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Hepatitis C virus NS3 RNA helicase activity is modulated by the two domains of NS3 and NS4A

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Abstract

To determine whether the two domains of hepatitis C virus (HCV) NS3 and the NS4A interact with each other to regulate the RNA unwinding activity, this study compares the RNA unwinding, ATPase and RNA binding activities of three forms of NS3 proteins—the NS3H protein, containing only the helicase domain, the full-length NS3 protein, and the NS3–NS4A complex. The results revealed that NS3 displayed the weakest RNA helicase activity, not because it had lower ATPase or RNA binding activity than did NS3H or NS3–NS4A, but because it had the lowest RNA unwinding processivity. A mutant protein, R1487Q, which contained a mutation in the helicase domain, displayed a reduced protease activity as compared to the wild-type NS3–NS4A. Together, these results suggest the existence of interactions between the two domains of NS3 and the NS4A, which regulates the HCV NS3 protease and RNA helicase activities.

Keywords: HCV; NS3; NS4A; RNA helicase; Protease

The hepatitis C virus (HCV) genome encodes a large polyprotein of 3008–3037 amino acids (aa) [1]. The polyprotein is proteolytically processed into at least 10 distinct products arranged in the following order in the genome: NH2–C–E1–E2–p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH. C, E1, and E2 are viral structural proteins, but the role of p7 is still unclear. The remaining proteins (NS2–NS5B) are non-structural proteins, which are presumably the essential components of viral replicative machinery. The NS3 protein of HCV has attracted much interest in relation to discoveries of antiviral drugs because of its importance in viral replication. The protein ranges from aa 1027 to 1657 of the polyprotein and is multifunctional. The N-terminus of NS3 is a serine protease [2,3], and two-thirds of the C-terminal region of the protein is an RNA helicase [4,5].

Several forms of recombinant HCV helicase have now been produced; these include a truncated NS3 that contains only the helicase domain [4,5], the full-length NS3 [6], and the complex of NS3–NS4A [6,7]. Numerous studies of the HCV protease and helicase activities were based on two separate domains [2–5]. However, no available evidence suggests that proteolytic processing in vivo separates the two domains of NS3. Additionally, in nature NS3 associates with NS4A to form a complex [8]. The truncated NS3 or the full-length NS3, though functional for helicase activity assays, may not represent a naturally biological relevant entity. In particular, information about the possible mutual influence between the various enzymatic activities of different subdomains may not be obtained using truncated NS3 helicase or the full-length NS3 alone.

This study describes the expression and purification of truncated NS3 helicase, full-length NS3 alone, and the complex of NS3–NS4A, as well as two helicase mutants derived from NS3–NS4A in baculovirus-infected Sf9 cells. ATPase, duplex RNA unwinding,

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substrate RNA binding, single processive cycle RNA unwinding, and protease activities were all compared across these proteins. Some notable differences among these proteins were detected, in terms of processive unwinding activity and protease activity. The data provide strong evidence of interdomain communication among the protease and helicase domains of NS3 and NS4A, modulating the various NS3 catalytic activities.

Materials and methods

Expression and purification of histidine-tagged helicase proteins. The cDNA coding for the protein sequences of NS3H (the HCV NS3 helicase domain, aa 1175–1657), full-length NS3 (aa 1027–1657), or NS3–NS4A (aa 1027–1711) was obtained from the HCV genotype 1b of Taiwanese strain [9] by PCR amplification. The upstream primer contained a tag sequence of hexahistidine added to the N-terminus of each polypeptide. The PCR products were cloned into the pVL1392 baculovirus expression vector (BD PharMingen). The mutant clones, K1236N and R1487Q, were generated by PCR-directed mutagenesis using NS3–NS4A as a template. The resulting histidine-tagged constructs were co-transfected with BaculoGold DNA (BD PharMingen) into Sf9 cells to generate recombinant baculoviruses according to manufacturer's instructions. The recombinant viruses were plaque-purified, and virus stocks were prepared and tittered.

To express and purify the histidine-tagged proteins, the recombinant viruses were used to infect ten 10-cm dishes of Sf9 cells (2×10^7 cells/dish) at an moi of 2. Recombinant proteins were purified from the viurs-infected Sf9 cells following the previously described procedures [6].

