A novel recombinant single-chain hepatitis C virus NS3-NS4A protein with improved helicase activity

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Abstract

Hepatitis C virus (HCV) nonstructural protein 3 (NS3) has been shown to possess protease and helicase activities and has also been demonstrated to spontaneously associate with nonstructural protein NS4A (NS4A) to form a stable complex. Previous attempts to produce the NS3/NS4A complex in recombinant baculovirus resulted in a protein complex that aggregated and precipitated in the absence of nonionic detergent and high salt. A single-chain form of the NS3/NS4A complex (His-NS4A_{21–32}-GSGS-NS3_{3–631}) was constructed in which the NS4A core peptide is fused to the N-terminus of the NS3 protease domain as previously described (Taremi et al., 1998). This protein contains a histidine tagged NS4A peptide (a.a. 21–32) fused to the full-length NS3 (a.a. 3–631) through a flexible tetra amino acid linker. The recombinant protein was expressed to high levels in *Escherichia coli*, purified to homogeneity, and examined for NTPase, nucleic acid unwinding, and proteolytic activities. The single-chain recombinant NS3-NS4A protein possesses physiological properties equivalent to those of the NS3/NS4A complex except that this novel construct is stable, soluble and sixfold to sevenfold more active in unwinding duplex RNA. Comparison of the helicase activity of the single-chain recombinant NS3-NS4A with that of the full-length NS3 (without NS4A) and that of the helicase domain alone suggested that the presence of the protease domain and at least the NS4A core peptide are required for optimal unwinding activity.

Keywords: helicase; hepatitis C; NS3; NS4A; recombinant

Hepatitis C is one of the world's most pandemic and insidious diseases. According to the World Health Organization, there are approximately 170 million carriers worldwide, with prevalence up to 0.5–10% (WHO, 1998). In the United States, 4 million individuals are afflicted with hepatitis C (Alter & Mast, 1994). The causative agent, hepatitis C virus (HCV), was identified in 1989, and accounted for 50–60% of the non-A, non-B transfusion associated hepatitis (Alter et al., 1989; Choo et al., 1989; Kuo et al., 1989). To date, interferon- α monotherapy and interferon- α 2b and ribavirin combination therapy (Rebetron, Schering-Plough, Kenilworth, New Jersey) are the only approved treatments. However, less than 10% of the patients responded to interferon- α monotherapy, and 41% of the patients responded to Rebetron combination therapy (Reichard et al., 1998). Therefore, it is important to develop effective antiviral agents against the various viral targets to effectively combat the disease.

HCV, a member of the *Flaviviridae* family, is a positive-sense, single-stranded RNA virus with genome size of ~9.4 kb (Takami-zawa et al., 1991; Heinz, 1992; Mizokami & Ohba, 1993; Ohba et al., 1996). The genomic RNA encodes a polyprotein of 3010–

3011 amino acid residues in the order of NH₂-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Lohmann et al., 1996; Simmonds, 1996). This polyprotein is processed by host and viral proteases (Grakoui et al., 1993; Shimotohno et al., 1995). NS3 has been the target of interest for antiviral discovery because of its important role in HCV replication. NS3 has two major functional domains: the amino terminal one-third of the protein is a serine protease responsible for some key aspects of polyprotein processing (Shimotohno et al., 1995), and the carboxyl terminal two-thirds of NS3 share sequence similarity with those of the DEAD box family of RNA helicases (Gorbalenya et al., 1988; Koonin & Dolja, 1993; Korolev et al., 1998). Virus-encoded helicases such as vaccinia NPH-II and plum pox potyvirus CI have been demonstrated to be important for viral replication and production of infectious virions (Gross & Shuman, 1996, 1998; Fernandez et al., 1997). There are two enzymatic activities associated with the HCV helicase—NTPase and nucleic acid unwinding; the former is believed to provide an energy source for the unwinding reaction through NTP hydrolysis (Suzich et al., 1993; Kim et al., 1997a, 1997b). Studies of the crystal structure of HCV helicase reveal that this molecule consists of three domains—domain I contains the ATP- and Mg⁺⁺-binding sites, domain II is speculated to be a nucleic acid binding site, and domain III is characterized as having extensive helical structure. In between domain I and II lies a cou-

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pling region speculated to be involved in transforming chemical energy into motion associated with unwinding (Kim et al., 1996; Yao et al., 1997; Yan et al., 1998).

Despite these different functions during HCV replication, there is no evidence that the NS3 protease and helicase are ever separated from one another in HCV infected cells. Likewise, only the 70 kDa NS3 protein containing both the protease and helicase domains has ever been detected in cells transfected with recombinant vaccinia expressing HCV nonstructural proteins (Grakoui et al., 1993). In addition, NS3 has been found to spontaneously associate with NS4A to form a stable noncovalent NS3/NS4A complex in vivo (Grakoui et al., 1993; Failla et al., 1995). The covalent fusion of the protease and helicase domains in NS3 as well as the spontaneous formation of a complex with NS4A suggests that there may be functional dependence between these polypeptides to achieve additional activities other than the fundamental enzymatic properties.

