RNA-Stimulated ATPase and RNA Helicase Activities and RNA Binding Domain of Hepatitis G Virus Nonstructural Protein 3

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Hepatitis G virus (HGV) nonstructural protein 3 (NS3) contains amino acid sequence motifs typical of ATPase and RNA helicase proteins. In order to examine the RNA helicase activity of the HGV NS3 protein, the NS3 region (amino acids 904 to 1580) was fused with maltose-binding protein (MBP), and the fusion protein was expressed in Escherichia coli and purified with amylase resin and anion-exchange chromatography. The purified MBP-HGV/NS3 protein possessed RNA-stimulated ATPase and RNA helicase activities. Characterization of the ATPase and RNA helicase activities of MBP-HGV/NS3 showed that the optimal reaction conditions were similar to those of other Flaviviridae viral NS3 proteins. However, the kinetic analysis of NTPase activity showed that the MBP-HGV/NS3 protein had several unique properties compared to the other Flaviviridae NS3 proteins. The HGV NS3 helicase unwinds RNA-RNA duplexes in a 3'-to-5' direction and can unwind RNA-DNA heteroduplexes and DNA-DNA duplexes as well. In a gel retardation assay, the MBP-HGV/ NS3 helicase bound to RNA, RNA/DNA, and DNA duplexes with 5' and 3' overhangs but not to blunt-ended RNA duplexes. We also found that the conserved motif VI was important for RNA binding. Further deletion mapping showed that the RNA binding domain was located between residues 1383 and 1395, QRRGRGRT-GRGRSGR. Our data showed that the MBP-HCV/NS3 protein also contains the RNA binding domain in the similar region.

Materials and Methods

Expression and purification of the HGV NS3 protein. The putative HGV NS3 open reading frame, encompassing amino acids 904 to 1580, was amplified from HGV cDNA PNF2161 by PCR. EcoRI and HindIII sites were created at the 5' and 3' ends, respectively, during the PCR process. The PCR product was digested with EcoRI and HindIII and cloned into the EcoRI and HindIII sites of pMAL-c2 vector (New England Biolabs, Beverly, Mass.). The recombinant plasmid was designated pMAL-c2/HGVNS3, and the protein product from this plasmid was designated MBP-HGV/NS3. The MBP-HGV/NS3 protein contains a portion of maltose-binding protein.
The MBP-HGV/NS3 protein, with a pET vector system (Novagen, Madison, Wis.) was unsuccessful because of the extreme insolubility of the recombinant protein. To increase the solubility of the HGV NS3 protein, we expressed it as an MBP fusion protein (see Materials and Methods). The preparation of MBP-HGV/NS3 protein fragment (amino acids 904 to 1510) that we expressed in *E. coli* by using the pMAL-c2 system contained the protease domain and the putative RNA helicase domain. The recombinant HGV NS3 protein fused with MBP at the NH2 terminus was expressed efficiently as a soluble form and was purified with an amylose resin column (Fig. 1A). This protein was designated MBP-HGV/NS3. The protein fractions from the amylose column were then loaded onto a Q-Sepharose column, and bound protein was eluted with an NaCl gradient. The fractions eluted with 250 to 400 mM NaCl contained an 117-kDa protein that reacted with anti-MBP antiserum. This protein was not detected in IPTG-induced *E. coli* cells harboring the pMAL-c2 parental vector (see Materials and Methods). We concluded that the 117-kDa protein was MBP-HGV/NS3. Analysis of the final protein preparation by SDS-PAGE indicated that the purity of the 117-kDa protein was >90%. Several other minor protein bands were detectable. It is likely that these contaminating proteins derived from the full-length MBP-HGV/NS3 protein, since they reacted with anti-MBP antiserum and were absent in fractions purified from *E. coli* cells harboring pMAL-c2 (data not shown). The ATPase assay with or without poly(U) was performed with 200, 250, 300, 400, 500, and 600 mM NaCl-eluted fractions (Fig. 1B). No ATPase activity was detected with affinity-purified MBP. Only ATPase activity was highest or decreasing pH (data not shown). The MBP-HGV/NS3 protein was expressed in *E. coli* cells harboring the pMAL-c2 parental vector (see Materials and Methods). We concluded that the 117-kDa protein was MBP-HGV/NS3. Analysis of the final protein preparation by SDS-PAGE indicated that the purity of the 117-kDa protein was >90%. Several other minor protein bands were detectable. It is likely that these contaminating proteins derived from the full-length MBP-HGV/NS3 protein, since they reacted with anti-MBP antiserum and were absent in fractions purified from *E. coli* cells harboring pMAL-c2 (data not shown). The ATPase assay with or without poly(U) was performed with 200, 250, 300, 400, 500, and 600 mM NaCl-eluted fractions (Fig. 1B). No ATPase activity was detected with affinity-purified MBP. Only ATPase activity was highest in the presence of poly(U), the ATPase activity was proportional to the concentration of MBP-HGV/NS3 protein, which was highest at 200 mM NaCl. ATPase activity was not detected at lower or higher NaCl concentrations (Fig. 1B). This indicated that the RNA-stimulated ATPase and RNA helicase activities of the HGV NS3 protein were not derived from contaminating *E. coli* proteins. RNA helicase activity was detected only in the presence of MBP-HGV/NS3 and was proportional to the amount of MBP-HGV/NS3 protein added to the reaction (Fig. 1C).

