Hepatitis C Virus Polyprotein Processing: Kinetics and Mutagenic Analysis of Serine Proteinase-Dependent Cleavage

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Hepatitis C virus (HCV) serine proteinase (Cpro-2) is responsible for the processing of HCV nonstructural (NS) protein processing. To clarify the mechanism of Cpro-2-dependent processing, pulse-chase and mutation analyses were performed by using a transient protein production system in cultured cells. Pulse-chase study revealed the sequential production of HCV-NS proteins. Production of p70(NS3) and p66(NS5B) were rapid. An 89-kDa processing intermediate protein (p89) was observed during the early part of the chase. p89 seemed to be cleaved first into a 31-kDa protein (p31) and a p58/56(NS5A). p31 was further processed into p4(NS4A) and p27(NS4B). Mutation analysis of cleavage sites of NS4A/4B, NS4B/5A, and NS5A/5B revealed that cleavage at each site is essentially independent from cleavage occurring at the other site.

The positive-stranded RNA genome of hepatitis C virus (HCV) (3, 12) contains a single long open reading frame encoding a polyprotein of 3,010, 3,011, or 3,033 amino acid residues (3, 4, 10, 15, 16, 18, 19) (Fig. 1). HCV proteins are considered to be produced from the precursor polyprotein by proteolytic processing conducted by both host and viral proteinases (2, 5-9, 13, 14, 21). The order of individual proteins in the polyprotein of HCV 1b (type II), the major HCV subtype in Japan (10), is reported to be NH2-p22(C)-gp35(E1)-gp70 (E2)-p21(NS2)-p70(NS3)-p4(NS4A)-p27(NS4B)-p2(NS5C)-p58/p56(NS5A)-p66(NS5B)-COOH, where C, E, and NS are the putative viral core, envelope, and nonstructural protein, respectively (9). Processing of the region downstream of p70(NS3) in the polyprotein appears to be dependent on the viral serine proteinase, Cpro-2, located between residues 1049 and 1215 of the HCV precursor polyprotein (20).

From determination of the N-terminal amino acid sequence of the Cpro-2-dependent processed viral products, a cysteine residue is suggested to be located at the P1 position of the putative cleavage sites except for the p70(NS3)/p4(NS4A) (abbreviated to 3/4A) junction, where a threonine residue is positioned (6, 11). Two different cleavage modes, intramolecular (cis) and intermolecular (trans) proteolytic cleavages, are proposed to be employed in the Cpro-2-dependent polyprotein processing (20, 21). The 3/4A junction is processed only in a cis cleavage manner. The cleavage at the amino (N) terminus of p58(NS5C+NS5A) is also suggested to occur in a cis-acting manner, because this product is not produced under the conditions of trans cleavage reaction (20). Furthermore, efficient cleavage at p4(NS4A)/p27(NS4B) (4A/4B) is shown to require the presence of the downstream p58/p56(NS5A) region of the polyprotein (9). This result suggests that proteolytic cleavage at these processing sites may be strictly regulated. Partially or completely processed nonstructural proteins may associate with other nonstructural protein components (9) and thereby influence proteolytic processing at various steps in the pathway. A pulse-chase experiment was conducted to determine the order of nonstructural protein production. Furthermore, the effect of mutation on the processing pathway was also analyzed.

Pulse-chase analysis of Cpro-2-dependent HCV polyprotein processing. To study the kinetics of Cpro-2-dependent HCV polyprotein processing, COS I cells transfected with pCMV/N729-3010, which encodes the entire HCV polyprotein nonstructural protein region (9) (Fig. 1), were used in pulse-chase analysis. The transfectant was pulse-labeled for 15 min with [35S]methionine and chased for various times after addition of excess amounts of cold methionine. The cell lysates at chase times of 0, 20, 60, 180, and 360 min were immunoprecipitated with α-p70, α-NS4, α-p27, α-NS5a, and α-p66 (Fig. 2). Production of p70(NS3) in the lysate was detected by α-p70 at 0 min of the chase period, and no precursor protein reactive with α-p70 was detected. The relative abundance of p70(NS3) was almost constant during the 360-min chase, indicating that the processing of p70(NS3) from the precursor polyprotein was almost completed within the 15-min pulse period and that p70(NS3) was stable in the cell (Fig. 2A). Although the production of p66(NS5B) was detected by α-p66 at the start of the chase, a small amount of precursor polypeptide of p66(NS5B) was detected, suggesting that processing to p66(NS5B) was not completed by the start of the chase (Fig. 2E, lane 3). However, after 20 min of chase, only an increased

