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The NS3 Proteinase Domain of Hepatitis C Virus Is a Zinc-Containing Enzyme

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NS3 proteinase of hepatitis C virus (HCV), contained within the N-terminal domain of the NS3 protein, is a chymotrypsin-like serine proteinase responsible for processing of the nonstructural region of the HCV polyprotein. In this study, we examined the sensitivity of the NS3 proteinase to divalent metal ions, which is unusual behavior for this proteinase class. By using a cell-free coupled transcription-translation system, we found that HCV polyprotein processing can be activated by Zn^{2+} (and, to a lesser degree, by Cd^{2+}, Pb^{2+}, and Co^{2+}) and inhibited by Cu^{2+} and Hg^{2+} ions. Elemental analysis of the purified NS3 proteinase domain revealed the presence of zinc in an equimolar ratio. The zinc content was unchanged in a mutated NS3 proteinase in which active-site residues His-57 and Ser-139 were replaced with Ala, suggesting that the zinc atom is not directly involved in catalysis but rather may have a structural role. Based on data from site-directed mutagenesis combined with zinc content determination, we propose that Cys-97, Cys-99, Cys-145, and His-149 coordinate the structural zinc in the HCV NS3 proteinase. A similar metal binding motif is found in 2A proteinases of enteroviruses and rhinoviruses, suggesting that these 2A proteinases and HCV NS3 proteinase are structurally related.

Hepatitis C virus (HCV) was identified as a major causative agent of posttransfusion and community-acquired non-A, non-B hepatitis throughout the world (see reference 17 for a review). HCV is an enveloped virus with a positive-stranded RNA genome of 9.4 kb which contains a single, large open reading frame (ORF) encoding a precursor polyprotein of about 3,010 amino acids. Based on comparison of deduced amino acid sequences and the extensive similarity in the 5′ untranslated region, HCV has been classified as a separate genus of the family *Flaviviridae*, distantly related to flaviviruses and pestiviruses (7, 26). As was determined by transient expression of cloned HCV cDNAs (11, 15), the precursor polyprotein is cotranslationally and posttranslationally processed into at least 10 viral structural and nonstructural proteins by the action of a host signal peptidase and by two distinct viral proteinase activities (Fig. 1). A novel Zn^{2+}-dependent activity (NS2-3 proteinase) appears to mediate autocatalytic cleavage at the NS2/3 site (10, 16). NS3 proteinase, located in the N-terminal one-third of the 70-kDa NS3 protein (the remaining two-thirds of NS3 encompasses a helicase domain) catalyzes cleavage at four downstream sites in the nonstructural region (5, 11, 15, 35).

The His-57, Asp-81, and Ser-139 residues of the NS3 proteinase are conserved among all sequenced HCV strains and have been proposed to constitute the characteristic serine proteinase catalytic triad, as in the NS3 protein of flaviviruses and pestiviruses (3, 8). That these three residues are essential for HCV NS3 proteinase activity was confirmed by site-directed mutagenesis (16). Based on inhibition studies using series of class-specific protease inhibitors, the NS3 proteinase has been classified as a chymotrypsin-like serine proteinase (12).

Recently, it was shown that NS3 proteinase requires another virus-encoded protein, NS4A, to cleave efficiently at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions (1, 6, 20, 34). In addition to this requirement for a protein cofactor, there were reports of NS3 proteinase sensitivity to divalent metal ions, behavior that is not expected for a chymotrypsin-like serine protease. These sometimes contradictory reports mention, e.g., mild activation by Zn^{2+} and inhibition by Cu^{2+} (13); a requirement for Mg^{2+} (4); inhibition by Zn^{2+}, Ni^{2+} and several chelators, such as EDTA and 1,10-phenantroline (27); and mild inhibition by EDTA (21). In this study, we explored the metal sensitivity of the NS3 proteinase activity in more detail. By using a cell-free transcription-translation system and several forms of purified recombinant protein, we have established that the NS3 proteinase domain of HCV contains a zinc atom which appears to have a structural role.

