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Substrate Specificities of DDX1: A Human DEAD-Box Protein

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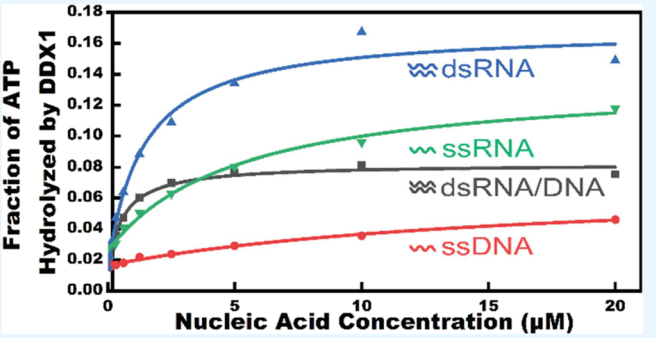
ABSTRACT: DDX1 is a human DEAD-box RNA helicase involved in various stages of RNA metabolism, from transcription to decay, and is consequently implicated in many human diseases. The nucleotides hydrolyzed by DDX1 and the structures of the nucleic acids upon which it acts in cells remain largely unknown. In this study, we identify the nucleic acid sequences and structures that support DDX1's nucleotide hydrolysis activity and determine its nucleotide hydrolysis specificity. Our data demonstrate that DDX1 hydrolyzes only ATP and deoxy-ATP in the presence of RNA. The ATP hydrolysis activity of DDX1 is stimulated by single-stranded RNA molecules as short as ten nucleotides, a blunt-ended double-stranded RNA, double-stranded RNA/DNA hybrid, and single-stranded DNA. Under our experimental conditions, single-stranded DNA stimulates DDX1's ATPase activity to a smaller extent compared to the other RNA constructs or the RNA/DNA hybrid. Given DDX1's involvement in numerous critical cellular processes and its implication in various human diseases, determining its substrate specificity not only enhances our understanding of its *in vivo* function, but also facilitates the development of novel therapeutic approaches.



INTRODUCTION

Helicases are enzymes that utilize the energy from nucleoside triphosphate (NTP) hydrolysis to unwind double-stranded DNA or RNA helices.[1] Based on their conserved amino acid motifs, helicases are classified into five superfamilies (superfamilies 1−5).[1] Members of superfamily 2 (SF2), which are further divided into ten families, participate in numerous processes of RNA metabolism and many aspects of DNA metabolism.[2,3] One of the families within SF2 is the DEAD-box RNA helicase family.[2,3] All DEAD-box proteins possess two covalently linked RecA-like domains, which form the ATP and nucleic acid binding pockets, as well as regions of communication between these pockets.[4−6] For the majority of DEAD-box proteins, the RecA-like domains use the energy of ATP binding and hydrolysis to unwind short RNA double helices, perform clamping, displace proteins from RNA, and/or anneal RNA.[4−6] Interestingly, although both the structure and sequence of DEAD-box proteins' RecA-like domains are conserved among this family of enzymes, members of the DEAD-box family show different nucleic acid substrate preferences (Table S1).[5,7−15] Moreover, a few DEAD-box proteins demonstrate a promiscuous NTP hydrolysis activity (Table S1).[9,16−18] Here, we systematically interrogate the NTP hydrolysis specificity of DDX1, a human DEAD-box RNA helicase, and determine the length, sequences, and structural properties of nucleic acid substrates supporting the DDX1 protein's NTP hydrolysis activity. The DDX1 protein engages in a wide range of cellular processes, including DNA double-strand break repair, RNA transcription, ribosomal RNA (rRNA), microRNA (miRNA), and transfer RNA (tRNA) processing, messenger RNA (mRNA) 3′-end maturation, translation initiation, and RNA transport, storage, and decay.[19−30] Consequently, this protein is implicated in the progressions of cancers, viral infections, and embryonic development.[20,31−36] Understanding how the catalytic activity of DDX1 is modulated by the NTPs and nucleic acid substrates is crucial for both comprehending DDX1's role in cellular processes and designing and developing novel therapeutic agents that target the DDX1 protein.

The modulation of DDX1 catalytic activity by various NTPs and deoxy-NTPs (dNTPs) is investigated in this study using the malachite green assay.[15,37] The modulation of DDX1 ATPase activity by nucleic acids of different sequences, lengths, and structural properties is investigated using thin-layer chromatography (TLC).[15,38,39] Combined, the experiments outlined in this study determine the NTPs and dNTPs that regulate DDX1 function and identify the structural features, sequences, and lengths of nucleic acids that stimulate DDX1's ATPase activity *in vitro*, which may correspond to the structural features, sequences, and lengths of its nucleic acid substrates *in vivo*.

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MATERIALS AND METHODS

Materials. 2-Mercaptoethanol (BME), Bradford reagents, and glacial acetic acid were obtained from Sigma-Aldrich. Dithiothreitol (DTT), Tris-base, concentrated HCl (12.1 M), $MgCl_2$, KCl, KOH, 40% acrylamide:bis-acrylamide solution (29:1 and 37.5:1), glycerol, and urea were obtained from Fisher. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Biosynth. The random RNA and/or DNA strands, as well as the $poly(A)_{10}$, $poly(C)_{10}$, $poly(G)_{10}$, and $poly(U)_{10}$ RNAs used in ATPase activity analysis, were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). [γ-^{32}P]-ATP (6000 Ci/mmol, 150 mCi/mL) was purchased from PerkinElmer. T4 polynucleotide kinase, CTP, GTP, UTP, dATP, dCTP, dGTP, and dTTP were purchased from New England Biolabs. The $tRNA^{Phe}$ from *Saccharomyces cerevisiae* (*S. cerevisiae*) was purchased from Sigma-Aldrich.

