An RNA Helicase, DDX1, Interacting with Poly(A) RNA and Heterogeneous Nuclear Ribonucleoprotein K*

Received for publication, July 12, 2002, and in revised form, August 12, 2002
Published, JBC Papers in Press, August 14, 2002, DOI 10.1074/jbc.M206981200

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Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a multifunctional protein known to be involved in the regulation of transcription, translation, nuclear transport, and signal transduction. To systematically obtain insight into mechanisms of hnRNP K activities, we set out to identify protein factors that interact with hnRNP K by using glutathione S-transferase-hnRNP K affinity chromatography followed by liquid chromatography/mass spectrometry/mass spectrometry analysis. Several partner proteins in the K562 cell lysates were identified through this method. One of them is a DEAD box-containing putative RNA helicase, DDX1. In vitro binding and co-immunoprecipitation studies confirmed the protein-protein interaction between hnRNP K with DDX1, and the region spanning amino acids 1–278 of hnRNP K is apparently responsible for its physical interaction with DDX1. Interestingly, their interaction was disrupted by the addition of poly(C), poly(A), and poly(U) RNA substrates. We found that DDX1 was a homopolymeric poly(A) RNA-binding protein. On the other hand, the ATPase activity of the purified recombinant DDX1 protein was stimulated by these homopolymeric RNAs and yeast total RNA but not by DNA. Moreover, the immunoprecipitated DDX1 complex but not purified DDX1 can unwind double-stranded RNA having single-stranded poly(A) overhangs.

The hnRNP K1 protein has been identified as a component of the heterogeneous nuclear ribonucleoprotein complexes. These hnRNP proteins bind pre-mRNAs directly and appear to facilitate various stages of mRNA biogenesis (1–3). hnRNP K can bind to RNA and single-stranded or double-stranded DNA. The nucleic acid binding activity of hnRNP K is mediated by three repeats of motifs termed the K homology domain, which consists of 65–70 residues (4). It contains both classical nuclear localization signal and the K nuclear shuttling domain that allow the protein to shuttle between nucleus and cytoplasm (5). hnRNP K appears to be involved in transcriptional regulation as exemplified by its binding to a C-rich sequence (the CT element) within the human c-myc promoter and subsequent activation of c-myc expression (6–8). Additionally, its association with CCAAT/enhancer-binding protein β results in the repression of CCAAT/enhancer-binding protein β target genes (9). hnRNP K was also reported to bind 3’ end of 15-lipoxygenase, and HPV 16 L2 mRNA was reported to trigger translation inhibition (10, 11). Moreover, direct protein-protein interactions between hnRNP K and some proto-oncogene products serve as clear evidence that hnRNP K acts as a docking platform to facilitate molecular interactions within signal transduction cascades (12–16).

Modulation of RNA structure is an essential step in many fundamental cellular processes, including RNA synthesis, splicing, export, turnover, and translation. Recently, an increasing number of RNA helicases from different organisms, ranging from bacteria to yeast, human, and virus, has been identified (17, 18). DEAD box proteins are a family of putative RNA helicases that are characterized by a core region of 300–360 amino acids that possesses eight conserved amino acid motifs, one of which is the ATP hydrolysis motif containing the core tetrapeptide DEXD/H (19). The eighth domain, HRI-GRXXR, is part of a basic region and is involved in RNA binding. DEAD box proteins are assumed to be ATP-dependent RNA helicases, based on the observed in vitro RNA helicase activities of the mouse translation initiation factor eIF4A (20), the human nuclear protein p68 (21), the Xenopus An3 (22), and the Drosophila vasa (23). The family is growing steadily, and each member differs in its size. The specificity and properties of each protein, including subcellular localization, RNA binding specificity, and regions required for the interaction with accessory proteins, are conferred by the additional N- and C-terminal sequences (17).

