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In Vitro Study of the NS2-3 Protease of Hepatitis C Virus

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Processing at the C terminus of the NS2 protein of hepatitis C virus (HCV) is mediated by a virus-encoded protease which spans most of the NS2 protein and part of the NS3 polypeptide. In vitro cotranslational cleavage at the 2-3 junction is stimulated by the presence of microsomal membranes and ultimately results in the membrane insertion of the NS2 polypeptide. To characterize the biochemical properties of this viral protease, we have established an in vitro assay whereby the NS2-3 protease of HCV BK can be activated posttranslationally by the addition of detergents. The cleavage proficiency of several deletion and single point mutants was the same as that observed with microsomal membranes, indicating that the overall sequence requirements for proper cleavage at this site are maintained even under these artificial conditions. The processing efficiency of the NS2-3 protease varied according to the type of detergent used and its concentration. Also, the incubation temperature affected the cleavage at the 2-3 junction. The autoproteolytic activity of the NS2-3 protease could be inhibited by alkylating agents such as iodoacetamide and *N*-ethylmaleimide. Metal chelators such as EDTA and phenanthroline also inhibited the viral enzyme. The EDTA inhibition of NS2-3 cleavage could be reversed, at least in part, by the addition of $ZnCl_2$ and $CdCl_2$. Among the common protease inhibitors tested, tosyl phenylalanyl chloromethyl ketone and soybean trypsin inhibitor inactivated the NS2-3 protease. By means of gel filtration analysis, it was observed that the redox state of the reaction mixture greatly influenced the processing efficiency at the 2-3 site and that factors present in the rabbit reticulocyte lysate, wheat germ extract, and HeLa cell extract were required for efficient processing at this site. Thus, the in vitro assay should allow further characterization of the biochemical properties of the NS2-3 protease of HCV and the identification of host components that contribute to the efficient processing at the 2-3 junction.

Hepatitis C virus (HCV) is the major etiologic agent of posttransfusion non-A, non-B hepatitis (4, 18). The enveloped virion contains a single-stranded positive-sense RNA genome of about 9,500 nucleotides encoding a polyprotein precursor of 3,010 to 3,011 amino acids (aa) with the following gene order: C-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b (4, 5, 10, 16, 20, 28, 30, 31). C is an RNA binding protein (26) and is believed to be the viral nucleocapsid, E1 and E2 are thought to be the virion glycoproteins, and p7 is a protein of unknown function inefficiently cleaved from the E2 polypeptide (19, 23). The NS2-NS5b proteins are putative nonstructural proteins involved in processing of the polyprotein precursor and viral RNA replication (16).

Upon synthesis, the HCV polyprotein is processed into functional subunits by a combination of host and viral proteases. The host signal peptidase seems to be responsible for the maturation of the C-NS2 region (14, 26). A trypsin-like serine protease encoded in the N-terminal region of the NS3 gene is responsible for cleavage at the NS3-4a, NS4a-4b, NS4b-5a, and NS5a-5b junctions (1, 2, 7, 9, 11, 20, 21, 31). The viral enzyme requires the central region of NS4a as a cofactor for processing at these sites (9, 21, 32) and requires zinc for structural integrity and activity (6). The NS2 protein extends from aa 810 to 1026, and cleavage at the 2-3 junction involves an additional viral protease which includes most of the NS2 region and the entire NS3 serine protease domain (aa 849 to 1237 [27]). The catalytic activity of the serine protease is not required for the 2-3 cleavage (11–13), but the NS3 portion of the viral enzyme cannot be substituted by other fragments of the HCV polypeptide (27). In vitro studies of the cleavage reaction were performed with cDNA of different HCV strains (27), and it was observed that these clones display various degrees of microsomal membrane dependency for efficient processing. These studies have indicated that the NS2-3 precursor is targeted via the signal recognition particle (SRP) and SRP receptor pathway to the endoplasmic reticulum (ER) membrane, where cleavage mediated by the viral protease occurs and leads to the membrane insertion of the NS2 protein (27). The mechanism of processing and membrane localization of NS2 is in agreement with the very hydrophobic profile of this polypeptide (16). No homology has been found between the NS2-3 protease and any other known proteases; however, the HCV NS2-3 protease has been proposed to be a metalloprotease on the basis of the observation that enzymatic activity is inhibited by EDTA and stimulated by $ZnCl_2$ (13). Site-directed mutagenesis studies have identified His-952 and Cys-993, numbered according to their location within the HCV polyprotein, as important residues for enzymatic activity (11–13). On the basis of this observation, the NS2-3 enzyme has been proposed to be a cysteine protease (9a). Additionally, it has been shown that cleavage could be inhibited by mutations that most probably perturb the local conformation of the polyprotein precursor, suggesting that the correct folding of the NS2-3 polypeptide greatly influences the proper cleavage at the 2-3 junction (12, 13, 25).

Although significant progress has been made in the genetic characterization of the HCV NS2-3 protease, the exact biochemical nature of this viral enzyme is still not understood primarily because of the lack of a suitable in vitro assay in which the autoproteolytic activity of the HCV enzyme can be

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separated from the synthesis of the protein precursor. In this study, we have established an in vitro assay that allows the HCV NS2-3 protease to be activated posttranslationally and have studied the effects of several common inhibitors on the enzymatic activity of this viral protease. Furthermore, we have observed that ubiquitous soluble factors are required for efficient proteolysis at the 2-3 junction.