FIG. 1. Schematic representation of HCV polyprotein structure and oligonucleotides used to introduce mutations. The structure of the nonstructural region from p21(NS2) to p66(NS5B) is shown enlarged below the HCV open reading frame with the scale for the amino acid position. Polypeptides encoded in pCMV/N729-3010 and its mutant pCMV/N729-3010M1 are indicated by bars. The position of the Ser to Ala mutation in N729-3010M1 is depicted by X. The sequence of the cDNA of pCMV/N729-3010 has been deposited in the DDBJ/EMBL/GenBank DNA databases under accession number D16435.
FIG. 2. Pulse-chase analysis of processing of viral precursor polyprotein in the lysate transfected with pCMV/N729-3010. Cells were pulse-labeled for 15 min in the medium supplemented with [35S]methionine (ICN) and then chased as indicated. The lysate at each time point was immunoprecipitated with α-p70 (panel A), α-NS4 (panel B and B'), α-p27 (panel C), α-NS5a (panel D), and α-p66 (panel E). Immunoprecipitation was performed with lysates transfected with pKS(+)/CMV (8), an original plasmid without HCV insert (lane 1); pCMV/N729-3010M1, a Cpro-2-defective mutant (lane 2); and pCMV/N729-3010 (lanes 3 to 7). Gel conditions were sodium dodecyl sulfate–8% polyacrylamide for panels A, B, C, D, and E and tricine–sodium dodecyl sulfate–16% polyacrylamide (17) for panel B'. The gels were dried and were exposed to imaging plates (1). Molecular mass markers (in kilodaltons) are shown on the left. The positions of processing products p70(NS3), p4(NS4A), p27(NS4B), p58/p56(NS5A), and p66(NS5B) and precursor polyproteins p31(p4-p27), p89(p4-p27-p58/p56), p125(p58/p56-p66), and p150(p4-p27-p58/p56-p66) are shown on the right with arrows. The α-NS4 and α-NS5a were generous gifts from A. Takamizawa (Osaka University, Osaka, Japan).
amount of p66(NS5B) was detected, suggesting that processing of p66(NS5B) was almost completed by this time. On the contrary, productions of p4(NS4A), p27(NS4B), and p58/p56(NS5A), which were detected by α-NS4, α-p27, and α-NS5a, respectively, were low at the start of the chase time, while the 89-kDa protein (named p89) was immunoprecipitated by these three antibodies (Fig. 2B, C, and D, lanes 3 to 7). Because p31 is composed of p27(NS4B) and p4(NS4A) (9), the decrease of the p31 level is related to the increase of p4(NS4A) and p27(NS4B) levels (Fig. 2B' and C). An 85-kDa protein which is expected to be produced when the cleavage between 4A/4B proceeds prior to the cleavage between 4B/5A was not detected in the chase period. Instead, the 31-kDa product of NS4A-NS4B was detected by both α-NS4 and α-p27 (Fig. 2B, B', and C) early in the chase. For the first 60 min of the chase time, p31 was detectable, and it was not present at 180 min. The further processed products, p4(NS4A) and p27(NS4B), accumulated at the late stage of the chase period. These observations suggested that p89 is processed into p4(NS4A), p27(NS4B), and p58/p56(NS5A) through suc-
cessive processing steps. It appears that p89 is processed into p58/p56(NS5A) and p31; p31, in turn, is cleaved into proteins p4(NS4A) and p27(NS4B). However, the existence of 125-kDa faint bands which were detected by both α-NS5a and α-p66 (Fig. 2D and E) suggested an alternative processing pathway in which cleavage at 4B/5A site proceeds prior to the cleavage at 5A/5B site.