MATERIALS AND METHODS

Expression constructs. ORFs encoding the NS3-4A-4B, NS4A-4B, and NS3 proteins were amplified by PCR from the plasmid template pBRTM/HCV1-3011 (11), which contains the entire HCV H strain ORF. A methionine codon present in the *Nde*I site was designed into the PCR primers to immediately precede the first codon of each ORF. The *Nde*I-*Eco*RI fragments were inserted into multicopy plasmid pGZ (25). A gene encoding the NS3 protease domain (amino acids 1 to 181) of the HCV J strain designed for expression in *Escherichia coli* was assembled from synthetic oligonucleotides in the pGZ vector. The nucleotide sequence of the gene was modified to reflect the codon usage for *E. coli* and to introduce several unique restriction sites (19a). Standard techniques were used for recombinant DNA manipulations. Splice overlap extension PCR (39) was used to introduce defined mutations into the nucleotide sequence. Each mutation was verified by DNA sequencing.

Cell-free transcription and translation. Cell-free transcription and translation of the HCV H sequences were performed in the TnT T7 Coupled Reticulocyte Lysate System (Promega) by using circular plasmid DNA templates in accordance with the manufacturer's instructions. No viral sequences, such as the 5′ nontranslated region of encephalomyocarditis virus, were present on the plasmid templates; rather, a consensus bacterial ribosomal binding site of the pGZ vector was used to direct translation in this system. The translation products were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the ^{35}S-labeled proteins were visualized by using a PhosphorImager (Molecular Dynamics) with ImageQuant software.

Protein preparation. Protein expression, purification, and enzymatic characterization will be described in detail elsewhere. Briefly, a pGZ plasmid construct encoding amino acids 1 to 181 of the HCV J strain NS3 protein was expressed in *E. coli* BL 21 (DE3) grown in a complex medium (2xYT) at 28°C in a 30-liter fermentor. After the presence of zinc was established in the first NS3 proteinase preparations, the 2xYT medium was routinely supplemented with 100 μM zinc acetate in the subsequent fermentation runs. A soluble cytoplasmic portion of

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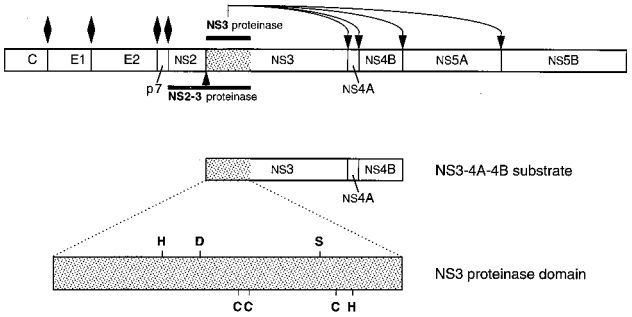

FIG. 1. HCV polyprotein, NS3-4A-4B polyprotein substrate, and NS3 proteinase domain. The full-length precursor polyprotein encoded by the HCV ORF is schematically depicted at the top, and the names of 10 cleavage products are included. Cleavage sites of a host signal peptidase are indicated by solid diamonds. The extent of the NS2-3 proteinase region is shown below the polyprotein; a single NS2-3 proteinase cleavage site is indicated by a short arrow. The NS3 proteinase region is shown above the polyprotein, and NS3 proteinase cleavage sites are indicated by curved arrows. The NS3 proteinase domain is shaded. Residues (in the single-letter amino acid code) of the presumed serine proteinase catalytic triad (His-57, Asp-81, and Ser-139) are above the bar, and residues of the putative zinc binding site proposed in this report (Cys-97, Cys-99, Cys-145, and His-149) are below the bar. The residue numbers are in the NS3 proteinase numbering, which can be converted to the HCV J strain polyprotein numbering by adding 1,026.

the induced *E. coli* cell paste was subjected to chromatography on Fast Flow SP Sepharose, FPLC Mono S, and Sepharose S-200. The purified protein was stored in 50 mM sodium acetate buffer (pH 6.0)–10 mM dithiothreitol–350 mM sodium chloride at −70°C until used for analysis. The protein concentration was determined with Pierce Coomassie Assay reagent by using serum albumin as the standard. Modification of the HCV NS3 proteinase with $HgCl_2$ was carried out in 20 mM morpholineethanesulfonic acid (MES) buffer, pH 6.0, for 30 min at 4°C in the presence of a twofold excess of $HgCl_2$ over protein. The unreacted $HgCl_2$ was removed on a Pierce Desalting Column in 20 mM MES, pH 6.0. For metal analysis, 0.4 to 1 mg of pure protein was treated with 5 mg of Chelex 100 resin (Bio-Rad), a divalent metal chelating resin, by mixing in suspension for 1 h at 4°C. Following centrifugation, the supernatant was lyophilized to dryness.