MATERIALS AND METHODS

Recombinant plasmids. Clones expressing HCV E2-NS2-NS3 sequences are derived from cDNA fragments of HCV BK (30) from nucleotides 2496 to 5303 (aa 722 to 1657) unless otherwise specified. The cDNA fragments were cloned in the desired expression vectors by PCR amplification of the area of interest, with synthetic oligonucleotides with the appropriate restriction sites, or by standard DNA methods. Plasmid pCD(810–1615) contains HCV sequences from nucleotides 2758 to 5177 inserted downstream of a T7 promoter in the expression vector pCDNA-3 (Invitrogen). pCITE(722–1325) (nucleotides 2496 to 4307), pCITE(849–1355) (nucleotides 2872 to 5177), pCITE(960–1615) (nucleotides 3208 to 5177), pCITE(722–1325)J (nucleotides 2496 to 4307 of the HCV-J strain [13]), and pCITE(849–1325) (nucleotides 2872 to 4307) were obtained by inserting the desired DNA fragment downstream of the 5′ untranslated region of encephalomycarditis virus and under the T7 promoter in the pCite-1 expression vector (Novagen) (8).

pGEM(810–1615:L1026ΔQ) (nucleotides 2758 to 5177) includes nucleotides 2758 to 5177; in the resulting protein, the amino acid sequence LLAP, spanning residues 1025 to 1028 and including the NS2-3 cleavage site (11, 13), was mutated into LQP with a net deletion of residue 1026 and an alanine-to-glutamine substitution at residue 1027. pCD(810–1615:C993A) (nucleotides 2758 to 5177) encodes a protein that contains a cysteine-to-alanine substitution at aa 993. Construction of these plasmids has been reported previously (27).

In vitro transcription and translation. All plasmids used as templates for in vitro RNA synthesis were linearized with the appropriate restriction enzyme and transcribed in vitro with T7 RNA polymerase as previously described (31). A transcript encoding aa 849 to 1137 was obtained by runoff transcription of pCITE(849–1615) digested with *Aat*II (nucleotide 3744).

In vitro translation reactions (in 50-μl reaction mixtures) were performed for 30 to 60 min at 30°C with nuclease-treated reticulocyte lysate (Promega). Translation products were labeled by incorporation of [^{35}S]methionine. When stated, canine rough microsomes were used at a concentration of 1 eq/25 μl. Alternatively, Triton X-100 or Nikkol was added to the translation reaction mixture at a final concentration of 1 or 0.5%, respectively. After the incubation period, translation was stopped by adding cycloheximide and unlabeled methionine to final concentrations of 2 mM and 80 μM, respectively. The samples were incubated for an additional 5 min at 30°C and were then either diluted in sample buffer and directly analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or treated as described below.

After termination of translation, detergent was added to 10-μl aliquots of the translation mixture. The detergents used were Triton X-100, Nikkol, Nonidet P-40, Tween 20, and 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS) at final concentrations of 0.05, 0.2, 0.5, 1, and 2%. The reaction mixtures were incubated at 20°C for 1 to 4 h. The time course analysis was performed by adding to 90 μl of the translation aliquots Triton X-100 to a final concentration of 1% and by incubating the reaction mixtures at 4, 15, 20, or 30°C for up to 6 h. At the desired time, 15-μl samples were taken, diluted with sample buffer, and subsequently analyzed by SDS-PAGE.

Incorporation of radioactivity into specific bands was determined by direct analysis of the dried gel with a PhosphorImager (Molecular Dynamics). The processing efficiency was estimated on the basis of the incorporation of radioactivity in translation products according to the following formula:

$$\text{percentage of total counts per minute (cpm)} = \frac{\text{NS}(a/b)}{\text{NS}(a/b) + P} \times 100$$

where NS is the cpm of the processed protein, a is the number of methionine residues in the precursor, b is the number of methionine residues in the processed protein, and P is the cpm of the unprocessed precursor.

Inhibition study. Inhibition of proteolysis was determined by the addition of a variety of proteinase inhibitors to the in vitro translation mixture during the chase period in the presence of 1% Triton X-100. The transcript derived from plasmid pCD(810–1615) was translated in vitro as described above. After termination of the translation reaction, 10-μl aliquots of the translation mixture were incubated for 30 min on ice in the presence or absence of proteinase inhibitors. Detergent was then added to a final concentration of 1%, and the reaction mixtures were incubated at 20°C for 4 h. The proteinase inhibitors used were aprotinin (0.5 mg/ml), antipain (0.15 mg/ml), dithiothreitol (DTT) (10 mM), E64 (10 μM), pepstatin (0.5 mg/ml), EGTA (2 mM), EDTA (2 mM), iodoacetamide (IAA) (0.5 mM), *N*-ethylmaleimide (NEM) (4 mM), 1,10-phenanthroline (2 mM), phenylmethylsulfonyl fluoride (PMSF) (3 mM), soybean trypsin inhibitor (1.5 mg/ml), tosyl lysyl chloromethyl ketone (TLCK) (0.5 mM), and tosyl phenylalanyl chloromethyl ketone (TPCK) (0.5 mM).

To determine the effect of metals on the EDTA inhibition of NS2-3 cleavage, Triton X-100 at 1% final concentration was added to 10-μl aliquots of the translation reaction mixture supplemented with cycloheximide and unlabeled methionine. EDTA (2 mM) was then added, and the samples were left on ice for 10 min. Metals were added, and the reaction mixtures were incubated for 4 h at 20°C. The metals used were $MgCl_2$ (1, 2, and 3 mM), $MnCl_2$ (1, 2, and 3 mM), $ZnCl_2$ (1, 2, and 3 mM), $CaCl_2$ (1, 2, and 3 mM), and $CdCl_2$ (0.5, 1, and 2 mM). The samples were then diluted with sample buffer and run on an SDS–11% polyacrylamide gel, and the bands were visualized by autoradiography. The percent inhibition was calculated dividing the efficiency of processing in the presence of the inhibitor by that obtained in the absence of inhibitors, subtracting this value from 1, and multiplying by 100.

