Poly(U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase

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Abstract **A non-structural protein of the hepatitis C virus (HCV), NS3, contains amino acid sequence motifs characteristic of serine-proteinases and RNA helicases. RNA binding activity of the NS3 protein with an apparent dissociation constant of 2×10^{-7} M was detected using a poly(U)-Sepharose resin. Competitive RNA binding analysis suggested that the NS3 protein binds preferentially to the poly(U) sequence, which is located at the 3′ end of HCV RNA. Mutational analysis of NS3 protein revealed the possibility that both the RNA helicase region and the serine-proteinase region were necessary for full RNA binding activity.**

Key words: Poly(U); RNA binding; Helicase; Hepatitis C virus; NS3 protein

1. Introduction

Hepatitis C virus (HCV) is a positive strand RNA virus. The RNA, approximately 9.4 kb in length, contains a single large open reading frame encoding about 3,010 amino acids and a poly(U) of 30 to 100 nucleotides at the 3′ end. Although cDNA sequences of several HCV strains have been determined [1–5], little is known about the virus-specific RNA replication. Furthermore, viral polypeptides involved in RNA replication of HCV have not been identified.

Comparative analyses using available nucleotide sequences of flaviviruses and pestiviruses have suggested that the NS3 and NS5b proteins of HCV may be possible enzymatic components of the viral RNA replicase. The HCV NS3 protein (about 70 kDa) contains motifs for serine-proteinase in the N-terminal third and of NTPases and RNA helicase in the C-terminal portion, respectively. Indeed, enzymatic activity of the serine-proteinase which mediates several cleavages in the viral proteins has been demonstrated [6–8] and partially purified C-terminal fragments of NS3 protein has been shown to have NTPase activity [9]. Recently, the NS3 protein of bovine viral diarrhea virus (BVDV), a member of pestivirus has been reported to possess RNA helicase activity [10].

In the present study, we have established an assay system for detecting the RNA binding activity of recombinant NS3 protein of HCV by using poly(U)-Sepharose resin. The NS3 protein has been shown to preferentially bind to the poly(U) sequence suggesting that the NS3 protein may have an affinity to the 3′ end of HCV RNA.

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2. Materials and methods

2.1. Construction of expression vectors

A DNA fragment corresponding to the NS3 protein (amino acid positions 1011 to 1608) of HCV strain R6 was prepared by polymerase chain reactions using the region-specific oligonucleotides as primers. These oligonucleotides were designed to provide the PCR product *Eco*RI and *Xho*I sites at the 5′ and 3′ termini, respectively. The amplified DNA fragments were cut with *Eco*RI and *Xho*I restriction enzymes and cloned into pET-21b vector (Novagen). All the mutants contained the T7·tag sequence coding for the 11 amino acid of the N-terminal peptide of T7 gene 10 protein at the 5′ termini, as well as the sequence for 6 consecutive histidine residues (His·tag) at 3′ termini as an affinity tag for purification. The structural organization of these mutants (termed pETNS3F and pETNS3δ1~δ9) were summarized in Fig. 1.

2.2. Expression and isolation of His·tagged NS3 protein

E. coli BL21(DE3) were transformed with expression plasmids. The transformed cells grew logarithmically in LB medium containing 50 μg/ml ampicillin and then supplemented with 0.4 mM isopropyl-γ-D-thiogalactopyranoside (IPTG). After 2 h of further growth at 37°C, the cells were harvested by centrifugation (5,000 × *g* for 5 min at 4°C) and suspended in buffer A (20 mM Tris-HCl (pH 7.9),500 mM NaCl, 5 mM imidazole) with brief sonication. Inclusion bodies of His-tagged NS3 proteins were collected by centrifugation (40,000 × *g* for 15 min at 4°C) and resuspended in buffer A containing 5 M guanidine-HCl with sonication. After incubation for one hour on ice, the extract was centrifuged as above.

NS3 proteins were purified batchwise using Ni^{2+}-Sepharose column chromatography in the presence of 5 M guanidine-HCl as described in the instruction manual (Novagen). The purified proteins were renatured by dialysis against buffer B (50 mM HEPES-KOH (pH7.4), 50 mM NaCl, 1mM EDTA, 0.02% 2-mercaptoethanol, 0.01% Triton X-100, 20% glycerol) containing 0.5 M guanidine-HCl overnight and then against buffer B without guanidine-HCl twice. The dialysates were cleared by centrifugation and stored at −80°C.

