Comparative characterization of two DEAD-box RNA helicases in superfamily II: human translation-initiation factor 4A and hepatitis C virus non-structural protein 3 (NS3) helicase

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Eukaryotic initiation factor 4A (eIF4A) is an ATP-dependent RNA helicase and is homologous to the non-structural protein 3 (NS3) helicase domain encoded by hepatitis C virus (HCV). Reported here is the comparative characterization of human eIF4A and HCV NS3 helicase in an effort to better understand viral and cellular helicases of superfamily II and to assist in designing specific inhibitors against HCV infections. Both eIF4A and HCV NS3 helicase domain were expressed in bacterial cells as histidine-tagged proteins and purified to homogeneity. Purified eIF4A exhibited RNA-unwinding activity and acted on RNA or RNA/DNA but not DNA duplexes. In the absence of cellular cofactors, eIF4A operated unwinding in both the 3' to 5' and 5' to 3' directions, and was able to unwind blunt-ended RNA duplex, suggesting that bidirectionality is an intrinsic property of

eIF4A. In contrast, HCV NS3 helicase showed unidirectional 3' to 5' unwinding of RNA and RNA/DNA, as well as of DNA duplexes. With respect to NTPase activity, eIF4A hydrolysed only ATP or dATP in the presence of RNAs, whereas HCV NS3 helicase could hydrolyse all ribo- and deoxyribo-NTPs in an RNA-independent manner. In parallel, only ATP or dATP could drive the unwinding activity of eIF4A whereas HCV NS3 could function with all eight standard NTPs and dNTPs. The observed differences in their substrate specificity may prove to be useful in designing specific inhibitors targeting HCV NS3 helicase but not human eIF4A.

Key words: ATPase, ATP hydrolysis, NTPase, unwinding activity.

INTRODUCTION

Proteins of the DEAD-box family are highly conserved from bacteria to humans and are involved as helicases in a wide range of cellular processes, including translation, ribosome biogenesis, mRNA splicing, and RNA maturation and degradation [1–3]. All DEAD-box proteins studied *in vitro* exhibit an NTPase activity that hydrolyses NTPs and provides the energy source for duplex unwinding [4,5]. Among the DEAD-box proteins is eukaryotic initiation factor 4A (eIF4A), an ATP-dependent RNA helicase that is thought to melt the 5' proximal secondary structure of eukaryotic mRNAs to facilitate attachment of the 40 S ribosomal subunit [5–7]. Three different isoforms, eIF4AI, eIF4AII and eIF4AIII, have been identified thus far [8,9]. It is believed that eIF4A functions *in vivo* in a complex termed eIF4F, with other initiation factors, including eIF4E, eIF4G and eIF4B, to operate this unwinding process [7,10–12]. However, it has been demonstrated recently that eIF4A can function independently *in vitro* as an ATP-dependent RNA helicase [13,14]. Detailed kinetic analyses of the ATPase and RNA helicase activities have been performed using either recombinant mouse eIF4A or the enzyme purified from rabbit reticulocyte lysate [13–16]. However, such characterization for human eIF4A has not been documented.

The DEAD-box protein family also contains several viral RNA helicases that are involved in viral replication, transcription and protein translation (see [1,17] for review). One of the viral RNA helicases is the non-structural protein 3 (NS3) encoded by

hepatitis C virus (HCV), the major aetiological agent of transfusion non-A, non-B hepatitis [18]. HCV NS3 is a multifunctional protein; its N-terminal 180 amino acids define a serine protease activity and its C-terminal ≈ 450 residues contain NTPase/helicase activities [19–21].

It is estimated that 1–2% of the world population is infected by HCV. HCV infections represent a serious health problem worldwide because most can become chronic, which can lead to liver cirrhosis and hepatocellular carcinoma. Since current treatments for HCV infections have not been found to be sufficiently effective, development of potent and specific antiviral agents for treatment of HCV infections is highly desirable. In recent years, extensive efforts have focused on the development of various inhibitors targeting the viral proteins/enzymes as well as the genomic RNA of HCV (see [22] for review). Among the proteins encoded by HCV, ATPase and helicase activities contained in the NS3 protein have been shown to be essential to viral replication and infection [23], and thus represent attractive targets for antiviral intervention [22–25].

Sequence alignment has shown that HCV NS3 is highly homologous to human eIF4A [1]. Therefore, possible cytotoxicity resulting from cross-reactivity may present a serious concern in the efforts of developing inhibitors targeting HCV NS3 helicase (NS3H). It is thus of great interest to gain more insight into the biochemical properties of eIF4A, particularly the human version, to assist not only HCV helicase inhibitor design but also to help minimize potential host toxicity. Documented here is the cloning and expression of human eIF4A in bacterial cells. Using highly

Abbreviations used: HCV, hepatitis C virus; eIF4A, eukaryotic initiation factor 4A; NS3, non-structural protein 3; NS3H, NS3 helicase; DTT, dithiothreitol.

