor-acceptor site accessibility. Nevertheless, since NS2S168A mutant is resistant to proteasome degradation, we hypothesize that NS2 phosphorylation might be important for its degradation.

NS2 is stabilized in the presence of a CK2 activity inhibitor. To determine whether CK2 activity might be involved in NS2 degradation, we wondered whether inhibition of this enzymatic activity would stabilize NS2. HepG2 cells transfected to express VSV-G-tagged NS2 or β-galactosidase were left untreated or treated with 100 μM curcumin. After 16 h, cell lysates were analyzed by Western blotting. Figure 6A shows, as expected, that in the absence of any inhibitors β-galactosidase could be detected (lane 1), whereas only a weak NS2 signal could be visualized even after long exposure (data not shown). In contrast, in the presence of 100 μM curcumin, NS2 degradation was inhibited (Fig. 6A, lane 3) since the protein level was similar to that detected in cells treated with MG132 (lane 4).

We then verified whether curcumin might inhibit NS2 degradation in a dose-dependent manner. HepG2-NS2 cells, stably expressing VSV-G-NS2, were pretreated with 10 μM MG132 for 16 h. The cells were then either directly lysed (control) or washed and exposed simultaneously to CHX alone or combined with different concentrations of curcumin (1, 10,

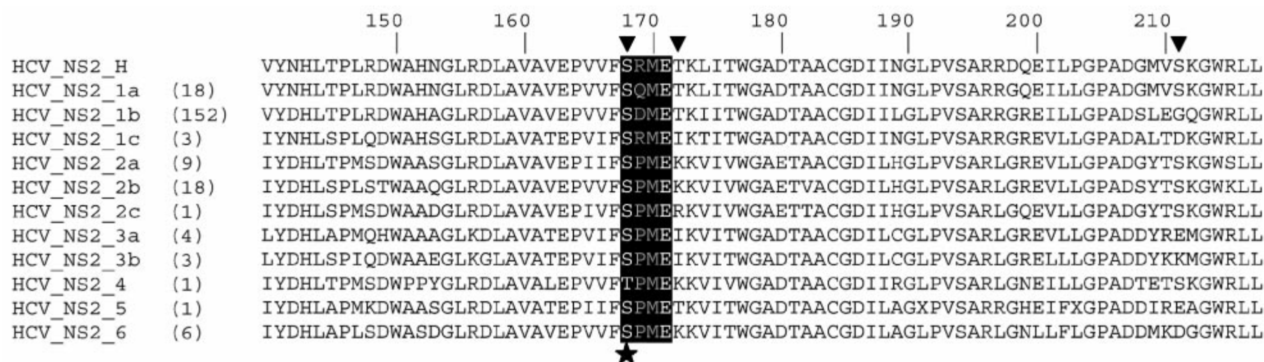

FIG. 4. Sequence alignments of the NS2 region from residues 140 to 217. Multiple sequence alignments of the protein region (residues 140 to 217) of NS2 primary consensus sequences from all HCV genotypes (i.e., 1 to 6). Numbers in parentheses correspond to the totality of HCV isolates recorded for each HCV genotype in the HCV database (http://hcvpub.icbp.fr), e.g., 18 HCV isolates of genotype 1a, 152 HCV isolates of genotype 1b, etc. These HCV isolates were aligned to determine the primary consensus sequence for each HCV genotype. Residue numbers above the sequences correspond to the amino acid position in NS2 from the HCV-H strain. The predicted phosphorylation sites (serine 168, threonine 172, and serine 211) in the HCV-H isolate are indicated by arrows. The serine residue at position 168 is indicated by an asterisk. The conserved consensus CK2 phosphorylation sequence motif (S/TXXE, where X is any amino acid) is represented in a black box.

and 100 μM) for 6 h. Cells were then harvested, and the NS2 protein level was analyzed by Western blotting with anti-VSV-G antibody. As shown in Fig. 6B, NS2 was mainly degraded in cells only exposed to CHX (lane 2) compared to the initial NS2 protein level detected in cells only pretreated with MG132 (lane 1). Interestingly, treatment of cells with curcumin inhibited NS2 degradation in a dose-dependent manner (Fig. 6B, lanes 3 to 5). This inhibition could be observed at 10 and 100 μM curcumin concentrations which were consistent with concentrations recently used in HeLa cells (39).