Determination of metal content. Analysis of metal content in HCV NS3 proteinase samples was performed by Elemental Research, Inc., North Vancouver, British Columbia, Canada. The protein sample was subjected to metal analysis by inductive coupled mass spectroscopy (ICPMS). The metal content was reported in nanograms per milliliter of sample. By using the calculated molecular mass of the protein (based on the primary amino acid sequence), the mass of the metal, and the protein concentration, the moles of metal per mole of protein were calculated.

RESULTS

Effect of divalent metal ions on autoprocessing of HCV polyprotein. HCV polyprotein fragment NS3-4A-4B (Fig. 1) was used as model substrate to explore the extent of autoprocessing by the NS3 proteinase activity. This substrate contains the NS3-NS4A cleavage site, believed to be processed in *cis*, and the NS4A-4B site processed in *trans* (2). The polyprotein was synthesized in a coupled transcription-translation system containing rabbit reticulocyte lysate and phage T7 RNA polymerase, from a plasmid encoding the NS3-4A-4B fragment. Different metal ions were added to the reaction mixture, and their effects on processing were monitored by separation of products by SDS-PAGE. Of the 12 divalent metal ions tested at a 100 μM final concentration as the respective chloride salts, Zn^{2+} was the most efficient at stimulating the basal level of polyprotein processing. Less efficient but still detectable stimulation was observed with Cd^{2+}, Pb^{2+}, and Co^{2+} (Fig. 2). Monitoring of the effect of increasing concentrations of Zn^{2+} on polyprotein processing showed a 50% stimulatory concentration of ~20 μM (Fig. 3). Expression of the NS3-4A-4B polyprotein in the absence of Zn^{2+}, followed by Zn^{2+} addition and further incubation for up to 4 h, showed no activation of polyprotein processing (data not shown). This suggests that Zn^{2+} needs to be present during protein folding to activate

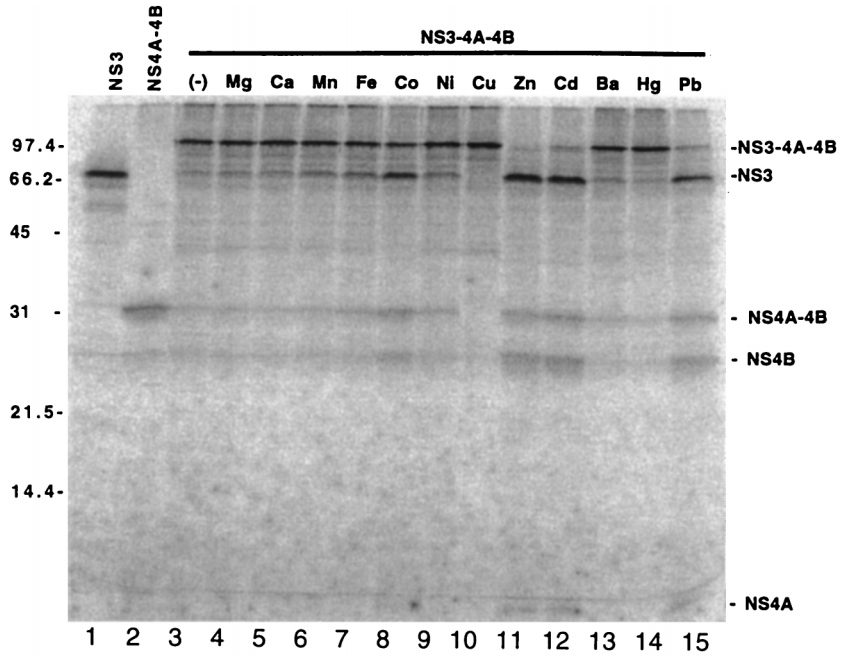

FIG. 2. Effects of divalent metal ions on autoprocessing of the NS3-4A-4B polyprotein substrate. Plasmids directing expression of the NS3 protein (lane 1) and the NS4A-4B protein (lane 2) in the coupled transcription-translation reactions were used as controls to indicate positions of an expected product and intermediate of NS3-4A-4B autoprocessing. The reactions expressing the NS3-4A-4B substrate were performed in the presence of various metal chlorides (indicated above) at a 100 μM final concentration. After 3 h of incubation at 30°C, the ^{35}S-labeled translation products were separated by SDS–12% PAGE. Molecular weights (in thousands) are shown on the left, and positions of the substrate, processing intermediate, and products are shown on the right.