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purified eIF4A, we characterized its ATPase and helicase activities in comparison with those of HCV NS3. A quantitative human eIF4A helicase assay was established which could be used as a counter-assay assisting in the development of HCV NTPase/helicase inhibitors.

MATERIALS AND METHODS

Materials

Regular and ^{32}P -labelled ribo- and deoxyribo-NTPs were purchased from Gibco BRL and NEN, respectively. Homopolymeric RNAs and DNAs were purchased from Sigma. Various RNA and DNA oligonucleotides were custom-synthesized by Cyber-Syn (Aston, PA, U.S.A.).

Molecular cloning, expression and purification of human eIF4A

Human eIF4A (isoform I) cDNA was amplified by PCR using an EST clone (592165), obtained from the A.T.C.C. (Manassas, VA, U.S.A.), as a template. The two primers used for PCR were eIF4A-1 (5'-GCTCGCTAGCATGTCTGCGAGCCAGGA-3') and eIF4A-2 (5'-GCTCAAGCTTTTCAGATGAGGTCAGCAACA-3'), synthesized using a Beckman Oligo 1000 DNA synthesizer. PCR reactions were performed in a total volume of 100 μl , containing 20 mM Tris, pH 8.8, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 0.1% Triton X-100, 0.1 mg/ml BSA, 200 μM dNTP, 500 nM primers, 250 ng of template DNA and 5 units of cloned *Pfu* polymerase (Stratagene). Following 25 cycles of PCR amplification, the resulting 1.2 kb product was subcloned into the *NheI/HindIII* sites of pRSET A (Invitrogen). The resulting expression vector, pRSET-HeIF4A, allowed us to express a N-terminal histidine-tagged human eIF4A protein. The cDNA sequence encoding human eIF4A was confirmed by DNA sequencing.

For generation of recombinant human eIF4A protein, *Escherichia coli* BL21 (DE3) cells were transformed by pRSET-HeIF4A vector, grown at 30 °C in LB medium to a D_{600} value of ≈ 0.6 , and induced with 500 μM isopropyl β -thiogalactoside for protein production. After 4 h of induction at 30 °C, cells were harvested by centrifugation. With respect to eIF4A purification, the cell pellet was resuspended in buffer A [25 mM Hepes, pH 7.5/10% glycerol/1 mM dithiothreitol (DTT)/0.5 M NaCl] with protease inhibitor cocktail tablets (Roche). Cells were lysed by sonication and the lysate was clarified at 75000 g for 30 min. The collected supernatant was mixed with Ni^{2+} -nitrilotriacetate resin (Qiagen) at 4 °C for 60 min in buffer A. The resin was pelleted and washed with 20 mM imidazole in buffer A to remove unbound proteins. Bound proteins were batch-eluted with 500 mM imidazole in buffer A. The elute was then diluted 10-fold with buffer B (25 mM Hepes, pH 7.5/10% glycerol/1 mM DTT) and loaded on to a 5 ml Hi-Trap heparin column (Pharmacia). The column was washed to the baseline of protein absorbance. A 50 ml gradient of NaCl from 0 to 1 M in buffer B was used to elute the bound proteins. Fractions containing eIF4A protein were identified by SDS/PAGE gels and Western-blot analysis using a monoclonal antibody against the histidine tag, and then pooled and dialysed overnight at 4 °C against buffer B. Purified eIF4A was aliquoted and stored at -20 °C for characterization.

Molecular cloning, expression and purification of the HCV NS3H domain

For expression of the HCV NS3H, PCR was performed under the conditions described above to amplify a 1.4 kb DNA fragment encompassing amino acids 1193–1658 from a cDNA clone of

HCV-1a [26]. The amplified PCR fragment was then inserted into an expression vector, pET30 (Promega), to yield the expression plasmid pET-hcvNS3H. The accuracy of the coding region for NS3H was confirmed by nucleotide sequencing.

Expression vector pET-hcvNS3H, which allowed us to produce a C-terminal histidine-tagged NS3H protein containing the helicase domain (465 amino acids) of HCV NS3, was transformed into *E. coli* BL21 (DE3) cells for generation of recombinant NS3H. Transformed bacterial cells were grown at 30 °C in LB medium to a D_{600} of ≈ 0.6 and isopropyl β -thiogalactoside was then added to a final concentration of 1 mM to induce protein production. After another 3 h of incubation, cells were harvested by centrifugation. For purification, the cell pellet was resuspended in PBS buffer plus a protease inhibitor cocktail tablet (Roche). Cells were lysed by sonication and the lysate was centrifuged at 75000 g for 30 min. Clarified supernatant was allowed to pass through a ≈ 50 ml DEAE-Sepharose resin and the flow-through was then loaded on to a 5 ml HiTrap Ni^{2+} -Sepharose column (Pharmacia). Bound proteins were eluted using a 100 ml gradient of 0–150 mM imidazole in PBS buffer. Fractions containing NS3H protein, with the expected molecular mass of 50 kDa, were identified by SDS/PAGE, then pooled and dialysed overnight at 4 °C against buffer B. Purified HCV NS3H was aliquoted and stored at -20 °C for characterization.