To study the enzymatic properties of HCV helicase with as much biological relevance as possible, it is desirable to generate as authentic a full-length NS3/NS4A complex as possible outside a cellular environment. Previous attempts to express a full-length NS3/NS4A protein complex in recombinant baculovirus resulted in a material with a propensity to aggregate, which limited its application (Sali et al., 1998). Based on the results from the co-crystal structure studies of the NS3_{1–181}/NS4A_{21–39} complex (Kim et al., 1996; Yan et al., 1998), it was reasoned that if an appropriate flexible spacer was provided, it might be possible to link the NS4A core peptide (a.a. 21–32) to the amino terminal region of the NS3 protease domain and mimic the interaction of the NS4A with the N-terminus of the NS3 protease (Taremi et al., 1998). In this paper, the construction and expression of an arguably more physiologically relevant single-chain recombinant NS3–NS4A protein are described. This purified recombinant His-NS4A_{21–32}–GSGS–NS3_{3–631} not only has properties similar to those described for the NS3/NS4A protein complex generated in eukaryotes (Morgens-tern et al., 1997), but also appears to be stable, more soluble, and more robust at unwinding RNA compared with the His-NS3/NS4A complex previously reported (Sali et al., 1998). In addition, comparison of the unwinding activity of the recombinant His-NS4A_{21–32}–GSGS–NS3_{3–631} with that of the helicase domain and that of the full-length NS3 (without NS4A) indicated that the presence of the protease domain and the core peptide of NS4A are important for optimal HCV helicase activity.

Results

Cloning and expression of a single-chain recombinant His-NS4A_{21–32}–GSGS–NS3_{3–631}

To produce a recombinant HCV NS3/NS4A protein complex with structural conformation similar to its biological counterpart, an expression plasmid encoding HCV (1b/BK strain) NS4A peptide GSVVIVGRIILS (NS4A a.a. 21–32) covalently joined to a NS3 protein via an amino acid linker GSGS was engineered. The cDNA fragment encoding this recombinant HCV protein was inserted into a pET-22b+ expression vector that provided an additional three amino acids and six histidine residues followed by a thrombin cleavage site at the amino terminus of the NS4A peptide (Fig. 1). Induction of the bacteria harboring this expression plasmid resulted in the production of a recombinant protein of ~72 kDa (Fig. 2, lane 2). Most of the expressed recombinant

Fig. 1. Schematic diagram of constructs expressing HCV His-NS4A_{21–32}–GSGS–NS3_{3–631} (1b/BK strain), His-NS3/NS4A (1a strain), His-NS3_{1–631}, and His-NS3_{182–631}. HIS, hexahistidine metal chelation tag; GSVVIVGRIILS, NS4A peptide (a.a. 21–32) and GSGS, an amino acid linker connecting the NS4A peptide to the NS3 protein. Boxes represent open reading frames encoding a full-length NS3 in NS4A_{21–32}–GSGS–NS3_{3–631} and His-NS3_{1–631}, a polyprotein protein of NS3, NS4A, and the amino terminus of NS4B in His-NS3/NS4A and the helicase domain in His-NS3_{182–631}. Protease and helicase domains of NS3 are indicated. Proteolytic cleavage sites of thrombin and NS3 are shown by arrows. Numbers of predicted amino acids present in the expressed proteins and the cleavage products are indicated. His-NS4A_{21–32}–GSGS–NS3_{3–631}, His-NS3_{1–631}, and His-NS3_{182–631} were expressed in *E. coli* [BL21(DE3)], and His-NS3/NS4A was expressed by recombinant baculovirus in SF9 insect cells.

protein existed in the soluble fraction of the bacterial lysate. Further purification of the expressed protein using nickel-affinity and size-exclusion chromatography yielded a product with greater than 95% homogeneity as measured by SDS-PAGE (Fig. 2, lanes 3 and 4). Approximately 6 mg of protein per liter of culture was obtained following purification. The purified protein was highly soluble and did not appear to aggregate following dialysis to remove the n-octyl- β -D-octyl glucoside (β OG) detergent. The identity of the purified protein was confirmed by (1) Western blot using an anti-NS3 polyclonal antibody, (2) Edman sequencing confirming the expected N-terminal 18 residues of the protein including the histidine tag (GSSHHHHHHSGLVPRGSH), and (3) MALDI mass spectrometry, which yielded a molecular mass of $70,689 \pm 10$ Da, in good agreement with the calculated value of 70,643 Da for the His-NS4A_{21–32}–GSGS–NS3_{3–631} (results not shown).

Confirmation of NS3 proteolytic activity

To confirm that the amino terminus of NS3 protein had undergone the folding events associated with NS4A peptide intercalation (Kim

Fig. 2. Expression of a single-chain recombinant HCV His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ complex. Aliquots of bacterial lysate or protein samples at different stages of purification were analyzed in a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue G. Lane 1, molecular weight standards in kDa; lane 2, induced precleared cell lysate; lane 3, pooled fractions following nickel-imidoacetate purification; lane 4, pooled fractions following sephacryl-200 chromatography.

et al., 1996), the proteolytic activity of the construct was compared with that of the full-length NS3/NS4A protein complex from the closely related 1a strain, generated using recombinant baculovirus (Sali et al., 1998). As seen in Table 1, the activity of the His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ was roughly comparable to that of the full-length His-NS3/NS4A protein complex. As the efficiency of the protease dropped approximately 40-fold in the absence of the NS4A cofactor (Shimizu et al., 1996; Landro et al., 1997), it would appear that, as with the NS4A₂₁₋₃₂-GSGS-NS3₃₋₁₈₁ protease do-

main (Taremi et al., 1998), the tethered NS4A peptide has reorganized the amino terminus of the His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ and restored full proteolytic activity.

