PRODUCTION OF TWO PHOSPHOPROTEINS FROM THE NS5A REGION OF THE HEPATITIS C VIRAL GENOME

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Received October 5, 1994

Hepatitis C virus produces about 12 viral proteins by proteolytic cleavage of the viral polyprotein precursor produced from the largest open reading frame in the viral genome. We have analyzed the production of viral nonstructural proteins with an *in vivo* transient expression system using COS-1 cells. Two proteins, a 56-kDa protein and a 58-kDa protein, were produced from the nonstructural region 5A (NS5A), which has the potential to produce a 49 kDa protein. We showed that these proteins are phosphorylated at the serine residues. The presence of the two proteins was reflected by different degrees of phosphorylation. Moreover, the hyper-phosphorylation of p58 was shown to depend on the presence of NS4A, another hepatitis C virus protein. © 1994 Academic Press, Inc.

Infection with hepatitis C virus (HCV) is closely associated with post-transfusion non-A, non-B chronic hepatitis. The HCV genome includes a single large open reading frame (ORF) encoding about 3,000 amino acid (aa) residues, the size of which depends on the HCV genotype (1,2). Functional viral proteins are produced by proteolytic processing of viral precursor polyprotein produced from the ORF. Using *in vitro* and *in vivo* expression systems for various regions of the viral genome, we showed that at least three independent processing pathways are involved in the production of viral proteins (3). HCV encodes two proteinases, a metalloproteinase and a serine proteinase. Viral metalloproteinase cleaves a site located between the nonstructural region (NS) 2 and NS3 in the viral precursor polyprotein, while viral serine proteinase cleaves all of the other sites located in the nonstructural region (4-7).

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Abbreviations: HCV, hepatitis C virus; ORF, open reading frame; NS, nonstructural; α-, anti-; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-291X/94 $5.00
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During analysis of virus proteins processed from the nonstructural region, we found that two proteins, a 56 kDa protein and a 58 kDa protein, are produced from NS5A (8). The presence of a protein produced from NS4A, p4, has been shown to be important for production of the 58 kDa protein (9,10). Since the p4 (NS4A) is required for cleavage between NS4B and NS5A by a serine proteinase, a product of NS3, we thought that additional cleavage upstream or downstream of NS5A might occur. Both mutation analysis and deletion analysis of these regions revealed that this was not the case.

By analyzing possible modifications of these proteins, we found that both proteins are phosphorylated and that p58 is a hyper-phosphorylated form of p56.

Materials and Methods

Plasmid construction The construction of plasmid pCMV/N729-3010, which produces all of the processed HCV nonstructural proteins of HCV-1bJ, and of plasmid pCMV/NS3/4NN, which also produces most of the processed viral proteins but has mutations at positions p1 and p1' of the NS3/NS4A cleavage site and thus produces only one type of protein from the NS5A region, is described elsewhere (8, 11). Plasmid pCMV/N1973-2419, which produces NS5A protein, was constructed as follows. PCR was carried out using pCMV/N729-3010 as the template and the synthetic oligonucleotides 5'-CCGAAGCTTCTCGAGATGTCCGGCTCGTGGCTA-3' (the underlined sequence represents 1973 to 1977 on the HCV ORF and the other region is the tagged sequence with a restriction enzyme site) and 5'-AAGGGATCCTTAGCAGCAGACGATGTC-3' (2415 to 2419) as the primers. The fragment obtained was digested with *Bam*HI and the ends were filled in with Klenow enzyme. The DNA fragment was further digested with *Hin*dIII and ligated into the *Hin*dIII-*Eco*RV site of pCMVS3. A cloning vector, pCMVS3, was derived from pKS(+)/CMV (11) by modifying it so that a *Pst*I fragment of 27 base pairs located downstream from the CMV promoter was deleted after the unique *Hin*dIII site was eliminated. The *Pst*I site was then converted to *Hin*dIII. Plasmid pCMV/N1658-1711, which produces NS4A protein was constructed as follows. A PCR fragment was obtained using pCMV/N729-3010 as the template and synthetic DNA fragments of 5'-GTCTGCAGATGAGCACCTGGGTGCTG-3' (1658 to 1662) and 5'-TCGAATTCTTAGCACGCTTCCATTTC-3' (1707 to 1711) as the primers. The fragment obtained was digested with *Pst*I and *Eco*RI, and then ligated into the *Pst*I-*Eco*RI site of pKS(+)/CMV. The procedures for constructing pCMV/N1973-2419 and pCMV/N1658-1711 are shown in Fig.1.