DDX1 Expression and Purification. The DDX1 clone was a gift from the Roseline Godbout laboratory at the University of Alberta.[22] The DDX1 protein is 720 amino acids long.[22,35,40] We attempted to express and purify the full-length enzyme but were unable to do so because it was digested by bacterial proteases. Consequently, we switched to a truncated, His-tagged construct, as previously employed by Kellner et al.[40]

The coding sequence of the DDX1 protein was PCR amplified and inserted into the pET28a vector (Novagen). A His-tag was placed in the N-terminus of DDX1, and a stop codon was inserted at the coding sequence of amino acid 708.

As described in the Kellner *et al.* study, four chromatography purification steps were necessary to obtain a homogeneous DDX1 protein sample.[40] *Escherichia coli* (*E. coli*) BL21 (DE3) CodonPlus was transformed with the pET28a vector bearing the coding sequence of the DDX1 protein, and cells were grown in Luria broth media containing 30 μg/mL of kanamycin and 34 μg/mL of chloramphenicol. Purification of the expressed DDX1 was carried out similar to procedures published by Kellner *et al.*[40] Briefly, cell pellets were resuspended in a nickel column equilibrating buffer (50 mM Tris−HCl (pH 8.0) at 4 °C, 250 mM KCl, 10 mM BME, and 1 mM PMSF) and supplemented with a protease inhibitor cocktail (cOmplete Protease Inhibitor Cocktail Tablets, EASYpack, Roche). Cells were lysed by sonication on ice (Branson Digital Sonifier 450, equipped with a standard flat tip, 0.5 in outer diameter, set at 50% amplitude) in six 30 s pulses and 5 min between pulses. The lysate was spun down at 14,000 rpm for 60 min. The supernatant was loaded onto an 8 mL Ni-NTA column. The protein was eluted with 250 mM imidazole. Pooled fractions were diluted 1:10 with the heparin column equilibrating buffer (50 mM Tris−HCl, pH 8.0 at 4 °C, 5 mM $MgCl_2$, 3 mM DTT, and 1 mM PMSF), loaded onto a 5 mL heparin column (HiTrap heparin HP, GE Healthcare Life Sciences), and washed with the heparin equilibrating buffer supplemented with 10 mM KCl. The protein was eluted on a linear gradient with the heparin equilibrating buffer supplemented with 1 M KCl. Fractions containing the desired protein were pooled and diluted 1:10 with an anion-exchange equilibrating buffer (50 mM Tris−HCl at pH 9.0 at 4 °C, 5 mM $MgCl_2$, 3 mM DTT, and 1 mM PMSF), loaded onto an anion-exchange column (HiTrap Q HP, GE Healthcare Life Sciences), washed with the anion-exchange equilibrating buffer supplemented with 30 mM KCl, and eluted with a linear gradient of an anion-exchange equilibrating buffer supplemented with 1 M KCl. Fractions containing the desired protein were pooled, concentrated, and further purified via a gel-filtration column (Superdex 200 10/300 GL, GE Healthcare), which was equilibrated with the gel-filtration buffer (10 mM HEPES-KOH, pH 8.0, 250 mM KCl, 5 mM $MgCl_2$, and 3 mM DTT) (Figure S1). Protein concentrations were determined via a Bradford assay using SpectraMax Plus 384 (Molecular Devices). Pooled fractions were concentrated via 10 kDa centrifugal filters (Amicon Ultra Ultracel-10K, Millipore) and stored at −80 °C in small aliquots. As described in the previous study, four chromatography purification steps were necessary to obtain a homogeneous DDX1 protein sample.[40]

Malachite Green Phosphate Assay. The malachite green-molybdate assay was employed to measure the DDX1 protein's NTP and dNTP hydrolysis activity.[15,37] $tRNA^{Phe}$ was employed as a substrate for these experiments because it was shown from a previous study to support the ATPase activity of DDX1.[35]

The malachite green-molybdate reagent was prepared by mixing 0.034% malachite green, 1.04% ammonium molybdate, and 1 M HCl, stirring constantly overnight at 4 °C, filtering the solution, and storing it at 4 °C. Poly(vinyl alcohol), which acts as a stabilizing reagent, was added to a concentration of 0.4%. The final mixture was equilibrated at room temperature before use.

The NTP and dNTP hydrolysis reactions were performed in a reaction mixture consisting of 50 mM Tris−HCl (pH 8.0), 250 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 1 μM DDX1, and 1 mM nucleotide or deoxynucleotide in the presence or the lack of 20 μM $tRNA^{Phe}$. The background control reactions lacked the DDX1 protein. Reactions were initiated by the addition of a nucleotide or a deoxynucleotide solution. 10 μL of DDX1 hydrolysis reactions were allowed to continue for 60 min at 23 °C in 384-well microplates. The hydrolysis reaction was quenched with 2.5 μL of 50 mM EDTA-KOH (pH 8.0) and incubated for an additional 5 min before adding 75 μL of the malachite green-molybdate reagent. After 1 min, the malachite green-molybdate reagent reaction was quenched with 10 μL of 34% sodium citrate and allowed to develop for 50 min, before absorbance was measured at 625 nm using SpectraMax Plus 384 (Molecular Devices).

The absorbance values for the background control and experimental samples were averaged. The average absorbance values of the background control reactions were then subtracted from those of the experimental samples. Errors were calculated by using error propagation for both the control and experimental samples.

The dependence of the malachite green assay on the inorganic phosphate concentration, reaction time, and DDX1 concentration is illustrated in Figures S2 and S3.