In the presence of poly(U), the ATPase activity was highest at pH 7.0 and approached basal activity levels with increasing or decreasing pH (data not shown). The MBP-HGV/NS3 ATPase activity was dependent on the presence of divalent ions. In addition, in the presence of poly(U), ATPase activity was
inhibited by increasing concentrations of NaCl. At 200 mM NaCl, ATPase activity decreased to basal levels. The optimal pH for the RNA helicase activity of MBP-HGV/NS3 was 7.5, which was slightly different from the optimal condition (pH 7.0) for the RNA-stimulated ATPase activity. At pH 7.5 and with 10 mM ATP in the reaction mixture, the RNA helicase activity of MBP-HGV/NS3 was stimulated at low divalent ion concentrations (up to 2 mM); however, the RNA helicase activity decreased in the presence of divalent ion concentrations higher than 2 mM. Helicase activity was inhibited by increasing concentrations of NaCl, and at 100 mM NaCl, the activity fell to basal levels. Thus, the RNA helicase activity was more sensitive to NaCl than was the RNA-stimulated ATPase activity.

**Kinetic analysis of NTPase and dNTPase activity of MBP-HGV/NS3 protein.** All NTPs and dNTPs except dTTP were hydrolyzed efficiently by the MBP-HGV/NS3 protein (Table 1). The kinetic parameters \( k_{cat} \) (turnover number) and \( K_m \) (Michaelis constant) were determined in the absence or presence of poly(U). In the presence of poly(U), the \( k_{cat} \) values increased 13- to 100-fold. The MBP-HGV/NS3 protein exhibited either no change or a two- to threefold increase in \( K_m \) in the presence of poly(U). The catalytic efficiency, i.e., the \( k_{cat}/K_m \) ratios, increased 15- to 40-fold in the presence of poly(U). Substrate specificity and directionality of the HGV helicase. In order to determine the substrate specificity of the MBP-HGV/NS3 helicase, three additional substrates were synthesized. These substrates included an RNA template strand annealed to a DNA release strand (R/D) and a long DNA template strand annealed to a short RNA release strand (D/R) and a short DNA release strand (D/D). All of these substrates were displaced by the MBP-HGV/NS3 protein (Fig. 2A). Therefore, the HGV RNA helicase can unwind RNA-DNA duplexes and DNA-DNA duplexes, as well as RNA-RNA duplexes.

To determine the directionality of RNA unwinding by the MBP-HGV/NS3 protein, we constructed three different RNA substrates, one with a 3' single-stranded tail (3'/3'), one with a 5' single-stranded tail (5'/5'), and one with no single-stranded tail (blunt). As shown in Fig. 2B, only the RNA substrate containing the 3' single-stranded region (3'/3') was displaced by MBP-HGV/NS3. Thus, we conclude that the MBP-HGV/NS3 protein unwinds RNA duplexes in a 3'-to-5' direction.

