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The DEAD-Box RNA Helicase DDX1 Interacts with RelA and Enhances Nuclear Factor kappaB-Mediated Transcription

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ABSTRACT

DEAD-box RNA helicases constitute the largest family of RNA helicases and are involved in many aspects of RNA metabolism. In this study, we identified RelA (p65), a subunit of nuclear factor-kappaB (NF-κB), as a cellular co-factor of DEAD-box RNA helicase DDX1, through mammalian two hybrid system and co-immunoprecipitation assay. Additionally, confocal microscopy and chromatin immunoprecipitation assays confirmed this interaction. In NF-κB dependent reporter gene assay, DDX1 acted as a co-activator to enhance NF-κB-mediated transcription activation. The functional domains involved were mapped to the carboxy terminal transactivation domain of RelA and the amino terminal ATPase/helicase domain of DDX1. The DDX1 trans-dominant negative mutant lacking ATP-dependent RNA helicase activity lost it transcriptional inducer activity. Moreover, depletion of endogenous DDX1 by specific small interfering RNAs significantly reduced NF-κB-dependent transcription. Taken together, the results suggest that DDX1 may play an important role in NF-κB-mediated transactivation, and revelation of this regulatory pathway may help to explore the novel mechanisms for regulating NF-κB transcriptional activity. J. Cell. Biochem. 106: 296–305, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: RNA HELICASE DDX1; NF-κB RelA; TRANSCRIPTION; DEAD-BOX; ACTIVATION

DEAD-box RNA helicases constitute the largest family of RNA helicases and are characterized by eight conserved amino acid motifs including the DEAD box (Asp-Glu-Ala-Asp) in Walker motif B [Lorsch, 2002; Rocak and Linder, 2004]. This family of RNA helicase plays important roles in many aspects of RNA metabolism, including transcription, pre-mRNA processing, RNA decay, ribosome biogenesis, RNA export and translation [Lorsch, 2002; Rocak and Linder, 2004]. The RNA helicases possess both ATPase and helicase activities, the former providing energy by nucleoside triphosphate hydrolysis and the latter utilizing the energy for the unwinding of RNA duplexes or modulation of complex RNA structures and large RNP complexes [Lorsch, 2002; Rocak and Linder, 2004].

DDX1 belongs to DEAD-box RNA helicase family and was first characterized for its over-expression in certain neuroblastoma (NB) and retinoblastoma (RB) cell lines [Godbout and Squire, 1993; Godbout et al., 1998, 2007]. However, the concrete function of DDX1 in this context remains to be clarified. The first evidence for a role of DDX1 in RNA metabolism was provided by showing that DDX1 is bound to an RNA cleavage stimulation factor involved in 3′-end processing of pre-mRNA [Bleoo et al., 2001]. DDX1 can also associate with poly(A) RNA and pre-mRNA processing heterogenous ribonuclear protein hnRNPK [Chen et al., 2002]. Moreover, DDX1 has been shown to bind to the 3′-untranslated region of hepatitis C virus (HCV) and the transcriptional control region of JC virus, suggesting a potential role of DDX1 in viral replication cycles [Tingting et al., 2006; Sunden et al., 2007a,b].

Recently, DDX1 has been observed in RNA transporting granules in dendrites of neurons, together with other related molecules, including DDX3 [Kanai et al., 2004]. The direct evidence for the involvement of DDX1 as well as DDX3 in RNA trafficking came from the studies on HIV-1 mRNA transport [Fang et al., 2004, 2005; Yedavalli et al., 2004]. It has been shown that DDX1 and DDX3 play an important role in the export of unspliced and singly spliced HIV-1 mRNAs from nucleus to cytoplasm in HIV-1 Rev-CRM1 pathway [Fang et al., 2004, 2005; Yedavalli et al., 2004]. However, it remains unclear whether DDX1 is directly involved in RNA transcription activation as is shown for some other RNA helicases

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like the DExH-box-containing RNA helicase A (RHA) [Nakajima et al., 1997; Aratani et al., 2001; Fujii et al., 2001; Tetsuka et al., 2004]. Therefore, it is interesting to screen for possible interaction partners of DDX1 among the activators and co-activators of RNA transcription machinery.

In present study, we show that DDX1 could associate with the RelA (p65) subunit of nuclear factor kappaB (NF-κB) and thus promote the transcriptional activation mediated by NF-κB. To our knowledge, this is the first report for the involvement of a DEAD-box RNA helicase in NF-κB-mediated transcriptional activation and regulation.