2.3. RNA binding assay

The RNA binding assay was performed with approximately 15 μg of partially purified NS3 protein fraction from Ni^{2+}-Sepharose column chromatography (the purity of all NS3 protein derivatives was about 50% on SDS-polyacrylamide gel electrophoresis). The protein solutions were incubated with 0.025 g of poly(U)-Sepharose beads (Pharmacia) in 600 μl of buffer B for 2.5 h at 4°C. Beads were centrifuged and washed five times with buffer B containing 0.5 M or 1.0 M NaCl. After washing, proteins were eluted in SDS-loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis (5–15%). NS3 protein were detected by staining with Brilliant blue G (Sigma) or immunoblotting using NS3 protein specific monoclonal antibody 3D3 (a gift from Dr Akira Hasegawa) and IgG coupled to alkaline phosphatase (Bio-Rad). The amount of all NS3 protein derivatives was estimated by scanning Brilliant blue G-stained SDS-polyacrylamide gels with a computing densitometer (ACI). The total amount of protein in each fraction was determined with Bradford assay using BSA as standard.

For competition analysis, an excess amount of homoribopolymers; poly(A), poly(U), poly(C) and poly(G) (Pharmacia) were pre-incubated with an NS3 protein fraction for 30 min on ice, and then poly(U)-Sepharose beads were added to the reaction mixture. After washing, NS3 proteins were detected by staining with Brilliant blue G.

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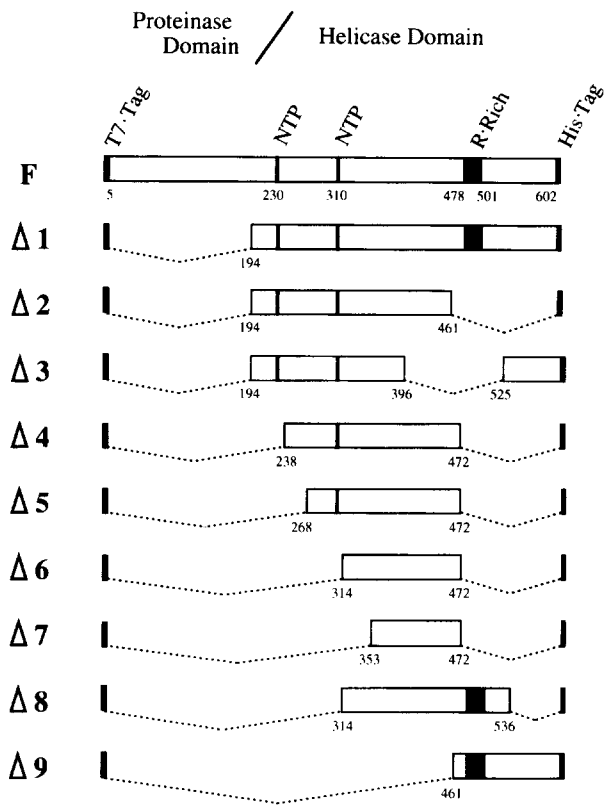

Fig. 1. Schematic presentation of NS3 protein-deletion mutants. The relative positions of the mutant NS3 proteins are shown. T7·Tag, tag sequence of T7 gene 10; NTP, NTP-binding consensus sequence; R·Rich, arginine-rich sequence; His·Tag, 6 consecutive histidine residues. Numbers indicate the amino acid position corresponding to the HCV NS3 protein [1,3,7].

3. Results and discussion

In order to obtain an abundant source of HCV NS3 protein for biochemical analysis, we subcloned a series of DNA fragments, encoding the NS3 protein into *E. coli* expression vector. All constructs are schematically represented in Fig. 1. We mainly used two constructs to establish the system of the RNA-binding assay. One was a plasmid pETNS3F containing most of the coding sequences of NS3 protein (both the serine-proteinase domain and the putative helicase domain). The other was the plasmid pETNS3δ1 containing the nucleotide sequence encoding only the putative helicase domain. All recombinant proteins were fused at their N-termini to the T7·Tag sequence and C-termini to the His·Tag sequence. The proteins, NS3F and NS3δ1 were expressed in *E. coli* BL21(DE3) in the presence of IPTG and partially purified using Ni^{2+}-Sepharose column chromatography. The NS3δ1 protein was expressed as a polypeptide at about 55 kDa (lanes resin(−)) and was detected by the NS3 protein-specific monoclonal antibody 3D3 (Fig. 2A, column b).