**Binding of the HGV NS3 protein to helicase substrates.** The inability of the MBP-HGV/NS3 protein to unwind the 5'/5' and blunt RNA substrates may indicate that the NS3 protein does not interact with them. Thus, we measured the ability of each substrate. Substrates were incubated with the MBP-HGV/NS3 protein in the helicase reaction mixture lacking

### Table 1. Kinetics of NTP and dNTP hydrolysis by the HGV NS3 protein

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Without poly(U)</th>
<th>With poly(U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>( k_{cat} ) (min⁻¹)</td>
</tr>
<tr>
<td>ATP</td>
<td>0.39</td>
<td>6.2</td>
</tr>
<tr>
<td>GTP</td>
<td>0.50</td>
<td>4.3</td>
</tr>
<tr>
<td>UTP</td>
<td>0.38</td>
<td>3.5</td>
</tr>
<tr>
<td>CTP</td>
<td>1.11</td>
<td>13.3</td>
</tr>
<tr>
<td>dATP</td>
<td>0.77</td>
<td>12.8</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.33</td>
<td>1.1</td>
</tr>
<tr>
<td>dCTP</td>
<td>–</td>
<td>–</td>
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<tr>
<td>dTTP</td>
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\( ^* \) Extent of hydrolysis of dTTP by the HGV NS3 protein was too low to determine the \( K_m \) and \( k_{cat} \) values.
ATP to bind to the MBP-HGV/NS3 protein in vitro, and the reaction mixtures were subjected to electrophoresis through a polyacrylamide gel (Fig. 3). This gel mobility shift assay revealed that the MBP-HGV/NS3 protein was able to bind to all but the blunt RNA substrate. The reactions containing only the MBP as a negative control did not show complex formation. Mapping of an RNA binding domain of the MBP-HGV/NS3 protein. To determine the RNA binding domain of the MBP-HGV/NS3 protein, we constructed various deletion mutants from the amino-terminal and carboxy-terminal ends (Fig. 4A and B). The amino-terminal amino-acid-deleted proteins, MBP-HGV/N970 and N1030, contained the same RNA helicase and RNA-stimulated ATPase activities as the MBP-HGV/NS3 protein, but all of the carboxy-terminal amino-acid-deleted proteins did not have any RNA-stimulated ATPase and helicase activities (data not shown). The RNA binding activities of these proteins were determined by Northwestern assay (Fig. 4C). MBP-HGV/NS3, N970, N1030, and only the MBP-HGV/C1460 among the carboxy-terminal amino-acid-deleted proteins bound to 32P-labeled ssRNA (98 nucleotides). These RNA binding activities were not sequence specific because other ssRNA (e.g., 32P-labeled 28-nucleotide RNA prepared from in vitro transcription of BamHI-digested pSP65 with SP6 RNA polymerase) showed similar results. From these data, we speculated that the conserved helicase motif VI was the RNA binding domain of MBP-HGV/NS3. In order to determine the RNA binding domain more precisely, further deletions were constructed around the motif VI (Fig. 5A and B). The amino-terminal amino-acid-deleted proteins, MBP-HGV/N1363 and N1383, still possessed RNA binding activity, however, MBP-HGV/N1391, which did not contain the conserved motif QR-RGRTGR, had no RNA binding activity (Fig. 5C). The carboxy-terminal amino-acid-deleted protein, MBP-HGV/C1390, which ended just after QRGRGTGR motif, could not bind to the RNA. MBP-HGV/C1395 protein, which has five additional carboxy-terminal amino acids from the QRGRGTGR motif, regained the RNA binding activity, and all of the carboxy-terminal amino-acid-deleted proteins with more additional carboxy-terminal amino acids than MBP-HGV/C1395 had the RNA binding activities. In order to determine whether this motif is sufficient for the RNA binding activity, we synthesized three peptides, SNTPHVNHHMPHC, QRRGRTGRGRSGR, and TRGRGSRGGRQRR. SNTPHVNHHMPHC is a 13-amino-acid peptide and has a random sequence. TRGRGSRGGRQRR has the same amino acid composition as QRRGRTGRGRSGR, but it has a randomized sequence. All three peptides did not bind to 98-nucleotide RNA when we test them by using Northwestern assay (data not shown). We conclude that the helicase motif VI is necessary for RNA binding; however, it is not sufficient for the RNA binding activity. To determine whether this fact could also be applied to the HCV NS3 protein, we made several carboxy-terminal amino-acid-deleted MBP-HCV/NS3 proteins (Fig. 6A and B). MBP-HCV/C1485, which had no helicase motif VI, and MBP-HCV/C1493, which had QRGRGTGR motif carboxy-terminal ends, had no RNA binding activity (Fig. 6C). MBP-HCV/C1503 and C1513, which each had 10 or 20 more carboxy-terminal amino acids, respec-
tively, than MBP-HCV/C1493, showed the RNA binding activity.

