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Analysis of N-Terminal Processing of Hepatitis C Virus Nonstructural Protein 2

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We determined the partial amino (N)-terminal amino acid sequence of hepatitis C virus p21(nonstructural protein 2 [NS2]). Cleavage at the p21 (NS2) N terminus depended on the presence of microsomal membranes. The amino-terminal position of p21(NS2) was assigned to amino acid 810 of the hepatitis C virus strain II_J precursor polyprotein. Mutation of the alanine residue at position P1 of the putative cleavage site inhibited membrane-dependent processing. This alteration in processing together with the fact that hydrophobic amino acid residues are clustered upstream of the putative cleavage site suggested the involvement of a signal peptidase(s) in the cleavage. Furthermore, mutation analysis of this possible cleavage site revealed the presence of another microsome membrane-dependent cleavage site upstream of the N terminus of p21 (NS2).

From analysis of the hepatitis C virus (HCV) genome and the encoded viral proteins, HCV is likely to belong to the family *Flaviviridae* (3, 4, 14, 15, 18). In this family, the precursor polyprotein translated from the long open reading frame of the genome undergoes proteolytic processing, resulting in mature viral proteins. At least three different pathways mediate processing of the HCV polyprotein. Cellular proteinases associated with the microsomal membrane fraction probably process the viral structural proteins (8). Virally encoded proteinases appear to process the C-terminal region of the HCV polyprotein (1, 5, 6, 10, 27). To date, about 10 viral proteins are predicted to be cleaved from the precursor polyprotein in virus-infected cells (7, 11, 23, 27). Although many of the cleavage sites have been estimated, some cleavage sites, including the junction between the putative structural and nonstructural regions, still remain to be clarified.

We partially determined the N-terminal amino acid sequence of a fusion protein consisting of the N-terminal portion of p21(nonstructural protein 2 [NS2]) and *Escherichia coli* dihydrofolate reductase (DHFR) (11, 21). From that, we estimated the N-terminal cleavage site of this protein in the HCV precursor polyprotein. We also examined processing of the putative cleavage site by mutation analysis.

Plasmids used in this study were constructed as follows. pHCN722-962 was obtained by deleting the 3′-terminal end of pHCN722-1325 by using exonuclease III digestion as previously described (11). The *Eco*RI-*Bss*HII fragment of pHCN722-962 was replaced by an *Nco*I linker (5′-CAGCCAT GGCTG-3′) after filling in of the cohesive ends of the restriction sites with T4 phage DNA polymerase to construct pHCN729-962. Plasmid pHCN752-962 resulted from replacement of the *Eco*RI-*Nco*I fragment of pHCN729-962 with the *Eco*RI-*Nco*I fragment of PCR product I (see below for generation of PCR products I to V). Deletion of the *Nco*I fragment of pHCN729-962 resulted in pHCN810-962. To yield pHCN752-840, the *Eco*RI-*Hin*dIII fragment of PCR product II was subcloned into the *Eco*RI-*Hin*dIII site of pTZ18U vector. The *Bss*HII-*Hin*dIII fragment of pN340 described previously (8) was replaced with the same fragment from PCR product V to make pHCN340-840. The primer combinations used to make PCR products I to V were as follows: product I, primers 1 and 2; product II, primers 1 and 3; product III, primers 4 and 5; product IV, primers 4 and 6; and product V, primers 3 and 7. The primer sequences were as follows: primer 1, 5′-GGAATTCATCATGGTGGTCCTCAA-3′; primer 2, 5′-GCAAGTGCGCCTCGGCTCTG-3′; primer 3, 5′-CAAGCT TGCGAGGAACACCTT-3′; primer 4, reverse primer; primer 5, 5′-CATTCTATAAGCTCGTGGTG-3′; primer 6, 5′-TCT GGCATAAGCTCGTGGTG-3′; and primer 7, 5′-CTTTTC CTTCTTCTGGGCGGA-3′. Single underlines denote the restriction sites used for subcloning, and double underlines indicate the initiation methionine codons. The *dhfr*-fused construct, pHCN752-840d, was made by inserting the *Hin*dIII fragment of pdhfrFX2SH into the *Hin*dIII sites of pHCN752-840 (11). The *Eco*RI-*Nco*I fragments of PCR products III and IV (see the legend to Fig. 1) were ligated into the *Eco*RI-*Nco*I site of pHCN729-962 after blunt ending of the *Nco*I site with mung bean nuclease, to obtain pHCN729-962 (A809R) and pHCN729-962 (M810R), respectively. Plasmids pHCN340-962 and pHCN340-962 (A809R) were constructed by replacing the *Bss*HII-*Hin*dIII fragment of pHCN340-840 with the *Bss*HII-*Hin*dIII fragments of pHCN729-962 and pHCN729-962 (A809R), respectively. The regions of the HCV genome that were translated from those plasmids are indicated in Fig. 1.