ATPase activity assays. ATPase activity assays were performed as previously described [4]. In brief, the reaction mixtures (10 μl) contained 20 mM Hepes–KOH (pH 7.0), 2 mM dithiothreitol, 1.5 mM $MgCl_2$, 5 μCi [α-^{32}P]ATP (3000 Ci/mmol, Amersham), 1 mg/ml poly(U), and 0.9 pmol of recombinant helicase proteins. For kinetic analysis, various concentrations of cold ATP ranging from 0.067 to 20 mM were added and the reaction was carried out at room temperature for just 5 min to constrain the reaction rate within the linear phase. Reaction was halted by adding EDTA to 20 mM and reaction products were analyzed by thin-layer chromatography.

RNA helicase assays. The radiolabeled partial double-stranded RNA (dsRNA) substrates were prepared and RNA helicase assays were conducted following the previously described procedures [4]. The reaction mixture (20 μl) contained 20 mM Hepes–KOH (pH 7.0), 2 mM dithiothreitol, 1.5 mM $MnCl_2$, 2.5 mM ATP, 0.1 mg/ml bovine serum albumin, 20 U RNasin, 90 nM of the helicase proteins, and 30 nM dsRNA substrate. The reaction was carried out at 37 °C for 1 h and then terminated by adding 5 μl of 5× RNA loading dye (0.1 M Tris–HCl, pH 7.4, 20 mM EDTA, 1% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol, and 50% glycerol). For kinetic analysis, various amounts of dsRNA substrate ranging from 0.33 to 10.56 pmol were added and the reaction was performed for just 5 min. The reaction products were analyzed on an 8% native polyacrylamide gel.

For analysis under single processive cycle conditions, assays were performed in the presence of heparin that was used as trapping molecules [10,11]. Reaction mixtures, containing 180 nM of the helicase proteins and 30 nM of the labeled dsRNA substrates in a helicase reaction buffer lacking the ATP, were pre-incubated at room temperature for 15 min. After the pre-incubation, the reactions were started by addition of ATP with or without the heparin (5 μg/ml). Unlabeled ssRNA (500 nM), complementary to the template strand, was added together with ATP to prevent product re-annealing during the reaction in the presence of heparin. Reactions were carried out at 37 °C. At the indicated time points, aliquots were withdrawn, mixed with the 5× RNA loading dye, and analyzed on an 8% native polyacrylamide gel.

RNA binding assays. The binding of the partial dsRNA substrate to the HCV helicase proteins was analyzed by gel mobility shift assay. Helicase proteins (3.6 pmol) was incubated with 0.66 pmol of radiolabeled partial dsRNA substrate and 8 pmol of cold single released strand in 10 μl of a helicase reaction buffer containing non-hydrolyzable ATP-γS. The binding reaction was incubated at 37 °C for 5 or 15 min. Reaction products were analyzed on a 4% polyacrylamide gel containing 5% glycerol. The bound complexes were visualized by autoradiography.

Protease assay. The intramolecularly quenched fluorogenic substrate (IQFS), containing an ortho-aminobenzoic acid (Abz) at the N-terminus and an *N*-(ethylene-diamine)-2,4-dinitrophenylamide (EDDnp) at the C-terminus of the peptide [12], was used for the protease activity assays. The reaction mixture (100 μl) contained 20 mM Hepes, pH 7.3, 100 mM NaCl, 0.1% Triton X-100, 10 mM DTT, 100 μM IQFS peptide, and 100 nM of the NS3–NS4A enzymes. Reactions were performed in a 96-well microtiter plate incubated at 31 °C. After the enzyme was added, the change of fluorescence was recorded continuously using a Labsystems fluorometer (Fluoroskan Ascent) with a plate reader accessory with excitation and emission wavelengths of 320 and 420 nm, respectively. The amounts of product released by proteolytic cleavage were determined by interpolating the fluorescence data into a standard curve of several known concentrations (6.25–100 μM) of synthetic Abz–peptide–OH, which was the final cleaved product. The percentages of cleavage were calculated by dividing the amounts of products released from the 100 μM substrate.