HPLC-based ATPase assay

A typical ATPase assay for human eIF4A was performed in a total volume of 100 μl of buffer C (25 mM Hepes, pH 6.5/0.1 mM ATP/0.1 mM MgCl_2) plus 4 $\mu\text{g}/\text{ml}$ poly-U and 5 $\mu\text{g}/\text{ml}$ enzyme for the time specified. An ATPase assay for HCV NS3 was conducted in buffer C plus 100 mM NaCl and 1 $\mu\text{g}/\text{ml}$ enzyme. All ATPase reactions were carried out at room temperature and stopped by addition of 5 μl of 1 M HCl. For separation of the reaction products by HPLC methods, terminated reaction mixture (100 μl) was fractionated with 0.1 M phosphate buffer, pH 6.0, using a C_{18} column (4.6 mm $\times$ 250 mm, 120 Å) at a flow rate of 1 ml/min. ATP and ADP were detected by their absorbance at a wavelength of 254 nm.

NTPase assay by TLC

Hydrolysis of NTPs or dNTPs by either eIF4A or HCV NS3H was performed under the conditions described above for ATP hydrolysis but using one of the following α - ^{32}P -labelled NTPs (ATP, CTP, GTP or UTP) or dNTPs (dATP, dCTP, dGTP or dTTP). The reaction mixture (0.5 μl) was analysed by TLC on polyethyleneimine cellulose sheets using 1.2 M LiCl buffer. Sheets were then air-dried and exposed to X-ray films. To mark positions of mono-, di- and tri-phosphate nucleotides on the sheets, α - ^{32}P -labelled NTP and dNTPs were also partially digested individually by tobacco acid pyrophosphatase (Roche), an enzyme known to hydrolyse NTPs and dNTPs to produce NDPs and NMPs. The digested mixtures were then analysed in the same way.

Helicase unwinding assay

Various duplexes, as shown in Table 1, were prepared by annealing one RNA or DNA fragment with the complementary ^{32}P -labelled RNA or DNA of 11 nucleotides (RNA_{11} or DNA_{11}). RNA_{11} or DNA_{11} was labelled at a concentration of 1 μM with T4 kinase (Gibco BRL) and $[\gamma$ - $^{32}\text{P}]$ ATP (3000 Ci/mmol) for 20 min and the reaction was stopped by heating at 65 °C for 10 min. Unincorporated $[\gamma$ - $^{32}\text{P}]$ ATP was removed by passing the labelled mixtures through MicroSpin G-50 columns (Amersham)

Table 1 Nucleic acids designed for eIF4A

Residues involved in hybridization during duplex annealing are underlined.

Oligonucleotide	Sequence
RNA ₂₅ -1	5'-AAAAACAAACUAGC <u>ACCGUAAAGC</u> -3'
RNA ₂₅ -2	5'- <u>CACCGUAAAGC</u> ACGCAAAAACAAA-3'
RNA ₁₁ -1	5'-GCUUUACGGUG-3'
RNA ₁₁ -2	5'- <u>CACCGUAAAGC</u> -3'
DNA ₂₅	5'-CACCGTAAAGCAGCAAAAACAAA-3'
DNA ₁₁	5'-GCTTTACGGTG-3'
AU14-1	5'-UUAAAAUUAAAAU-3'
AU14-2	5'- <u>AUUUUAAUUUUUAA</u> -3'

and collecting the flow-through liquid. For annealing, 15 pmol of unlabelled RNA₂₅, DNA₂₅ or RNA₁₁ were mixed with 7.5 pmol of labelled complementary RNA₁₁ or DNA₁₁ in buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA and 100 mM KCl [13]. The mixture was heated at 95 °C for 5 min and cooled to room temperature in a 2 h period before being transferred to 4 °C.

Unwinding assays were performed at 35 °C in a total volume of 20 µl of buffer, containing 25 mM Hepes, pH 7.5, 70 mM KCl, 2 mM DTT, 1 mM ATP, 1 mM MgCl₂, 2 nM labelled duplex, 1 mg/ml BSA and 0.4 µM enzyme [13]. For NTP utilization assays, individual NTPs or dNTPs were added to replace ATP at the same concentration. Reaction was stopped by addition of 4 µl of 6 × Tris/borate/EDTA sample buffer (Novex; where 1 × TBE = 45 mM Tris/borate/1 mM EDTA). The reaction products were resolved on pre-cast 20% TBE gels (Invitrogen) at 200 V and 4 °C for 1.5 h. The gel was then dried and the radioactivity of the bands representing duplexes or unwound products were analysed using a PhosphorImager (Molecular Dynamics). Unwinding efficiency was calculated using the following formula: unwinding (%) = 100 × [c.p.m. of unwound single-stranded RNA₁₁ or DNA₁₁/(c.p.m. of unwound single-stranded RNA₁₁ or DNA₁₁ + c.p.m. of residue duplex)]. The amount of thermal melting over the time course was subtracted based on control reactions with no enzyme. Quantification analyses were performed using Typhoon PhosphorImager and ImageQuant software.