Gel filtration analysis. Transcript derived from plasmid pCD(810–1615) was translated in vitro as described above, and after the addition of 2 mM cycloheximide and 80 μM unlabeled methionine, the translation mixture was passed through a Sephadex G-50 spin column (5 Prime-3 Prime Inc., West Chester, Pa.) by centrifugation at 1,100 × *g* for 4 min at 4°C. Prior to centrifugation, the column was equilibrated by repeated washes with centrifugation buffer (0.1 M morpholinepropanesulfonic acid [MOPS; pH 7.5], 0.4 mM $MgCl_2$, 0.5 mM magnesium acetate, 0.1 M potassium acetate). Where indicated, the centrifugation buffer was supplemented with 2 mM DTT. After centrifugation, the flowthrough fraction was aliquoted in 10-μl samples and the NS2-3 cleavage proficiency was determined by the addition of Triton X-100 to a 1% final concentration. The reaction mixture was incubated at 20°C for 4 h, diluted with sample buffer, and analyzed by SDS-PAGE. Where indicated, 400 μg of protein equivalent of either rabbit reticulocyte lysate (RRL) S-60 supernatant, dialyzed RRL S-60 supernatant, wheat germ extract S-60 supernatant, or HeLa cell lysate S-60 supernatant was added to the reaction mixture prior to incubation at 20°C for 4 h. The HeLa cell lysate was obtained by resuspending 1.5×10^7 cells in 200 μl of centrifugation buffer supplemented with 2 mM DTT, followed by cell lysis by repeated freezing and thawing. The cell lysate was clarified by centrifugation for 5 min at 1,000 × *g* in a Haereus benchtop centrifuge. The S-60 supernatants were obtained by centrifugation of the lysate at 60,000 rpm in a Beckman TLA-100 rotor for 15 min at 4°C in a 100-μl volume. Then 100 μl of the RRL S-60 supernatant was dialyzed for 12 h at 4°C in 500 ml of centrifugation buffer supplemented with 2 mM DTT. The Bradford method was used for a rough calculation of the protein concentration in each sample. Cycloheximide and unlabeled methionine were added to all the lysates to final concentrations of 2 mM and 80 μM, respectively, prior to utilization in the NS2-3 processing assay.

RESULTS

Detergent-activated cleavage of the NS2-3 protease. We have shown that processing at the NS2-3 junction requires the SRP receptor for proper targeting of the nascent polypeptide precursor to the ER membrane (27). Additionally, we have proposed that microsomal membranes enhance cleavage by providing a hydrophobic environment that facilitates the proper folding of the NS2-3 precursor polypeptide. To test this hypothesis, we determined whether membranes could be substituted by the addition of detergents to the in vitro translation reaction mixture. As shown in Fig. 1A, translation of a transcript encoding aa 810 to 1615 of HCV BK in the presence of 0.5% Nikkol yielded an 88-kDa unprocessed precursor as well as two proteins of 65 and 23 kDa corresponding to the cleaved NS3 and NS2 polypeptides, respectively (Fig. 1A, lane 3). The identity of each of the translation products was confirmed by immunoprecipitation with specific antisera (data not shown). Thus, cleavage at the NS2-3 site occurred in the presence of detergent in a similar fashion to what could be observed upon addition of microsomal membranes (lane 2), albeit at a lower efficiency. In contrast, processing was not evident when translation was carried out in the absence of membranes or detergents (lane 1), consistent with the observation that processing at the NS2-3 junction of the HCV-BK strain requires a hydrophobic environment for proper activation and folding of the viral protease (27).

We next determined whether the detergent could activate the processing of the NS2-3 junction if added posttranslationally. Thus, translation of the transcript encoding aa 810 to 1615 was carried out in the presence of [^{35}S]methionine for 30 min at 30°C and the protein synthesis was terminated by the addition of cycloheximide and unlabeled methionine. Subsequently, microsomal membranes or detergent were added to the