TLC ATPase Assay. Hydrolysis of ATP was monitored using TLC as previously described.[15,38,39] First, the RNA was annealed, if necessary, by incubating it with 50 mM HEPES-KOH (pH 7.5) and 50 mM KCl at 95 °C for 1 min, 65 °C for 3 min, and cooled to 25 °C for 1 min, before adding $MgCl_2$ to a final concentration of 10 mM. The annealed solution was incubated at 23 °C for 20 min before placing on ice. Subsequently, varying concentrations of the RNA substrate were mixed with 25 mM Tris−HCl (pH 8.0), 2 mM $MgCl_2$, 250 mM KCl, 2 mM DTT, 0.5 mM EDTA-KOH (pH 8.0), 1 μM DDX1, 6 mM ATP, and 2.5 nM γ-^{32}P-labeled ATP and then incubated at 37 °C for 60 min. After incubation, the reactions were quenched with 0.5 M EDTA-KOH (pH 8) and

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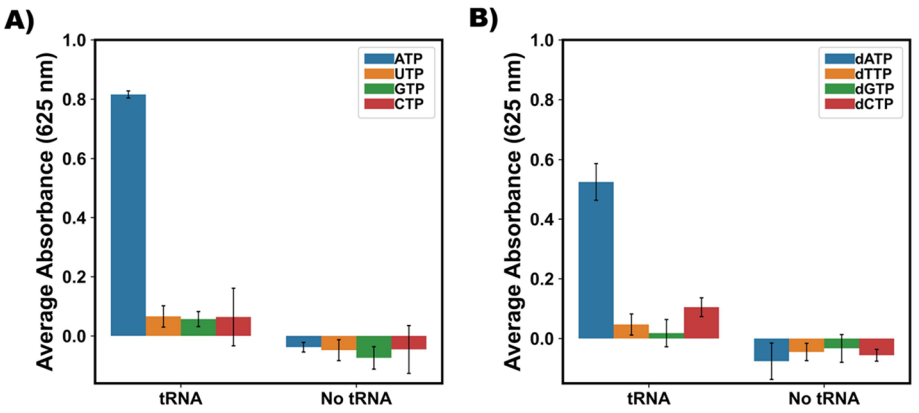

Figure 1. The DDX1 protein hydrolyzes ATP and dATP in the presence of $tRNA^{Phe}$ from *S. cerevisiae*. The malachite green colorimetric assay was employed to examine the DDX1 protein's specificity for the four nucleotides and deoxynucleotides. (A) NTPs employed to investigate the DDX1 protein's specificity are ATP (blue), UTP (brown), GTP (green), and CTP (red). The absorbance values shown were background-corrected by subtracting from the absorbance of reactions performed in the presence of DDX1, $tRNA^{Phe}$ and NTP, or from the absorbance of reactions performed in the presence of DDX1 and NTP, the absorbance of reactions performed in the presence of $tRNA^{Phe}$ and NTP only. (B) dNTPs employed to investigate the DDX1 protein's specificity are dATP (blue), dTTP (brown), dGTP (green), and dCTP (red). The data are background-corrected as in (A). For both panels (A) and (B), the depicted absorbance values represent the background-corrected averages from three independent experiments. The errors were calculated through error propagation.

spotted on PEI-cellulose F-coated TLC plates. The TLC plates were developed using a solvent system consisting of 0.75 M LiCl and 1 M acetic acid. TLC plates were left to dry and then exposed to phosphor imaging screens. The screens were scanned using a Personal Molecular Imager System (Bio-Rad Laboratories) and analyzed using Quantity One (Bio-Rad Laboratories). The fraction of ATP hydrolyzed was calculated as the ratio of the counts on the inorganic phosphate band over the total counts on the lane. The ATP hydrolysis values in the absence of RNA were not subtracted from the reaction samples that contained RNA. The Michaelis–Menten equation was employed to fit the fraction of ATP hydrolyzed versus the RNA concentration (eq 1). OriginLab was used to fit the data.

$$f_B = f_B(0) + \left[f_B(\max) - f_B(0)\right]\left\{\frac{[\text{substrate}]}{K_m + [\text{substrate}]}\right\} \quad (1)$$

In eq 1 f_B is the fraction of ATP hydrolyzed, $f_B(0)$ and $f_B(\max)$ are the lower and upper baselines of ATP hydrolysis, K_m is the Michaelis constant, and [substrate] is the nucleic acid concentration. The ATP hydrolysis signal in the absence of RNA was not subtracted from the ATP hydrolysis signal obtained in the presence of RNA.

The amount of inorganic phosphate observed in the absence of the nucleic acid substrate could be due to inorganic phosphate contamination in [γ-^{32}P]-ATP, spontaneous ATP hydrolysis, or errors in background radioactive count correction. To correct for the background in the data, we subtract from each area containing counts for [γ-^{32}P]-ATP or $^{32}PO_4$, the counts from an area of the same size in a randomly selected location on the phosphorimager screen.[15,38,39,41,42] However, different areas on the screen may have varying background levels, which could also affect the inorganic phosphate counts in the absence of RNA or DNA. The $f_B(0)$ term in eq 1 accounts for the inorganic phosphate present in the ATP mixture, spontaneous ATP hydrolysis, and potential inaccuracies in the performance of background correction. An example of a TLC plate is shown in Figure S4.

Hazards/Risks Associated with the Methods. The ^{32}P isotope emits high-energy beta particles that can cause significant damage to human cells. Lab coats, safety glasses, gloves, a radiation monitoring badge, and a thick acrylic shield should be used when handling ^{32}P-labeled ATP in the experiments described here. A Geiger counter should also be used to check the working area for contamination.

Polyacrylamide is a potential human carcinogen. Lab coats, gloves, and safety glasses should be worn when performing the experiments described in this manuscript involving polyacrylamide.