RESULTS

Expression and purification of human eIF4A and HCV NS3H

A cDNA encoding human eIF4A isoform I was subcloned into a pRSET expression vector for high-level expression in bacterial cells as a histidine-tagged protein. After induction by isopropyl β-thiogalactoside, a 45 kDa protein, consistent with the expected molecular mass of eIF4A, was identified from the soluble fraction of the transformed bacterial cell lysates (Figure 1, lane 2). This protein could be highly enriched using a two-step purification protocol as described in the Materials and methods section. Under the conditions employed, we obtained ≈ 7 mg of purified eIF4A, with a purity over 95 %, per litre of cell culture (Figure 1, lane 4). The identity of eIF4A was confirmed by N-terminal amino acid sequencing.

Molecular cloning and expression of the HCV NS3H were achieved as described in the Materials and methods section. This protein, containing only the C-terminal 465 amino acids of the NS3 derived from HCV genotype-1a, was also expressed as a histidine-tagged protein to aid in its purification. Using combination of DEAE-Sepharose and Ni²⁺-chelating columns, as described in the Materials and methods section, recombinant

Figure 1 Analysis of purified human eIF4A protein and HCV NS3H

eIF4A samples were resolved on a 4–20% gradient gel and stained with Coomassie Brilliant Blue (lanes M and 1–4) or detected by Western blotting using polyclonal anti-(histidine tag) antibodies as described in the text (lanes 5–7), whole-cell lysate (lane 1), soluble (lanes 2 and 5) and insoluble (lanes 3 and 6) fractions, and purified eIF4A protein (lanes 4 and 7). Purified HCV-1a NS3H is shown in lane 8.

NS3H was purified to homogeneity with a yield of ≈ 5 mg/l of culture (Figure 1). Both purified HCV NS3H and eIF4A were dialysed against the same buffer and then subjected to comparative analysis.

ATPase activity of human eIF4A and HCV NS3H

Previous studies revealed that eIF4A purified from rabbit reticulocytes or recombinant mouse eIF4A demonstrate ATP-hydrolytic activity [6,16]. Since nucleic acids might affect the efficiency of ATP hydrolysis [6], we first examined the ATPase activity of human eIF4A by measuring the hydrolysis of ATP to ADP in the absence or presence of RNA. These experiments were performed side by side using purified HCV NS3H. As can be seen in Figure 2, human eIF4A protein showed only RNA-dependent ATPase activity, with no ATP hydrolysis in the absence of RNA, whereas HCV NS3H could hydrolyse ATP without the assistance of RNA, as reported previously [19]. It was found that inclusion of poly-U up to a concentration of 16 µg/ml did not significantly affect the efficiency of ATP hydrolysis catalysed by HCV NS3H (results not shown). Hydrolysis of ATP by both enzymes was dependent on Mg²⁺ (Table 2), which could not be replaced by other bivalent cations such as Mn²⁺ or Zn²⁺ (results not shown). The two enzymes showed different responses to monovalent cations. When tested at concentrations of less than 250 mM, Na⁺ and K⁺ salts had stimulatory effects on ATP hydrolysis catalysed by HCV NS3H, but were inhibitory to eIF4A (Table 2). Interestingly, NS3H was less sensitive to high temperature than eIF4A (Figure 2C). Although both enzymes demonstrated their highest activity at pH ≈ 6.5, HCV NS3H was less sensitive to alkaline pH (Figure 2D). A summary of these analyses is shown in Table 2.

Steady-state kinetic analysis of the ATPase activity associated with human eIF4A and HCV NS3H was also performed and compared. The ATP-hydrolytic efficiency of eIF4A, expressed as k_{cat}/K_m , was lower than that of HCV NS3H by two orders of magnitude, as seen in Table 2. The enzyme turnover number, k_{cat} , representing the maximal catalytic rate, was determined as 3.4 min⁻¹ for human eIF4A and 349.2 min⁻¹ for HCV NS3H. Therefore, the observed low catalytic efficiency of eIF4A resulted

Figure 2 Comparison of the ATPase activity catalysed by human eIF4A and HCV NS3H

(A) ATP hydrolysis time course using 1 $\mu\text{g/ml}$ HCV NS3H, with (●) or without (◆) poly-U, or 5 $\mu\text{g/ml}$ eIF4A in the absence (▲) or presence (■) of 4 $\mu\text{g/ml}$ poly-U. (B) Enzyme concentration-dependence curve. Reactions were allowed for 60 min for each concentration using purified NS3H (●), eIF4A alone (▲) or eIF4A plus 4 $\mu\text{g/ml}$ poly-U (■). For (A) and (B), ATPase activity is expressed as a percentage of the conversion of 100 μM ATP to ADP. (C) Effect of temperature. (D) pH dependence of HCV NS3H (●) and eIF4A (■). For (C) and (D), the relative ATP hydrolysis activity is shown expressed as a percentage of the maximum activity obtained for each enzyme. All ATPase reactions were performed under the conditions described in the text with the changes specified.