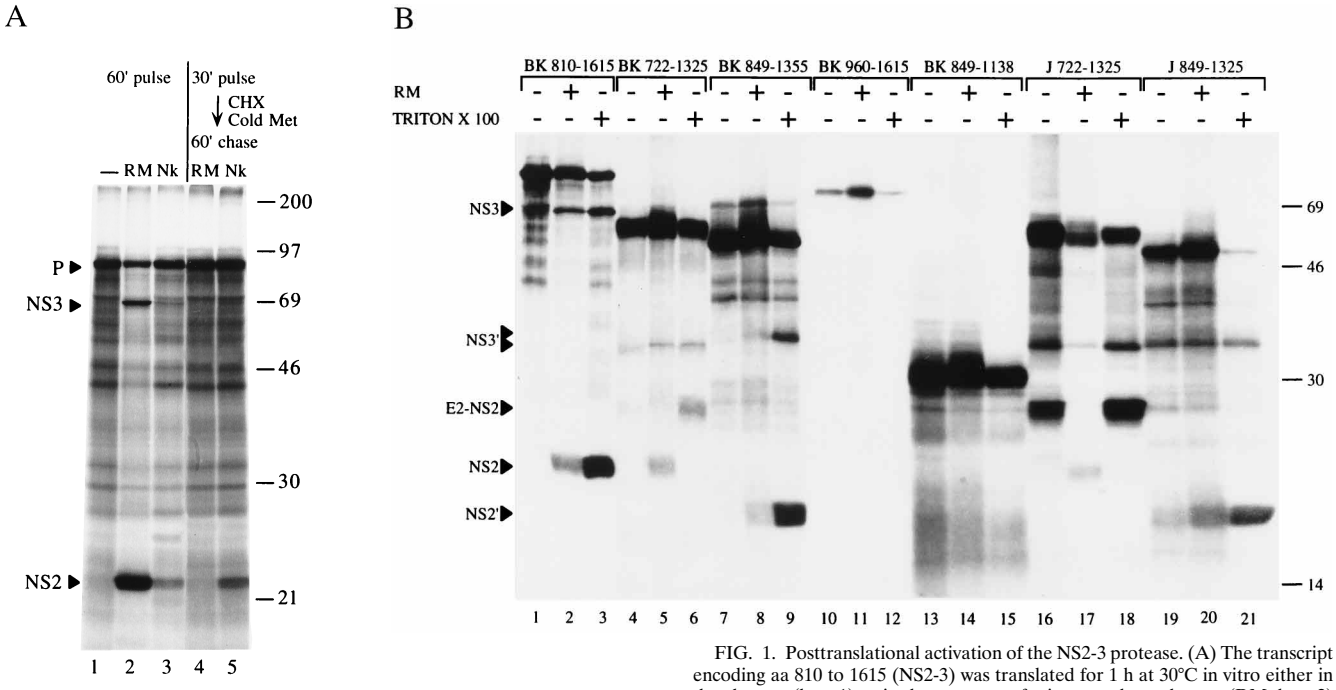

FIG. 1. Posttranslational activation of the NS2-3 protease. (A) The transcript encoding aa 810 to 1615 (NS2-3) was translated for 1 h at 30°C in vitro either in the absence (lane 1) or in the presence of microsomal membranes (RM; lane 2) or 0.5% Nikkol (Nk; lane 3). Alternatively, the translation was carried out in the absence of microsomes for 30 min at 30°C (lane 4 and 5). The translation reaction was terminated by the addition of cycloheximide (CHX) and unlabeled methionine, and aliquots of the translation reaction mixture were incubated for 1 h at 30°C in the presence of microsomal membranes (lane 4) or 0.5% Nikkol (lane 5). (B) Transcripts encoding aa 810 to 1615 (lanes 1 to 3), aa 722 to 1325 (lanes 4 to 6), aa 849 to 1355 (lanes 7 to 9), and aa 960 to 1615 (lanes 10 to 12) of HCV BK and aa 722 to 1325 (lanes 16 to 18) and aa 849 to 1325 (lanes 19 to 21) of the HCV J were translated in vitro with a rabbit reticulocyte lysate for 30 min at 30°C in the absence (lanes 1, 4, 7, 10, 13, 16, and 19) or presence (lanes 2, 5, 8, 11, 14, 17, and 20) of rough microsomes (RM). Alternatively, the samples were translated in the absence of rough microsomes and, after addition of cycloheximide and unlabeled methionine, Triton X-100 was added to the translation mixture at a final concentration of 1% (lanes 3, 6, 9, 12, 15, 18, and 21). The reaction mixtures were then incubated for 2 h at 20°C. (C) RNAs of the mutants L1026ΔQ (lanes 3 and 4) and C993A (lanes 5 and 6) were translated in vitro in the absence of microsomal membranes, aliquots were incubated for 2 h at 20°C in the presence of 1% Triton X-100 (lanes 2, 4, and 6), and their processing patterns were compared to that of the HCV BK parental transcript encoding aa 810 to 1615 (lanes 1 and 2). Translation products labeled with ^{35}S were analyzed on an SDS–11% polyacrylamide gel. The positions of the processed NS3, NS3′, E2-NS2, NS2, and NS2′ polypeptides and of the uncleaved precursors (P) are indicated. The positions of the molecular mass standards (in kilodaltons) are indicated.

reaction mixture, which was incubated for an additional 60 min at 30°C. As can be seen in Fig. 1A, lane 5, the addition of Nikkol to the translation product resulted in the proper cleavage of the polypeptide precursor, yielding the processed NS2 and NS3 proteins. In contrast, no processing could be detected upon addition of microsomal membranes to the reaction mixture (lane 4).

To determine whether processing of the NS2-3 precursor observed in the presence of detergent maintained the same amino acid requirements as those necessary for cleavage in the presence of microsomal membranes, we compared the processing proficiency of several polypeptides spanning aa 722 to 1615 of HCV BK in the presence of microsomal membranes during protein synthesis and of detergents added postranslationally. The precursors used in this comparison comprise part of the E2 glycoprotein as well as most of the NS2-3 region. Thus, generation of a mature NS2 polypeptide requires cleavage at the E2-NS2 junction by the ER signal peptidase and at the NS2-3 site by the NS2-3 protease. Additionally, precursors of HCV J spanning aa 722 to 1325 were analyzed. This series of experiments was performed with Nikkol (data not shown) or Triton X-100 at a final concentration of 1%, which gave the highest efficiency of cleavage (see below).

As shown in Fig. 1B, the addition of 1% Triton X-100 to the translation products allowed proper processing at the NS2-3 junction of the HCV BK similarly to what has been observed with Nikkol. The transcript encoding aa 810 to 1615 yielded an 88-kDa precursor as well as 65- and 23-kDa processed polypeptides corresponding to cleaved NS3 and NS2 proteins, respectively (Fig. 1B, lane 3). Similarly, processing of a 56-kDa precursor extending from aa 849 to 1355 could also be detected upon addition of Triton X-100, yielding two polypeptides, a cleaved 36-kDa NS3 and a truncated 19-kDa version of NS2 (NS2′) (lane 9). With both polypeptide precursors, the processing efficiency seemed greater than what was observed when the translation reaction was carried out in the presence of

microsomal membranes (Fig. 1B, compare lane 2 with 3 and lane 8 with 9). As expected, little or no processing could be detected in the absence of either membrane or detergent (lanes 1 and 7).

Cleavage was limited to the NS2-3 junction when performed in the presence of detergent, in agreement with the involvement of ER signal peptidase in the processing of the E2-NS2 site. In fact, processing of a 66-kDa precursor extending from aa 722 to 1325 and thus including part of E2, all of NS2, and part of NS3 (NS3′) in the presence of detergent yielded two cleaved polypeptides of 32 and 26 kDa corresponding to the NS3′ and E2-NS2 proteins, respectively (Fig. 1B, lane 6). The 26-kDa protein disappeared and the 23-kDa polypeptide appeared when translation was carried out in the presence of microsomal membranes, indicating that the cleavage mediated by the signal peptidase had occurred and that the NS2 product had been released from the E2 protein (lane 5).

Processing of the HCV-J polyproteins occurred at the NS2-3 site in the absence of microsomal membranes or detergents, yielding the cleaved products of the expected size. This is in agreement with the observation that the HCV J NS2-3 processing is membrane independent (27). More specifically, the HCV J transcript encoding aa 722 to 1325 yielded a 66-kDa precursor, a 32-kDa protein corresponding to the cleaved NS3′, and a 26-kDa polypeptide corresponding to E2-NS2 (Fig. 1B, lane 19). Similarly, the HCV J transcript encoding aa 849 to 1325 yielded a 53-kDa unprocessed precursor, as well as two proteins of 32 and 19 kDa corresponding to NS3 and NS2′ (lane 19). However, the addition of detergent increased the efficiency of cleavage at the NS2-3 site (lanes 18 and 21) similarly to what was observed with the HCV BK polypeptides. Notably, when transcripts encoding aa 960 to 1615 or aa 849 to 1138 of the HCV BK polyprotein were used in the experiment, no processing could be induced when detergent was added or if membranes were added to the translation reaction mixtures, and thus only the uncleaved precursors of 72 and 32 kDa could be detected (lanes 10 to 15), in agreement with the observation that the minimal region of the HCV BK precursor required for processing at the NS2-3 site extends from aa 849 to 1237 (27) and thus indicating that this sequence requirement was also maintained when processing occurred in the presence of detergent.