hnRNP K is involved in multiple regulatory processes of gene expression. To gain more insight into the mechanisms of hnRNP K action, we set out to identify its associated proteins through the hnRNP K affinity column. One of them was DDX1. DDX1 (originally named HuDBP-RB for human DEAD box protein identified in retinoblastoma cells) was isolated from a screen for cDNA clones of transcripts preferentially expressed in the retinoblastoma cell lines (24). Co-amplification of DDX1 and the proto-oncogene MYCN has been demonstrated in both retinoblastoma and neuroblastoma cell lines (24, 25). DDX1 protein levels in MYCN/DDX1-amplified neuroblastoma and retinoblastoma cell lines correlate well with DDX1 gene copy number and its transcript levels (26). Although there have been extensive research efforts directed toward DDX1 and MYCN, the enzyme activity of DDX1 protein remains to be investi-
gated. Here, we found that DDX1 could interact with hnRNPK and poly(A) RNA. The ATPase and RNA unwinding activities of DDX1 were also further examined and characterized.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—The full-length hnRNPK cDNA was cloned as described (9). The EcoRI fragment of hnRNPK was subcloned into pEGFP-C (Clontech) for eukaryotic GFP-hnRNPK protein expression and also subcloned into pGEX (Amersham Biosciences) for GST fusion protein production. To construct truncated mutants, the EcoRI-HindIII (amino acids 1–180), Xbal-EcoRI (amino acids 277–446), HindIII-XhoI (amino acids 179–464), and InuIII-XhoI fragments (amino acids 1–524) of myeloid leukemia kinase (amino acids 1–524) were cloned into pEGFP-C (Clontech) and pGEX (Amersham Biosciences) vectors. DDX1s were cloned by RT-PCR using the specific primers (sequence shown in the following method) and K562 cDNA. After being cloned into pCRI-TOPO vector (Invitrogen) and confirmed by nucleotide sequencing, the DDX1 cDNA was further subcloned into pCMV-Tag2 vector (Stratagene) for mammalian cell expression and pNLV829 (Pharmingen) for baculovirus/insect cell expression. Several deletion fragments including the BamHI-ScaI (amino acids 1–524), ScaI-XhoI (amino acids 525–740), BamHI-Smal (amino acids 1–295), and EcoRI-ScaI fragments (amino acids 185–524) were subcloned into the pCMV-Tag2 vector.

RT-PCR—RT-PCR was undertaken to clone the DDX1 cDNA. This is done by using total RNA from K562 cells, reverse-transcribed into cDNA using RT-PCR (Amersham Biosciences), and specific primers for DDX1: primer A, 5′-ATG-GGGRAAATGGCTTGGAGG3′; and primer B, 5′-GAATAATGAAAAATACTCGAGG-3′. Affinity Chromatography—Whole cell extracts of K562 cells were prepared in buffer containing 25 mM HEPES, pH 7.7, 0.4 M NaCl, 1 mM MgCl2, 0.2 mM EDTA, 0.5 M glycerophosphate, 0.1 mM Na3VO4, 2 μg/ml leupeptin, and 100 μg/ml PMSF. For GST fusion protein column, 0.5 ml of glutathione-Sepharose beads (Amersham Biosciences) carrying 5 mg of immobilized GST-hnRNPK K or GST proteins are used. 100 μg of whole cell extracts are eluted by boiling in SDS sample buffer and checked by SDS-PAGE. DNA-binding reactions included in vitro translated 32P-labeled protein and 10 μg of RNA resin in 0.5 ml of binding buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 0.5% Triton X-100, and 0.1 mg/ml bovine serum albumin) and was incubated for 30 min with rotating at 4 °C. The beads were washed three times with ice-cold binding buffer. The proteins bound to the RNA beads were released by boiling in SDS protein sample buffer, resolved on SDS-PAGE, and visualized by fluorography.

Cell Culture and Transfection—Human renal 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and 100 μg/ml penicillin-streptomycin at 37 °C in humidified incubator containing 5% CO2. Human erythroleukemic K562 cells and retinoblastoma Y79 cells were cultured in RPMI 1640 with 10% fetal bovine serum. Whole cell extracts were purchased from Sigma. The binding reactions included in vitro translated 32P-labeled protein and 10 μg of RNA resin in 0.5 ml of binding buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 0.5% Triton X-100, and 0.1 mg/ml bovine serum albumin) and was incubated for 30 min with rotating at 4 °C. The beads were washed three times with ice-cold binding buffer. The proteins bound to the RNA beads were released by boiling in SDS protein sample buffer, resolved on SDS-PAGE, and visualized by fluorography.