The RNA binding activity of the NS3 protein was detected using poly(U)-Sepharose beads. As shown in Fig. 2A, in the partially purified fraction of the Ni^{2+}-Sepharose column, only the NS3δ1 protein was bound to the poly(U)-Sepharose beads and the protein was immuno-reactive to the NS3-specific antibody. Conversely, the NS3δ1 protein did not bind with control Sepharose beads. This demonstrates that the system could detect NS3 protein-specific RNA binding activity in the solution. To establish the basis for delineating the RNA binding activity of the NS3 protein, we determined the affinity (apparent equilibrium dissociation constant, K_d) of NS3F and NS3δ1 proteins. Fig. 2B shows the normalized binding curves. NS3F protein had a five times higher affinity ($K_d = 2 \times 10^{-7}$ M) than NS3δ1 protein ($K_d = 1 \times 10^{-6}$ M). Since we washed the poly(U)-Sepharose beads with buffer containing 0.5 M NaCl (see section 2) to detect the NS3-specific RNA binding activity in the system, the real Kd value of NS3F protein was probably more than 2×10^{-7} M. The results suggested that the putative RNA helicase region of the NS3 protein may be responsible for the specific RNA binding activity and showed how the serine-

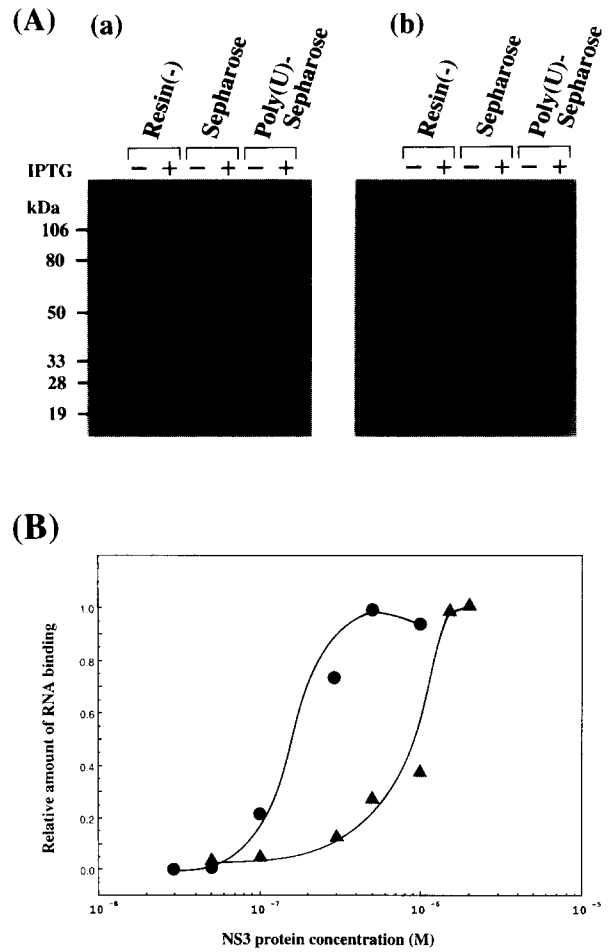

Fig. 2. (A) Detection of the RNA binding activity in a partially purified fraction of the HCV NS3 protein. (a) Plasmid pETNS3δ1 (see Fig. 1) transformed into *E. coli*. The cell lysate of cultures either uninduced (−) or IPTG induced (+) were prepared and partially purified using Ni^{2+}-Sepharose column chromatography. The partially purified protein (Lanes of resin (−)) were incubated with Sepharose or poly(U)-Sepharose beads. After washing with buffer B containing 0.5 M NaCl (see section 2), bound proteins were eluted and analyzed by SDS-polyacrylamide gel electrophoresis (5–15%). The gel was identified using Brilliant blue G staining. An arrow indicates the position of the NS3δ1 protein. (b) Immunoblot analysis of the same fraction in (a). (B) RNA binding curves of the NS3 protein. RNA binding activity was measured using poly(U)-Sepharose beads. The data for each curve were normalized to the saturation point for each protein, NS3F(●) and NS3δ1 (▲). The K_d value is equal to the protein concentration at which 50% of the highest RNA binding activity.