The protein level of mature NS2 is increased in HCV replicon cells exposed to a proteasome inhibitor and curcumin. Since NS2 is part of a multiprotein complex, in vivo (22), we could not exclude that other viral proteins might have an effect on the individual NS2 protein stability. To determine the protein turnover of unmodified NS2 issued from the complete HCV polyprotein, we took advantage of an HCV replicon system kindly provided by C. M. Rice (2). This system consists in an autonomous efficient RNA replication of the full-length HCV genome which produces all viral proteins upon transfection in the highly permissive Huh-7.5 hepatoma cell line. Huh-7.5 and Huh-7.5 replicon cells were left untreated or exposed to a 10 μM concentration of proteasome inhibitor MG132. After 24 h, protein levels were examined by Western blotting. Since a commercial anti-NS2 antibody is still not available, we used a polyclonal antiserum kindly provided by C. M. Rice. We first verified that this antiserum recognized our transiently expressed NS2 fusion protein. As shown in Fig. 7A, VSV-G-tagged NS2 could only be detected by Western blotting in the presence of proteasome inhibitor MG132 (upper panel, lanes 1 and 2). Moreover, as expected, no NS2 was visualized in Huh-7.5 control cells (Fig. 7, lanes 3 and 4). However, in Huh-7.5 replicon cells, the mature NS2 protein level was significantly increased when cells were pretreated with MG132 (lane 6) compared to the weak signal visualized in untreated cells (lane 5). Interestingly, compared to NS2, the protein level of the structural E2, another viral protein, was not modified by the presence of MG132. Indeed, the expression level of E2 detected in untreated Huh-7.5 replicon cells was similar to that revealed in MG132-exposed cells (lower panel, lanes 5 and 6). These data indicate that HCV proteins differ in their sensitivity to proteasome-mediated degradation, thus suggesting different viral protein stabilities.

We further analyzed the effect of the CK2 inhibitor curcumin on NS2 issued from the complete HCV polyprotein. Huh-7.5 and Huh-7.5 replicon cells were left untreated or exposed to the indicated concentrations of curcumin for 24 h. Cells were then harvested, and the NS2 protein level was analyzed by Western blotting. As shown in Fig. 7B, only very weak NS2 signals could be detected in untreated or 25 μM curcumin-exposed replicon cells (lanes 1 and 2). However, mature NS2 was stabilized at curcumin concentrations ranging from 50 to 100 μM (lanes 3 to 5).

Therefore, we suggest that the CK2 inhibitor curcumin influences NS2 stability in the context of the HCV polyprotein.

DISCUSSION

In this study we investigated the turnover of HCV mature NS2 protein. We found that NS2 is a short-lived protein that is rapidly degraded by the proteasome. Proteolytic processing of NS2 is accomplished by a liver-nonspecific mechanism since it could be demonstrated in primary culture of human hepatocytes and HepG2 cells, as well as in nonhepatic HeLa cells. Proteasome-mediated degradation was already described for several HCV proteins, such as NS5B (14), the unglycosylated cytosolic form of E2 (27), a truncated form of the core protein (36), or the recently identified F protein (40). Therefore, our results in combination with data in the literature suggest that proteasome-mediated degradation seems to control the half-life of several HCV proteins. This proteolytic mechanism could be an explanation for the observation that HCV proteins are not present in amounts detectable in the livers of many chronically HCV-infected patients (31).

Phosphorylation of cellular proteins by CK2, e.g., IκBα (35), but also viral proteins, e.g., Vpu of HIV-1 (33), was shown to modulate protein turnover. Here we show that inhibition of CK2 activity by curcumin, one of the strongest recently iden-