Whole Cell Extract Preparation, Immunoprecipitation, Western Blot Analysis, and Protein Purification—To prepare whole cell extract, 1 × 106 cells were resuspended in 400 μl of lysis buffer (20 mM HEPES, pH 7.6, 0.4 M NaCl, 1.5 mM MgCl2, 1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 100 μg/ml PMSF). The cell suspension was rotated at 4 °C for 30 min and clarified by centrifugation at 10,000 × g for 10 min. The protein concentration was estimated by Bio-Rad protein assay. The cell lysates were incubated with the FLAG-DDX1 affinity purifying beads for 4 h at 4 °C. The beads were washed four times with a lysis buffer containing 20 mM HEPES, pH 7.6, 100 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 M NaCl, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) at 4 °C. The resulting immune complexes were washed three times with washing buffer (20 mM HEPES, pH 7.6, 0.2 or 0.5 M NaCl, 0.5 mM MgCl2, 0.1 mM EDTA, 0.1% or 1% Triton X-100, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) and then resuspended in binding buffer (20 mM HEPES, pH 7.6, 100 mM NaCl, 2.5 mM MgCl2, 0.5% Triton X-100, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) at 4 °C. The bead mixture were evaluated from the dried plates using a Fujifilm BAS 1000 phosphor imager. The bound RNA was extracted with phenol/chloroform.

ATPase Activity Assay—The reactions were carried out in a total volume of 10 μl, which contained 50 mM Tris, pH 8.0, 2.5 mM MgCl2, 1 μg of [γ-32P]ATP (3000 Ci/mmol), and various amounts of protein samples and, unless otherwise stated, polyribonucleotides. The reaction mixtures were incubated for 1 h at 37 °C and stopped by adding EDTA to a final concentration of 20 mM. One microliter of the reaction product was spotted onto polyethyleneimine-cellulose thin layer chromatography plates (Merck). The chromatography was performed in 0.375 M potassium phosphate buffer, pH 5.5, and analyzed by autoradiography.

RNA Binding Assays—The reactions used in unwinding assays were prepared as follows: A, pSP64/poly(A) vector (Promega) was linearized with EcoRI and transcribed with SP6 RNA polymerase; B, pGEM3 vector (Promega) was linearized with EcoRI and transcribed with SP6 RNA polymerase; C, pGEM3 vector was linearized with EcoRI and transcribed with SP6 RNA polymerase; and D, pGEM3 vector was linearized with Smal and transcribed with T7 RNA polymerase. RNA probes B and D were incorporated with [γ-32P]UTP. Two sets of RNA fragments, A and B or C and D, were annealed in buffer containing 10 mM Tris-HCl, pH 7.5, and 1 mM MgCl2 for 30 min at room temperature. The reaction mixture were evaluated from the dried plates using a Fujifilm BAS 1000 phosphor imager.
bound proteins were eluted stepwise by 0.5 M (lanes 1, 2 and 3), and then the flow-through was further brought down by GST-hnRNP K bound beads. After extensive washing, the bound proteins were eluted stepwise by 0.5 M (lanes 3 and 6), 0.7 M (lanes 2 and 5), and 1 M (lanes 1 and 4) NaCl containing binding buffer. B, Peptides identified by LC/MS/MS.

Fig. 2. hnRNP K interacts with a putative RNA helicase DDX1. A, GST binding assay. The in vitro translated 35S-labeled DDX1 protein was incubated with GSH beads which were prebound with GST (lane 2) or GST-hnRNP K (lane 3). After washing, the proteins retained on the beads were analyzed by SDS-PAGE and autoradiography. (lane 1 is direct loading). B, in vitro binding assay was performed by preincubation of beads bound GST-hnRNP K with 10 μg/ml of poly(C), poly(A), poly(G), and poly(U) at room temperature for 10 min, and then 35S-labeled DDX1 was added for a further 30 min. The gel was Coomassie Blue-stained and autoradiographed. Lane 1 is half of the input of 35S-labeled DDX1.