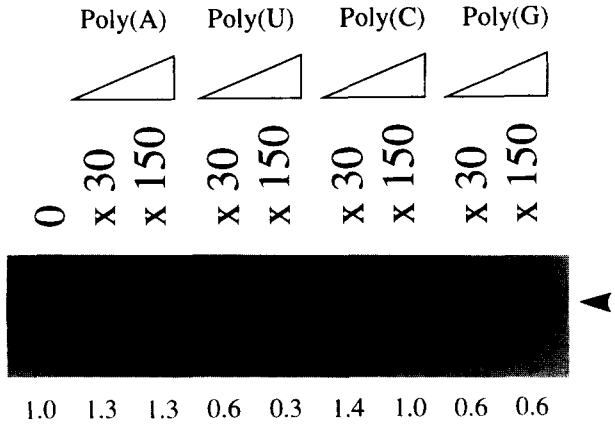

Fig. 3. Competitive RNA binding experiment. The NS3δ1 protein was pre-incubated with 30 or 150 molar excess of homoribopolymers to that of NS3δ1 protein followed by the addition of poly(U)-Sepharose beads. The NS3δ1 protein was analyzed as described in Fig. 2a. The relative RNA binding activities are indicated at the bottom. Similar results were obtained from the several independent experiments.

proteinase region enhanced activity. Thus, the whole NS3 protein is likely required for stable RNA binding.

To examine the specificity of RNA binding activity in the NS3 protein, we carried out the competitive RNA binding experiments. An excess amount of the four different homoribopolymers were pre-incubated with the NS3 protein and degree of NS3 protein binding to the poly(U)-Sepharose beads was analyzed. Little or no competition was observed when poly(A) or poly(C) was used as a competitor. Poly(G) partially blocked the poly(U) binding to the NS3 protein. Conversely, as shown in Fig. 3, the binding of NS3 protein to poly(U)-Sepharose beads was blocked in a dose-dependent manner by the presence of poly(U). This suggests that the NS3 protein has a binding activity specific for the poly(U) sequence.

In order to determine the region(s) responsible for the RNA binding activity of the NS3 protein, we constructed a series of NS3 protein-deletion mutants (Fig. 1) and analyzed their RNA binding activity. Fig. 4A shows the expressions and partial purifications of the mutant proteins using Ni^{2+}-Sepharose column chromatography. All mutants were expressed and purified as the major protein in each fraction. After the RNA binding reaction followed by washing with a buffer containing 0.5 M NaCl, the NS3F protein was positively bound to poly(U)-Sepharose beads. Deletion of the serine-proteinase region (NS3δ1 protein) had no effect on RNA binding activity. However, deletions from any other region of the helicase domain (NS3δ1 protein) led to a significant reduction in RNA binding activity (Fig. 4B). Conversely, when the binding beads were washed with a buffer containing 1.0 M NaCl, only the NS3F protein was significantly retained in the beads (Fig. 4C). Similar results of the RNA binding activity of NS3 protein was observed using a Sepharose beads coupled with synthetic oligonucleotides corresponding to the 3′ end of HCV (data not shown). These results support our observations in Fig. 2B and further suggested that whole the NS3 protein is necessary for stable RNA binding.