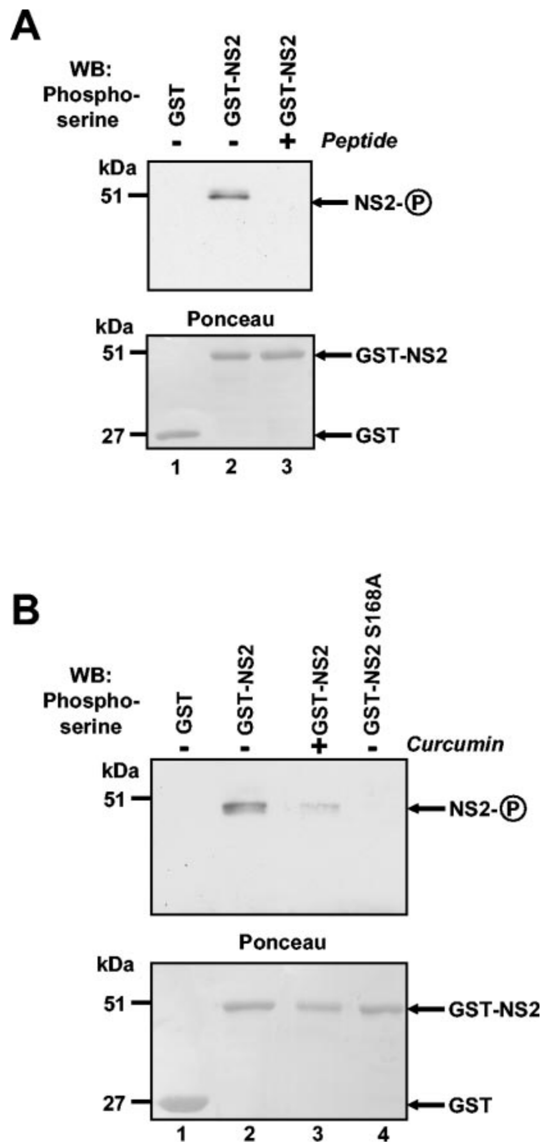

FIG. 5. CK2 phosphorylates NS2 on the serine residue at position 168 as demonstrated by in vitro. (A) For the upper panel, recombinant CK2 was mixed with equal amounts of purified GST or GST-NS2, and in vitro kinase assays were carried out with or without 500 μM concentrations of CK2 peptide substrate prior to Western blot analysis with anti-phosphoserine antibody. The phosphorylated GST-NS2 band is indicated (phosphorylation is indicated by a capital P in a circle). (B) For the upper panel, kinase assays were performed for purified GST, GST-NS2, and GST-NS2S168A, with (+) or without (−) 100 μM curcumin, and protein phosphorylation was verified as described for panel A. The lower portions of panels A and B show Ponceau staining of nitrocellulose using the amounts of recombinant proteins used for the kinase assay in the upper panels.

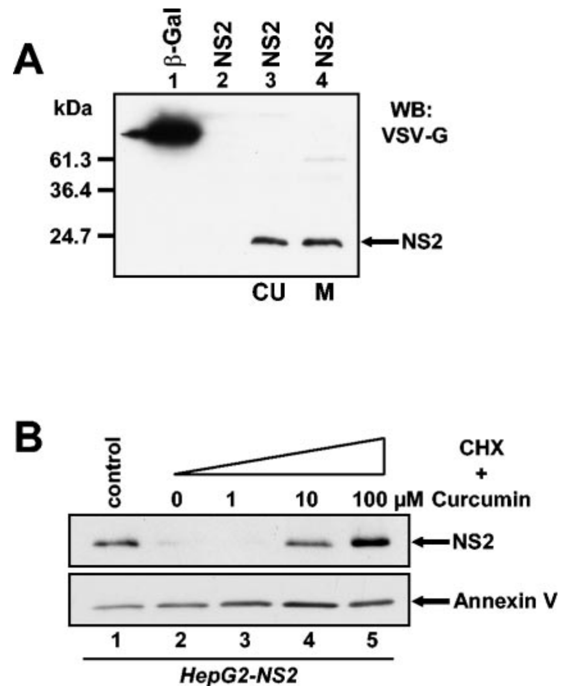

FIG. 6. Curcumin, a CK2 inhibitor, affects NS2 degradation. (A) Curcumin affects NS2 degradation. HepG2 cells were transfected with 24 μg of different pVM6 plasmids to express VSV-G-tagged β-galactosidase (lane 1) or VSV-G-tagged NS2 (lanes 2 to 4). Cells were left untreated (lanes 1 and 2) or exposed to either 10 μM MG132 (M) (lane 4) or 100 μM curcumin (lane 3) for 16 h. Cell lysates were analyzed by Western blotting with anti-VSV-G antibody. (B) The HepG2-NS2 cell line, continuously expressing VSV-G-NS2, was pretreated with 10 μM MG132 for 16 h. Cells were then exposed simultaneously to CHX and the indicated concentrations of curcumin for 6 h. Total cell lysates were analyzed by immunoblotting with anti-VSV-G or anti-Annexin V antibodies, as shown in the lower and upper panels, respectively. Control, lysates of HepG2-NS2 cells only exposed to MG132 for 16 h.