The regions and names of the peptides produced from these plasmids on the HCV polyprotein are shown in Fig.2 together with the genomic organization of HCV.

Transient expression in COS cells DNA transfection was performed as described previously (3). Lysate of COS cells transfected with a plasmid was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and this was followed by immunoblot analysis. The antibodies used were anti-NS4A antibody (α-NS4A) and anti-NS5A antibody (α-NS5A). α-NS4A and α-NS5A were a generous gift of Dr. A. Takamizawa (Osaka University, Japan).

Analysis of phospho-amino acids Cells transfected with plasmids which produce HCV proteins were metabolically labelled with ^{32}P-ortho-phosphate. The cell lysate was immunoprecipitated with α-NS5A, and the immunoprecipitate was fractionated with SDS-PAGE and blotted on a nylon membrane filter. The bands corresponding to p56 and p58 were recovered and treated with 6N HCl for 2 hours. Phospho-amino acids were electrophoretically separated and detected by autoradiography.

Dephosphorylation by treatment with alkaline phosphatase Cells transfected with plasmids were lysed and immunoprecipitated with α-NS5A. Samples bound to Protein G sepharose were suspended in 10 mM Tris-HCl(pH8)-2 mM $MgCl_2$-1 mM PMSF-1 mM DTT,

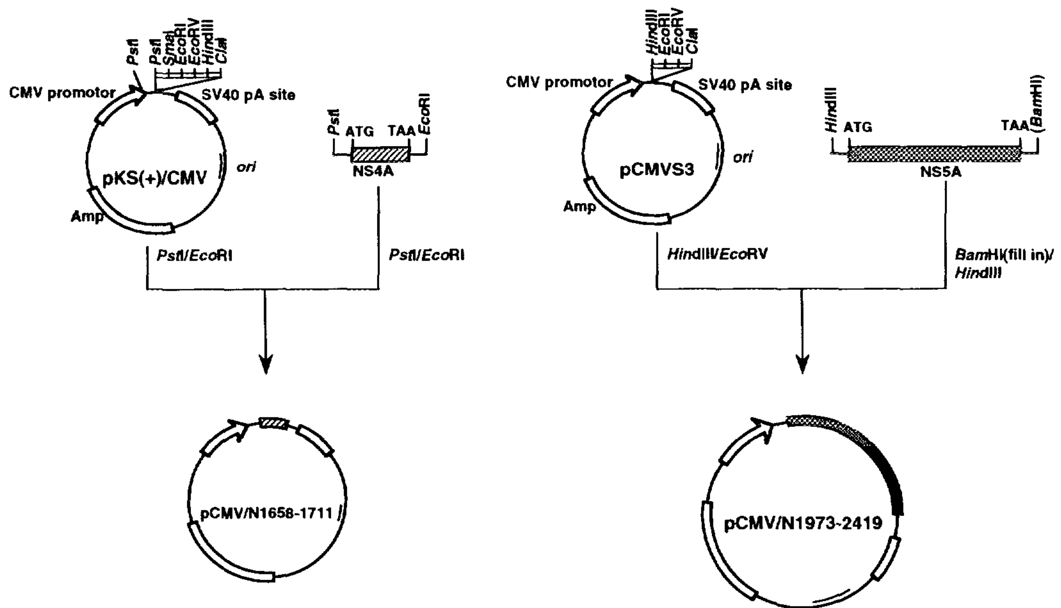

Fig.1. Construction of expression plasmids pCMV/N1658-1711 and pCMV/N1973-2419. The detailed procedures of construction are described in Materials and Methods.

and after adding 0.3 units of *E. coli* alkaline phosphatase, they were incubated at 56 °C. The reaction was terminated at various time points by the addition of Laemmli sample buffer and samples were subjected to SDS-PAGE, followed by immunoblot analysis or autoradiography.