To confirm that processing at the NS2-3 site in the presence of detergent did not alter the structural requirements of the viral protease, we analyzed the effect of detergent on the processing efficiency of mutants C993A and L1026ΔQ. These mutations abolished processing at the NS2-3 junction both in in vitro translation and in transfection studies, affecting in an equal manner HCV H, J, and BK (12, 13, 27). Mutant C993A carries a cysteine-to-alanine substitution at residue 993. Cysteine 993 is thought to play an important role in the cleavage reaction. Mutant L1026ΔQ carries a net deletion of aa 1026 and an alanine-to-glutamine substitution of aa 1027, thus affecting the NS2-3 cleavage site (aa 1026 to 1027). As expected, in contrast to the wild-type polyprotein, neither mutant underwent cleavage when incubated in the presence of detergent (Fig. 1C). Taken together, these data indicate that the detergent did not alter the processing requirements of the NS2-3 protease but, rather, contributed to a greater cleavage efficiency by probably facilitating proper folding of the viral precursor.

Effect of detergents on NS2-3 cleavage. The data reported above demonstrated that the viral NS2-3 protease can be posttranslationally activated in vitro by the addition of detergents to the translation reactions. To further investigate the effect of detergents on the NS2-3 cleavage, we compared the efficiency

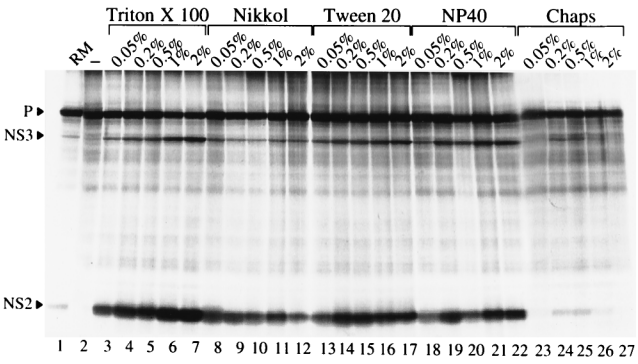

FIG. 2. Effect of detergent concentration on NS2-3 processing efficiency. The transcript encoding HCV BK aa 810 to 1615 was translated in vitro with a rabbit reticulocyte lysate in the presence (lane 1) or absence (lanes 2 to 27) of rough microsomes (RM) for 1 h at 30°C. After termination of the translation reaction, aliquots of the reaction mixture were incubated for 4 h at 20°C in the presence of detergent. Translation products labeled with ^{35}S were analyzed on an SDS–11% polyacrylamide gel. The positions of the processed NS3 and NS2 polypeptides and of the uncleaved precursor (P) are indicated.

of processing of a precursor encompassing aa 810 to 1615 upon posttranslational addition of increasing amounts of several detergents. As shown in Fig. 2, the zwitterionic detergent CHAPS had little stimulatory effect on the NS2-3 processing at low concentrations and inhibited cleavage at the NS2-3 junction at higher concentrations (Fig. 2, lanes 23 to 27). In contrast, a significative enhancement of processing efficiency was observed when the nonionic detergents Triton X-100, Nikkol, Tween 20, and Nonidet P-40 were used (lanes 3 to 22). Of these, Triton X-100 gave the highest efficiency of protease activation and was used throughout the rest of these studies at a final concentration of 1% (lanes 3 to 7).

We next determined the effect of incubation time and temperature on the processing efficiency at the NS2-3 junction upon detergent activation. To this end, the in vitro-translated polyprotein precursor encompassing aa 810 to 1615 was incubated in the presence of 1% Triton X-100 at various temperatures for a period ranging from 0 to 6 h. The results revealed that the NS2-3 protease was poorly active at 4°C (Fig. 3A, lanes 1 to 6), whereas its activity increased at 15°C (lanes 7 to 12) and 20°C (lanes 13 to 18), reaching 75% processing efficiency (Fig. 3B), but significantly decreased at 30°C (Fig. 3A, lanes 19 to 24). In addition, incubation of the translation products at 37°C resulted in extensive degradation of the polyprotein precursor (data not shown). The decrease in protease activity at temperatures above 20°C was probably due to loss of cleavage competence of the precursor due to aggregation or misfolding; in fact, we observed a similar time- and temperature-dependent decrease in processing efficiency when the precursor was preincubated at the above temperature before the addition of detergents (data not shown).

Effect of proteinase inhibitors and of metals on NS2-3 cleavage. Many protease inhibitors block translation in vitro; therefore, so far it has been difficult to analyze the effect of protease inhibitors on the NS2-3 cleavage. We took advantage of the posttranslational activation of NS2-3 processing by detergent to test the action of several common proteinase inhibitors on the cleavage activity of the HCV NS2-3 protease. To this end, the in vitro-translated precursor encompassing aa 810 to 1615 was incubated with various protease inhibitors in the presence of 1% Triton X-100 for 4 h at 20°C. As shown in Fig. 4, metal chelators such as EDTA (2 mM) and phenanthroline (2 mM) and alkylating agents such as IAA (0.5 mM), and NEM (4 mM)