tified inhibitors of CK2 activity (39), decreased significantly NS2 phosphorylation in vitro and correlated directly with increased NS2 stability observed in our established HepG2-NS2 cell line. In addition, mutation of the serine residue 168 in NS2, the identified CK2 serine phosphorylation site, resulted in a stabilized NS2S168A mutant that was resistant to proteasome-mediated degradation. Our results show that NS2 is a substrate of CK2 and thus favor the idea that CK2-directed phosphorylation is directly involved in targeting NS2 for degradation to the proteasome.

NS2 is phosphorylated by CK2 in vitro, on serine residue 168 which belongs to a consensus CK2 phosphorylation sequence motif (S/TXXE). Interestingly, sequence alignments between NS2 proteins from all HCV genotypes revealed that this CK2 phosphoacceptor motif was highly conserved. Therefore, we suggest that NS2 phosphorylation by CK2 seems to be a common mechanism to regulate NS2 stability from different HCV genotypes.

A variety of phosphorylated proteins are targeted for degradation to the proteasome pathway in an ubiquitin-dependent manner (28). Thus, as a protein is degraded, multiple ubiquitin molecules are attached to internal lysine residues of the target substrate (15). Furthermore, modifying these ubiquitin acceptor sites by site-directed mutagenesis has already been shown to restore the stability of protein substrates, e.g., Bid (5). However, in the case of NS2, we could neither detect any ubiquitin conjugation to NS2 nor stabilize the NS2 protein by mutations introduced in potential ubiquitin acceptor sites (data not shown). Interestingly, it was already described that a small number of proteins, e.g., CDK inhibitor p21 (18), ornithine

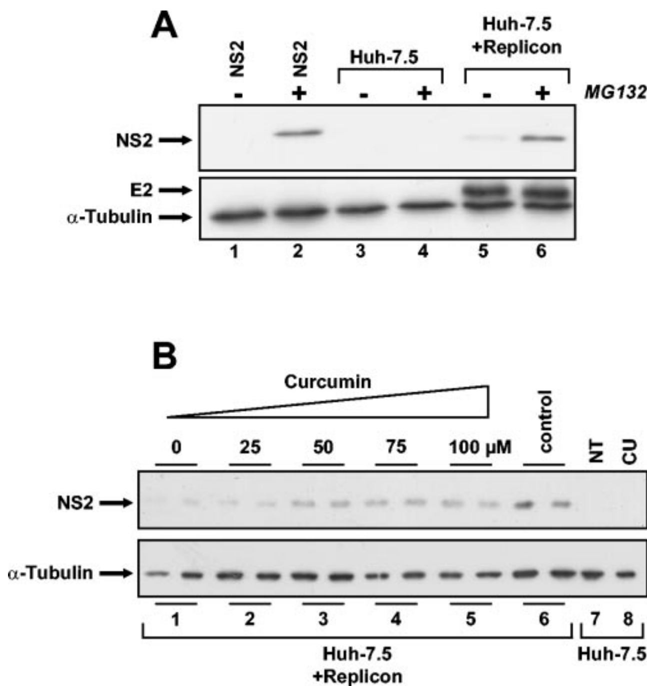

FIG. 7. Increased protein level of mature NS2 in Huh-7.5 replicon cells exposed to MG132 and curcumin. (A) Huh-7.5 cells (lanes 3 and 4) and Huh-7.5 replicon cells (lanes 5 and 6) were left untreated (−) or exposed to 10 μM MG132 for 16 h (+). Total cell lysates were analyzed by immunoblotting with anti-NS2 antiserum (upper panel) and with anti-E2 antibody (lower panel). To ensure comparable loading of the samples, the blot was also probed with anti-α-tubulin antibody (lower panel). Huh-7.5 cells transfected with 2 μg of pVM6-NS2 plasmid to express VSV-G-tagged NS2 (lanes 1 and 2) were left untreated or exposed to MG132 as indicated. These cell lysates were then used as controls for specific anti-NS2 antibody recognition. (B) Huh-7.5 replicon cells were treated with the indicated concentrations of curcumin (lanes 1 to 5). Total cell lysates were analyzed by Western blotting with anti-NS2 and anti-α-tubulin antibodies. Control, lysates of cells exposed to MG132; NT, lysates of nontreated cells; CU, lysates of cells treated with 100 μM curcumin.