His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ possesses ATPase activity comparable to His-NS3/NS4A

The purified single-chain recombinant His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ was evaluated for its ability to hydrolyze ATP in a standard ATPase assay. As shown in Figure 3A, His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ exhibited poly U-dependent ATPase activity, which was proportional to the concentration of the enzyme. A greater than 10-fold increase in ATP hydrolysis was observed using 1 nM of enzyme with the addition of poly U. Only minimal ATP hydrolysis, however, was detected in the absence of poly U under the same experimental conditions. The detected ATPase activity was specific to His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ because no ATP hydrolysis was seen in a sample prepared from bacteria that expressed only the NS3 protease domain (data not shown). The level of ATPase activity found in His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ was comparable to that present in His-NS3/NS4A complex (Fig. 3B). Approximately $2-3 \times 10^5$ pmol of ATP per pmol of enzymes was hydrolyzed in the presence of nucleic acid polymers under the assay conditions.

Characterization of His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ RNA unwinding activity

The single-chain recombinant His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ was investigated for the ability to unwind double stranded RNA (dsRNA). A dsRNA with 5' and 3' overhangs was incubated with increasing concentrations of the purified enzyme, and the reaction product was analyzed in 15% SDS-PAGE. Native duplex and heat denatured single-stranded RNAs displayed different mobilities in SDS-PAGE, as shown in Figure 4, lanes 1 and 2. His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ displaced single-stranded RNA (ssRNA) from the dsRNA substrate in a concentration-dependent manner (Fig. 4, lanes 3–5). As a negative control, a truncated NS3 (His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₁₈₁) containing only the protease domain that was expressed and purified in the same manner as that of the His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ was used. No displacement of ssRNA from dsRNA was detected in the sample, excluding the possibility of contamination by a bacterial helicase activity (result not shown).

To determine the directionality of the His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ unwinding activity, dsRNA substrates with 5'/5', 3'/3' overhangs or blunt ends were used in the unwinding assay. As illustrated in Figure 4, His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ unwound only substrates containing 3' overhangs (5'/3' and 3'/3', Fig. 4, lanes 3–5 and lanes 13–15). Both 5'/5' or blunt-ended dsRNA were not utilized by the enzyme (Fig. 4, lanes 8–10 and lanes 18–20). These data suggested that His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ acted unidirectionally from 3' to 5', consistent with the property of the truncated NS3 HCV helicase domain (Tai et al., 1996). More unwound product was detected in the sample containing the 3'/3' substrate than that containing the 3'/5' substrate (compare Fig. 4, lanes 3–5 to lanes 13–15), which might be due to a higher availability of the 3' overhangs as well as a shorter duplex region present in the 3'/3' substrate (21 bp in the 3'/3' substrate vs. 29 bp in the 3'/5' substrate).

The effect of pH, ATP-MgCl₂, and salt on nucleic acid unwinding activity of the His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ were also in-

Table 1. Comparison of proteolytic activity of His-NS3/NS4A and His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁^a

	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
His-NS3/NS4A	14 ± 1	18 ± 1	13,000 ± 2,000
His-NS4A ₂₁₋₃₂ -GSGS-NS3 ₃₋₆₃₁	18 ± 2	51 ± 4	6,000 ± 1,000

^aThe kinetic constants for proteolytic activities were determined from Lineweaver-Burk plots by the peptide cleavage assay as described in Materials and methods. 5A/5B peptide DTEDVVCC*SMSYTWG-K was used as the substrate.

vestigated using a scintillation proximity assay (SPA). Optimal conditions required for the unwinding occurred at pH 7.0 with ATP and MgCl_2 concentrations of 1–3 and 0.25–0.5 mM, respectively, while 50 mM K^+ completely abolished unwinding. In addition, the enzyme was found to be very stable, and no significant reduction of unwinding activity was observed with up to seven freeze/thaw cycles (data not shown).

Comparison of helicase activity between His-NS4A_{21–32}–GSGS-NS3_{3–631} and His-NS3/NS4A protein complex

To determine if the helicase activity of His-NS4A_{21–32}–GSGS-NS3_{3–631} was comparable to the natural NS3/NS4A protein complex, the nucleic acid unwinding activity of the recombinant single-chain enzyme was compared with that of the His-NS3/NS4A protein complex, expressed in recombinant baculovirus (Sali et al., 1998), under the same experimental conditions in a scintillation proximity assay. When saturating substrate was provided, His-NS4A_{21–32}–GSGS-NS3_{3–631} was found to be six- to sevenfold more active than the His-NS3/NS4A protein complex (Fig. 5A). Because the conditions used in the previous experiment were similar to the optimal conditions for the His-NS4A_{21–32}–GSGS-NS3_{3–631}, the two enzymes were also compared under their corresponding optimal conditions. In the linear range of the assay for both enzymes (1–10 pM), about sixfold to sevenfold more product

was released by the His-NS4A_{21–32}–GSGS-NS3_{3–631} than that from an equivalent concentration of the His-NS3/NS4A protein complex (Fig. 5B). In addition, 10-fold less His-NS4A_{21–32}–GSGS-NS3_{3–631} was required to yield a similar percentage of unwound products compared with the full-length His-NS3/NS4A protein complex in the corresponding reactions (Fig. 5B).