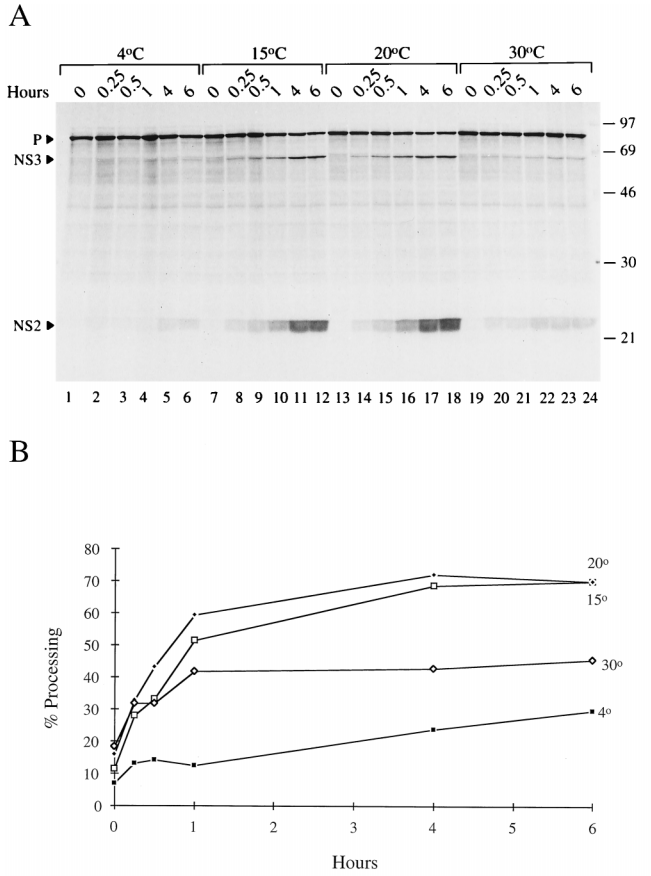

FIG. 3. Temperature effect on NS2-3 cleavage. The transcript encoding aa 810 to 1615 of HCV BK was translated in vitro in the absence of microsomal membranes for 30 min at 30°C. After termination of the translation reaction, aliquots of the reaction mixture were incubated for up to 6 h at various temperatures in the presence of 1% Triton X-100. Translation products labeled with ^{35}S were analyzed on an SDS–11% polyacrylamide gel. (A) Unprocessed precursor (P) and cleaved NS2 and NS3 proteins are indicated. Positions of the molecular mass standards (in kilodaltons) are indicated. (B) The percentage of processing was calculated as described in Materials and Methods and plotted as a function of incubation time.

strongly inhibited processing at the NS2-3 junction. Additionally, a significant inhibition of the cleavage reaction was observed with the serine protease inhibitor TPCK (0.5 mM) and with soybean trypsin inhibitor (1.5 mg/ml). A slight decrease in processing was detected with the serine protease inhibitor TLCK (0.5 mM) and with EGTA (2 mM). Other protease inhibitors, even at the highest concentration tested, had no detectable effect on protease activity. These include antipain (0.15 mg/ml), aprotinin (0.5 mg/ml), E64 (10 μM), PMSF (3 mM), and pepstatin (0.5 mg/ml). Also, the addition of reducing agents such as DTT (10 mM) had no inhibitory effect on the processing reaction.

As shown in Fig. 5, NS2-3 processing inhibition mediated by EDTA (2 mM) could be reversed by the addition of $CdCl_2$ (1 mM) and $ZnCl_2$ (2 mM). Little or no reactivation of the viral protease could be detected upon addition of $MgCl_2$, $MnCl_2$, or $CaCl_2$ (3 mM). Although we could reproduce these results several times, the extent of inhibition by EDTA, and consequently the extent of reactivation by $ZnCl_2$ and $CdCl_2$, varied among experiments, possibly because of the reversible chelating activity of EDTA and of the variable amounts of endogenous metal(s) in the solutions and reticulocyte lysate used in

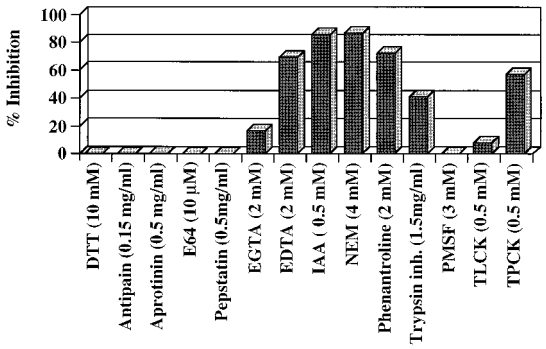

FIG. 4. Effect of proteinase inhibitors on NS2-3 processing. The transcript encoding aa 810 to 1615 of HCV BK was translated in vitro in the absence of microsomal membranes for 30 min at 30°C. After termination of the translation reaction, aliquots of the reaction mixture were incubated for 4 h at 20°C in the presence of 1% Triton X-100 and in the presence of DTT, antipain, aprotinin, E64, pepstatin, EGTA, EDTA, IAA, NEM, phenanthroline, soybean trypsin inhibitor, PMSF, TLCK, or TPCK at the indicated concentration. Translation products labeled with ^{35}S were analyzed on a SDS–11% polyacrylamide gel. The percentage of inhibition was calculated as described in Materials and Methods.

these experiments. Nonetheless, the data obtained are in agreement with the protease inhibitor profile of NS2-3 and support the involvement of metals in the processing of the NS2-3 junction (13). The observation that cadmium reverses the EDTA inhibition to an extent similar to or greater than that by zinc suggests that the metal probably plays a structural rather than a catalytic role.