Results

Expressing and purifying recombinant HCV NS3 proteins in baculovirus-infected Sf9 cells

Three forms of NS3 proteins, NS3–NS4A, NS3, and NS3H (Fig. 1A), were produced to investigate the roles of NS4A and the protease domain of the HCV NS3 protein in the RNA helicase activity. NS3H contains only the helicase domain of the HCV NS3 protein; NS3 encompasses both the protease and the helicase domains; and NS3–NS4A is a fusion protein which can undergo autocleavage and form a heterodimeric complex between NS3 and NS4A upon synthesis [7,8]. Two helicase mutants, K1236N and R1487Q, in the context of NS3–NS4A, were produced in parallel to examine the possible influence of the C-terminal helicase domain on the N-terminal proteolytic activity. The K1236N mutant has a mutation at Lys-1236 of the HCV NS3 protein, which is a residue of Walker A motif, severely reducing the ATP- and Mg^{2+}-binding activities [13]. The R1487Q mutant has a mutation at Arg-1487 of motif VI. An earlier study demonstrated that mutation at this residue in the context of an *Escherichia coli*-expressed helicase domain protein caused significantly reduced ATP hydrolysis and RNA unwinding activities [13].

The above proteins were expressed as N-terminal histidine-tagged forms, with a baculovirus expression system in Sf9 cells. Fig. 1B shows the purities of the purified proteins. The proteins were further analyzed