Presence of protease domain and NS4A in His-NS4A_{21–32}–GSGS-NS3_{3–631} enhances helicase RNA unwinding activity

Several laboratories have previously demonstrated helicase activity in amino terminally truncated NS3 retaining the helicase domain (Suzich et al., 1993; Kim et al., 1995; Tai et al., 1996); however, results from studies of HCV polyprotein processing have suggested that the HCV helicase domain is fused to the protease domain, which, in turn, complexes with NS4A (Grakoui et al., 1993; Shimotohno et al., 1995; Tanji et al., 1994). The formation of this protein complex might affect HCV helicase activity. To demonstrate that His-NS4A_{21–32}–GSGS-NS3_{3–631} represents a construct with physiological relevance, the effect of NS4A and the amino terminal region (protease domain) of NS3 on helicase activity was investigated. Expression plasmids encoding a HCV (1b/BK strain) full-length NS3 (His-NS3_{1–631}) and an amino terminally truncated NS3 (His-NS3_{182–631}), in which the protease domain was deleted, were cloned and expressed in *Escherichia*

Fig. 5. Comparison of nucleic acid unwinding activity between His-NS4A_{21–32}–GSGS-NS3_{3–631} and NS3/NS4A complex. Nucleic acid unwinding activities of His-NS4A_{21–32}–GSGS-NS3_{3–631} and His-NS3/NS4A complex were compared under the same (A) and corresponding optimal (B) reaction conditions in a SPA assay as described under Materials and methods. **A:** Under the same reaction condition. Nucleic unwinding activities of His-NS4A_{21–32}–GSGS-NS3_{3–631} and His-NS3/NS4A complex were compared in two concentrations (10 and 50 pM) in the presence of 100 mM MOPS (pH 7.0), 2 mM ATP, 0.5 mM MgCl_2 , 0.5 mM DTT, 100 $\mu\text{g}/\text{mL}$ BSA, 1 U RNase inhibitor, and 1 nM of double-stranded RNA substrate. The reaction was incubated at 37 °C for 1 h. **B:** Under corresponding optimal conditions. The assay conditions for the His-NS4A_{21–32}–GSGS-NS3_{3–631} were 100 mM MOPS (pH 7.0), 0.5 mM MgCl_2 , 2 mM ATP, 0.5 mM DTT, 100 $\mu\text{g}/\text{mL}$ BSA, 2% DMSO, and 1 U RNase inhibitor. For the His-NS3/NS4A complex, the assay conditions were 100 mM PIPES (pH 6.0), 1 mM MgCl_2 , 2 mM ATP, 0.6 mM DTT, 100 $\mu\text{g}/\text{mL}$ BSA, 2% DMSO, and 1 U RNase inhibitor. In both reactions, 500 pM ^{33}P -labeled double-stranded RNA was used.

coli (Fig. 1). RNA unwinding activity of these recombinant proteins was examined in the scintillation proximity assay. Compared with the full-length NS3 (His-NS3_{1–631}) and the single-chain recombinant NS3–NS4A (His-NS4A_{21–32}–GSGS–NS3_{3–631}), the amino terminally truncated NS3 (His-NS3_{182–631}) showed significantly reduced helicase activity. Nucleic acid unwinding of the duplex RNA could only be detected at high enzyme concentrations (2–10 nM) (Fig. 6). The reduced activity seen in the His-NS3_{182–631} was not due to the disordered structure introduced into the amino terminus of the truncated protein as a result of the deletion, as about twofold higher ATPase activity compared with that of the full-length NS3 was observed, suggesting that the His-NS3_{182–631} has an ordered amino terminus with an intact ATP binding domain (Fig. 7). Because the nucleic acid unwinding activity in His-NS3_{182–631} was barely detected at 1 nM, at which significant ATP hydrolysis was observed (compare Fig. 7 to Fig. 6), it was concluded that the reduced unwinding activity seen in His-NS3_{182–631} was not due to inefficient ATP hydrolysis. Absence of the protease domain or part of the protease domain appeared to account for the reduced unwinding activity present in the amino terminally truncated NS3.

The effect of NS4A on NS3 helicase activity was investigated by comparing the unwinding activity of the single-chain recombinant NS3–NS4A (His-NS4A_{21–32}–GSGS–NS3_{3–631}) with that of the full-length NS3 (His-NS3_{1–631}). Fourfold enhancement of the unwinding activity was observed in the single chain recombinant NS3–NS4A (Fig. 6), suggesting that the NS4A core peptide might also promote helicase activity in some fashion—most likely through its impact on the protease domain.

In summary, the evidence suggests that both the protease domain and the NS4A cofactor play a significant role in promoting HCV NS3 helicase activity. Absence of either the protease domain

Fig. 6. Effect of protease domain and NS4A on HCV helicase activity. Nucleic acid unwinding activity of His-NS4A_{21–32}–GSGS–NS3_{3–631}, His-NS3_{1–631}, and His-NS3_{182–631} was compared in the presence of 2 nM duplex RNA under the reaction conditions as described in Materials and methods. The amount of unwound ssRNA was quantified using a TOPCount reader.