RESULTS

Identification of hnRNP K-associated Proteins—To investigate the proteins that may interact with hnRNP K, we performed GST-hnRNP K affinity chromatography using proleukemic K562 whole cell extracts. The protein fractions from control GST and GST-hnRNP K chromatography are separated on SDS-PAGE followed by silver staining (Fig. 1A). Several specific protein bands were identified through comparison between the protein fractions from GST and GST-hnRNP K columns. These specific polypeptides purified from the GST-hnRNP K column were subsequently trypsinized and fractionated by capillary HPLC. Fractions eluted from the capillary HPLC column were directly analyzed by LC/MS/MS. Several proteins were identified by this method (Fig. 1B). We were particularly interested in one of them, the putative RNA helicase termed DDX1, because of the increasingly important role of helicases in mRNA maturation and translation and their potential functional link to hnRNP K.

Protein-Protein Interaction between hnRNP K and DDX1—All eight conserved motifs characteristic of DEAD box proteins are present in DDX1. To further explore the physical and functional interaction between hnRNP K and DDX1, we first cloned the complete open reading frame of DDX1 by RT-PCR.

To test whether DDX1 directly interacts with hnRNP K, pull-down assay and co-immunoprecipitation experiments were performed. By using GST pull-down assay, the in vitro translated DDX1 protein was tested for binding to the GSH beads pre-bound with GST or GST-hnRNP K. The results, as shown in Fig. 2A, indicate that the in vitro translated DDX1 could associate with GST-hnRNP K. The molecular weights of GST-hnRNP K and DDX1 are very similar, hence a doublet of protein bands was visible on the gel when the GST-hnRNP K was abundant. Because both hnRNP K and DDX1 have RNA-binding domains, their interaction might be mediated through RNA binding. However, we observed that the interaction between hnRNP K with DDX1 seemed to be better in the presence of RNase A (data not shown). To further clarify the possible effects of RNA on their interaction, the binding of 35S-labeled DDX1 to GST-hnRNP K was performed in the presence of several homopolymeric RNAs. Poly(C), poly(A), and poly(U) weakened the interaction of DDX1 with hnRNP K, whereas poly(G) did not affect this binding (Fig. 2B).

To further establish the in vitro interaction between DDX1 and hnRNP K, co-immunoprecipitation was performed using cell lysates derived from 293T cells and insect cells. Whole cell extracts from FLAG-hnRNP K-expressed 293T cells were immunoprecipitated with anti-FLAG antibody beads and analyzed by Western blotting with antibody against DDX1. DDX1 protein was found to co-immunoprecipitate with FLAG-hnRNP K (Fig. 3A). In addition, insect cell SF21 were co-infected with viruses expressing recombinant FLAG-tagged DDX1 and GFP-tagged hnRNP K. In this system, hnRNP K was also detected through immunoblotting as a component of the FLAG-tagged immunocomplex (Fig. 3B). Taken together, these results indicate that DDX1 can...
interact with hnRNPK both in vitro and in vivo.

To map the interacting region within hnRNPK that facilitates binding to DDX1, we generated a series of deletion mutants of the hnRNPK protein. Whole cell extracts of 293T cells previously transfected with various GFP-hnRNPK derivatives were next incubated with anti-FLAG beads preabsorbed with FLAG-DDX1. The in vitro binding was then analyzed by SDS-PAGE. As shown in Fig. 4A, DDX1 was found to co-precipitate with the K (1–464), K (1–180), K (179–276), and K (179–464) constructs, but no binding was observed with the K (277–464) mutant and control GFP protein. GST pull-down assay yielded the same results (Fig. 4B). These data pinpoint the DDX1 interaction region of hnRNPK K to an area between amino acids 1 and 276.

Using a panel of DDX1 deletion mutants, we next mapped the region within DDX1 that is responsible for binding to hnRNPK K. The results of the in vitro binding assay (shown in Fig. 5) demonstrate that two separate regions of DDX1, amino acids 1–295 and amino acids 525–740, can independently bind to hnRNPK K, although their relative binding efficiencies are lower than that of full-length DDX1.