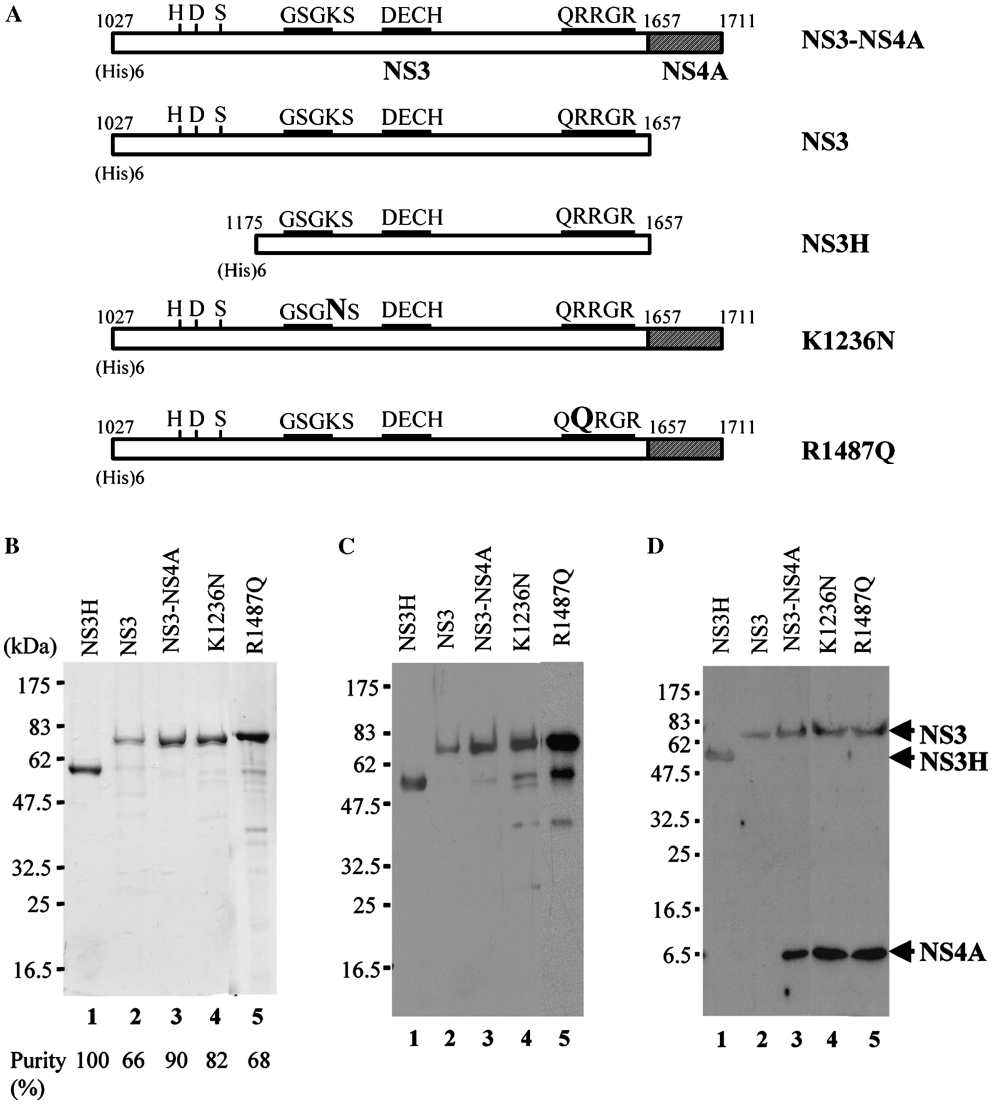

Fig. 1. Expression of the NS3 proteins. (A) HCV NS3 expression constructs. Constructs generated for the expression of NS3–NS4A, NS3, or NS3H are indicated. H, D, and S represent triad amino acid residues, His, Asp, and Ser, respectively, that are important for serine protease activity. GSGKS and QRRGR are the consensus motifs I and VI, respectively, for RNA helicase. The K1236N mutant has a Lys → Asn mutation at motif I, and the R1487Q mutant has a Arg → Gln mutation at motif VI. Mutation residues are shown in bold. A hexahistidine sequence is tagged to the N-terminus of each protein. (B) Purity of the recombinant proteins. Two micrograms of each of the purified proteins was resolved by SDS–12.5% PAGE and stained with Coomassie brilliant blue. The purity shown below each lane was determined using a densitometer. The proteins were further subjected to Western blot analysis with a rabbit anti-NS3 antiserum (C), or a mouse antiserum that recognized the C-terminal region of NS3 and NS4A (D). The bands that represent NS3, NS3H, and NS4A are indicated on the right. Molecular size markers (in kilodaltons) are indicated on the left. Lanes 1–5 correspond to proteins NS3H, NS3, NS3-NS4A, K1236N, and R1487Q, respectively.

by Western blot with a rabbit anti-NS3 antiserum (Fig. 1C) or with a mouse antiserum that recognized NS4A and the C-terminus of NS3 (Fig. 1D). The results confirmed the ~56- and ~72-kDa protein species as NS3H and NS3, respectively, and the ~7-kDa protein species, present in NS3–NS4A and the two mutants, as NS4A. The results thus indicate that NS4A was autocleaved from the NS3–NS4A precursor protein, spontaneously associated with NS3 to form a stable complex, and then co-purified with the His–NS3 protein.

Comparison of duplex RNA unwinding activities among the NS3 proteins

Fig. 2 shows the duplex RNA unwinding activities of these NS3 proteins. Under the optimized conditions [4], the data revealed that NS3–NS4A and NS3H displayed significantly higher RNA unwinding activity (81% and 79%, respectively) than NS3 (47%). As expected, the K1236N mutant completely lost RNA unwinding activity, whereas the R1487Q mutant exhibited only 40% RNA unwinding activity.

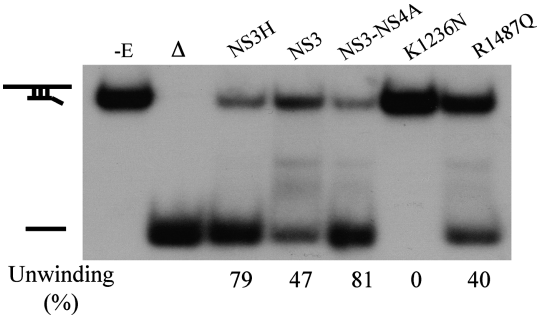

Fig. 2. RNA helicase activities of the NS3 proteins. The reactions were carried out as described in Materials and methods. Lane 1 (−E), the reaction did not contain the enzyme and the substrate was left native; lane 2 (Δ), the reaction did not contain the enzyme and the substrate was heat denatured. All other lanes, the reactions contained 30 nM of the partial dsRNA substrate and 90 nM of each of the indicated enzymes. The reaction products were analyzed on a native 8% polyacrylamide gel. Numbers below indicate the unwinding percentages, which have been normalized with the purity of each purified protein.