Results and Discussion

Production of NS5A products in COS-1 cells

Viral nonstructural proteins are produced by proteolytic processing of HCV precursor polyprotein. Viral proteinase, Cpro-2, is responsible for this cleavage (4-7). Previously, 6

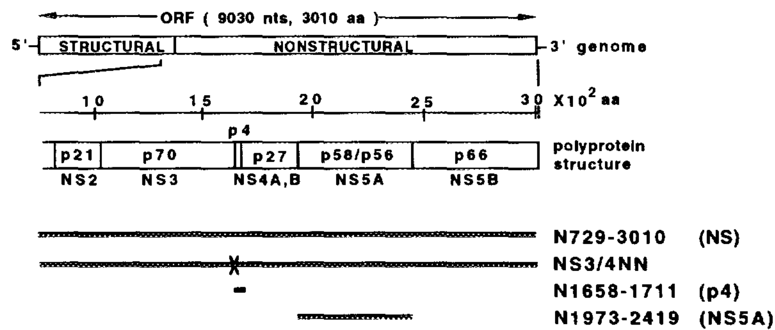

Fig.2. Schematic maps of the expression plasmids. The HCV genomic ORF is represented as a box. The nonstructural region of the HCV precursor polyprotein structure is shown as a box with the names of the processing products. The cDNA fragments for the expression plasmids are represented by bars with the names of produced primary proteins. Mutations at p1 and p1' of NS3/NS4A sites were described(8).

major viral nonstructural proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B, were shown to be produced using a vaccinia virus vector system. Potential cleavage sites on the viral precursor polyprotein have been identified based on the results of an analysis of the N-terminal amino acid sequences of these products, and a protein with 447 amino acid residues (from amino acid 1973 to 2419 on HCV ORF) is expected from the NS5A region (4,5,12). Using a plasmid-based transient expression system for HCV nonstructural proteins in COS-1 cells, however, we observed that two proteins, p56 and p58, are produced from the NS5A region (8). Because of a lack of information concerning the C-terminus of the NS5A product, we thought that products of different sizes might be derived based on differences in their C-termini. However, this was not the case. When the plasmid which produces the C-terminally deleted form of NS5A was transfected to COS-1 cells, two NS5A specific proteins were produced (data not shown). The possibility of the presence of an alternative cleavage site upstream of the authentic N-terminus of NS5A is unlikely because we always observed production of a single discrete band of the juxtaposed product (NS4B) upstream of the NS5A region.

These findings suggest that production of two proteins from the NS5A region is not caused by different cleavages during proteolytic processing, but that it is probably caused by some modification of the primary translated peptide backbone from NS5A. This speculation is supported by examination of the difference between the actual molecular weights (56 and 58 kDa) of the NS5A products and the calculated molecular weight (49 kDa). Thus, we analyzed the nature of the modification of the NS5A products.

NS5A products are phosphorylated

The plasmids which we used in this study produce proteins from the region coding the entire nonstructural region of the viral genome. When plasmid pCMV/N729-3010 is transfected to COS-1 cells, viral proteins p21(NS2), p70(NS3), p4(NS4A), p27(NS4B), p56/p58(NS5A), and p66(NS5B) are produced, in that order, from the amino-terminus of the viral precursor polyprotein. Production of p56 and p58 from NS5A was detected with the NS5A specific antibody α-NS5A (Fig.3B, lane 1). COS-1 cells transfected with pCMV/NS3/4NN, which covers the same region of HCV ORF as pCMV/N729-3010 but has mutations at the NS3/NS4A cleavage site, produced all viral nonstructural proteins except p58 (Fig. 3B, lane 2).

To analyze the possible modification of these proteins by phosphorylation, COS-1 cells transfected with these plasmids were metabolically labeled by culturing in medium containing ^{32}P-ortho-phosphate. The lysate was immunoprecipitated with α-NS5A, and the precipitate was analyzed for phosphorylation by SDS-PAGE followed by autoradiography (Fig. 3A, lanes 1 and 2). Two proteins, p56 and p58, of the same size observed by Western blot using α-NS5a, were detected in their phosphorylated form. Since the C-terminal amino acid sequence of the NS5A product has the characteristics of a certain Ras-related protein farnesylated at the site (13), the possibility of modification with fatty acids by palmytoylation or farnesylation was analyzed. However, we were unable to detect any such modifications (data not shown).

p58 is a hyper-phosphorylated form of p56

To determine whether the difference in molecular weight of the two proteins encoded in NS5A is attributable to different degree of phosphorylation, the immunoprecipitates with α-