Fig. 7. ATPase activity of His-NS3_{1–631} and His-NS3_{182–631}. ATPase activity of increasing concentrations of His-NS3_{1–631} and His-NS3_{182–631} was measured in the presence or absence of 65 μg/μL poly U at 37 °C for 1 h. The amount of hydrolyzed ATP was estimated by quantifying the amounts of ³²P-labeled ADP and ATP following the hydrolysis using a Phospho-Imager.

or the NS4A core peptide cofactor resulted in reduced nucleic acid unwinding activity. Although the experimental data could not delineate the mechanism of action, it clearly implicated the importance of the NS3/NS4A interaction and suggests that the single-chain recombinant NS3–NS4A may be at least an equivalent to, if not a more appropriate rendering of, the NS3/NS4A complex for in vitro evaluation and study.

Discussion

From the inception of modern biochemistry and molecular biology, researchers have faced with the issue of the validity of the reductionist paradigm. Whether in pursuit of a target molecule for the development of therapeutics or the unraveling of a biological process, the isolation of a single component from the integrated organic web has raised the question of what is the best representation of the natural phenomenon. Although the default position has typically been to duplicate Nature's details to the letter (nucleotide or amino acid) in many cases, this strategy has been problematic, dissection and analysis of membrane-associated protein complexes being one of the most notable examples.

Although containing relatively few components, positive-strand RNA viruses such as picornavirus, HCV, etc., are believed to form higher order replication structures with host membranes and proteins (Wimmer et al., 1993; Xiang et al., 1995). Isolation and overexpression of certain individual components of these “replisomes” (e.g., the 3A protein of poliovirus or the NS4A protein of HCV) have been difficult if not impossible without their compan-

ion molecules to provide the necessary context for their proper behavior/function, limiting detailed understanding of their role or mode of action (Lama et al., 1994; Sali et al., 1998). In an effort to obtain deeper insight into their mechanism of action, various reconstitution strategies have been employed—the ultimate being the recombinant molecular reconstitution experiment where individual components are grafted together in a manner to regenerate known function (i.e., single-chain antibodies, hormones, receptor, etc.) (Altenschmidt et al., 1996; Finney et al., 1998; Mousli et al., 1998). In this study a similar approach was used to generate a fully functional NS3/NS4A unit from what was a relatively intractable two-protein membrane-associated complex.

In the HCV NS3/NS4A complex, NS4A has been shown to (1) activate the NS3 protease activity (Lin & Rice, 1995; Tomei et al., 1996), (2) promote membrane association and cytoplasmic retention of NS3 (Tanji et al., 1995; Koch et al., 1996; Muramatsu et al., 1997), and (3) stabilize NS3 from cellular degradation (Tanji et al., 1995). Crystallographic studies of the NS3 protease domain complexed with a synthetic NS4A peptide confirmed that NS4A was an integral structural component of NS3, and played a significant role in structuring residues 1–28 of the protease domain and stabilizing the entire NS3 protein (Yan et al., 1998). The intercalated portion of NS4A was determined to be at the central hydrophobic region spanning from residues 21 to 32 (Kim et al., 1996; Yan et al., 1998). Hitherto, several recombinant versions of an overexpressed HCV helicase have been available; these include a truncated NS3 containing only the helicase domain (Suzich et al., 1993; Jin & Peterson, 1995; Gwack et al., 1996, 1997; Tai et al., 1996), the full-length NS3 (Gwack et al., 1995; Gallinari et al., 1998), and the complex of NS3/NS4A (Sali et al., 1998). Truncated NS3 helicase or full-length NS3 alone, although functionally active, might not represent the true biological entity as suggested by the transfection and biochemical studies (Grakoui et al., 1993; Failla et al., 1995). Previous attempts to express a full-length NS3/NS4A protein complex in recombinant baculovirus suffered from poor yield due to protein aggregation (Sali et al., 1998). High detergent and salt concentrations were required to maintain the expressed protein in soluble form. Based on the results from the co-crystal structure studies of the NS3_{1–181}/NS4A_{21–39} complex, a single-chain recombinant NS3–NS4A protein in which the NS4A peptide (a.a. 21–32) was fused through a flexible tetrapeptide linker to the amino terminus of the NS3 protein was constructed first using only the protease domain (Taremi et al., 1998) and subsequently using the full-length NS3 protein. The resultant constructs allow the interaction of the NS4A peptide with the amino terminus of NS3 in a way that appears by all measures of proteolytic activity to mimic the natural intercalation (Table 1). Furthermore, results from characterization of the ATPase (Fig. 3A,B) and the nucleic acid unwinding activities (Fig. 4) in the His-NS4A_{21–32}–GSGS-NS3_{3–631} support the notion that this single-chain recombinant NS3–NS4A represents a bona fide HCV helicase.