To be more accurate, ATPase and duplex RNA unwinding activities were measured kinetically and expressed as K_{cat}/K_m, determined from Lineweaver–Burk plots of the enzymatic activity assays (Table 1). The relative enzymatic activities were compared to that of NS3–NS4A, which was arbitrarily taken as 100%. Table 1 illustrates that NS3 exhibited only 30.6% of the RNA unwinding activity of NS3–NS4A, and K1236N exhibited no RNA unwinding activity; R1487Q exhibited a reduced RNA unwinding activity to 5.3% of that of NS3–NS4A, and NS3H had similar RNA unwinding activity (95.2%) to that of NS3–NS4A. However, all the NS3 proteins, except K1236N, retained significant levels of ATPase activity. Interestingly, NS3H displayed even weaker ATPase activity than NS3, 64% vs. 91.6%. Apparently, little correlation exists between the ATPase activity and the duplex RNA unwinding activity of the proteins. The data imply that the reduced-RNA unwinding activity of NS3 and the R1487Q mutant was not attributable to reduced-ATPase activity.

RNA binding activities of the NS3 proteins

RNA binding activities of the NS3 proteins were compared by examining the RNA–enzyme complex formation in the presence of a non-hydrolyzable ATP-γS. The formation of RNA–enzyme complexes steadily increased with time, reaching a plateau at about 10 min (data not shown). The results shown in Fig. 3 concern the RNA binding at 5 and 15 min, respectively. At 5 min, when binding was changing linearly, both NS3 and mutant R1487Q had higher affinity for RNA binding than did NS3–NS4A or NS3H (Fig. 3B, black bars). The difference, however, was less obvious when the binding reached the plateau at 15 min (gray bars).

Processivity of duplex RNA unwinding of the NS3 proteins

Previous helicase reactions were all conducted in a manner that allowed recycling of the helicase to rebound and unwind the duplex RNA. Under such conditions, the above data indicated that NS3 exhibited lower RNA helicase activity than NS3–NS4A or NS3H (Fig. 2 and Table 1). However, NS3 did not display lower ATPase activity or RNA binding activity than did NS3H or NS3–NS4A (Fig 3 and Table 1). Analyzing whether the lower helicase activity of NS3 is due to a lower processivity of duplex unwinding is therefore of interest. The duplex RNA unwinding activities of the NS3 proteins were therefore compared in time-course experiments under single processive cycle conditions, using a method described previously by Paolini et al. [10,11]. To this aim, heparin was used to trap both free enzyme molecules and molecules dissociated from the substrate during the reaction. In these experiments, the concentration of enzyme was increased to 180 nM to ensure that the enzyme bound all dsRNA substrates. Heparin was added together with ATP after the proteins were pre-incubated with RNA substrates. In addition, excess amounts of unlabeled ssRNA, complementary to the template strand, were added to prevent product re-annealing during the reaction in the presence of heparin.

Table 1
Summary of RNA helicase, ATPase, and RNA binding activities of the NS3 proteins

Enzyme	Helicase		ATPase		RNA binding	
	k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	Percentage of NS3–NS4A	k_{cat}/K_m ($mM^{-1}\,min^{-1}$)	Percentage of NS3–NS4A	RNA binding (%)[a]	Percentage of NS3–NS4A
NS3H	6110.4	95.2	1283.7	64	51	106.3
NS3	1961.8	30.6	2005.9	91.6	80	166.7
NS3-NS4A	6417.5	100	2189.5	100	48	100
K1236N	UD[b]	0	UD	0	44	91.7
R1487Q	338.2	5.3	2193.3	100.2	76	158.3

[a] The data were taken from the 5-min binding reactions in the absence of K^+ (Fig. 3).
[b] UD, undetectable. The mutant completely lost enzymatic activity.