EXPERIMENTAL PROCEDURES

PLASMIDS, ANTIBODIES, AND CELL LINES

pM-DDX1 (full length) and deletion mutants pM-DDX1 (1–525), pM-DDX1 (525–740), pM-DDX1 (1–185), pM-DDX1 (185–525), and pM-DDX1 (1–295) were constructed by inserting the PCR generated DDX1 [Godbout et al., 1998] in fusion with Gal4 DNA binding domain (DB) in pM vector and pVP16-RelA plasmid by cloning the RelA cDNA [Benezra et al., 2003] in fusion with transcriptional activation domain in pVP16 (Clontech). pFlag-DDX1 (full length) and deletion mutants pFlag-DDX1 (1–525), pFlag-DDX1 (525–740), pFlag-DDX1 (1–185), pFlag-DDX1 (185–525), and pFlag-DDX1 (1–295) were reported previously [Chen et al., 2002]. DDX1 ATPase/RNA helicase-inactive mutant (generated by mutating the DEAD motif to DGAD) under the control of CMV promoter were obtained by cloning the respective mutated cDNA into vector pCMV2A (Stratagene). pHA-RelA (1–551), pHA-RelA (1–332), and pHA-RelA (299–550) were described previously [Benezra et al., 2003]. The coding sequences of wild-type RelA and its mutants were amplified by PCR and cloned in fusion with Gal4 DB domain in pM vector, resulting in the following constructs: pGal4-RelA (1–551), pGal4-RelA (1–325), pGal4-RelA (286–551), pGal4-RelA (286–443), pGal4-RelA (430–551), pGal4-RelA (286–521), pGal4-RelA (521–551), and pGal4-RelA (430–525). The expression plasmids for IKKα, MEKK1 and the luciferase reporter plasmids p5xNF-κB-luc, pIFNβ-luc, pELAM-luc, and pAP-1-luc were kindly provided by Dr Hong-Bing Shu [Xu et al., 2004; Tian et al., 2007]. Mouse monoclonal anti-Flag (M2) and anti-HA antibody were purchased from Sigma. DDX1 N-terminal polyclonal antibody was a kind gift from Roseline Godbout [Godbout et al., 1998]. Mouse monoclonal anti-RelA was purchased from Santa Cruz Biotechnology. HEK293T cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone), 100 U/ml of penicillin and 100 μg/ml streptomycin (Gibco Invitrogen Corporation) in a humidified incubator containing 5% CO_2 at 37°C.

LUCIFERASE REPORTER ACTIVITY ASSAYS

293T cells were seeded on 24-well dishes and transfected on the following day by Lipofectamine 2000 (Invitrogen) using standard protocols. Renilla luciferase reporter plasmid pRL-TK (Promega, Madison, WI) was used as an internal control to normalize transfection efficiency. Luciferase activity was measured 24 h after transfection using a Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was obtained by comparing various protein-expression constructs with empty vector. For the NF-κB induction experiments, cells were treated with tumor necrosis factor (TNF) α (10 ng/ml) for 8 h and relative luciferase activity was obtained by comparing with untreated cells.

MAMMALIAN TWO-HYBRID ASSAY OF PROTEIN–PROTEIN INTERACTIONS

Mammalian two-hybrid assay was performed by co-transfecting the DNA binding domain (DB) and activation domain (AD) fusion constructs together with a Gal4 luciferase reporter according to the standard protocols (Clontech). Cells were transfected with indicated doses and combinations of mammalian expression plasmids, and luciferase activity was measured 48 h post-transfection.

IMMUNOPRECIPITATION

293T cells (3×10^6) were plated 1 day prior to transfection in 10-cm^2 dishes. Cells were transfected with indicated combination/concentrations of expression plasmids or control vectors by Lipofectamine 2000 (Invitrogen). Cells were treated with TNFα (10 ng/ml) 48 h after transfection for 1 h at 37°C and lysed in Nonidet P-40 lysis buffer. Cell lysates were incubated with anti-Flag antibody for 2 h at 4°C. Immunocomplexes were captured by incubation with protein A/G-agarose beads for 2 h at 4°C. The immunoprecipitated proteins were resolved by 10% SDS–PAGE and analyzed by Western blotting with anti-HA or anti-Flag antibody. For immunoprecipitation of endogenous proteins, cells were also treated with TNFα (10 ng/ml) for 1 h at 37°C and cell lysates were incubated with polyclonal anti-DDX1 antibody or non-specific IgG as a control. Western blot was performed using anti-RelA or anti-DDX1 to detect the endogenous RelA and DDX1 protein.