Estimation of the N-terminal region of p21 (NS2). Direct sequencing of the N terminus of p70 (NS3) (12) indicates that the cleavage site between p21 (NS2) and p70 (NS3) is between residues 1026 and 1027 in the HCV-II_J precursor polyprotein. The N-terminal end of p21 (NS2) is estimated to be at residue 800 (7, 10), and microsomal membrane-dependent cleavage occurs at this site in an HCV polypeptide spanning residues 722 to 1019 of the precursor polyprotein (N722-1019) (10).

We wanted to continue to define the microsomal membrane-dependent cleavage site of the p21 (NS2) N terminus. To do so, we constructed several expression plasmids containing deletions of N722-1019 and determined whether processing of their protein products required microsomal membrane-dependent cleavages (Fig. 2A). The first of these plasmids, N729-962, was the N- and C-terminal deletion mutant of

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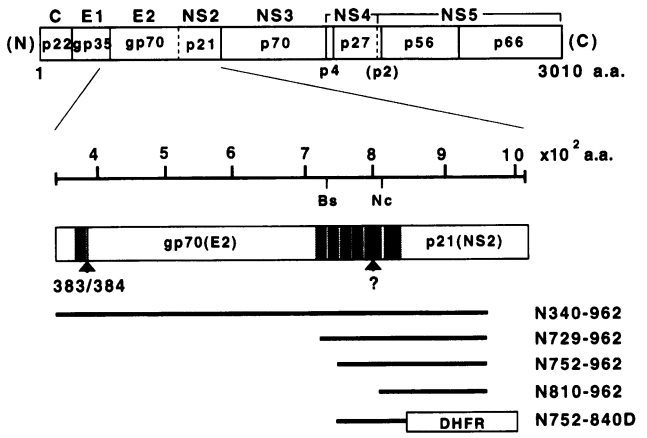

FIG. 1. Map of the HCV polypeptide regions expressed in vitro. The 3,010-aa open reading frame of HCV-II_J and its products are shown in the top panel. The shaded regions are rich in hydrophobic amino residues. Numbering of amino acid residues begins from the initiator methionine of the HCV-II_J precursor polyprotein. At the bottom, the coding region of each plasmid is shown by black bars. The coding regions of the plasmid expressing an HCV fusion protein, fused at the C terminus with *E. coli* DHFR, is also shown. Each polypeptide region is identified at the right; numbers correspond to the amino acid positions of the N and C termini of each region in the HCV-II_J precursor polyprotein.