DDX1 Interacts with Poly(A) RNA—Fig. 2B showed that the interaction of DDX1 with hnRNPK K could be interfered by the presence of poly(A), poly(C), and poly(U) homopolymeric RNAs. Because poly(A) RNA has never been mentioned to affect interaction between hnRNPK and other associated proteins, we tested whether DDX1 can bind to poly(A) RNA. RNA binding assay showed that DDX1 bound to poly(A) beads but not to poly(C) beads (Fig. 6A, lanes 1–3). The RNA-binding domain-deleted mutant of DDX1 lost the RNA binding activity (Fig. 6A, lanes 4–6). Consistent with a previous report (28), hnRNPK K possessed a specific poly(C) RNA binding ability (Fig. 6A, lanes 7–9). Fig. 6B showed that DDX1 could be brought down by poly(A) beads from the Y79 cytoplasm extracts. Interestingly, we noticed that hnRNPK K was absent in this DDX1-containing poly(A) RNA-binding protein complexes (lane 2) and vice versa (lane 3).

ATPase Activity of DDX1 Protein—Most but not all of the DEAD box proteins have ATPase activity. DDX1 protein has an ATPase motif similar to other DEAD box proteins. However, the ATPase activity of the purified DDX1 protein has not been fully characterized. Recombinant FLAG-hnRNPK K was ex-
pressed in 293T cells, and recombinant FLAG-DDX1 was expressed in SF21 insect cells (Fig. 7A). To examine the ATPase activity in vitro, [γ-32P]ATP was incubated along with FLAG-DDX1, and ATP hydrolysis was measured based on the production of [γ-32P]PP from [γ-32P]ATP as observed on the polyethyleneimine-cellulose TLC plates. As shown in Fig. 7B, recombinant protein DDX1 was found to possess ATPase in the presence of various polynucleotides. We compared the stimulatory effect of a variety of RNAs and DNAs on such enzymatic activity. Generally speaking, the ATPase activity of DDX1 was stimulated efficiently by poly(A), poly(U), poly(C), and total RNA from yeast (1.5–3.7-fold increases from the initial rates in the absence of polynucleotides), whereas DNA and poly(G) had no effect and even an inhibitory effect on the ATPase activity of DDX1, respectively.

We have demonstrated that hnRNP K can form a complex with DDX1 in cell lysates (Fig. 3). We further examined the ATPase activity of hnRNP K-DDX1 complex. Overexpressed FLAG-K (full-length) or truncated FLAG-K (277–464, a DDX1-interacting mutant as shown in Fig. 4) from 293T cell lysates was immunoprecipitated with anti-FLAG antibody agarose. After washes with high salt or low salt buffer, the protein complexes were eluted with FLAG peptide and were subjected to Western blot and ATPase assays. The result showed that under the lower salt wash condition the full-length hnRNP K could bring down the DDX1 but truncated hnRNP K could not and that the co-immunoprecipitated DDX1 had ATPase activity. Under the more stringent conditions, hnRNP K could not interact with DDX1, and thus no ATPase activity was detected from the eluents (Fig. 8).

**RNA Unwinding Activity of DDX1 Protein**—Several DEAD box proteins exhibit RNA unwinding activities in vitro. To evaluate the RNA helicase activity of DDX1, we carried out the RNA unwinding activity assay by first immobilizing DDX1 or hnRNP K immunocomplexes on protein A-Sepharose by anti-

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**Fig. 5.** Mapping of DDX1 protein domain responsible for binding to hnRNP K. Beads bound GST-hnRNP K (lanes 2, 5, 8, 11, and 14) or GST alone (lanes 3, 6, 9, 12, and 15) were incubated with in vitro translated full-length (lanes 1–3) and truncated DDX1: amino acids 1–524 (lanes 4–6), amino acids 525–740 (lanes 7–9), amino acids 1–295 (lanes 10–12), and amino acids 185–524 (lanes 13–15). Lanes 1, 4, 7, 10, and 13 are half of the input of the in vitro translated product. The gel was autoradiographed (upper panel) and Coomassie Blue-stained (lower panel). The relative efficiency of each DDX1 deletion mutant binding to hnRNP K was measured by a phosphorus imager.