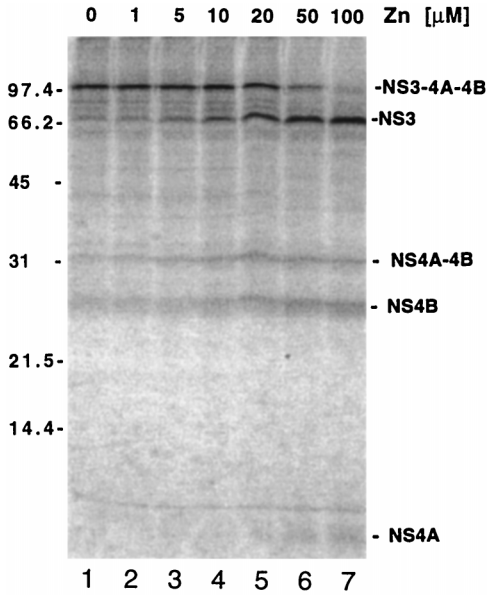

FIG. 3. Effect of increasing concentrations of $ZnCl_2$ on the extent of NS3-4A-4B substrate autoprocessing. The final Zn^{2+} concentrations in the reaction mixtures are shown at the top. The other experimental conditions for the coupled transcription-translation reactions and the lane designations are the same as in Fig. 2.

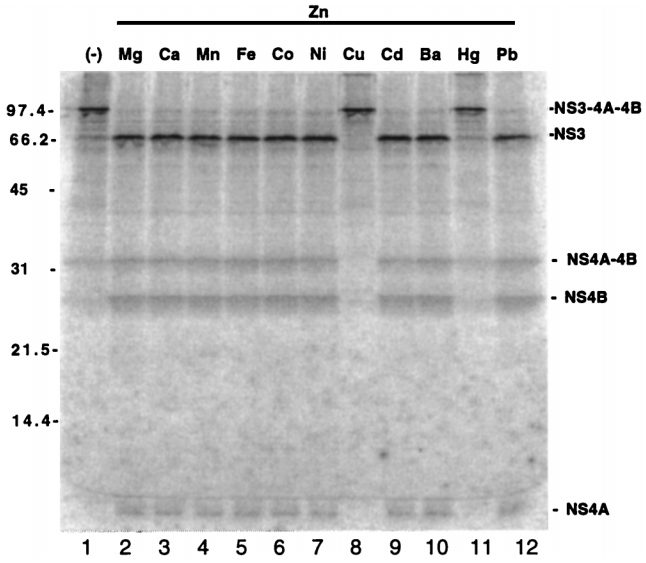

FIG. 4. Effects of divalent metal ions on Zn^{2+}-activated autoprocessing of the NS3-4A-4B substrate. Plasmid-directed expression of the NS3-4A-4B substrate was performed in the presence of 100 μM $ZnCl_2$ and different metal chlorides at 100 μM as indicated at the top. Lane 1 contained a control reaction to which no metals were added. The other experimental conditions for the coupled transcription-translation reaction and the lane designations are the same as in Fig. 2.

NS3 proteinase and has no effect when added posttranslationally. Elemental analysis of the transcription-translation reaction mixture before metal addition indicated that there is approximately a 40 μM concentration of total zinc. However, this analysis cannot distinguish how much of that zinc is in a free form and how much is protein bound in components of the rabbit reticulocyte lysate. The concentration of available Zn^{2+} in the reaction mixture is apparently not sufficient for complete activation of the NS3 proteinase but may explain the basal level of polyprotein processing (e.g., Fig. 2, lane 3).

Inhibition of NS3-4A-4B polyprotein processing by Cu^{2+} and Hg^{2+} was clearly visible in the reaction mixture supplemented with 100 μM Zn^{2+} (Fig. 4). Further characterization of this effect in experiments with various concentrations of inhibitory metals showed the approximate 50% inhibitory concentrations in this system to be ~7 μM for Cu^{2+} and ~20 μM for Hg^{2+} (data not shown). These results confirm and extend previous observations that HCV polyprotein processing by NS3 proteinase can be influenced by divalent metal ions and suggest that Zn^{2+} is an activator, while Cu^{2+} and Hg^{2+} are potent inhibitors, of this enzyme.