Table 2 Comparison of the ATPase activities of human eIF4A and HCV NS3H

For V_{max} , the units $\text{pmol/min} \times \text{pmol}$ refer to the amount of product (in pmol) that a pmol of enzyme can produce per min.

Substrate description	eIF4A	HCV NS3H
RNA dependency	Yes	No
Metal requirement	Mg^{2+}	Mg^{2+}
Na^+ or K^+ effects	Inhibitory	Stimulatory ($< 300 \mu\text{M}$)
Optimum pH	6.5	6.5–9.5
Optimum temperature	30 °C	37 °C
Kinetic parameters		
$k_{\text{cat}}/K_{\text{m}}$	$\approx 6200 \text{ M}^{-1} \cdot \text{s}^{-1}$	$505800 \text{ M}^{-1} \cdot \text{s}^{-1}$
k_{cat}	3.4 min^{-1}	349.2 min^{-1}
K_{m}	$9.2 \pm 1.5 \mu\text{M}$	$11.5 \pm 2.2 \mu\text{M}$
V_{max}	$1.7 \text{ pmol/min} \times \text{pmol}$	$139.7 \text{ pmol/min} \times \text{pmol}$

from a low enzyme turnover (k_{cat}) rather than its affinity towards the ATP substrate, as both eIF4A and HCV NS3H had similar K_{m} values (Table 2).

Regulation of eIF4A ATPase activity by polynucleotides

Since the ATPase activity of human eIF4A depended on input RNAs such as poly-U (Figure 2), it was interesting to determine the characteristics of nucleic acids required to stimulate eIF4A ATPase activity. First, we examined various RNAs and DNAs for their abilities to stimulate human eIF4A ATPase activity. As shown in Figure 3, no ATPase activity of eIF4A was observed in any reaction containing DNAs, indicating that DNA could not activate eIF4A. Most homopolymeric and heteropolymeric RNAs, including globin mRNA, stimulated eIF4A ATPase activity, although their efficiencies varied (Figure 3). Of all the RNAs examined, no stimulatory effect was detected with poly-G at several concentrations (Figure 3 and results not shown). Similar results were obtained using smaller RNAs. For example, eIF4A could be activated to cleave ATP in the presence of oligonucleotide U_{14} , but not G_{14} or dU_{14} (Figure 3). In addition, we found that eIF4A could also be activated by double-stranded RNAs (Figure 3B).

To have a better understanding of the differential effects of RNAs on eIF4A ATPase activity, RNA-binding analysis was performed using homopolymeric RNAs. As seen in Figure 4(A), poly-G, despite being unable to activate eIF4A, had stronger

Figure 3 Effects of various nucleic acids on human eIF4A ATPase activity

Various nucleic acids, as specified, were examined individually at 4 μ g/ml for their effects on ATPase activity of human eIF4A. The effects of long nucleic acids and synthetic oligonucleotides are shown in (A) and (B), respectively. All ATPase reactions were performed using 5 μ g/ml eIF4A for 60 min and the reaction products were analysed by HPLC as described in the text.

binding to eIF4A than poly-A, poly-C or poly-U, suggesting that its tight binding might be the cause of its lack of stimulation of eIF4A activity. To test this hypothesis, we measured the effect of different concentrations of oligonucleotide G₁₄ on the ATP hydrolysis catalysed by eIF4A in the presence of U₁₄. As shown in Figure 4(B), G₁₄ could reverse the stimulatory effect of U₁₄ on eIF4A activity, even at low concentrations. As a comparison, oligonucleotide dT₁₄ did not generate a significant effect on the eIF4A reaction at the same concentrations (Figure 4B). These results indicated that G₁₄ was able to compete with U₁₄ for enzyme binding and suggested that the inability of poly-G or G₁₄ to stimulate eIF4A ATPase activity was not due to a lack of interaction with eIF4A. In addition, we found that RNA-dependent ATPase activity of eIF4A was sensitive to nucleotide analogues; for example, a complete inhibition of eIF4A was seen with adenylyl imidodiphosphate, a non-hydrolysable ATP analogue, at a concentration of 25 μ g/ml (results not shown).