RESULTS AND DISCUSSION

The DDX1 Protein's NTP and dNTP Specificity. The majority of DEAD-box protein family members hydrolyze only ATP and deoxy-ATP (dATP).[5,7,8] On the other hand, the human DEAD-box protein DDX3X and the *S. cerevisiae* DEAD-box protein Mss116 bind and hydrolyze all four NTPs and deoxynucleotides (dNTPs).[16−18] In addition, the *S. cerevisiae* DEAD-box protein Dhh1 binds all four nucleotides and dATP but hydrolyzes only ATP and dATP.[9] The NTP and dNTP specificity of DDX1 has not been previously examined. Here, the specificity of the DDX1 protein for NTPs and dNTPs was examined by employing the malachite green phosphate assay.[15,37]

The malachite green phosphate assay quantifies the complex formed among malachite green, molybdate, and the inorganic phosphate produced by DDX1-mediated hydrolysis of nucleotides or deoxynucleotides. Our data show that the DDX1 protein specifically hydrolyzes ATP and dATP, but only in the presence of $tRNA^{Phe}$ (Figure 1). The DDX1 protein is unable to hydrolyze other NTPs or dNTPs, nor can it hydrolyze nucleotides in the absence of RNA. Thus, the DDX1 protein exhibits a nucleotide specificity similar to that of the majority of the investigated DEAD-box protein family members (Table S1).[5,7,8]

A 10-Nucleotide Long RNA Substrate Is Sufficient for Supporting the ATPase Activity of DDX1. The ATPase activity of different DEAD-box proteins is stimulated by varied-length single-stranded RNA (ssRNA). A survey of the DEAD-

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Table 1. Nucleic Acid Molecules Employed for the TLC ATPase Assay

Nucleic Acid Molecules	Sequences
ssRNA [a] 10-mer	5'CUCAACUCAA
ssRNA [a] 18-mer	5'CUCAACUCAACCCUUCAU
ssRNA [a] 36-mer	5'CUCAACUCAACCCUUCAUCUCAACUCAACCCUUCAU
poly(A)	5'AAAAAAAAAA
poly(C)	5'CCCCCCCCCC
poly(G)	5'GGGGGGGGGG
Poly(U)	5'UUUUUUUUUU
dsRNA[b]	5' CUCAACUCAACCCUUC AUCUCAACUC AACCCUUCAU 3'GAGUUGAGUUGGGAAGUAGAGUUGAGUUGGGAAGUA
ssDNA [c] 36-mer	5'dCdTdCdAdAdCdTdCdAdAdCdCdCdTdTdCdAdTdCdTdCdAdAdCdTdCdAdAdCdCdCdTdTdCdAdT
dsRNA/DNA [d]	5'dCdTdCdAdAdCdTdCdAdAdCdCdCdTdTdCdAdTdCdTdCdAdAdCdTdCdAdAdCdCdCdTdTdCdAdT 3' G A G U U G A G U U G G G A A G U A G A G U U G A G U U G G G A A G U A

[a]The ssRNA 18-mer RNA molecule contains the sequence of 10-mer and eight extra bases. The ssRNA 36-mer is the 18-mer ssRNA sequence repeated twice. [b]The dsRNA molecule consists of the 36-mer ssRNA and its reverse complement. [c]The ssDNA 36-mer molecule consists of the same sequence as the ssRNA 36-mer molecule. [d]The dsRNA/DNA molecule has the same sequence as the dsRNA molecule.

box protein crystal structures in complex with ssRNA reveals that the footprint of the DEAD-box protein catalytic core on RNA is between 6 and 10 nucleotides.[43−46] In addition, the length of ssRNA was shown to modulate the Dhh1 protein's binding affinity for poly(U) molecules.[9] The Dhh1 protein binds a 12-nucleotide long poly(U) 15-fold tighter than a 10-nucleotide long poly(U), while it binds a 20-nucleotide long poly(U) 30-fold tighter than a 10-nucleotide long poly(U).[9] The ssRNA molecule length required to support the DDX1 ATPase activity was not previously examined. Here, employing TLC assays, we examined the ability of different-length ssRNA molecules to support the DDX1 protein's ATPase activity.[15,38,39]

The lengths of the molecules used for the TLC assay were 10-nucleotide long, 18-nucleotide long, and 36-nucleotide long (Table 1). The first ten residues in the 18-nucleotide long RNA molecule are the same as in the 10-nucleotide long RNA molecule, while the 36-nucleotide long RNA construct is the repeat of the 18-nucleotide long RNA molecule. Lastly, Mfold was used to investigate the propensity of the 10-nucleotide long, 18-nucleotide long, and 36-nucleotide long molecules to form secondary structures.[47] Mfold predicted no stable secondary structure formation for the RNA molecules. Thus, the 10-nucleotide long, 18-nucleotide long, and 36-nucleotide long molecules employed here are predicted to be single-stranded in solution.

The dependence of ATP hydrolysis on the concentration of RNA, as measured by TLC, reveals that all three RNA constructs, independent of their lengths, stimulate the ATPase activity of DDX1 to the same extent and with a similar K_m (Figure 2 and Table 2). Thus, a 10-nucleotide long RNA construct is sufficient to support the ATPase activity of DDX1.

10-Nucleotide Long Poly(A), Poly(C), and Poly(U) Molecules Support the ATPase Activity of the DDX1 Protein, While a 10-Nucleotide Long Poly(G) Molecule Does Not. A large number of DEAD-box proteins show sequence nonspecific RNA-dependent ATPase activity.[5,7,42,48−50] However, the *Thermus thermophilus* (*T. thermophilus*) DEAD-box protein Hera is unable to form the closed, ATPase-competent conformation in the presence of poly(G).[10] Moreover, poly(G) and poly(U) molecules support the ATPase activity of the *S. cerevisiae* DEAD-box protein Ded1p to a significantly lesser extent when compared to poly(A) and poly(C) molecules.[11] Lastly, poly(A), poly(C), and poly(U) stimulate the ATPase activity of Dhh1, *S. cerevisiae* Dbp5, and human Dbp5 (DDX19) to a greater extent