**DISCUSSION**

On the basis of sequence motif analysis, the HGV NS3 protein is predictive of a multifunctional protein containing serine protease, ATPase, and RNA helicase activities (2, 14). Recently, it was found that the HGV NS3 protein has the ATPase and DNA helicase activities (12). In this study we confirm that the HGV NS3 protein has an RNA-stimulated ATPase activity and an RNA helicase activity, and we characterize the biochemical properties of the protein in detail. The kinetic analysis of the NTPase activity showed that the MBP-HGV/NS3 protein had several unique properties compared with the other *Flaviviridae* viral NS3 proteins. In the presence of poly(U), the HGV NTPase, like the HCV and BVDV NTPases, showed a little increase in $K_m$ values, while the YFV NTPase showed either no change or a two- to threefold reduction (22, 24, 26). The $k_{cat}$ values of HGV NTPase increased more than the $k_{cat}$ values of any other *Flaviviridae* NTPase in the presence of poly(U). For ATP hydrolysis, the increase in the $k_{cat}$ values in the presence of cofactors was 40-, 11-, 15-, and 5-fold for the HGV, HCV, BVDV, and yellow fever virus (YFV) NTPases, respectively. Thus, the increases in the catalytic efficiency ($k_{cat}/K_m$) of HGV NTPase were greater than with the HCV, BVDV, and YFV NTPases.

We found that the MBP-HGV/NS3 protein is able to unwind both RNA and DNA duplexes and is thus the fifth such enzyme to be identified. The other four enzymes are simian virus 40 large T antigen, nuclear DNA helicase II, the vaccinia virus protein 18R, and the HCV NS3 protein (1, 17, 7, 29). All of these helicases displace RNA or DNA duplexes in a 3'–5' direction. In addition, they all possess oligonucleotide-stimulated ATPase and helicase activities that are sensitive to monovalent ions. Despite these common characteristics, these enzymes do not share a conserved primary amino acid sequence or an identical mechanism for displacement of the duplexes.

Since it had not yet been proved experimentally that the *Flaviviridae* viral helicases contained a RNA binding motif, we tried to determine the presence of a RNA binding motif of the HGV helicase. By using deletion mutants and Northwestern assay, we showed that the RNA binding domain of the HGV NS3 protein was located at the conserved helicase domain VI and five additional amino acids after this domain, i.e., QRRGRTGRGRSGR. The amino acid motif QRRGRTGR in the RNA binding domain was well conserved among the *Flaviviridae* viral helicases. The five-additional-amino-acid motif GRSGR...
was less conserved, but most Flaviviridae viral helicases contained at least one positively charged amino acid in this domain. We also determined that the MBP-HCV/NS3 protein has the RNA binding domain at the same position, i.e., the helicase motif VI and several additional amino acids at the carboxy-terminal end. The RNA binding domain of the plum pox potyvirus CI protein was previously determined by Northwestern assay to be around the helicase motif VI, although the exact RNA binding motif was not defined in detail (5). The HIRIGXR region, the conserved motif VI of the eIF-4A, was proved to be required for RNA binding and ATP hydrolysis (15). So it is not surprising that the helicase motif VI of the HGV helicase was necessary for RNA binding activity. This means that the RNA binding motifs of the HCV helicase are dispersed throughout the protein rather than being limited to a few amino acids that were important for RNA binding. In fact, another RNA binding motif of the plum pox potyvirus CI protein was found with a subtle change of the assay condition assay (e.g., pH) (4). In conclusion, the helicase motif VI of the HCV and the HGV NS3 proteins seems necessary for the RNA binding activity of these proteins, but it could not be excluded that another RNA binding motif exists.

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REFERENCES