decarboxylase (25), or the viral pp71 protein of the RB family of tumor suppressors (19), are substrates of the proteasome but do not require ubiquitin for degradation. Therefore, we suggest that NS2 seems to be a novel candidate for proteasome-dependent, ubiquitin-independent degradation. However, it has not been fully elucidated how the 26S proteasome recognizes nonubiquitinated substrates (1). In the case of NS2, elements involved in a potential ubiquitin-independent pathway remain to be determined.

Since NS2 is part of a multiprotein complex, in vivo (22), we analyzed NS2 expression in a more physiologically relevant context. Thus, we showed that unmodified NS2 expressed in the context of the entire HCV polyprotein in Huh-7.5 replicon cells was also sensitive to both proteasome-mediated degradation and CK2 inhibitor treatment. Indeed, the NS2 protein level was significantly increased in Huh-7.5 replicon cells pretreated with MG132 or with curcumin compared to those left untreated. These results are therefore consistent with our data obtained with the independently expressed NS2 fusion protein, suggesting that NS2 protein properties seem to be similar in both expression systems. Furthermore, NS2 was described to be an integral membrane protein anchored to the ER (32, 41). Interestingly, we also found by fluorescence double-staining experiments with the ER-Tracker as an ER marker that the independently expressed NS2 fusion protein was associated with ER membranes. Our observations were thus in accordance with data of the literature showing that NS2 in the presence or absence of other viral proteins is always membrane associated (41).

Altogether, these observations raise questions regarding the molecular mechanism by which NS2, a transmembrane protein anchored to the ER, is phosphorylated and targeted to the proteasome. Since the exact membrane topology of NS2 has still not been elucidated (23, 26, 32, 41), we suggest that for NS2 degradation the C-terminal half of NS2 harboring the CK2 phosphoacceptor motif has to be directed to the cytosol. Alternatively, it was proposed that the C-terminal half of NS2 could also be directed into the ER lumen (32, 41). This alternate membrane topology could protect NS2 from CK2-directed phosphorylation and thus contribute to modulate NS2 stability. However, in our study, we could only detect a short-lived NS2 protein.

Proteasome-mediated degradation was described as a mechanism used by viruses to eliminate cellular proteins. Indeed, HIV-1 Vpu degradation has been shown to mediate CD4 downregulation, the primary receptor for HIV-1 (for a review, see reference 30). As a consequence, downregulation of CD4 avoids superinfection of the host cell, which is deleterious for the HIV (for a review, see reference 4). Nevertheless in the case of HCV, although important progress was made with the development of the replicon system. which consists of autonomous efficient RNA replication of the full-length HCV genome (2, 29), research is still hampered by the lack of a cell culture system that permits the production of recombinant HCV virions (6). Thus, the impact of NS2 protein turnover on the HCV life cycle remains to be determined. However, we recently described that NS2 acts as an inhibitor of CIDE-B-induced apoptosis (12). Therefore, we suggest that NS2 degradation might interfere with CIDE-B apoptotic activities by triggering CIDE-B to the degradation pathway.

A second biological function recently described for NS2 is its ability to interfere with cellular gene transcription (11). A similar situation has already been described for HIV-1 Vpu, for which two independent functions were also found. These different biological activities are associated with a differential phosphorylation status of this protein (34). In the case of NS2, further investigations are necessary to analyze the possible impact of phosphorylation in the different functions attributed to NS2.

In conclusion, we described novel regulating properties for NS2 consisting in CK2-directed phosphorylation and its accelerated turnover. Since NS2 phosphorylation could be directly correlated with its stability, this strongly suggests that NS2 degradation is regulated in a phosphorylation-dependent manner. The regulation of NS2 protein turnover could be one of the mechanisms used by the HCV to interfere with both CIDE-B-induced apoptosis and cellular gene transcription and thus to escape from host cell defenses.

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