Requirements for efficient NS2-3 cleavage. To further characterize the biochemical features of the NS2-3 protease, the translation reaction of the transcript encoding aa 810 to 1615 was subjected to gel filtration on a Sephadex G-50 spin column prior to protease activation by addition of detergent. Notably, after gel filtration, addition of Triton X-100 was no longer sufficient to fully activate cleavage at the NS2-3 junction (Fig. 6A, compare lanes 2 and 4). However, processing was reestablished upon addition of RRL S-60 supernatant to the reaction mixture (lane 5). Similarly, the addition of 2 mM DTT had a comparable stimulatory effect on cleavage at the NS2-3 site (lane 6), indicating that the processing efficiency is strongly influenced by the presence of DTT in the translation reaction

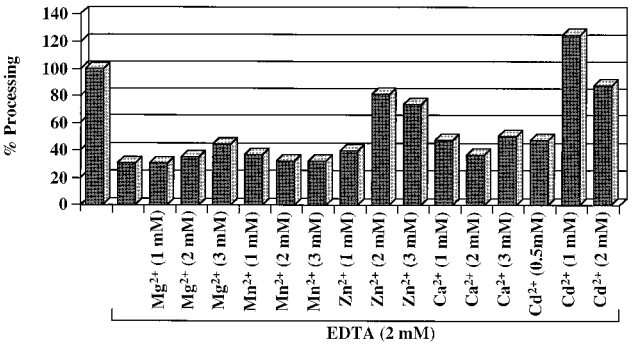

FIG. 5. Effect of metals on NS2-3 protease activity. The transcript encoding aa 810 to 1615 was translated in vitro in the absence of microsomal membranes. After termination of the translation reaction, aliquots of the reaction mixture were incubated for 4 h at 20°C in the presence of 1% Triton X-100 or 1% Triton X-100–2 mM EDTA. Where indicated, metals were added at concentrations ranging from 0.5 to 3 mM. The percentage of processing was calculated as described in Materials and Methods and normalized to processing in the absence of EDTA, which was assumed to be 100%.

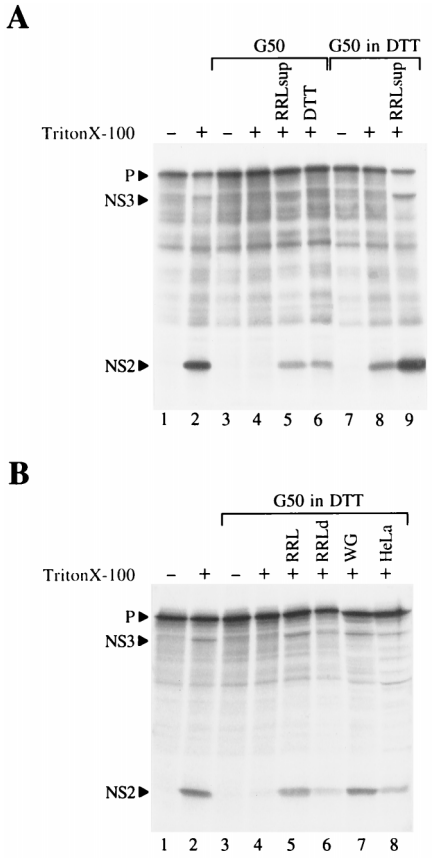

FIG. 6. NS2-3 protease requires a soluble ubiquitous factor. The transcript encoding aa 810 to 1615 of HCV-BK was translated in vitro for 30 min at 30°C. After termination of the translation, the reaction mixture was passed through a Sephadex G-50 spin column in the presence or absence of 2 mM DTT. Samples of the translation reaction mixture were then incubated for 4 h at 20°C. (A) Gel filtration was carried out in the presence (lanes 7 to 9) or absence (lanes 3 to 6) of 2 mM DTT. The reaction mixture was then aliquoted and incubated in the presence (lanes 2, 4 to 6, 8, and 9) or absence (lanes 1, 3, and 7) of 1% Triton X-100. Additionally, 2 mM DTT (lane 6) or RRL S-60 supernatant (lanes 5 and 9) was added to the reaction mixture. (B) Aliquots of the reaction mixture were incubated in the presence (lanes 2 and 4 to 8) or absence (lanes 1 and 3) of 1% Triton X-100. RRL S-60 supernatant, either unprocessed (lane 5) or dialyzed (RRLd; lane 6), Wheat germ S-60 (lane 7) and HeLa cell S-60 (lane 8) supernatants were also added. Translation products labeled with ^{35}S were analyzed on an SDS–11% polyacrylamide gel. The positions of the processed NS3 and NS2 polypeptides and of the uncleaved precursor (P) are indicated.

and that DTT is removed by the gel filtration procedure. This conclusion was supported by the observation that a basal level of processing proficiency in the presence of detergent was retained when gel filtration was performed in the presence of 2 mM DTT (compare lanes 4 and 8). Interestingly, in this case the addition of RRL S-60 supernatant resulted in an additional increase in the cleavage efficiency of NS2-3 (lane 9), suggesting that RRL S-60 supernatant contains another factor(s) which is lost upon gel filtration. Processing at the NS2-3 site could also be reestablished in the presence of detergent upon addition of S-60 supernatant of wheat germ extract and to a lesser extent of HeLa cells (Fig. 6B, compare lane 5 with lanes 7 and 8). Strikingly, the increase in NS2-3 cleavage detected upon addition of RRL S-60 supernatant that had been dialyzed under reducing conditions with a membrane cutoff of 30 kDa was not as high as that observed with untreated RRL S-60 (compare lanes 5 and 6). Taken together, these data suggest that in addition to the proper redox state of the cleavage reaction mixture, another factor(s) with a molecular mass less than 30 kDa is required for efficient processing at the NS2-3 junction.

DISCUSSION

In this study, we have examined the biochemical features of the HCV-BK NS2-3 protease by using an in vitro assay for posttranslational activation of the viral protease. The data presented here support the involvement of metals in the cleavage reaction and suggest that ubiquitous soluble factors are required for efficient proteolysis.

Processing of the NS2-3 junction is mediated by a viral autoprotease that displays unique characteristics. Recent published data have demonstrated that cleavage at this site can occur, although with low efficiency, as a bimolecular reaction (25). Additionally, the required portions of both the NS2 and NS3 polypeptides for cleavage at the 2-3 junction suggest that these two regions may interact in the processing event (25). The distinct degree of membrane dependency for efficient in vitro cleavage displayed by the HCV BK and HCV J precursors may reflect a different capacity of these two polypeptides to either properly fold or interact with host components to achieve processing at the 2-3 junction (27). It is likely that the membrane contribution to processing efficiency in HCV BK is involved in part in assisting the proper folding of the viral precursor, particularly in view of its hydrophobic nature (16). Similarly, the role of nonpolar detergents on the processing reaction can be interpreted as facilitating the establishment of the optimal folding of the viral precursor and allowing the correct protein-protein interactions required for cleavage at this site. Additionally, in view of the differences in the structure and permeability between the membrane bilayer and the detergent, the observation that processing can be obtained posttranslationally by addition of detergent indicates that the interaction of the viral precursor with the hydrophobic environment of the detergent is sufficient to induce proper cleavage at this site, thus bypassing the SRP-SRP receptor membrane-targeting pathway without altering the sequence requirements and possible structural constraints necessary for proteolysis (Fig. 1).