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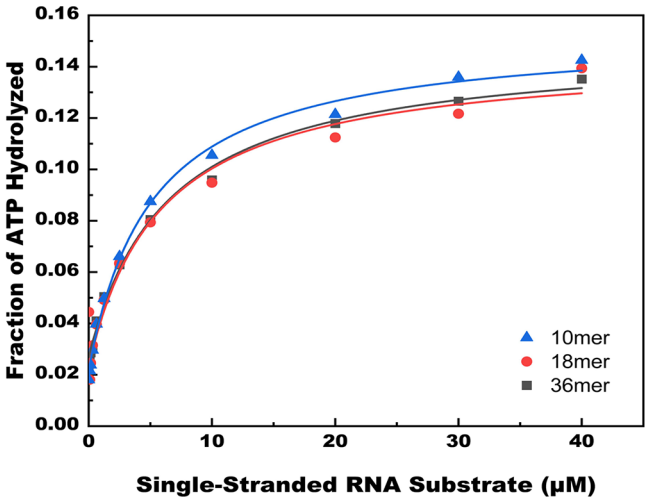

Figure 2. The single-stranded 10-nucleotide long RNA substrate supports the ATPase activity of the DDX1 protein to the same extent as the longer single-stranded RNA substrates. TLC was employed to determine the dependence of the DDX1 protein's ATP hydrolysis activity on RNA concentration. The graphs represent the dependence of the DDX1 protein's ATPase activity on the RNA concentration. The fraction of ATP hydrolyzed was calculated as described in the Materials and Methods section and corresponds to the 60 min duration of the reaction. Eq 1 was used to fit the data. The average value for K_m and the extent of maximum ATP hydrolyzed obtained from at least two independent experiments are shown in Table 2. The sequences of the single-stranded 10-, 18-, and 36-nucleotide long RNA molecules are shown in Table 1. The dependence of DDX1 ATP hydrolysis versus the 10-nucleotide long RNA concentration is shown in blue, the 18-nucleotide long RNA in red, and the 36-nucleotide long in gray.

Table 2. Stimulation of ATP Hydrolysis by RNA and DNA Constructs

Nucleic Acid Molecules	K_m (μM)[a]	Maximum Fraction[b] of ATP Hydrolyzed	k_{cat} (min^{-1}[c])
ssRNA 10-mer	5.14 ± 0.10	0.12 ± 0.01	12 ± 1
ssRNA 18-mer	8.15 ± 4.78	0.15 ± 0.03	15 ± 3
ssRNA 36-mer	6.10 ± 0.46	0.12 ± 0.003	12 ± 0.3
poly(A)	6.06 ± 0.56	0.11 ± 0.004	11 ± 0.4
poly(C)	7.68 ± 0.71	0.12 ± 0.004	12 ± 0.4
poly(G)[d]			
poly(U)	15.58 ± 6.33	0.11 ± 0.004	11 ± 0.4
dsRNA	0.92 ± 0.62	0.18 ± 0.03	18 ± 0.3
ssDNA 36-mer	24.92 ± 11.95	0.05 ± 0.001	5 ± 0.1
dsRNA/DNA	0.55 ± 0.06	0.07 ± 0.004	7 ± 0.4

[a]ATP hydrolysis in the presence of different nucleic acid molecules was determined using TLC. The K_m of ATP hydrolysis was calculated using eq 1 in the Materials and Methods section of the paper. The data shown are the averages obtained from at least two independent experiments. The error bars are the standard deviations from the averages. [b]The fraction of ATP hydrolyzed was calculated as described in the Materials and Methods section and corresponds to the 60 min duration of the reactions. The maximum fraction of ATP hydrolyzed was obtained by fitting eq 1 in the Materials and Methods section to TLC data. The data shown are the averages and the standard deviations obtained from at least two independent experiments. [c]The k_{cat} was determined by calculating the maximum fraction of 6 mM ATP hydrolyzed during a 60 min reaction in the presence of 1 μM DDX1. The maximum fraction of ATP hydrolyzed was calculated as described in eq 1. [d]The poly(G) molecule does not stimulate the ATPase activity of the DDX1 protein.

than poly(G) molecules.[9,12] The regulation of the DDX1 protein's ATPase activity by various RNA homopolymers has not been fully investigated.

Initially, Chen *et al.* investigated the stimulation of DDX1 protein's ATPase activity in the presence of poly(A), poly(C), poly(U), and poly(G), which had different lengths from hundreds to thousands of nucleotides.[19] They found that poly(A), poly(C), and poly(U) molecules supported the ATPase activity of DDX1, while the poly(G) molecules did not.[19] Since the Chen *et al.* experiments were performed in the presence of different-length RNA homopolymers, these experiments could not offer insights into the regulation of the DDX1 protein's ATPase activity by a specific homopolymer separated from the homopolymer length. More recently, Kellner *et al.* found that both 10-nucleotide long poly(A) and poly(U) molecules support the ATPase activity of DDX1.[40] The 10-nucleotide long poly(C) and poly(G) constructs were not investigated in the Kellner *et al.* study.[40] Here, we systematically examined whether 10-nucleotide long poly(A), poly(C), poly(G), and poly(U) molecules differently regulate the ATPase activity of the DDX1 protein.

TLC was employed to investigate the preference of DDX1 for 10-nucleotide long RNA homopolymers (Figure 3, Tables 1, and 2).[15,38,39] All of the RNA homopolymers, with the exception of poly(G), support the ATPase activity of DDX1.