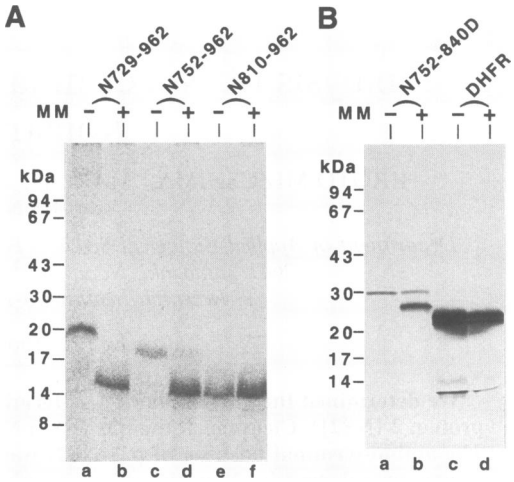

FIG. 2. Microsomal membrane-dependent processing of HCV polypeptides. In vitro translation was performed as described previously (8). The in vitro translation products were separated by tricine–sodium dodecyl sulfate–16% polyacrylamide gel electrophoresis and detected by fluorography (8, 9, 16) (A) or by radioimmunoprecipitation using the antibody against DHFR followed by fluorography (B). (A) Processing of the [^{35}S]methionine-labeled translation products. Translation was carried out in the absence (lanes a, c, and e) or presence (lanes b, d, and f) of the microsomal membrane (MM) fraction. Shown is processing of primary products from aa 729 to 962 (lanes a and b), 752 to 962 (lanes c and d), and 810 to 962 (lanes e and f). (B) Processing of a ^{35}S-labeled fusion protein. A transcript of a plasmid with the HCV DNA sequence covering aa 752 to 840 of the HCV-II_J precursor polyprotein, fused at the 3′ terminus with the *E. coli dhfr* gene, was translated in the absence (−; lane a) or presence (+; lane b) of microsomal membranes. Translation from the *dhfr* gene transcript served as a positive control (lanes c and d).

N722-1019; its size decreased from 19 to 14 kDa in the presence of microsomal membranes (Fig. 2A, lanes a and b). When RNA transcribed from pHCN752-962 was translated in vitro, we detected 17- and 14-kDa proteins in the absence and presence, respectively, of the microsomal membrane fraction (Fig. 2A, lanes c and d). The molecular size of the in vitro-translated polypeptide, N810-962, however, remained 14 kDa in the absence and presence of the microsomal membrane fraction (Fig. 2A, lanes e and f). These observations indicated that the microsomal membrane-dependent cleavage occurred around amino acid (aa) 810. We could not detect a processed N-terminal protein from the primary HCV translation product of pHCN729-962, possibly because of a low methionine content in that region.

To facilitate detection of small cleavage products, we constructed plasmids that encoded a fusion protein of HCV linked to the 5′ end of the *dhfr* gene; DHFR was used as an epitope tag as described previously (11). We designed plasmid pHCN752-840d to contain the cDNA fragment corresponding to aa 752 to 840 of the HCV precursor polyprotein and to be attached to DHFR. In the absence of microsomal membranes, the reaction product detected by radioimmunoprecipitation against DHFR was a 30-kDa protein (Fig. 2B, lane a). The 30-kDa protein was converted to a 25-kDa protein when microsomal membranes were present in the reaction. This result clearly indicated that the HCV polypeptide fused with DHFR was cleaved in a microsomal membrane-dependent manner. From the size differences between the unprocessed and processed proteins, the cleavage site in the fused protein was estimated to be very near the C terminus of the inserted HCV polypeptide as predicted above. The C-terminal 186 amino acid residues of p21 (NS2) were not necessary for cleavage because N752-840D, in which most of p21 (NS2) was deleted, was susceptible to microsomal membrane-dependent cleavage. Clearly, a small region from aa 752 to 840 of the HCV precursor polyprotein contained enough information to act as substrate, because the DHFR molecule itself was not processed by the addition of microsomal membranes in the reaction mixture (Fig. 2B, lanes c and d).

Microsequencing of radiolabeled p25-DHFR fusion protein. Using the Edman degradation procedure described previously (8), we partially determined the N-terminal amino acid sequence of the p25-DHFR fusion protein labeled with [^{35}S]methionine. We detected radioactivity in the degradation products of the first and fifth cycles of the Edman reaction (Fig. 3A). After comparing this result with the putative amino acid sequence of N752-840D, we designated the first methionine residue as residue 810 of the HCV-II_J precursor polyprotein. A comparison of amino acid sequences around the cleavage site of various HCV isolates is shown in Fig. 3B. Amino acid residues Ala-807, Tyr-808, and Ala-809, corresponding to P3, P2, and P1, respectively, of the possible cleavage site, were conserved in all isolates. In all isolates, amino acid sequences corresponding to residues 782 to 804, upstream of the possible cleavage site, were rich in hydrophobic amino acid residues. The characteristics of this region were identical to those of a signal peptide (22, 28–30), suggesting that this microsomal membrane-dependent cleavage was mediated by a signal peptidase(s) in microsomes. The conserved characteristic features of amino acid sequences upstream of this cleavage site implied that all HCV genotypes share the cleavage site identified here.

Mutation analysis of the p21 (NS2) N-terminus cleavage site. Positions P1 and P3 of the cleavage site recognized by a signal peptidase are usually rich in amino acids with noncharged and small side chain moieties, and a polar amino acid residue is often seen at position P2 (22, 28–30). Mutation of an amino acid residue at P1 to a charged amino acid such as glutamic acid or arginine (24) greatly suppresses cleavage by a signal peptidase(s).