Substrate specificity for nucleotide hydrolysis

Previous study has shown that HCV NS3H can hydrolyse various nucleotide triphosphates [19,27]; however, such analysis has not been performed on eIF4A. To examine eIF4A substrate specificity, NTPase assays were performed using ³²P-labelled nucleotide triphosphates, including ATP, CTP, GTP, UTP, dATP, dCTP, dGTP and dTTP. As a comparison, NTPase assays were also performed using purified HCV NS3H. These reactions

Figure 4 Differential RNA binding and activation of human eIF4A

(A) RNA-binding efficiency of eIF4A. Experiments were performed using ³²P-labelled poly-A (●), poly-C (▲), poly-G (■) and poly-U (◆) as described in the text. (B) Inhibitory effect of oligo-G₁₄ on eIF4A catalytic activity. ATPase assay was performed in the presence of 2.5 μ M of U₁₄ alone (▲) or in combination with oligo-G₁₄ (●) or oligo-dU₁₄ (■) at the concentrations indicated.

Table 3 NTP hydrolysis and utilization by human eIF4A and HCV NS3H

Nucleotide	NTP hydrolysis		Utilization for unwinding	
	eIF4A	HCV NS3H	eIF4A	HCV NS3H
ATP	+	+	+	+
CTP	—	+	—	+
GTP	—	+	—	+
UTP	—	+	—	+
dATP	+	+	+	+
dCTP	—	+	—	+
dGTP	—	+	—	+
dTTP	—	+	—	+
ADP	—	—	—	—

were resolved by TLC and the results are summarized in Table 3. Under the conditions examined, human eIF4A could hydrolyse only ATP and dATP in the presence of RNA, whereas HCV NS3H could act on all NTPs and dNTPs examined without RNA being present, suggesting that HCV NS3H has a less restricted substrate requirement than eIF4A. In addition, neither eIF4A nor HCV NS3H could hydrolyse nucleotide diphosphates such as ADP (results not shown).

Table 4 Unwinding substrates and specificity

Duplex	Substrate description	Unwinding specificity	
		eIF4A	HCV NS3H
RNA ₂₅ -1/RNA ₁₁ -1	5' Overhang RNA duplex	+	—
RNA ₂₅ -2/RNA ₁₁ -1	3' Overhang RNA duplex	+	+
RNA ₁₁ -2/RNA ₁₁ -1	Blunt-ended RNA duplex	+	—
RNA ₂₅ -1/DNA ₁₁	3' RNA-overhang RNA/DNA hybrid	+	+
DNA ₂₅ /RNA ₁₁ -1	3' DNA-overhang DNA/RNA hybrid	+	+
DNA ₂₅ /DNA ₁₁	3' Overhang DNA duplex	—	+

Intrinsic RNA-unwinding activity of human eIF4A

Previous studies showed that eIF4A purified from rabbit reticulocyte lysate could unwind an RNA duplex of 10–12 nucleotides with a 5' overhang [13]. To characterize the human eIF4A unwinding activity, a duplex of 11 nucleotides with a 5' overhang of 14 nucleotides was designed for the initial experiment (Table 1). This duplex was made by annealing a 25-nucleotide RNA with a ³²P-labelled complementary 11-nucleotide RNA (Table 4). When labelled RNA duplex was incubated with purified human eIF4A, as described in the Materials and methods section, duplex unwinding occurred in the reactions containing eIF4A (Figure 5A), and this activity increased with eIF4A concentration (results not shown). In addition, no unwinding was observed in the absence of ATP (Figure 5, lanes 2), indicating that human eIF4A possesses an intrinsic ATP-dependent helicase activity in the absence of any cofactors.

Unwinding polarity and specificity of human eIF4A and HCV NS3H

The helicase activity of eIF4A has been characterized as bidirectional [28], in contrast with HCV NS3H, which catalyses unidirectional unwinding [20]. To test their unwinding polarity, 3'-overhang and blunt-ended RNA duplexes were prepared as shown in Table 4 and tested against purified human eIF4A and HCV NS3H. Unwinding of duplexes with 5' and 3' overhangs was observed in the reactions with purified human eIF4A (Figures 5A and 5B), indicating that the human version also catalysed bidirectional unwinding, as revealed for eIF4A isolated from rabbit reticulocytes. Interestingly, blunt-ended RNA duplex could also serve as a substrate (Figure 5C), suggesting that no overhang was needed for eIF4A unwinding. Further quantification analysis revealed that the unwinding efficiency for the blunt-ended RNA duplex was similar to those for 5'- and 3'-overhang duplexes, indicating that a single-stranded overhang did not stimulate eIF4A unwinding activity (Figure 5). In addition, no helicase activity was observed in the absence of enzyme or ATP (Figure 5), consistent with the notion that eIF4A is an ATP-dependent helicase.