Elemental analysis of the purified NS3 proteinase domain. To explore the possibility that NS3 proteinase contains a metal cofactor, the purified recombinant NS3 proteinase domain was subjected to ICPMS and analyzed for the presence of 70 elements. Of the metals, only zinc had a significant presence in the protein samples. There were only traces of calcium and iron (0.17 and 0.04 mol/mol of protein, respectively) and no significant presence of Cd, Pb, Co, Mg, Mn, Cu, or any other metal. The zinc atom was apparently taken up in the course of NS3 proteinase synthesis and folding in the bacteria, as no additional zinc ions were added to the sample during or after protein purification. As seen in Table 1, the wild-type form of the NS3 proteinase domain contains about 1.05 mol of zinc per mol of protein. The active-site double mutant, which has two presumed active-site residues, Ser-139 and His-57, changed to alanine (H57A, S139A) contains about 1 mol of zinc per mol of protein, suggesting that zinc ion is not bound at the active site. The results of this analysis, as summarized in Table 1, indicate that the NS3 proteinase domain contains one zinc atom per protein molecule.

Zinc binding site. The sulfhydryl group of cysteine and the imidazolyl group of histidine are the most common ligands in structural zinc binding sites, whereas acidic side chains are more frequent ligands at catalytic zinc sites (36). In our search for possible zinc binding residues in the NS3 proteinase sequence, we therefore focused on cysteine and histidine residues. There are seven cysteines in the NS3 proteinase domain of the HCV J, BK, and H strains, four of which are conserved in all known HCV sequences. Mutation analysis by Hijikata et al. (16) suggested that, in contrast to Cys-16, Cys-47, Cys-52, and Cys-159, which appear to be dispensable, Cys-97, Cys-99, and Cys-145 are critical for efficient polyprotein processing. The latter three residues thus became prime candidates for involvement in zinc coordination. The zinc contents of the NS3 proteases with mutations in dispensable cysteines (C159S and

TABLE 1. Zinc content[a] in the wild type and several mutated forms of the purified NS3 proteinase domain of HCV

Mutation(s)[b]	Zn/enzyme molar ratio
None (wild type)	1.05[c]
H57A, S139A	1.02
C159S	0.86
C16A, C47S, C52L, C159S	1.04
C16A, C47S, C52L, C159S/$HgCl_2$	0.16[d]
H149A	ND[e]

[a] Zinc content was determined as described in Materials and Methods.
[b] The single-letter amino acid code is used to describe mutations introduced into the NS3 proteinase domain.
[c] Average of 14 measurements for different protein preparations.
[d] Zinc content determined in the quadruple mutant covalently modified with $HgCl_2$.
[e] ND, not detectable; see text for details.

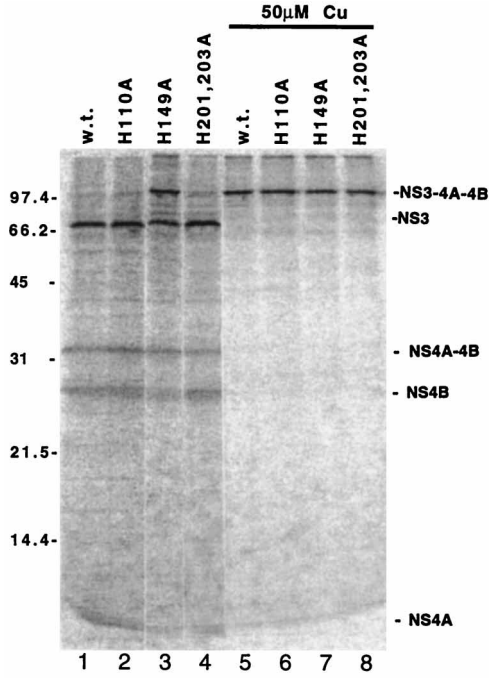

FIG. 5. Effect of His substitutions in the N-terminal region of NS3 on autoprocessing of the NS3-4A-4B substrate. His-to-Ala substitutions were introduced into the plasmid directing expression of the NS3-4A-4B substrate at the positions indicated at the top. $ZnCl_2$ (100 μM) was present in all of the reaction mixtures, and 50 μM $CuCl_2$ was added to the reaction mixtures in lanes 5 to 8. The other experimental conditions for the coupled transcription-translation reaction and the lane designations are the same as in Fig. 2. w.t., wild type.