To confirm the possibility that the cleavage occurring between residues 809 and 810 of the HCV precursor polyprotein

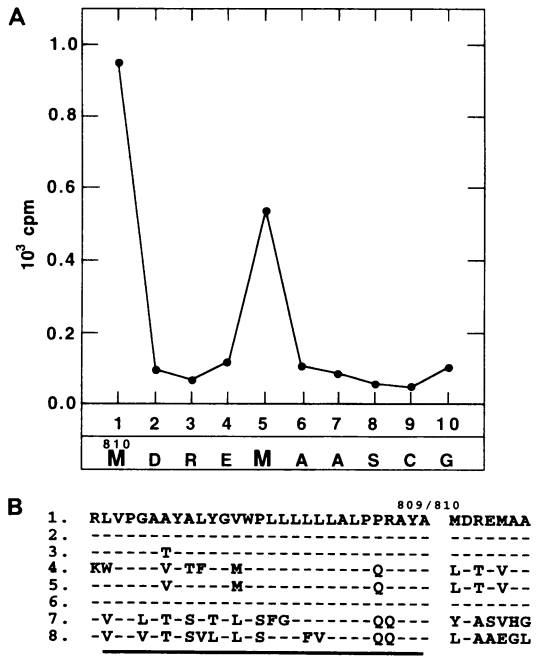

FIG. 3. (A) Microsequencing of radiolabeled N752-840D product processed in a microsomal membrane-dependent manner. Numbers on the abscissa indicate Edman degradation cycles. The HCV-II_J amino acid sequence assigned from the result of microsequencing is indicated below each cycle number. The values of the ordinate indicate uncorrected counts per minute. RNA transcribed in vitro from pHCN752-840d was translated in vitro in the presence of [^{35}S]methionine and microsomal membranes. The microsomal membrane-dependent processing product was used for determination of the N-terminal amino acid sequences after purification by immunoprecipitation using antibody to *E. coli* DHFR as previously described (10). (B) Alignment of amino acid sequences surrounding the possible cleavage site, between aa 809 and 810, of various HCV isolates. The putative signal sequence upstream of the cleavage site is underlined. Dashes indicate the same amino acid residue as in the top line. Amino acid sequences 1 to 8 were taken from references 14, 26, 25, 4, 13, 2, 20, and 19, respectively.

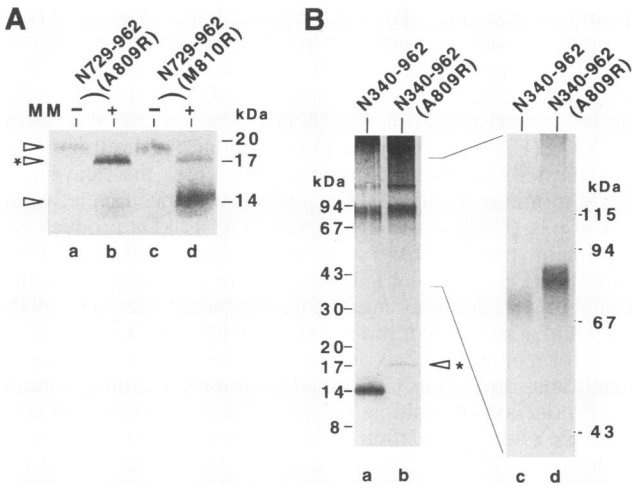

FIG. 4. Mutation analysis of the possible N-terminal cleavage site of p21 (NS2). Transcripts with possible cleavage site mutations of Ala to Arg at position P1 and Met to Arg at position P1′ were translated in the presence or the absence of microsomal membranes (MM). The reaction products were separated on a tricine–sodium dodecyl sulfate–16% polyacrylamide gel and fluorographed. (A) Mutation analysis of the cleavage at the p21 (NS2) N terminus. Transcripts from pHCN729-962 (A809R) (lanes a and b) and pHCN729-962 (M810R) (lanes c and d) were used. The translation reaction was conducted in the presence (+; lanes b and d) or absence (−; lanes a and c) of microsomal membranes. Arrowheads show processed and unprocessed products. (B) In vitro translation reaction products derived from pHCN340-962 (lanes a and c) and pHCN340-962 (A809R) (lanes b and d). In vitro translation was carried out in the presence of microsomal membranes. Better resolution, using a sodium dodecyl sulfate–11% polyacrylamide gel, of the high-molecular-weight proteins observed in lanes a and b is shown in lanes c and d.