Next, hybrid RNA/DNA duplexes containing either an RNA 3' overhang (RNA₂₅-2/DNA₁₁) or a DNA 3' overhang (DNA₂₅/RNA₁₁-1) were designed to characterize eIF4A helicase specificity further (Table 4). For comparison, a DNA₂₅/DNA₁₁ duplex was also prepared and examined. When human eIF4A was tested against these duplexes in the presence of ATP/Mg²⁺, unwinding of the hybrids with either an RNA or a DNA overhang, but not the DNA/DNA duplex, was seen (Figures 5D–5F). No detectable unwinding of the DNA/DNA duplex was seen when tested at a higher eIF4A concentration (results not shown). These data indicated that human eIF4A was an RNA helicase that could function only when at least one strand of RNA was present in the

Figure 5 Unwinding specificity of human eIF4A helicase

RNA duplexes with a 3' overhang (**A**), with a 5' overhang (**B**) or that were blunt-ended (**C**), or RNA/DNA duplex with an RNA overhang (**D**), DNA/RNA duplex with a DNA overhang (**E**) and DNA/DNA duplex (**F**) were tested for unwinding efficiency by human eIF4A. Unwinding assays were performed for the time specified (2–60 min as shown in lanes 4–8) under the conditions described in the text. The first three lanes in each panel represent the boiling products (lane 1) of the indicated duplex or reactions performed without eIF4A (lane 2) or ATP (lane 3).

Figure 6 Comparison of substrate specificity of human eIF4A and HCV NS3H

RNA duplexes with a 3' overhang, a 5' overhang, or that were blunt-ended (A), or RNA/DNA hybrid duplex and DNA/DNA duplex (B) were tested for unwinding by human eIF4A and HCV NS3. Unwinding assays were performed under the standard conditions for 60 min and analysed as described in the text. Lane Δ represents the boiling product of the specified duplex substrates.

duplex substrate, and this activity did not require an overhang at either end.

In order to systematically compare the two helicases of the same family, we also analysed the helicase activity of HCV NS3H in parallel with that of human eIF4A. To do so, various RNA, DNA and hybrid RNA/DNA duplexes, as mentioned above, were tested for HCV NS3H. Consistent with previous reports [20,29], purified HCV NS3H could unwind duplexes with 3' overhangs, but not 5' overhangs or blunt ends (Figure 6A), indicating that HCV helicase only catalysed unidirectional unwinding. However, unlike eIF4A, NS3H could act on DNA/DNA duplexes efficiently as long as the duplexes contained a 3' overhang (Figure 6B). As summarized in Table 4, these data suggested that human eIF4A is a bidirectional RNA helicase whereas HCV NS3H is a unidirectional RNA and DNA helicase.

Differential utilization of NTPs and dNTPs by eIF4A and HCV NS3H for unwinding

It is believed that the intrinsic ATPase activity of helicases is to hydrolyse ATP, thus providing energy for duplex unwinding. Since human eIF4A and HCV NS3H demonstrated different specificities with respect to nucleotide hydrolysis, we investigated whether there was a correlation between their preference for NTP hydrolysis and duplex unwinding. Therefore, duplex unwinding efficiency in the presence of different nucleotide triphosphates, NTP and dNTP, was examined using 3'-overhang RNA₂₅-1/RNA₁₁-1 as a substrate. The unwinding activity of eIF4A was seen only when ATP or dATP, but not other NTPs, was included in the reaction (Figure 7A). However, HCV NS3H could utilize all NTPs and dNTPs to drive duplex unwinding (Figure 7B). Our results also show that ADP could not be

Figure 7 NTP-dependent RNA helicase activity of human eIF4A and HCV NS3H

RNA unwinding reactions for eIF4A (A) and HCV NS3H (B) were performed for 60 min in the presence of various nucleotide triphosphates under the conditions described in the text. Lane Δ represents the products of the boiled duplex.

utilized by either enzyme (Figure 7). As summarized in Table 3, these results revealed that human eIF4A and HCV NS3H had a direct correlation between their preference for NTP and their efficiency of duplex unwinding.

DISCUSSION

Among the proteins encoded by the HCV genome, the ATPase and helicase activities contained in the C-terminal ≈ 450 amino acids of the NS3 protein represent attractive targets for antiviral intervention. At least partially for this reason, HCV NS3H has been studied extensively. Sequence analysis classified HCV NS3H in the DEAD-box proteins of the RNA helicase superfamily II [1]. Both sequence and structural analyses revealed that NS3H shares striking homology with another DEAD-box RNA helicase, eIF4A, an important RNA helicase involved in the initiation of protein translation in eukaryotic cells. In compliance with the need to develop specific inhibitors targeting viral but not human counterparts, we cloned and expressed the human eIF4A in bacteria and characterized this protein in an attempt to compare its catalytic activity with that of HCV helicase and other reported eIF4As.