		97 99		139	145 149
HCV-J	(NS3)	SMTP**C**T**C**GSSD	.....(35 aa).....	*S*GGPLL**C**PSG**H**V	
HGV	(NS3)	SLTP**C**T**C**QAES	.....(34 aa).....	*S*GSPVL**C**DEG**H**A	
GBV-A	(NS3)	CLQA**C**K**C**QPTG	.....(33 aa).....	*S*GSPIL**C**DEG**H**A	
GBV-B	(NS3)	SLTR**C**S**C**GETK	.....(35 aa).....	*S*GAPIL**C**SSG**H**V	
HRV-2	(2A)	YIPS**C**D**C**TQAT	.....(47 aa).....	*C*GGKLL**C**--K**H**G	
PV-1	(2A)	SIAR**C**N**C**NAGV	.....(47 aa).....	*C*GGILR**C**--H**H**G	
		55 57		109	115 117

FIG. 6. Alignment of selected NS3 and 2A proteinases. A representative amino acid (aa) sequence was chosen for each genus of the *Flaviviridae* and *Picornaviridae* families that contains the presumed zinc binding motif in the respective NS3 and 2A proteinases. The GenBank accession or reference numbers of the sequences shown are as follows: HCV-J, D90208; hepatitis C virus strain J, reference 18; HGV, U44402; hepatitis G virus, reference 22; GBV-A, U22303; GBV-B, U22304; GB viruses A and B, reference 31; HRV-2, X02316; human rhinovirus type 2, reference 32; PV-1, P03399; poliovirus type 1, reference 19. Residues presumably involved in zinc coordination are in boldface type. The active-site nucleophiles (Ser for NS3 proteinases and Cys for 2A proteinases) are italicized and underlined. Residues are numbered according to the amino acid sequence of the HCV-J NS3 proteinase (top), or the 2A proteinase of the Mahoney strain of type 1 poliovirus (bottom). The HRV-2 and PV-1 sequences were taken from the alignment of 2A proteinases of rhinoviruses and enteroviruses (38).

the C16A, C47S, C52L, C159S quadruple mutant) were equivalent to that of the wild type (Table 1), suggesting that none of these four residues participates in zinc binding.

Because we expected four-coordinate tetrahedral geometry around the zinc ion and only three candidate cysteine ligands were identified, we considered a histidine residue as a possible fourth ligand. The sequence of the core domain of the NS3 proteinase (residues 1 to 181) contains three histidine residues, His-57, His-110, and His-149. Of these, His-57 was confirmed by mutational analysis to be essential for NS3 proteinase activity (9, 16), consistent with its being a member of the serine proteinase catalytic triad. As described above, the wild-type zinc content of the purified H57A, S139A double mutant form of the NS3 proteinase domain (Table 1) argues against involvement of His-57 in zinc coordination and against a catalytic role for the zinc ion. To explore the roles of the remaining His residues (His-110 and His-149) in polyprotein processing that would suggest zinc binding, His-to-Ala substitutions were introduced into the NS3-4A-4B polyprotein substrate. In addition, His-201 and His-203, which lie outside the amino acid sequence of the minimal NS3 proteinase, were also included in this experiment because of similarity to a possible HXH metal binding motif and their immediate proximity to the NS3 proteinase core domain. As is apparent from Fig. 5, the H110A mutant and the H201A, H203A double mutant show the wild-type level of polyprotein processing, while the processing of the substrate carrying the H149A mutation is reduced. The possibility that His-149 has a role in zinc binding was further explored by analysis of the purified recombinant protein. Expression in *E. coli* of the NS3 proteinase domain carrying the H149A mutation resulted in accumulation of practically all protein in the insoluble fraction. No zinc was detected in this material after it was solubilized and subjected to ICPMS metal analysis (Table 1), suggesting that absence of zinc correlates with improper protein folding. Interestingly, a small amount, representing less than 1% of the total expressed NS3 H149A proteinase, could be recovered from the soluble fraction of the bacterial lysate and, based on preliminary analysis, this soluble protein has a wild-type zinc content. Impaired protein folding can thus explain the incomplete processing of the NS3-4A-4B polyprotein substrate carrying the H149A mutation observed in the reticulocyte lysate. This phenotype is consistent with the possibility that His-149 is a fourth residue of the proposed Zn binding site, although its contribution to the metal coordination appears to be less important than that of any of the three cysteine residues (Cys-97, Cys-99, and Cys-145) (16).