was mediated by a signal peptidase(s), we constructed mutant plasmids pHCN729-962 (A809R) and pHCN340-962(A809R), which have mutations of Ala-809 to Arg, and pHCN729-962(M810R), which has a mutation of Met-810 to Arg. We analyzed the microsomal membrane-dependent processing of these mutated polypeptides by in vitro transcription and translation as described above. When the transcripts from pHCN729-962(A809R) and pHCN729-962(M810R) were translated in the absence of microsomal membranes, we detected an unprocessed primary translation product of 19 kDa (Fig. 4A, lanes a and c). In the processed product of pHCN729-962(A809R), we could hardly detect a 14-kDa protein, in contrast to the case for its parent, N729-962 (Fig. 2A, lane b), which produced an abundance of a 14-kDa protein. The translation reaction mixture containing the transcript from pHCN729-962(M810R) produced a 14-kDa product (Fig. 4A, lane d). This result indicated that alanine at position P1 was important for cleavage, but methionine at position P1′ was not.

These aspects of the N-terminal cleavage of p21(NS2) agreed well with characteristics of a signal peptidase(s) as proposed by von Heijne (28–30) and Perlman and Halvorson (22). We concluded that the cleavage of this site in the HCV precursor polyprotein is mediated by a host signal peptidase(s).

Previously we estimated a possible cleavage site of the C terminus of gp70 (E2) to be around aa 740 in the HCV precursor polyprotein; this was based on the deletion analysis of gp70(E2) (8). The difference between the estimated cleavage site and the position determined here raises the possibility that there is space for a polypeptide between the C terminus of gp70(E2) and the N terminus of p21(NS2). Others also reported the possible presence of such a small polypeptide (7).

When the transcripts derived from pHCN729-962(A809R) were translated in the presence of microsomal membranes, we detected an approximately 17-kDa product, smaller than the unprocessed primary product of 19 kDa (Fig. 4A, lane b, arrowhead with an asterisk). Accumulation of the 17-kDa protein indicated the presence of another microsomal membrane dependent-cleavage site in the region upstream of the cleavage site at aa 809 and 810. We obtained almost the same results when transcripts from pHCN340-962 and pHCN340-962(A809R), which additionally includes all of gp70 (E2) plus its possible signal sequence, were used as mRNAs (Fig. 4B, lanes a and b). Thus, the additional microsomal membrane-dependent cleavage in the region upstream of the N terminus of p21 (NS2) was not an artifact caused by using a truncated mRNA, in which the region encoding the N-terminal part of gp70 (E2) was deleted. This was the direct evidence of microsomal membrane-dependent processing taking place in the region upstream of the N terminus of p21 (NS2).

Although the absence of a 14-kDa protein in the reaction product derived from N340-962(A809R) led to the expectation that the 17-kDa product would accumulate, it did not; we saw only a small amount the 17-kDa protein (Fig. 4B, lane b). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis under conditions which gave better resolution for proteins of

around 80 kDa (Fig. 4B), a polypeptide larger than gp70 (E2) accumulated in the translation product derived from the mutated transcript (Fig. 4B, lane d). This product of about 80 kDa is likely to be the uncleaved protein composed of gp70 (E2) plus the N-terminal region of p21 (NS2). However, we also found less gp70 (E2) relative to the larger product in the translation reaction derived from the mutant. Apparently, the cleavage of the N terminus of the 17-kDa product was incomplete, while that of p21 (NS2) was quite efficient. The 17-kDa product was the major product from the translation of pHCN729-962 (A809R) (Fig. 4, lane b). However, the susceptibility of this protein to microsomal membrane-dependent cleavage might differ if it lacked an N-terminal region of gp70 (E2), because the intact form of gp70 (E2) is probably anchored in the microsomal membranes through its C-terminal membrane-spanning domain (8). Production of multiple polypeptides was observed in consequence of deglycosylation of the E2 glycoprotein produced in cultured cells (7, 17). Data presented in this report suggest the possibility that those multiple E2 products resulted from differences in their C-terminal sequences.

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