The single-chain recombinant His-NS4A_{21–32}–GSGS-NS3_{3–631} has several advantages over the His-NS3/NS4A complex from recombinant baculovirus. First, the expressed His-NS4A_{21–32}–GSGS-NS3_{3–631} was found to be present in the soluble fraction of the bacterial lysate, and the purified protein was readily soluble in the absence of detergent. The lack of the hydrophobic amino and carboxyl terminal regions of the NS4A in this recombinant His-NS4A_{21–32}–GSGS-NS3_{3–631} most likely accounts for the im-

proved solubility profile compared with that of the full-length NS3/NS4A protein complex (Sali et al., 1998). Second, the construction of the single-chain recombinant His-NS4A_{21–32}–GSGS-NS3_{3–631} circumvented the problem of dissociation of the NS4A peptide from NS3 protein as with the protease domain (Taremi et al., 1998). The dissociation constant of NS3 and NS4A peptide has been estimated to be between 3.8 and 7.7 μ M, and the affinity was observed to be further reduced by buffers of low ionic strength (Bianchi et al., 1997), a condition that is required for measuring nucleic acid unwinding activity of HCV helicase *in vitro*. In recombinant His-NS4A_{21–32}–GSGS-NS3_{3–631}, the NS4A peptide has been covalently linked to NS3, allowing study of the enzymatic properties with a structure equivalent to that of the NS3/NS4A protein complex but under physiologically relevant conditions (i.e., without detergent and in physiological ionic strength buffer). Third, the His-NS4A_{21–32}–GSGS-NS3_{3–631} appears to be very stable. Repeated freezing and thawing did not affect the enzymatic activity making this reagent a viable tool for antiviral drug discovery. Finally, it is interesting to note that the single-chain recombinant His-NS4A_{21–32}–GSGS-NS3_{3–631} is fivefold to sevenfold more active than the full-length NS3/NS4A complex (Fig. 5A,B). Whether this is due to an increase in solubility, stability, or due to the absence of inappropriate interactions of the amino or carboxy regions of the NS4A with either NS3 or the substrate remains unclear.

Characterization of HCV helicase using constructs containing the minimal helicase domain has been extensively studied by several laboratories (Suzich et al., 1993; Jin & Peterson, 1995; Kim et al., 1995, 1997a, 1997b; Tai et al., 1996). However, because NS3 (containing protease and helicase domains) and NS4A exist as a membrane-associated complex in nature, it is not clear if the protease domain, the NS4A, or the membrane itself affect helicase activity. Experimental results obtained from comparing the unwinding activity of the single-chain recombinant NS3–NS4A, the full-length NS3 (without NS4A), and the helicase domain alone suggest that the presence of the protease domain and the NS4A core peptide appear to enhance helicase activity (Fig. 6). The mechanism leading to the enhancement of the activity is not clear. However, addition of nucleic acid polymers has been shown to inhibit (Gallinari et al., 1998) and stimulate (Morgenstern et al., 1997) protease activity in NS3 and NS3/NS4A, respectively, suggesting that RNA may interact both directly and indirectly with the protease domain. It is, therefore, conceivable that such an RNA binding site in the protease domain might conversely facilitate the RNA unwinding process. As an alternative, the association of the protease and the helicase domains with the NS4A core peptide might lead to a more active conformation with a higher stability under physiological conditions. Crystallographic studies of this single-chain recombinant NS3–NS4A are in progress and should lead to a better understanding to the mechanism of enhanced unwinding.

In conclusion, the presence of the protease domain, the helicase domain, and the NS4A core peptide cofactor are essential for optimal HCV helicase activity. The construction of the single-chain recombinant His-NS4A_{21–32}–GSGS-NS3_{3–631}, therefore, represents a more relevant surrogate for the NS3/NS4A membrane complex found *in vivo* than does the NS3/NS4A complex artificially maintained in detergent and high salt. This novel recombinant protein is not only a viable tool for the discovery of antiviral agents, but also advances our understanding of the subtle structure–function relationships within this complex multidomain bifunctional enzyme.

Materials and methods

Reagents

Restriction enzymes, T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, Massachusetts). Ready-to-Go T4 DNA Ligase was obtained from Pharmacia Biotech (Piscataway, New Jersey). GTG agarose was purchased from FMC Corporation (Rockland, Maryland). The Qiaquick gel extraction kit and Qiaquick PCR purification kit were purchased from Qiagen Inc. (Chatsworth, California). pET vectors and BL21(DE3) cells were obtained from Novagen (Madison, Wisconsin). DH5 α cells and Terrific Broth were purchased from Gibco, BRL (Gaithersburg, Maryland). Plasmid BK138-1, which encoded the entire NS3 region of the 1b/BK strain of HCV, was obtained from Dr. Okayama (Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan). ATP and [α - ^{32} P]ATP were purchased from United States Biochemicals (Cleveland, Ohio) and NEN Life Science Products (Boston, Massachusetts), respectively. Polyethyleneimine-cellulose sheets were purchased from SA Scientific Adsorbents, Inc. (Atlanta, Georgia). RNase inhibitor was purchased from 5 prime $\rightarrow$ 3 prime, Inc. (Boulder, Colorado). Biotinylated capture oligomer (5'-biotin-GCT-CGC-CCG-GGG-ATC-CTC-TAG-3') was custom prepared by Gibco/BRL (Grand Island, New York), and SPA beads were obtained from Amersham Life Science Inc. (Arlington Height, Illinois). All other chemicals were purchased from either Boehringer Mannheim (Indianapolis, Indiana) or Sigma (St. Louis, Missouri).