The cleavage efficiency was highly dependent on the detergent concentration in the assay mix, declining at concentrations below the critical micelle concentrations (data not shown). However, the lack of significant cleavage activation and the concomitant inhibitory effect by zwitterionic detergents such as CHAPS suggest that parameters such as the micelle size, detergent structure, and/or overall charge strongly affect the NS2-3 cleavage proficiency (Fig. 2). Perhaps, in view of the membrane targeting and translocation of the processed polypeptide, the greater efficiency of cleavage obtained in the presence of nonpolar detergents can be explained by ability of the micelle of these detergents to more closely reproduce the hydrophobic microenvironment established upon interaction of the protein precursor with the lipid bilayer, thus allowing a folding of the polypeptide consonant with autoproteolysis (Fig. 2). Interestingly, a similar detergent effect has been observed with the NS3 protease activity in vitro. In fact, although the purified serine protease has been shown to work in vitro in the presence of CHAPS (29), the NS3 produced in mammalian cells with herpes simplex virus amplicon was shown to be inhibited by CHAPS at concentrations of 0.5% or more and was not affected by nonionic detergents (15). This observation suggests that the nonpolar detergents are in fact capable of reproducing the correct environment, which is optimal for the activity of both viral proteases. Lastly, the observation that the NS2-3 processing efficiency is highest at 20°C suggests that

the proper folding of the viral precursor achieved by means of the interaction of the newly synthesized polypeptide with the hydrophobic environment provided by the detergent is stabilized by the lower temperature and thus results in a greater enzymatic activity (Fig. 3).

The sensitivity of NS2-3 protease to EDTA and phenanthroline (Fig. 4), as well as the capacity of $ZnCl_2$ and $CdCl_2$ to reverse EDTA inhibition of cleavage (Fig. 5), is suggestive of the involvement of metals in the enzyme activity, in agreement with published data (13). In zinc-containing enzymes, the zinc ion performs a catalytic role and is usually coordinated by nitrogen and oxygen atoms exposed to the solvent. However, several enzymes bind zinc ions for a strictly structural role (3). In this case the metal ion is more often ligated by cysteine thiolates (33). The possible involvement of cysteine residues in determining the enzyme functionality is supported by the sensitivity to alkylating agents such as NEM and IAA, as well as to TPCK, and is in agreement with the mutagenesis of Cys 993 (11–13, 27). This finding is also supported by the observation that the presence of DTT greatly affects the enzyme functionality (Fig. 6A), again suggesting that the proper redox state of cysteines plays a role in the cleavage reaction. However, the data presented here do not indicate whether cysteine residues play a catalytic role in enzyme activity or whether these residues as well as the metal ions determine the structural integrity of the HCV autoprotease. Interestingly, the recent elucidation of the NS3 serine protease crystal structure has indicated that the viral enzyme contains a zinc binding site coordinated by three Cys residues (Cys 1123, 1127, and 1171) and by His 1175 (17, 22). The role of the zinc ion appears to be strictly structural, because it can be readily substituted by cadmium or cobalt ions (6). Mutagenesis of any of these metal-coordinating cysteine residues to alanine has a strong negative impact on the serine protease activity and a measurable effect on the NS2-3 autoprotease activity (13). These data suggest the possibility that these two proteases share the same metal binding site. However, whether the metal binding site located on the NS3 region is also responsible for the NS2-3 protease integrity and functionality remains to be determined.

The involvement of host factors in the biogenesis of the NS2 and NS3 polypeptides has already been demonstrated by the observation that targeting of the uncleaved NS2-3 precursor to the ER membrane requires SRP and SRP receptor (27). However, additional host factors may be involved in the cleavage reaction either by performing catalytic functions or by contributing to the proper folding of the viral polyprotein precursor. In the experiments described in this work, the contribution to the cleavage reaction of the SRP and SRP receptor were not examined in view of the lack of microsomal membranes in the translation and cleavage reaction. Thus, the SRP receptor normally present on the ER membrane is not included in the processing event and plays no role in the posttranslational cleavage of the NS2-3 precursor. However, the data obtained suggest the involvement of additional host components in the efficient processing of the NS2-3 junction. This possibility is supported by the observation that addition of S-60 supernatants derived from wheat germ extracts or from HeLa cell lysate results in a moderate reactivation of processing at the 2-3 site (Fig. 6B), suggesting that a ubiquitous factor is involved in the processing reaction. Interestingly, we have recently observed that the loss of efficient processing resulting from either gel filtration of the translation reaction mixture or from dialysis of the RRL S-60 supernatant can be partially corrected by the addition of ATP (data not shown). Thus, although the function of the ATP in this cleavage reaction requires further investigation, we can postulate two possible roles of the ATP in the processing reaction either as a direct cofactor of the viral autoprotease or as a component of the cellular machinery involved in establishing the proper folding of the viral precursor. This latter possibility is in agreement with the observation that several host chaperones such as Hsp70, Hsp60, and TRiC bind nascent polypeptides in an ATP-dependent manner to ensure the proper folding of the newly synthesized protein and correct interaction of different protein domains (24). Therefore, it is entirely possible to envision the involvement of such host proteins in the biogenesis of mature HCV polypeptides, in particular of NS2 and NS3.

The exact nature of the NS2-3 protease remains unclear in terms of its biochemical and structural requirements, as well as of its role in the biological functionality of the proteins involved. However, the establishment of an in vitro posttranslational activation of this viral enzyme should allow a better understanding of this enzyme.

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