**Fig. 6.** The RNA binding properties of DDX1. A, in vitro translated 35S-labeled full-length DDX1 (lanes 1–3), DDX1 (amino acids 185–524) (lanes 4–6), or hnRNP K (lanes 7–9) were incubated with homopolymeric A or C RNA beads as indicated. Proteins bound to the RNA beads were resolved by SDS-PAGE and visualized by fluorography. IP represents the direct input of translated proteins. B, Y79 cytosolic extracts were incubated with poly(A) (lane 2) or poly(C) (lane 3) RNA beads. The protein complexes pulled down by the beads were analyzed by Western blotting using anti-DDX1 (upper panel) or anti-hnRNP K (lower panel) antibody. a.a., amino acids; IP, immunoprecipitation.

**Fig. 7.** ATPase activity of DDX1 protein. A, the FLAG-tagged hnRNP K (lane 1) purified from 293T cells, and FLAG-tagged DDX1 (lane 2) purified from SF21 cells were subjected on SDS-PAGE and silver staining. B, the ATP hydrolysis activity of recombinant DDX1 protein and control eluent were tested by measuring the production of [32P]PP from [γ-32P]ATP on polyethyleneimine-cellulose TLC plates in the presence of 1 μg of various polynucleotides. Lane 2, polyA; lane 3, poly(U); lane 4, poly(C); lane 5, poly(G); lane 6, yeast total RNA; lane 7, HeLa total RNA; lane 8, DNA. The reactions shown here contain 100 ng of FLAG-DDX1, and the results were quantified by a Fujifilm BAS 1000 phosphorus imager. The ATPase activity in the reaction without any polynucleotides was set at 1 (lane 1). Three independent experiments were done.
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Fig. 8. hnRNP K-DDX1 complex contains ATPase activity. A, 293T cells were transfected with FLAG-K (full-length) (lanes 2, 4, and 6) or FLAG-K (277–464) (lanes 1, 3, and 5). The whole cell extracts (WCE) were isolated and were immunoprecipitated by anti-FLAG-agarose. The immunoprecipitated complexes were washed with 0.5 M NaCl and 1% Triton X-100 (lanes 3 and 4) or 0.2 M NaCl and 0.1% Triton X-100 (lanes 5 and 6) and then eluted with 0.5 μg/μl FLAG peptide in phosphate-buffered saline. The eluents from Western blotting with anti-DDX1 antibody and anti-FLAG antibody. Lanes 1 and 2 are direct loading of 100 μg of whole cell extracts. B, ATPase activity assay of hnRNPK complex. The eluents from A were used for the ATPase activity analysis. Lane 1, protein-free; lane 2, from FLAG-K (277–464) and high salt wash; lane 3, from FLAG-K (full-length) and high salt wash; lane 4, from FLAG-K (277–464) and low salt wash; lane 5, from FLAG-K (full-length) and low salt wash; lane 6, recombinant DDX1 control. All of the ATPase reactions are demonstrated in the presence of 1 μg of poly(C).

DDX1 or anti-hnRNP K antibody, respectively, and subsequent incubation with duplex RNA. Two pairs of duplex RNA were prepared as described under “Experimental Procedures,” and the RNA pair (A + B) has 30 bases of rATP overhangs. Fig. 9 showed that recombinant DDX1 alone exhibited no RNA unwinding activity (lanes 7 and 8), but protein complexes coimmunoprecipitated by DDX1 (lane 5) were able to reconstitute the RNA unwinding activity on the duplex RNA (A + B) in vitro. ATP is required in this reaction (data not shown). Compared with the activity of DDX1 complexes, that of immunoprecipitated hnRNP K protein complexes was very weak (lane 6).

DISCUSSION

The fundamental processes of the eukaryotic cell, such as transcription initiation, RNA metabolism, replication, and DNA repair, are catalyzed by multiple protein complexes consisting of up to many dozens of subunits that possess enzymatic as well as regulatory functions. To learn more about the molecular mechanisms of these processes and the nature of the multiprotein complexes involved, the identification of their subunits and the characterization of their interaction are essential. hnRNP K is a component of the hnRNP complex, and a large amount of literature has implicated it in the regulation of gene expression on different levels. Although many hnRNP K-interacting proteins have been identified (8, 12, 15, 29–32), the exact roles of hnRNP K-containing complexes in gene expression regulation are currently unclear. In this study, we identified a putative RNA helicase DDX1 from GST-hnRNP K affinity chromatography as a new hnRNP K-interacting factor. The GST binding assay and immunoprecipitation analysis further confirmed the physical interaction between these two proteins. RNA helicases have been thought to be ATP-driven switches for modulating RNA secondary structure or for rearranging RNA-protein complexes (33). We thus hypothesize that DDX1 may assist hnRNP K and/or hnRNPs during RNA processing steps.