Double-stranded RNA (dsRNA) substrates with 3' overhangs (3'/3') or blunt ends were custom prepared by Oligos, Etc., Inc. (Wilsonville, Oregon). 3'/3' substrate was comprised of a template 5'-GCU CGC CCG GGG AUC CUC UAG GAA UAC ACG UUC GAU-3' annealed to a primer 5'-CUA GAG GAU CCC CGG GCG AGC CCU AUA GUG AGU CGU-3' (complementary sequences of the template and the primer are underlined). Blunt-ended substrate was prepared by annealing two complementary RNA oligomers 5'-CUA GAG GAU CCC CGG GCG AGC GAC AAC GUG-3' and 5'-CAC GUU GUC GCU CGC CCG GGG AUC CUC UAG-3'. Double-stranded RNA substrates with 5' and 3' overhangs (5'/3') or 5' overhangs only (5'/5') were generously provided by Dr. J. Hurwitz (Memorial Sloan-Kettering Cancer Center, Sloan-Kettering Institute, New York). The 5'/3' substrate contained a primer 5'-GAA UAC ACG GAA UUC GAG CUC GCC CGG GGA UCC UCU AG annealed to a template 5' GAA UAC AAG CUU GGG CUG CAG GUC GAC UCU AGA GGA UCC CCG GGC GAG CUC GAA UUC GGG UCU CCC UAU AGU GAG UCG UAU UAA UUU CGA UAA GCC AG (complementary sequences are underlined). The 5'/5' substrate consisted of a template 5'-GAA UAC AAG CUU GCA UGC CUG CAG GUC GAC UCU AGA GGA UCC CCG GGU ACC G-3' annealed to a primer 5'-GGG AGA CCG GAA UUC GAG CUC GGU ACC CGG GGA UCC UCU AGA GUC G-3' (underlined sequences represent complementary regions of the two oligomers). All duplex RNAs used for the study of substrate specificity were end labeled with [γ - ^{32} P]ATP. The 3'/3' substrate was used for the scintillation proximity assay (SPA, Amersham Life Science Inc., Arlington Height, Illinois) and was end labeled with [γ - ^{32} P]ATP. The end-labeling reaction was performed by using T4 polynucleotide kinase according to standard procedures (Sambrook et al., 1989).

Construction of expression plasmids

pNS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ was derived from plasmid BK138-1, which encoded the entire NS3 region of the 1b/BK strain of HCV (Takamizawa et al., 1991). The recombinant pNS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ was constructed in two steps by first generating the pHis-NS4A₂₁₋₃₂-GSGS-NS3₃₋₁₈₁, which encoded the HCV NS3 protease domain (residues 3-181) (Taremi et al., 1998), followed by subcloning into an expression vector containing the NS3 (residues 49-631) via a cut-and-paste strategy. For the construction of pNS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁, a 270-bp fragment was generated by restricting pHis-NS4A₂₁₋₃₂-GSGS-NS3₃₋₁₈₁ with XbaI/BspMI. This fragment contained the sequence encoding the histidine tag, the thrombin site, the NS4A peptide, GSVVIVGRILS (NS4A a.a. 21-32), the linker, glycine-serine-glycine-serine (GSGS), and the NS3 a.a. 3-48. A second fragment (7111 bp) was generated by restricting a pET-22b+ plasmid containing the full-length NS3 (encoding a.a. 1-631 derived from BK138-1 plasmid) with XbaI/BspMI. This fragment encompassed the pET 22b+ expression vector in addition to the sequence encoding NS3 a.a. 49-631. These two fragments were ligated together to form pHis-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁. The resulting plasmid was used to transform competent *E. coli* DH5 α which were selected on LB agar plates with ampicillin (100 μ g/mL). Recombinant clones were identified by restriction enzyme mapping and sequencing. The resulting single-chain recombinant His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ is illustrated in Figure 1. Expression and purification of His-NS3/4A were performed as previously described (Sali et al., 1998).

pHis-NS3₁₈₂₋₆₃₁ and pHis-NS3₁₋₆₃₁ were constructed by generating cDNA fragments containing HCV NS3 (1b, BK strain) residues 181-631 and NS3 residue 1-631, respectively, using BK138-1 as a template and a pair of 5' and 3' primers. The 5' primer used for the pHis-NS3₁₈₂₋₆₃₁ was 5'-GAT ATA CC ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC TCT CCG GTC TTC ACG GAC-3', whereas the 5' primer used for the His-NS3₁₋₆₃₁ was 5'-CAT ATG GCT AGC GCG CCC ATC ACG GCG TAC GCC-3'. Underlined sequences represent NcoI and NheI restriction sites, respectively. The 3' primer (5'-GAA TTC CTA CGT AAC GAC CTC CAG GTC GGC -3') with a EcoRI site (underlined) was used for both constructs. The PCR products were cloned into a pET-28a vector digested with the corresponding enzymes and transformed into *E. coli* DH5 α .

Expression and purification of recombinant proteins in *E. coli*

A single colony from *E. coli* BL21(DE3) transformed with the recombinant plasmids was used to initiate growth in Terrific Broth supplemented with 100 μ g/mL ampicillin. Recombinant proteins were expressed and purified according to the procedures as described by Taremi et al. (1998).

N-terminal amino acid sequencing

N-terminal sequence analysis was performed on an Applied Biosystems Inc. (Foster City, California), 494 pulsed-liquid protein sequencer with an on-line PTH analyzer (ABI model 140C). Samples were spotted onto PVDF membranes (ProSorb, ABI, California) and were subjected to a minimum of 18 cycles of analysis.