The poly(G) employed here, due to its small size, is predicted by QGRS Mapper to be unable to form a G-quadruplex.[51] The poly(G) molecules used in Chen *et al.*, which also did not support the ATPase activity of DDX1, were

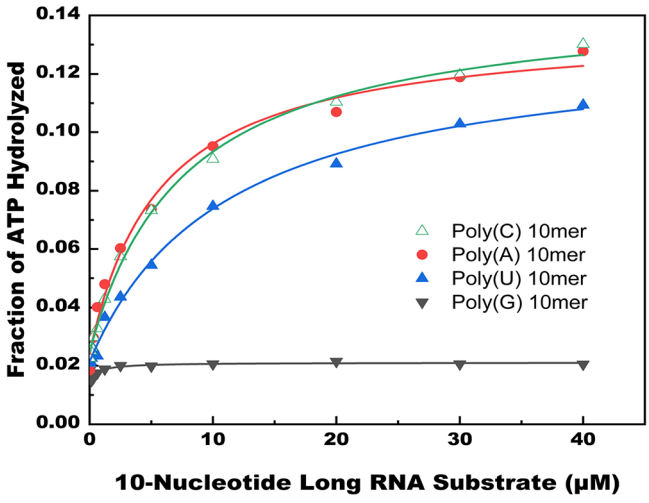

Figure 3. The dependence of the DDX1 protein's ATPase activity on the RNA homo-oligonucleotides. TLC was employed to determine the dependence of the DDX1 ATP hydrolysis activity on 10-nucleotide-long poly(A), poly(C), poly(G), and poly(U) concentrations. The graphs represent the dependence of the DDX1 protein's ATPase activity on 10-nucleotide-long poly(A), poly(C), poly(G), and poly(U) concentrations. The fraction of ATP hydrolyzed was calculated as described in the Materials and Methods section and corresponds to the 60 min duration of the reaction. Eq 1 was used to fit the data and determine the K_m and the maximum fraction of ATP hydrolyzed. The average values for K_m and the maximum fraction of ATP hydrolyzed obtained from at least two independent experiments are shown in Table 2. The dependence of DDX1 ATP hydrolysis versus poly(A) RNA concentration is shown in red circles, poly(C) in green triangles, poly(G) in gray triangles, and poly(U) in blue triangles.

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several hundred to a thousand nucleotides in length.[19] These molecules could potentially form G-quadruplexes in the presence of Na^+, K^+, or Li^+ ions.[52] However, Chen *et al.* did not use Na^+, K^+, or Li^+ ions in their ATP hydrolysis assay; as a result, the poly(G) molecules in that study could not form G-quadruplexes either.[19,52] Recently, it was shown that the DDX1 protein binds to a G-quadruplex but not to an RNA sequence rich in G nucleotides, which was unable to form the G-quadruplex structure.[8] Thus, an attractive hypothesis based on our data and previous results is that DDX1, during its cellular functions, has evolved to recognize G-quadruplex structures and interacts poorly with single-stranded G-rich sequences.

The inability of the single-stranded poly(G) molecule to support the ATPase activity of DDX1, while single-stranded poly(A), poly(C), and poly(U) molecules do support its activity, may be due to the conformation of single-stranded poly(G) in solution. The single-stranded poly(A) and poly(C) RNA molecules form ordered coil and helical structures in solution, while the poly(U) RNA samples form random coil conformations.[53] On the other hand, the solution structural conformation of single-stranded poly(G) remains unknown and may differ from the ordered coil, random coil, or helical conformations adopted by poly(A), poly(C), and poly(U).[53] This difference in structural conformation could render poly(G) refractory to DDX1 binding and/or incapable of stimulating its ATPase activity.

The ability to discriminate between different conformations of nucleic acid substrates may not be unique to DDX1 but could represent a general mechanism employed by DEAD-box proteins during their catalytic activity. Another DEAD-box protein, Mss116, has been shown to discriminate between different nucleic acid conformations. The A-form RNA and DNA double helices support the unwinding activity of Mss116, whereas the B-form DNA does not.[18] Thus, during its catalytic cycle, the Mss116 protein is able to distinguish between A-form and B-form double-stranded DNA.[18]

Blunt-Ended Double-Stranded RNA (dsRNA) and Double-Stranded RNA/DNA (dsRNA/DNA) Hybrid Molecules Support the DDX1 Protein's ATPase Activity. The ATPase activity of a large number of DEAD-box proteins is stimulated by blunt-ended dsRNA molecules.[5] However, single-molecule fluorescence experiments revealed that the *Caenorhabditis elegans* (*C. elegans*) LAF-1 DEAD-box protein does not bind to blunt-ended dsRNA.[13] In addition, the human ortholog of LAF-1, the DDX3X protein, was unable to bind to blunt-ended dsRNA molecules; thus, these helices did not support the DDX3X protein's ATP hydrolysis activity.[14,15] Here, employing the TLC assay, we investigated whether a blunt-ended dsRNA molecule supports the ATPase activity of DDX1.[15,38,39]

The dependencies of the DDX1 protein's ATP hydrolysis activity in the presence of various concentrations of nucleic acid molecules reveal that the blunt-ended dsRNA and a blunt-ended dsRNA/DNA hybrid stimulate the ATP hydrolysis activity of DDX1. However, the extent of the stimulation of the DDX1 protein's ATP hydrolysis activity is partially reduced in the presence of the dsRNA/DNA hybrid molecule (Figure 4 and Table 2).

Since both dsRNA and dsRNA/DNA hybrid molecules share the same sequence and adopt an A-form conformation in solution, the reduced ATPase activity observed with the dsRNA/DNA hybrid compared to the dsRNA molecule could be a consequence of the absence of 2′OH groups in the hybrid.