The published data on mutagenesis of Cys residues and our histidine mutagenesis data, combined with zinc content determination in selected mutated forms of the purified domain, lead us to propose that Cys-97, Cys-99, Cys-145, and His-149 constitute a structurally important zinc binding site in the HCV NS3 proteinase.

Sequence comparison of HCV NS3 proteinase with proteinases of other RNA viruses. Mutational analysis of Cys and His residues in the poliovirus 2A proteinase revealed that in addition to His-20 and Cys-109, presumed members of the catalytic triad, there are four other Cys and His residues (Cys-55, Cys-57, Cys-115, and His-117) whose alteration eliminates enzymatic activity (38). The four latter residues form a CXC. . .CXH motif (X = any amino acid) that is conserved among 2A proteinases of known enteroviruses and rhinoviruses and was suggested to maintain the active conformation of the 2A proteinase structure and implies the binding of a metal ion, such as Zn^{2+} (38). Later, it was demonstrated that the purified rhinovirus 2A proteinase, indeed, contains a zinc atom that is required for the correct folding and stability of an active enzyme (33, 37), although binding of the zinc atom by the CXC. . .CXH motif was not directly demonstrated.

As seen in Fig. 6, the motif CXC. . .CXXXH, which we propose to be the HCV NS3 proteinase zinc binding site, is quite similar to the proposed zinc binding motif in enteroviruses and rhinoviruses. The first half of the motif, CXC, is commonly found in multiple repeats in metallothioneins, a class of proteins that contain several tetrahedrally bound Zn and Cd ions (30). The Cys residue in the second half of the

motif has the same position with respect to the presumed active-site nucleophile (Ser or Cys) in all of the aligned sequences, suggesting the same three-dimensional arrangement and possibly a common evolutionary origin of the NS3 and 2A proteinases.

The similarity of the conserved CXC. . .CXH motif in the zinc containing rhinovirus 2A proteinase to the CXC. . .C XXXH motif in the HCV NS3 proteinase strengthens our suggestion that Cys-97, Cys-99, Cys-145, and His-149 represent a zinc binding site in HCV NS3 proteinase. The same CXC . . .CXXXH motif can also be found in the recently identified GB viruses GBV-A and GBV-B (31) and the GBV-C/hepatitis G virus (22) (Fig. 6), each of which constitutes a new genus of *Flaviviridae* closely related to HCV (28, 31). Interestingly, no similar Zn binding motif is present in the NS3 proteinase domains of other members of the *Flaviviridae* family, such as in yellow fever virus, dengue virus, and tick-borne encephalitis virus of the classic flaviviruses, nor in bovine viral diarrhea virus and hog cholera virus of animal pestiviruses.

DISCUSSION

In this study, a cell-free coupled transcription-translation system was used to examine the effects of several divalent metal ions on the autocatalytic processing of a polyprotein substrate. The effects ranged from significant activation (by Zn) to strong inhibition (by Cu). Analysis of the metal content of the purified NS3 proteinase domain indicated the presence of a single zinc atom per molecule of enzyme which, in turn, implied the existence of a metal binding site. We propose that this site is formed by Cys-97, Cys-99, Cys-145, and His-149, based on results from site-directed mutagenesis and sequence comparisons with 2A proteinases. The notion that Cys-97, Cys-99, Cys-145, and His-149 constitute a Zn binding site is further supported by a two-beta-barrel trypsin-like homology model of the NS3 proteinase domain (22a) based on the rhinovirus 3C proteinase structure (24). In this model, the Cys-97, Cys-99, Cys-145, and His-149 residues cluster close together but distant from the presumed active site, which is consistent with a structural rather than a catalytic role for the bound zinc.

The existence of a metal binding site, which presumably coordinates a single zinc atom under physiological conditions, may explain most, if not all, of the observed effects of various divalent metal ions in vitro. Depending on their affinities and atomic properties, they may bind at the zinc site in either a productive way, resulting in activation, or in a nonproductive way, resulting in inhibition. In the protein treated with $HgCl_2$, free sulfhydryl groups of Cys residues are covalently modified by mercury, which prevents them from zinc atom coordination (Table 1). The observed inhibition by Hg ions would then be explained by elimination of ligands, resulting in an inability to form a zinc site. Inhibition by Cu^{2+} may have a similar explanation. It may also be that Cu replaces Zn while maintaining coordination of Zn ligands but without optimum geometry.