Mass spectrometry

Matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained on a PerSeptive Voyager RP reflector-type time-of-flight mass spectrometer equipped with a nitrogen laser (wavelength 337 nm). The His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ samples analyzed were concentrated to 5 mg/mL in 25 mM HEPES, pH 7.6, 1.0 M NaCl, 10% glycerol, and 10 mM β ME. Matrix solution (10 μ L) was added to 1 μ L of the protein solution; 1 μ L of the resulting mixture was applied onto the sample plate and air dried prior to its transfer into the source of the mass spectrometer.

Protease activity assay

The activities of all protease constructs were determined utilizing the peptide cleavage assay previously described (Sali et al., 1998). In brief, various amounts of the 5A/5B peptide substrate DTEDVVCC*SMSYTWG-K were incubated with an appropriate amount of the recombinant proteases in 25 mM; 3-[N-morpholino]propanesulfonic acid (MOPS) (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 10 mM DTT, 10% glycerol, and 0.05% bOG at room temperature for 15 min and quenched with an equal volume of 10% phosphoric acid. The resulting 5B cleavage product SMSYTWG-K was quantified by reverse-phase high-performance liquid chromatography. Peak areas were converted to nanomoles using a standard curve of the 5B cleavage product. Kinetic parameters were obtained using nonlinear regression analysis of initial rates as a function of substrate concentration (Enzyme Kinetics; Trinity Software, Campton, New Hampshire). All parameters represent the weighted average of three independent determinations.

ATPase assay

ATPase activities of the recombinant enzymes were monitored by direct measurement of [α -³²P]ATP hydrolysis using thin-layer chromatography. The ATPase assay was carried out in a reaction mix containing 1 mM ATP mixed with [α -³²P]ATP, (3,000 Ci/mmol, ~0.5 μ Ci per reaction), 50 mM HEPES (pH 7.3), 10 mM KCl, 0.5 mM dithiothreitol (DTT), 100 μ g/mL bovine serum albumin, fraction V (BSA) and 1 mM MgCl₂ in a final volume of 10 μ L. The reaction was carried out at 37 °C for 1 h and terminated by an addition of 1 μ L of 0.5 M EDTA. Half a microliter of the reaction mix was spotted onto a polyethyleneimine-cellulose sheet and developed by ascending chromatography in 0.375 M potassium phosphate buffer (pH 3.5). The cellulose sheet was dried and ³²P-labeled ATP and ADP were quantified with a Storm 860 Phosphor-Imager (Molecular Dynamics, Sunnyvale, California). Basal and stimulated ATPase activities were examined in the absence or presence of 65 μ g/ μ L polyuridylic acid (poly U) (~1 mM for poly U of 200 bases), respectively.

Nucleic acid unwinding assay

His-NS4A₂₁₋₃₂-GSGS-NS3₃₋₆₃₁ or other recombinant NS3 enzymes was incubated with dsRNA in a reaction mix containing 100 mM MOPS (pH 7.0), 0.5 mM MgCl₂, 2 mM ATP, 0.5 mM DTT, 100 μ g/mL BSA, 2% dimethylsulfoxide (DMSO) and 1 U ribonuclease (RNase) inhibitor. For the His-NS3/NS4A complex, the optimal reaction conditions were 100 mM piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES) (pH 6.0), 1 mM MgCl₂, 2 mM ATP, 0.6 mM DTT, 100 μ g/mL BSA, 2% DMSO, and 1 U RNase inhibitor. The amounts of enzymes and substrates used for each

experiment are indicated in the corresponding figure legends. The reaction (final volume of 50 μ L) was incubated at 37 °C for 1 h and terminated by an addition of either 10 μ L of 0.5 M EDTA for SPA analysis or 5 μ L of RNA loading buffer (0.1 M Tris-HCl (pH 7.4), 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 50% glycerol) for SDS-PAGE analysis. 3'/3' dsRNA used for the SPA was end labeled with [γ -³³P]ATP whereas all dsRNAs used for the SDS-PAGE were end labeled with [γ -³²P]ATP using T4 polynucleotide kinase according to standard procedures (Sambrook et al., 1989).

For SDS-PAGE analysis, an aliquot (25 μ L) of the reaction was loaded onto a 15% SDS-polyacrylamide gel and electrophoresed at 7 mA for 16 h. The gel was dried and subjected to autoradiography.

For SPA analysis, single-stranded RNA (ssRNA) released from the unwinding reaction was captured by adding 60 μ L of a 100-nM solution of biotinylated capture oligomer (5'-biotin-GCTCGC-CCG-GGG-ATC-CTC-TAG-3') in 2 $\times$ hybridization buffer (40 mM HEPES (pH 7.3), 2M NaCl, 2 mg/mL BSA) after termination of the reaction. The hybridization mix was incubated at 37 °C for 1 h. The primer-oligomer complex was retained by Streptavidin coated SPA beads, filtered, and washed thoroughly with wash buffer [20 mM HEPES (pH 7.3), 15 mM NaCl, 1.5 mM sodium citrate, and 0.05% SDS]. The amount of released labeled ssRNA was quantified using a TopCount reader (Packard A991200, Meriden, Connecticut). All SPA reactions were performed in triplicate, and boiled ³²P-RNA substrate in the absence of enzyme was used to estimate the amount of total input RNA.

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