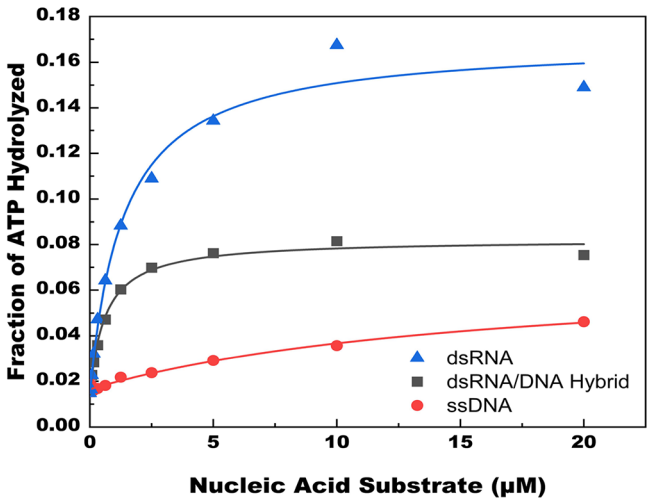

Figure 4. dsRNA, dsRNA/DNA hybrid, and ssDNA stimulate the ATPase activity of the DDX1 protein. TLC was used to measure the extent of the DDX1 protein's ATP hydrolysis in the presence of a blunt-ended dsRNA molecule, a blunt-ended dsRNA/DNA hybrid molecule, or an ssDNA molecule (Table 1). The graphs represent the dependence of the DDX1 protein's ATPase activity on the dsRNA, dsRNA/DNA, and ssDNA concentrations. The fraction of ATP hydrolyzed was calculated as described in the Materials and Methods section and corresponds to the 60 min duration of the reaction. Eq 1 was used to fit the data and determine the K_m and the maximum fraction of ATP hydrolyzed. The average values for K_m and the maximum fraction of ATP hydrolyzed obtained from at least two independent experiments are shown in Table 2. The dependence of DDX1 ATP hydrolysis versus dsRNA concentration is shown in blue triangles, the dsRNA/DNA hybrid in gray squares, and ssDNA in red circles.

The 2′OH groups have been shown to promote the formation of the ATPase-competent conformation in several DEAD-box proteins.[5,43,54,55] The structure of the DDX1 protein in complex with an RNA or DNA substrate remains undetermined. Future structural studies of DDX1 in complex with RNA and DNA substrates could provide insights into the structural changes modulated by RNA 2′OH moieties during the DDX1 catalytic cycle.

An ssDNA Molecule Supports the DDX1 Protein's ATPase Activity. With a few exceptions, the ATP hydrolysis activity of DEAD-box proteins is stimulated only by RNA molecules.[5,7,8] DEAD-box proteins are RNA-dependent ATPases.[5,7,8] The exceptions are the human DEAD-box proteins DDX3X, DDX43, and Dbp5p (DDX19), *S. cerevisiae* DEAD-box proteins Dbp5p and Dbp9p, and the *Pisum sativum* DEAD-box protein p68.[12,17,56−59] ATP hydrolysis activities of the above DEAD-box proteins are stimulated by both RNA and DNA molecules. The TLC experiments in Figure 4 and Table 2 reveal that the DDX1 protein's ATPase activity is also stimulated by both RNA and DNA molecules.

The finding here that a DNA molecule supports the ATPase activity of DDX1 contradicts a previous study by Chen *et al.*, which reported that DNA does not stimulate the ATPase activity of this protein.[19] The discrepancy between the two studies could be due to the lower concentration of ATP used in the previous study. In the Chen *et al.* study, only 50 μM ATP was used to investigate the stimulation of DDX1 ATPase activity by DNA, whereas in this study, we performed the reaction with 6 mM ATP.[19] Since the K_m of ATP hydrolysis by the DDX1 protein in the presence of different RNA molecules

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and reaction conditions was measured to be between 1000 and 1750 μM, the concentration of ATP employed by Chen *et al.* would be too low to produce a detectable signal for the stimulation of DDX1 ATPase activity in the presence of DNA.[19,35,40]

The *E. coli*-Purified DDX1 Protein Shows No Detectable Helicase Activity *In Vitro*. Gel electrophoresis experiments performed by Li *et al.* showed that the *E. coli*-expressed DDX1 proteins possessed ADP-dependent helicase activity and ATP-dependent single-stranded RNase activity.[22] Employing Li *et al.*'s reaction conditions, gel electrophoresis, and RNA molecules, we did not observe detectable DDX1 RNase or ADP-dependent helicase activity (Figures S5 and S6). Thus, the helicase activity of DDX1 in the presence of ADP and the RNase activity in the presence of ATP are below the detection limit of our assay.

The difference observed between our study and the Li *et al.* study could be a consequence of the differences in the DDX1 constructs employed in the two studies.[22] The construct employed here, as explained in the Materials and Methods section, contained an N-terminal histidine tag and lacked 12 residues from its C-terminal.[40] The final construct used by Li *et al.* did not have an N-terminal tag and contained the entire coding sequence of the DDX1 protein.[22] Furthermore, there were differences in the protein purification protocols. For the DDX1 protein purification, Li *et al.* employed solely S-transferase tag affinity.[22] In our experience, as well as in the publication by Kellner *et al.*, all four column chromatography steps detailed in the Materials and Methods section of the paper were necessary to obtain highly pure DDX1 protein.[40] Since Li *et al.* employed solely the S-transferase tag affinity purification step, it is possible that other proteins with RNase and ADP-dependent helicase activity were copurified with the DDX1 protein in that study.[22]

The helicase activity of DDX1 was also investigated by Chen *et al.*[19] They demonstrated that an RNA molecule, consisting of a double-stranded region and a long poly(A) tail, supported the helicase activity of DDX1 protein immunoprecipitated from Y79 human cells.[19] However, the same RNA molecule did not support the helicase activity of recombinant DDX1 protein purified from *Spodoptera frugiperda*21 (*Sf21*) insect cells.[19] In our study, we investigated the helicase activity of the *E. coli*-purified DDX1 protein using the same RNA molecule and reaction conditions employed by Chen *et al.*[19] Consistent with the findings from *Sf21* insect cells, the *E. coli*-purified DDX1 protein in our study also lacked detectable helicase activity (Figure S7). Furthermore, Drino *et al.* recently examined the helicase activity of the *E. coli*-purified DDX1 protein under various buffer conditions and in the presence of ATP, but they too were unable to detect helicase activity.[60]