Recently, it has been reported that a short (10 to 20 amino acids) arginine-rich sequence and the motif defined as closely spaced Arg-Gly-Gly (RGG) repeats in viral, bacteriophage, and ribosomal protein-mediated RNA binding (for review see [11]). In the NS3 protein sequence, one cluster of basic amino acids (R-rich) was found between residues 478 and 494, RSQRRGRTGRGRRGIYR (Fig. 1). However, as shown in Fig. 4B,C, the two constructs (NS3δ8 and NS3δ9) containing R-rich region did not bind effectively to poly(U)-Sepharose beads, suggesting that the region might not be responsible for RNA binding activity in the whole NS3 protein. We synthesized several oligopeptides, one of which corresponded to the R-rich region of the NS3 protein, and tested them for RNA binding activity using gel shift analysis. The R-rich peptide region bound strongly to several RNA sequences. However, the NS3δ1 protein failed to bind to RNA sequences in the gel shift assay (data not shown). This suggests that the gel shift assay may be useful for detecting potential regions of RNA binding activity, but this region was not always responsible for activity in the whole RNA-binding molecule. Recently, the RNA binding activity of the cellular helicase, eIF-4B [12] and viral heli-

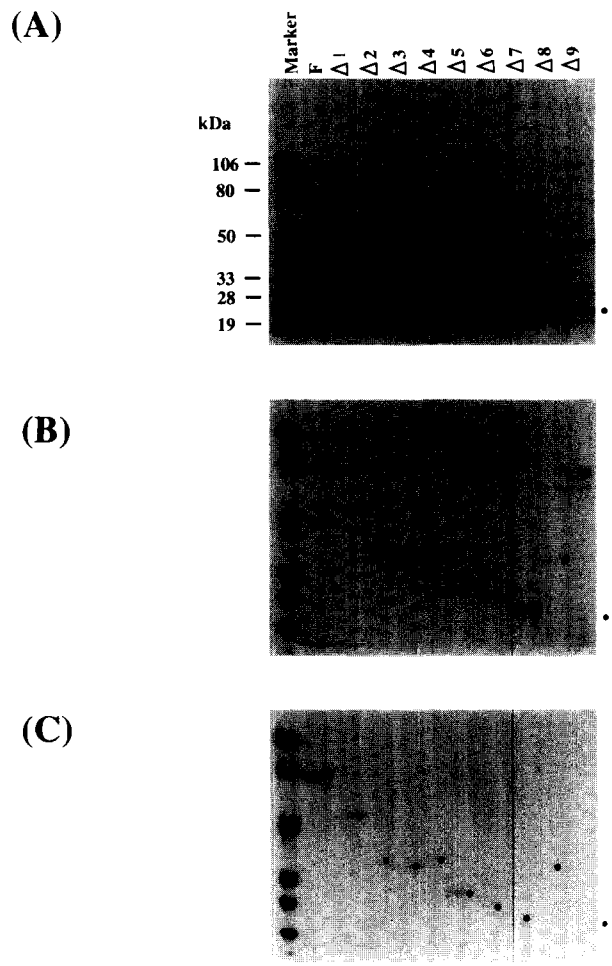

Fig. 4. RNA binding activity of the mutant NS3 proteins. (A) Expression and the partial purification (Ni^{2+}-Sepharose column bound) of mutant proteins. Mutant NS3 proteins were analyzed using SDS-polyacrylamide gel electrophoresis (5–15%), stained with Brilliant blue G. (B) Mutants proteins bound to poly(U)-Sepharose resin after washing with buffer B containing 0.5 M NaCl. (C) Mutants proteins bound to poly(U)-Sepharose resin after washing with buffer B containing 1.0 M NaCl. Products corresponding to individual deletion-mutants are marked with dots.

cases [10,13] has been detected by using a filter-binding assay. However, the binding specificity remains unclear. It is likely that the filter-binding assay is not suitable for determing the RNA-binding specificity. Thus, a binding assay using a resin coupled with the specific RNA sequence may be a useful tool for detecting the specificity of RNA-binding proteins such as RNA helicase.

What is the biological role of poly(U) binding activity in the NS3 protein? NTPase activity of the helicase domain is known to be regulated by the presence of polyribonucleotides. In the case of the HCV NS3 protein, the NTPase activity was enhanced by adding poly(U) to the reaction mixture [9]. HCV RNA may contain stable secondary structure in the sequence found just before the poly(U) stretches at the 3′ end of the HCV genome [14]. The single-stranded 3′ terminus is required for helicase activity in a BVDV NS3 protein [10] or a cellular helicase [16]. Based on these results and our observations, it is possible that the HCV NS3 protein binds to the 3′ end of the HCV genome and regulates the positive strand replication of viral RNA.

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