There are reports describing the nucleic acid-mediated interaction between hnRNP K and certain associated proteins. Poly(C) RNA, which binds to hnRNP K, blocked Zik1 binding to hnRNP K, whereas poly(A), which does not bind to hnRNP K, did not affect such binding (29). Poly(U) decreased the interaction of YB-1 with hnRNP K, whereas the other polynucleotides had no effect (30). Previously, we also reported that the interaction between hnRNP K and the dengue virus core protein was altered in the presence of poly(C) and poly(U) (32). Fig. 4 showed that the DDX1-interacting region in hnRNP K contains the N-terminal K homology or RGG domain that is known to confer nucleic acid binding. We performed the binding experiments in the presence of polyhomonucleic acids and found that the interaction of DDX1 and hnRNP K was weakened in the presence of poly(C), poly(A), and poly(U) (Fig. 2E). We further demonstrated that DDX1 could bind to poly(A), presumably sequence to facilitate its RNA unwinding activity. Fig. 6B shows that binding of DDX1 to poly(A) and hnRNP K or of hnRNP K to poly(C) and DDX1 seemed to be mutually exclusive. It suggests that protein-protein interactions of DDX1 and hnRNP K could modulate their RNA binding activity and sub-
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subsequently regulate their biological functions. The biological function of DDX1 is not known. Recently, Bleo et al. (34) reported that DDX1 associated with a pre-mRNA 3' end cleavage protein CstF-64. With our finding that DDX1 is one of the poly(A) RNA binding proteins, it can be postulated that DDX1 is involved in the 3' end processing of pre-mRNA.

Most DEAD box family proteins have been reported to be associated with ATP hydrolysis activity, and for many of them the activity is RNA-dependent. Although RNA binding assay showed that DDX1 preferred to bind to poly(A), the ATPase activity of recombinant DDX1 was stimulated by several RNAs (such as poly(A), poly(U), poly(C), and yeast total RNA) to various degrees (Fig. 7). Among the known DEAD box proteins, only some have RNA substrate specificity, and most of them either do not have any substrate specificity or have specificities that are unrelated to their assumed in vivo roles. For example, mammalian eIF-4A, which is required for translation initiation of all mRNAs, is stimulated more efficiently by poly(U) than by globin mRNA (20). For E. coli DbpA and yeast Slt2, only specific RNA can stimulate their ATP hydrolysis activity (23 in vitro). Most RNA helicases unwind dsRNA in either a 5' to 3' or a 3' to 5' direction when tested in vitro. The designed substrates provide the double-stranded region with either 5' or 3' single-stranded overhangs (19). These overhangs enable the helicase to load onto the substrate. Our data indicate that purified recombinant DDX1 does not possess RNA unwinding activity, but the immunoprecipitated DDX1 complexes exhibit such activity to unwind a substrate with poly(A) overhang. This finding implies that, just like eIF-4A (20) and Dbp5p (27), DDX1 requires co-factors to unwind RNA duplexes. It was also demonstrated that DDX1 exhibits its RNA unwinding activity only for a specific RNA substrate, as previously observed in the case of An3 (37). Taken together, DDX1 may function as a RNA helicase in the presence of a specific RNA substrate and other potential accessory proteins. The enzymatic properties of DDX1 will be further investigated.

hnRNP K is up-regulated in SV40-transformed cells (38) and in human breast cancer, and it positively controls the growth rate of human breast cancer cells (39). DDX1 is co-amplified with MYCN in several retinoblastoma and neuroblastoma cells and may be involved in either the formation or the progression of a subset of these tumors. Thus, the interaction of hnRNP K with DDX1 and the associated RNA unwinding activity may play significant roles in the physiology of both normal and tumor cells.

Acknowledgment—We thank Bertrand Chin-Ming Tan for the critical reading of the manuscript.

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