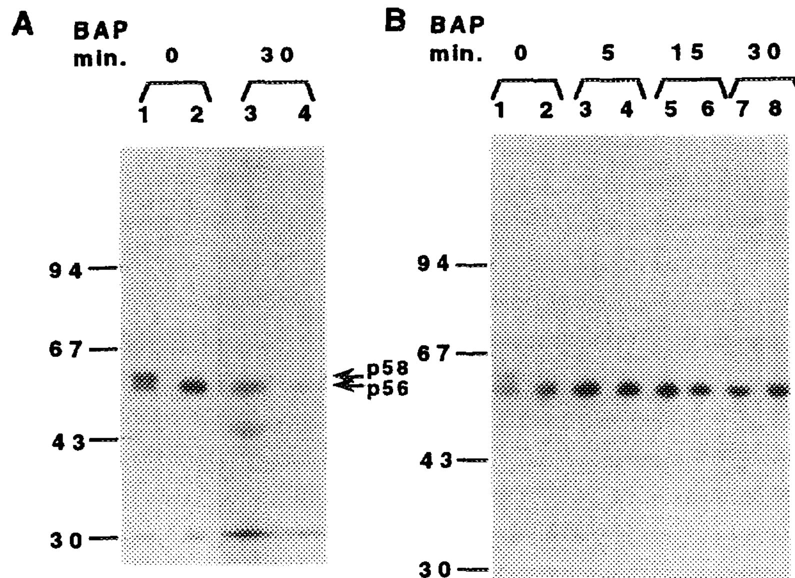

Fig.3. Detection of HCV NS5A proteins expressed in COS-1 cells. Autoradiograms of immunoprecipitated NS5A metabolically labelled with ^{32}P-ortho-phosphate (A). pCMV/N729-3010(A, lanes 1 and 3) and pCMV/NS3/4NN (A, lanes 2 and 4) were transfected into COS-1 cells. The proteins produced and processed in the cells were immunoprecipitated, treated with *E. coli* alkaline phosphatase for 0 minutes (lanes 1 and 2), or for 30 minutes (lanes 3 and 4), separated by SDS-PAGE on 7.5% gel and subjected to autoradiography. The 45-kDa band in lane 3 is likely to be *E. coli* alkaline phosphatase, which is believed to be intermediately bound to ^{32}P. Immunoblot detection of *E. coli*-alkaline-phosphatase-treated NS5A(B). COS-1 cells were transfected with pCMV/N729-3010 (lanes 1, 3, 5 and 7) and with pCMV/NS3/4NN (lanes 2, 4, 6 and 8). Immunoprecipitated samples were treated with *E. coli* alkaline phosphatase for the times indicated above the lanes and separated by SDS-PAGE.

NS5A were treated with *E. coli* alkaline phosphatase for 30 minutes at 56 °C. The radioactivity of p56 and p58 was lower after treatment, and the p58 band was no longer detectable after treatment (Fig. 3A lanes 3 and 4). To confirm that the loss of radioactivity was not due to degradation of p56 and p58 by a contaminating proteinase during phosphatase treatment, the phosphatase-treated sample was fractionated by SDS-PAGE and analyzed by Western blot using α-NS5a antibody. The 58 and 56 kDa bands were clearly detected in extracts obtained from COS-1 cells transfected with pCMV/N729-3010 and pCMV/NS3/4NN (Fig. 3B, lanes 1 and 2). After 5 minutes of digestion with alkaline phosphatase, only the p56 band was detected. The p56 band was still detectable after prolonged treatment (Fig. 3B lanes 3 to 8, up to 150 minutes data not shown). Since no degraded products of NS5A appeared during incubation, disappearance of p58 was not caused by proteolytic cleavage during incubation. This finding indicates that p56 and p58 were indistinguishable in size after dephosphorylation, that p58 is a hyper-phosphorylated form of p56, and that the mobility of p56 is unaffected by dephosphorylation. These findings strongly suggest that p56 and p58 have identical peptide backbone which is produced from NS5A. The fact that p58 is the hyper-phosphorylated form of p56 was further supported by the experiment described later.