Our results, combined with Chen *et al.* and Drino *et al.* findings, suggest two possibilities: (i) post-translational modifications made by unique human enzymes, and/or (ii) a protein cofactor associated with the DDX1 protein, immunoprecipitated from human cells, stimulates DDX1 helicase activity.[19,60] Protein cofactors have been shown to regulate the ATPase and helicase activities of various DEAD-box proteins, while post-translational modifications of DDX1 have been implicated in regulating its functions during miRNA processing, translation, DNA repair, and the oxidative stress response.[6,22,24,31,32] The regulation of the DEAD-box protein catalytic cycle by post-translational modifications remains an unexplored area of research.[6] Future helicase assays using DDX1 protein isolated from human cells, conducted in the presence or absence of potential DDX1 cofactors, will provide insights into how post-translational modifications or protein cofactors regulate the DDX1 catalytic cycle.

CONCLUSIONS

The sequence and structure of the RNA and DNA regions which the DDX1 protein directly contacts during its vast and diverse cellular functions, similar to most members of the DEAD-box family of enzymes, remain largely uncharacterized.[5,6,19−32] The experiments outlined here reveal that the ATPase activity of the DDX1 protein, unlike that of the human DDX3X protein and *C. elegans* LAF-1 protein, is stimulated by blunt-ended dsRNA (Figure 4).[13−15] Thus, blunt-ended dsRNA molecules likely serve as DDX1 substrates *in vivo*. In addition, we find that a blunt-ended dsRNA/DNA hybrid molecule, an ssRNA molecule, and an ssDNA molecule stimulate the ATPase activity of DDX1. Therefore, all of these molecules are also potential *in vivo* substrates for the DDX1 protein.

The interaction of the DDX1 protein with blunt-ended dsDNA/RNA and ssDNA molecules could be necessary for its molecular functions during transcription regulation and DNA repair.[22,25] Our *in vitro* experiments show that ssDNA stimulates the ATPase activity of the DDX1 protein to a smaller extent than all the other nucleic acid constructs investigated here (Figure 4). Because protein cofactors have been shown to regulate the ATPase activity of several DEAD-box proteins, it is plausible that, *in vivo*, a protein cofactor could enhance the DDX1 protein's ATPase activity in the presence of ssDNA.[6]

A limitation of this study is that the investigation of the activation of the DDX1 protein's NTP/dNTP hydrolysis activity was performed at one concentration of NTP/dNTP and at one time point in the reactions (Figure 1). Thus, the conclusions concerning the inability of the DDX1 protein to hydrolyze other nucleotides in addition to ATP and dATP are correct only for the described reaction conditions. It is possible that under higher concentrations of NTP/dNTP, reactions performed at longer time points, or in the presence of different buffers, the DDX1 protein could hydrolyze other NTPs/dNTPs. The ATPase activity of many DEAD-box proteins is enhanced by cofactors.[6] Hence, it is also possible that a cofactor could alter the DDX1 preference for NTP/dNTP hydrolysis. Future experiments measuring the extent of DDX1 hydrolysis activity at different reaction time points and different concentrations of NTP/dNTP will determine whether, at higher concentrations of NTP/dNTP and longer reaction times, the DDX1 protein can hydrolyze other NTPs/dNTPs in addition to ATP and dATP. Additionally, if cofactors are found that regulate the DDX1 ATPase activity, then the ability of these factors to regulate the DDX1 preference for NTP/dNTP hydrolysis will be investigated.

Lastly, we do not have a positive control for DDX1 enzymatic activity to confirm the absence of helicase activity under our reaction conditions. We only have a heat-denatured control showing how the unwinding product would appear if DDX1 completely unwound its RNA substrate (Figures S6 and S7). Thus, it is possible that the DDX1 protein purified from *E. coli* exhibits minimal helicase activity undetectable by our assay, or that it performs only one round of unwinding and fails to complete a second cycle. Future experiments aimed at detecting a small fraction of unwound RNA will be performed

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in the presence of a high concentration of ^{32}P-labeled RNA double-stranded substrate relative to DDX1.[39,61] Moreover, in these reactions, an excessive concentration of an unlabeled RNA competitor to the double-stranded RNA substrate will be employed. The unlabeled RNA competitor would prevent reannealing of the double helices that DDX1 might unwind under these conditions.[39,61]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c07522.

Comparison of known DDX1 activities with those of other DEAD-box proteins; sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified DDX1 protein; dependence of malachite green assay signal on phosphate concentration; dependence of DDX1 ATPase activity on time and DDX1 concentrations: example of thin-layer chromatography (TLC); DDX1 RNase activity assay; DDX1 helicase activity assay in the presence of ADP and ATP employing Li *et al.* RNA molecules;[22] DDX1 helicase assay employing Chen *et al.* RNA molecules[19] (PDF)

Accession Codes

Human DDX1: Uniprot ID Q92499, human DDX3X: Uniprot ID O00571, human DDX43: Uniprot ID Q9NXZ2, *S. cerevisiae* Dbp5p: Uniprot P20449, human Dbp5p (DDX19): Uniprot ID Q9NUU7, *S. cerevisiae* Dbp9p: Uniprot Q06218, *Pisum sativum* p68: Uniprot ID Q9LKL6, *S. cerevisiae* Mss116Uniprot ID P15424, *C. elegans* LAF-1: Uniprot ID D0PV95, *S. cerevisiae* Dhh1: Uniprot ID P39517, *T. thermophilus* Hera: Uniprot ID O07897.

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Notes

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