Compared with HCV helicase, the reactions catalysed by eIF4A are much less understood. eIF4A isoform I, containing 407 amino acids, is very conserved among different species; for example, human eIF4A is longer than the rabbit version by 12 residues located at the N-terminus (results not shown). The amino acids of this protein are 44% identical and 66% similar to those of HCV NS3H. Characterization of the helicase activity of eIF4A purified from rabbit reticulocyte lysate has been reported recently [13,14]; however, little is known about the human version of eIF4A. Our results reveal that the unwinding properties of the human eIF4A were similar to those of the rabbit enzyme, but different from the HCV NS3H of the same family in several ways. First, in the absence of any cofactors, eIF4A showed bidirectional unwinding even with a blunt-ended duplex, whereas HCV helicase was unidirectional and only unwound duplexes in the 3' to 5' direction. Together with other observations [20], these data support the notion that HCV NS3H catalyses directional and processive unwinding, whereas eIF4A might operate through a different mechanism; that is, non-processively melting short RNA duplexes present in mRNAs [14]. Another

striking difference is that eIF4A is an RNA helicase and only unwinds duplexes containing RNA, whereas HCV NS3H demonstrated both RNA and DNA helicase activity. Such a lack of RNA selectivity over DNA may suggest that the key residue(s) involved in RNA binding may be missed in HCV NS3H. Other supporting evidence includes the activation of ATPase activity of eIF4A by RNA but not DNA (Figure 3), contrasting with HCV NS3H, whose ATPase activity could be stimulated by both RNA and DNA [19,27]. Therefore, it would be interesting to see whether mutations of the key RNA-binding residues in eIF4A or HCV NS3 would change their selectivity. The ability of HCV helicase to bind and unwind DNA may also hint that this protein could interact with host-cell genomic DNA to exert certain regulations to cellular metabolic processes.

The analysis herein of the ATPase activity of eIF4A in the presence of various nucleic acids confirms and extends previous results. Previous studies and our results presented here indicate that only RNA can effectively stimulate eIF4A ATPase activity [6,11,16]. In contrast to a previous report [15], the ATPase activity of eIF4A could be stimulated by both single-stranded and double-stranded RNAs, although their efficiencies were slightly different. In conjunction with the ability of eIF4A to bind and unwind blunt-ended RNA duplexes in the absence of any cofactors ([14], and Figures 5 and 6), our data suggest that human eIF4A contains a double-stranded-RNA binding site. If single-stranded RNA activates the ATPase activity of eIF4A by inducing a conformational change in eIF4A [15], we would argue that double-stranded RNA may exert stimulatory effects through a similar mechanism.

It is worth noting that poly-G or small oligo-Gs, at all concentrations examined, did not show detectable stimulation of eIF4A ATPase activity. RNA-binding analysis revealed that eIF4A had a stronger interaction with poly-G than with other homopolymeric RNAs, suggesting that failure of ATPase stimulation by poly-G was not due to its lack of efficient binding to the enzyme. This conclusion is supported further by data generated from competitive inhibition experiments; oligo-G₁₄ inhibited ATP hydrolysis catalysed by eIF4A with an apparent K_i value of 0.1 μ M. This parameter is essentially equal to the dissociation constant (K_d) for its binding to eIF4A, since the concentration of the activating RNA was sub-saturating in the reaction. Therefore, binding of eIF4A to oligo-G₁₄ is estimated to be 5-fold stronger than that for oligo-U₁₄. On the basis of these data, we propose that a strong RNA-enzyme interaction could lock the enzyme conformation at the RNA-binding state, which could block the substantial conformational change required for ATP binding and hydrolysis.

In addition, our analysis revealed that eIF4A could hydrolyse not only ATP but also dATP in the presence of RNA, and, more importantly, it could utilize the energy released from dATP hydrolysis to drive RNA unwinding. These results suggest that the ribose 2'-OH group in a given nucleotide is not an important determinant for binding to eIF4A; rather, the purine base adenine seems to contain the essential group(s) interacting with the active-site residues of the enzyme. As a comparison, HCV NS3H demonstrated a broader specificity; it hydrolysed all NTPs and dNTPs examined and used all these nucleotides as an energy source to drive unwinding (Figure 7). Although it is not clear if this is the case *in vivo*, such a broad specificity of the HCV NS3H may confer the virus to utilize any energy source possible for unwinding during viral replication.

The significant differences demonstrated by HCV NS3H and human eIF4A with respect to their substrate specificity suggest that development of specific HCV helicase inhibitors without much cross-reactivity with human enzymes is possible. As a

matter of fact, we have identified a few compounds that specifically inhibit HCV NS3H activity but not that of eIF4A (results not shown). However, given the facts that HCV NS3 demonstrated a lack of selectivity on both NTP binding/hydrolysis and DNA/RNA unwinding, designing potent inhibitors targeting either NTP hydrolysis or helicase unwinding activities of HCV NS3H would be challenging. The expected difficulties may explain, at least partially, why few potent HCV helicase inhibitors have been disclosed thus far compared with HCV protease and polymerase.

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