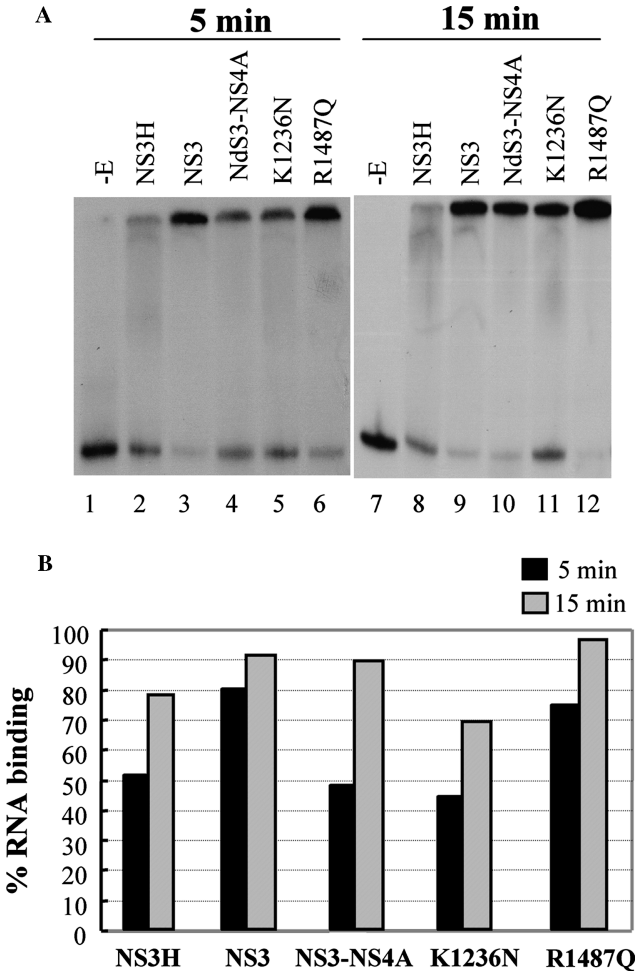

Fig. 3. RNA binding activities of the NS3 proteins. (A) Gel-shift assays. The reactions contained 360 nM of each of the purified enzymes and 8 μM unlabeled single-stranded RNA plus 0.66 μM radiolabeled partial dsRNA substrates. Reaction was carried out at the indicated time (5 or 15 min), and the bound complexes were analyzed on a native 4% polyacrylamide gel. (B) Quantitative comparison of the RNA binding activity. The percentages of RNA binding activity were determined by bound complex/bound complex + free substrate × 100% and are presented as bars.

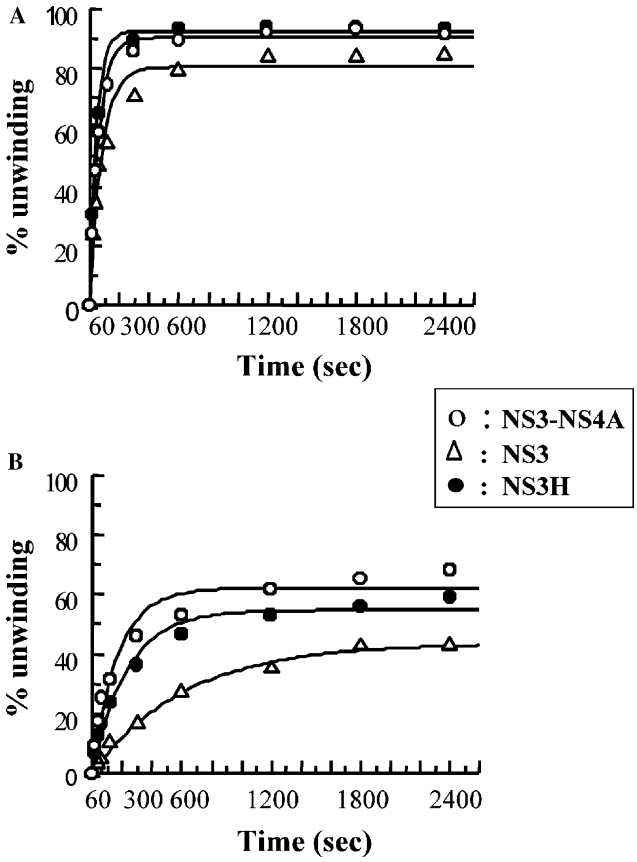

Fig. 4. Time-course of duplex RNA unwinding under single or multiple cycles of unwinding conditions. (A). Reaction without heparin—multiple cycle conditions. The reactions contained 180 nM enzymes and 30 nM labeled dsRNA substrate. After pre-incubation for 15 min at room temperature, the reactions were started by adding ATP together with 500 nM unlabeled single-stranded release strand RNA and performed at 37 °C. At the indicated times, aliquots were withdrawn, mixed with 5× loading dye to stop the reaction, and analyzed on a native 8% polyacrylamide gel. (B) Reaction with heparin—single processive cycle conditions. The reactions were carried out as described in (A), except in that 5 μg/ml heparin was added together with ATP to start the reactions.