To analyze phospho-amino acid(s) in p56 and p58, the bands corresponding to p56 and p58 were excised from the gel after SDS-PAGE, and the proteins extracted from the gel were treated

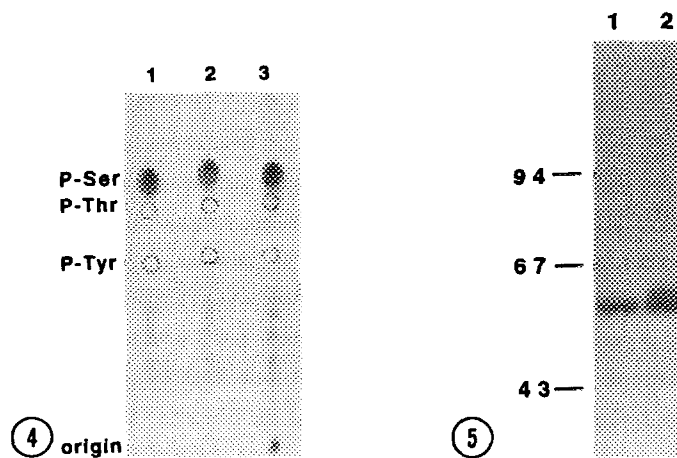

Fig.4. Autoradiogram of ^{32}P-labelled phospho-amino acids separated by thin-layer electrophoresis. p58 (lane 1), p56 (lane 2) expressed from pCMV/N729-3010, and p56 (lane 3) from pCMV/NS3/4NN were analyzed. Dotted circles represent the position of standard phospho-amino acids.

Fig.5. Immunoblot analysis of individually expressed NS5A with or without coexpression of NS4A. pCMV/N1973-2419 (lane 1), pCMV/N1973-2419 plus pCMV/N11658-1711 (lane 2) were expressed in COS-1 cells. Samples were separated by SDS-PAGE and subjected to immunoblot detection.

with 6N HCl for 2 hours. The hydrolysate was then analyzed by electrophoresis followed by autoradiography (Fig. 4). Only serine residues were phosphorylated in both proteins. Since no phosphotyrosine was detected when the phospho-amino acid(s) in proteins of p56 and p58 were analyzed after treatment by alkaline digestion (data not shown), it is concluded that phosphoserine is the unique phosphorylated amino acid in these proteins.

The NS4A product is required for hyper-phosphorylation of p56

When a plasmid which produces an HCV nonstructural polyprotein, except the region for p4(NS4A), is transfected into COS-1 cells, production of most processed viral proteins is observed. The only exception is production of a p85(NS4B-NS5A) intermediate (10). When p4(NS4A) is co-produced in this system, cleavage at NS4B/NS5A is completed and production of NS5A is detectable, however, when the level of production of p4 is lower than that of other nonstructural proteins, p56 is only the major product of NS5A. Production of p58 becomes detectable when a stoichiometrical amount of p4(NS4A) to that of other nonstructural proteins is produced. The reason for the lack of p58 in pCMV/NS3/4NN transfected COS-1 cells is unknown. Production of processed NS4A product has been shown to occur normally from this construct. However, aberrant cleavage at the mutated site may produce non-functional NS4A product or the level of NS4A production may be insufficient to convert p56 to p58.

When NS5A was co-transfected with p4(NS4A) into COS-1 cells, production of both p58 and p56 was observed (Fig. 5, lane 2). Thus, p4(NS4) is directly involved in the production of the hyper-phosphorylated form of NS5A, although whether p4(NS4) itself possesses kinase activity remains to be clarified.

The function of the NS5A of HCV in viral replication remains to be determined, however, the function of NS5A is probably modulated by hypo- or hyper-phosphorylation. Viruses of the Flaviviridae family, such as bovine virus diarrhea virus (BVDV), have a similar region corresponding to HCV NS5A, but the function of the product is unknown.

The HCV NS5A product is localized in the peripheral membrane fraction of cells transfected with plasmids which express viral nonstructural proteins (unpublished data). We previously demonstrated the mutual interaction of most of the nonstructural viral proteins, and that this complex is associated with the microsomal membrane (11). Thus the p4(NS4A)-dependent kinase which hyper-phosphorylates p56 is probably associated with membranes. In this regard, cAMP-dependent protein kinases, casein kinases, protein kinases C and calmodulin kinases, which are known to be associated with microsomal membranes (14), are likely candidates, although an unidentified kinase may be involved in the phosphorylation of the NS5A product. Possible sites for proline-directed protein kinase, and for casein kinase II were detected in a search for consensus phosphorylation sites by amino acid sequence homology. Information on the site of phosphorylation in p58 may be important in further clarifying the mechanism of phosphorylation of NS5A products.

Acknowledgments

This work was supported in part by a grant-in-aid from the Ministry of Health and Welfare Comprehensive 10-Year Strategy for Cancer Control, a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

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