**Effects of amino acid substitutions introduced at each putative cleavage site by Cpro-2 on the processing to cleavages of other sites.** To investigate the effect of mutations at each cleavage site on processing of the other sites, amino acid substitutions were introduced into each of the Cpro-2-dependent cleavage sites in the HCV polyprotein encoded by pCMV/N729-3010. Amino acid substitutions were introduced by using PCR as described previously (8). Cleavage at the putative 5A/5B site occurred rapidly in the transient expression system using COS-1 cells as mentioned above. In *Escherichia coli* the amino acid substitutions in the P1 position (Cys) of this site to Ala or Asn are not cleaved by Cpro-2; however, a mutation at P1', Ser to Asn, shows little effect on the cleavage (11). Therefore, we introduced the same amino acid substitutions in the cleavage site of the construct that could be analyzed in a eukaryotic transient expression system (Fig. 3A). When polyproteins NS5A/BP1A and NS5A/BP1'N, encoded in pCMV/NS5A/BP1A and pCMV/NS5A/BP1'N, respectively, were produced in COS-1 cells, production of p66(NS5B) was unexpectedly observed. The cleavage seemed to be as efficient as that of wild-type polyprotein, because there was no detectable amount of precursor polypeptide. This suggests that the substitutions with Ala and Asn at positions P1 and P1', respectively, do not dramatically impair the cleavage at the 5A/5B site (Fig. 3A, lanes 4 and 6). This observation was a contrast to what was seen in an *E. coli* expression system. However, when NS5A/BP1N and NS5A/BNN, whose polyproteins have Asn substitutions at the P1 and the P1 plus P1' positions, respectively, were expressed in COS-1 cells, very little of the p66(NS5B) protein and a significant amount of a possible 125-kDa processing intermediate accumulated (Fig. 3A, lanes 5 and 7). This result indicated that the cleavage at this site was diminished in these mutated polyproteins. More of the 125-kDa protein was found in the pCMV/NS5A/BNN transfectant than in the pCMV/NS5A/BP1N transfectant, which indicated that substitutions of Asn at both P1 and P1' of the putative cleavage site 5A/5B are likely to be more deleterious for Cpro-2 cleavage than a single amino acid substitution at position P1 would be. We further analyzed the effect of mutation at positions P1 and P1' of other sites for Cpro-2 cleavage. Amino acids substitutions to asparagine at both the P1 and P1' positions of the putative 3A/4A, 4A/4B, 4B/5A cleavage sites of the nonstructural precursor polyprotein were independently introduced, and cleavage at each site as well as the effects on the other cleavage sites was analyzed. The level of production of p70(NS3), p4(NS4A), and p27(NS4B) from NS5A/BNN was almost the same as that from N729-3010. Thus, this mutant polyprotein probably does not impair other cleavages at 3A/4A, 4A/4B, and 4B/5A sites (Fig. 3B, C, and E, lanes 7). Since NS3/4NN encoded in pCMV/NS3/4NN produced p70(NS3) and p4(NS4A) (Fig. 3B and E, lanes 4), cleavage of the 3A/4A site was abolished in this mutant polyprotein. However, relative production of p58 to p56 from NS3/4NN was reduced (Fig. 3F, lane 4). NS4A/BNN encoded in pCMV/NS4A/BNN showed cleavages at 3A/4A, 4B/5A, and 5A/5B sites which were as efficient as that of the wild-type polyprotein, while the cleavage efficiency at 4A/4B was apparently reduced (Fig. 3B to G, lanes 5). The accumulation of an uncleaved product of p31 was observed in the lysate of the transformant (Fig. 3C, D, and E, lanes 5).

FIG. 4. Schematic illustration of the possible major cleavage pathway of the polyprotein N729-3010. Putative structural and nonstructural regions of HCV polyproteins are illustrated at the top. Dashed lines represent the putative border between structural and nonstructural proteins. Polyproteins derived from pCMV/N729-3010 are indicated by a box in the second row from top with the name of the primary product. The minimum domain required for Cpro-2 is indicated by shading (20).

The production of p58 from the mutant polypeptide NS4A/BNN was also reduced as seen in NS3/4NN (Fig. 3D, lane 5). Production of p70(NS3), p4(NS4A), and p66(NS5B) from NS4/5NN encoded in pCMV/NS4/5NN suggested that cleavage at 3A/4A, 4A/4B, and 5A/5B occurred efficiently in this polyprotein (Fig. 3E, F, and G, lanes 6). In contrast, NS4/5NN generated an 85-kDa polyprotein that was detected by α-NS5a (Fig. 3F, lane 6). This may imply a reduced efficiency of 4B/5A cleavage. These results suggested that the decrease of cleavage efficiencies at the 4A/4B, 4B/5A, and 5A/5B sites do not impair cleavages of the other sites except that the substitutions of amino acids at 3A/4A and 4A/4B sites reduce the production of p58.

The difference in susceptibility to Cpro-2 of the mutated 5A/5B cleavage sites in COS cells and in *E. coli* remains to be resolved. Cleavage of this site by Cpro-2 expressed in *E. coli* was assayed by using a chimera protein composed of the substrate and a 30-amino-acid HCV sequence containing the 4A/4B junction and dihydroforate reductase in this order from the N terminus (11). Thus it may be possible that conformation of the cleavage site in the chimeric substrate might differ from that used in this work. Alternatively an additional viral protein(s) produced in this expression system might change the biological character of Cpro-2 or the substrate.

In vitro translation-processing analysis of the HCV nonstructural polyprotein shows that p4(NS4A) helps to anchor p70(NS3) on the surface of microsomal membranes (9). Furthermore, this complex is likely to mediate association with p27(NS4B), p58(NS5A), and p66(NS5B) to form a complex structure (9) which may be involved in virus replication. On the basis of these observations, Cpro-2-dependent processing may be located on the membrane. During the cleavage process, the association of various protein precursors with their products in various stages of processing may increase the efficiency of cleavage by Cpro-2 and may in effect regulate viral replication. A possible processing pathway of the HCV nonstructural protein is shown in Fig. 4.
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