The proportion of duplex unwinding activity measured in the presence of heparin is indicative of a single processive cycle of unwinding and represents an index of helicase processivity [10,11]. As shown in Fig. 4A, the maximal strand unwinding in the absence of heparin was about 80%, 91%, and 90% for NS3, NS3H, and NS3–NS4A, respectively. The reactions rapidly reached a plateau after about 5 min of incubation. Fig. 4B shows that the maximal unwinding in the presence of heparin was about 42%, 58%, and 62% for NS3, NS3H, and NS3–NS4A, respectively. Moreover, about 10 min was required for NS3–NS4A and NS3H to reach the plateau of the reaction, but more than 30 min was required for NS3 to reach the plateau. The results suggest that the NS3 enzyme is less processive than NS3–NS4A or NS3H, producing smaller amounts of unwound product before dissociating from the substrate.

Protease activity of the NS3–NS4A protein and its helicase mutant proteins

Numerous studies have demonstrated the strong dependence of the proteolytic activity of NS3 on NS4A [14,15]. This study examined only whether mutations in the helicase domain affect the N-terminal proteolytic activity of NS3–NS4A. An intramolecularly quenched fluorogenic substrate (IQFS) peptide [12], based on fluorescence resonance energy transfer between *ortho*-aminobenzoic acid (Abz) and *N*-(ethylene-diamine)-2,4-dinitrophenylamide (EDDnp) (Fig. 5A), was utilized herein to assess the proteolytic activities of NS3–NS4A

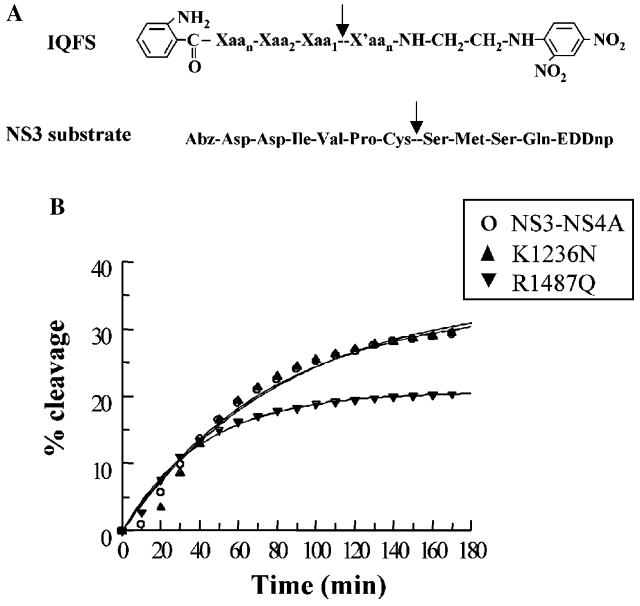

Fig. 5. Protease activity assays with an IQFS peptide. (A) Chemical structures of the IQFS peptide. (B) Time-course of proteolytic cleavage of the NS3–NS4A and mutant proteins. The reactions contained 100 μM of fluorogenic substrates and 100 nM of the NS3–NS4A or mutant proteins. The cleavage of IQFS peptide by the enzymes was continuously monitored using a fluorescence plate reader. The fluorescence data were converted into the concentrations of cleaved products using a standard curve made up from several known concentrations of the Abz–peptide–OH. The percentages of peptide cleaved from the 100 μM substrate were then calculated.

and its mutant proteins. The peptide substrate sequences were based on the HCV NS5A/NS5B junction. Upon cleavage of the IQFS peptide, the fluorophore was separated from the quenching group, generating a fluorescence signal [12]. The data shown in Fig. 5B demonstrate the time-course proteolytic reactions of NS3–NS4A and the two mutant proteins. The K1236N mutant had a proteolytic activity comparable with that of the wild-type NS3–NS4A, whereas the R1487Q mutant had reduced proteolytic activity. The proteolytic activity of NS3 was too low to be detected in this assay (data not shown). The results described here, therefore, indicate that the protease activity of the HCV NS3 protein could be influenced not only by NS4A but also by the C-terminal helicase domain, suggesting the possibility of interactions among NS4A, the N-terminal and the C-terminal domains of NS3.