However, since Cu^{2+} remains the most potent inhibitor of HCV NS3 proteinase reported thus far, its effect deserves further consideration. NS3 proteinase activity is completely inhibited by Cu^{2+} at low micromolar concentrations which, in addition to interference with a structural site, may also invoke direct interaction with the active-site residues. Interestingly, a single mutation was described in trypsin that made this prototype serine protease susceptible to Cu^{2+} inhibition (14). In that study, the Arg-96 to His substitution was introduced into the recombinant rat trypsin, which resulted in the placement of a new imidazole group on the surface of the enzyme near the essential active site, His-57 (coincidentally, the His residue of the presumed catalytic triad of the HCV NS3 proteinase has the same number). The spatial orientation of these two His side chains enables formation of a stable metal-binding site that chelates divalent first-row transition metal ions. The presence of Cu^{2+} at this site prevents the imidazole group of His-57 from participating as a general base in catalysis. The Cu^{2+} inhibition of the R96H trypsin is reversible by EDTA.

It is not clear whether the observed inhibition of the NS3 proteinase by Cu^{2+} acts through a similar mechanism that would involve active site His-57 and some other naturally occurring copper ligand, e.g., another histidine. The complete inhibition of processing by Cu^{2+}, even in the presence of a large molecular excess of Zn^{2+} (data not shown), may suggest participation of active site His-57. However, none of the other His residues present in the NS3 proteinase domain seems to be critically involved in Cu^{2+} binding, as both the H110A and H149A mutants (and also the H201A, H203A double mutant) are still susceptible to inhibition by Cu^{2+} (Fig. 5). Binding of Cu^{2+} away from the catalytic residues, most probably at the Zn binding site, and exertion of its allosteric inhibitory effect by disruption of structural features in the NS3 proteinase normally stabilized by Zn^{2+} thus remains the most likely explanation for the Cu^{2+} inhibition.

Our finding that the NS3 proteinase domain contains a zinc atom should be related to the observation by Hijikata et al. (16) that NS2-3 proteinase, a second virus-encoded activity that is responsible for autocatalytic cleavage at the NS2-NS3 site, is stimulated by $ZnCl_2$ and inhibited by EDTA, a chelator of divalent metal ions. This observation led the investigators to propose that NS2-3 is a novel zinc-dependent metalloproteinase. However, as emphasized also by Reed et al. (29), these results are not sufficient evidence for classification of NS2-3 as a metalloproteinase, since the observed stimulation of proteinase activity by $ZnCl_2$ and inhibition by EDTA could indicate a structural rather than a catalytic role for zinc (similar to our proposal for the role of zinc in the NS3 proteinase domain).

The NS2-3 proteinase was mapped to a region encompassing 129 amino acids of NS2 and the whole NS3 proteinase domain (10, 16). It is not clear whether NS2 has a different zinc binding site or whether the zinc atom present in the NS3 domain is identical to a zinc required for NS2-3 activity. In their mutagenesis study, Hijikata et al. (16) noted that the C97A, C99A, and C145A mutations in the NS3 domain, which are the residues we propose to constitute a zinc binding site, reduce both NS2-3 and NS3 proteinase activities, which is consistent with a single zinc. As NS2-3 is both an enzyme and a substrate in the autocatalytic cleavage at the NS2-3 site, the zinc atom within the NS3 proteinase may have a structural role in stabilizing the NS3 domain so that it can be efficiently recognized as a substrate by NS2-3 activity. On the other hand, the Cys-993 and His-952 (polyprotein numbering) residues present in the NS2 protein were suggested to coordinate a zinc atom important for NS2-3 proteinase activity (16). If confirmed, it would mean that there is a separate zinc binding site in the NS2 protein.

While this report was being prepared, a refined crystal structure of the HCV NS3 proteinase domain became available (23). The structure supports the main conclusions of this report, namely, the presence of a structural zinc atom coordinated by Cys-97, Cys-99, and Cys-145. The fourth ligand is a water molecule which is hydrogen bonded to His-149. Although His-149 appears not to play a direct chelation role in this crystal form, it is positioned to readily coordinate the metal as a substitute for the water ligand. A reduced level of polyprotein processing seen with the H149A mutant (Fig. 5, lane 3), which is less dramatic than the effects of any of the C97A, C99A, and C145A mutations (16), is thus consistent

with the possibility that His-149 is an integral part of zinc coordination only during initial folding.

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