Discussion

The data of this study demonstrate that the presence of the protease domain in the full-length NS3 reduces the RNA unwinding activity below that of NS3H. Associating NS3 with NS4A, however, reverses the inhibitory effects. However, the lower efficiency of the duplex RNA unwinding activity of NS3 is not attributable to a decreased ATPase activity, but is due to a lower processivity, as compared to NS3–NS4A or NS3H. Moreover, when the RNA binding activity was elucidated by gel shift assay, NS3 exhibited even higher binding affinity than did NS3–NS4A or NS3H. Taking together, we hypothesize that the overly strong binding activity of NS3 may impede its efficient motion along the ssRNA strand. In the absence of the protease domain, as in the case of NS3H, or when the protease domain is removed by association with NS4A, as in the case of NS3–NS4A, the interaction of the enzyme with the RNA substrate may be slightly reduced, thus allowing the efficient motion of the enzyme. This hypothesis, however, does not rule out the possibility that NS4A might virtually stabilize the NS3 protein. The various conformations of NS3H, NS3, and NS3–NS4A are thus associated with the inherently different processivities.

NS4A is a 54-residue protein that has been shown to be an integral structural component of NS3 [16] and to play a significant role in activating the N-terminal protease activity [14,15] and stabilizing the entire NS3 protein [17]. Yet, its role in modulating the C-terminal helicase activity is quite controversial. Some reports have demonstrated that NS4A peptide inhibited helicase activity in a dose-dependent manner [18,19]. However, Howe et al. [7] reported that the single-chain NS3–NS4A or the NS3–NS4A complex exhibited better duplex RNA unwinding activity than NS3 or NS3H. A recent study of Pang et al. [20] also reported that NS3 alone was a surprisingly poor helicase on RNA, but that the RNA helicase activity was promoted by cofactor NS4A.

The reason for the controversy of the results is unclear, but may be related to the systems that the recombinant proteins were produced, the ways in which the NS3–NS4A proteins were expressed—single-chain, complex form, or association with a synthetic NS4A peptide, and the conditions under which the helicase activity was measured. The results obtained here argue against that NS3 has stronger helicase activity than NS3–NS4A, and that NS4A inhibits the RNA helicase activity [19]. On the other hand, although we agree with Pang's et al. [20] assertion that NS3 is a poor RNA helicase, we offer a different explanation for the results. Pang et al. demonstrated that the presence of NS4A did not change the single-cycle processivity of NS3 on RNA unwinding, whereas NS4A acted before unwinding to promote NS3 binding to RNA. However, our data showed the opposite conclusion (Figs. 3 and 4). The reason for this discrepancy is not clear, but might be related to the different methods of measuring the amounts of enzyme–RNA complexes and of the single-cycle processive unwinding. The authors believe that their data did exhibit some enhancement of the single-cycle processive unwinding of NS3–NS4A over that of NS3 [20].

Some previous studies compared the protease activities of the isolated protease domain and the full-length NS3, and indicated that the presence of the C-terminal helicase domain did not significantly influence the proteolytic activity [10,18]. This study compares the proteolytic activities of two helicase mutants on the NS3–NS4A backbone. They turned out to behave differently (Fig. 5B). It is conceivable that mutation at Arg-1487 indirectly causes a large global change in the conformation, affecting not only RNA helicase activity, but also protease activity. However, the K1236N mutant and several other helicase mutants, as reported by others [10,18], may not undergo large conformational changes, and so do not significantly influence the N-terminal protease activity.

In conclusion, the presence of protease and helicase domains in a single polypeptide and the strong association of NS4A with NS3 not only represent an economical packaging of essential replicative components, but could also be associated with functional dependence among these domains, maximizing activity in nature. Consequently, we propose that the NS3–NS4A complex constitutes a more biologically relevant enzyme than NS3 or NS3H in screening antivirus drugs.

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