INDIRECT IMMUNOFLUORESCENCE ANALYSIS

In order to examine the subcellular localization of DDX1 and RelA, 293T cells were seeded on coverslips. Non-transfected cells were treated or untreated with TNFα (20 ng/ml) for 30 min. To determine the localization of RelA and DDX1, cells were immunostained with mouse monoclonal anti-RelA and rabbit polyclonal anti-DDX1 antibody. Rhodamine isocynate labeled goat anti-mouse and FITC-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) were used as secondary antibody. Immunofluorescence analysis for the intracellular localization of red fluorescence and green fluorescence light were examined using confocal microscopy as described previously [Watashi et al., 2001]. For nuclear staining, cells were incubated in DAPI (4′,6-diamidine-2′-phenylindole, dihydrochloride) solution.

QUANTITATIVE RT-PCR ANALYSIS

Total RNA was extracted from 293T cells using Trizole reagent (Invitrogen) as described in the protocols provided by the manufacturers. The reverse transcription (RT) was performed with total RNA as the template using the Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) and quantitative gene expression was determined by using SYBR Premix ExTaq TM (perfect real time) PCR kit as described previously [Ishaq et al., 2008]. The primer sequences were: p65-F 5′-CCCCACGAGCTTGTAG-GAAAG-3′, p65-R 5′-CCAGGTTCTGGAAACTG TGGAT-3′.

RNAi KNOCKDOWN OF DDX1

Endogenous DDX1 expression was inhibited by using specific anti-DDX1 siRNA duplex (sense 5′-C A A G C C C U C U U U C C U G C C U G U U-3′ and antisense 3′-U U G U U C G G G A G A A A G G A C G G A C (5′-P)-5′) that were prepared commercially (AUGC BioTech.) [Fang et al., 2005]. Cells were transfected with reporter plasmids after treatment with siRNA (200 nM) for 24 h. The transfected cells were harvested after 24 h and lysed in SDS sample buffer for Western blot analysis or in passive lysis buffer (Promega) for luciferase activity assay.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAYS

ChIP assays were performed by using an Ez-Zyme Chromatin Prep. Kit (17-375, Millipore). Briefly, 1×10^6 cells were grown in a 10-cm^2 culture dishes and fixed with 1% formaldehyde. EZ-Zyme enzymatic cocktail was used to cleave the DNA. Protein–DNA complexes were immunoprecipitated with either the anti-Flag or anti-HA antibody or non-specific IgG control antibody. The DNA–protein immuno-complexes were collected with protein A/G-agarose beads, washed and eluted with 250 µl freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) with rotation at room temperature. The mixture was further incubated with sodium chloride (final concentration of 0.2 M) at 65°C for 4 h to reverse crosslink DNA–protein complexes. Two microliters of proteinase K (10 mg/ml) was added to the samples and incubated for 1 h at 45°C. DNA samples were then purified with phenol/chloroform, precipitated with ethanol and resuspended in 40 µl of TE buffer. PCR was then performed on the purified DNA, according to manufacturer's protocol (TOBOYO), using the specific primers to the promoter of 5xNF-κB-luc construct: NF-κB-luc-F 5′-ACCGAAACGCGCGAGGCAGGATCAGCCATA-3′, NF-κB-luc-R 5′-GCTCTCCAGCGGTTCCATC-3′ and Gal4-luc construct: pGal4-F 5′-CTCGAGGACA GTACTCCG CT-3′, pGal4-R 5′-TTAGCTTCCTTAG-CTCCT-3′.

RESULTS

ASSOCIATION OF DDX1 WITH NF-κB SUBUNIT RelA

In the pursuit of the molecular mechanisms that engage DEAD-box RNA helicases in RNA metabolic processes, we used DDX1 as a bait and screened for cellular interaction partners, mainly focusing on the transcriptional factors and co-factors. A panel of proteins which are known to be involved in transcription activation (NF-κB1 [p50/p105], RelA [p65], mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase-1 (MEKK1), NF-{kappa}B-inducing kinase (NIK), COMMD1 and in RNA transport (Importin beta, CRM1, RanGTPase, eukaryotic initiation factor 5A [eIF5A], RNA helicase A [RHA])) were selected and tested for interaction with DDX1 in a mammalian two-hybrid system, and we found that NF-κB subunit RelA could strongly interact with DDX1 in the test system. As shown in Figure 1A, co-transfection of DDX1 fused with Gal4 DNA binding domain (pM-DDX1) and RelA in fusion with VP16 activation domain (pVP16-RelA) activated the luciferase reporter gene significantly as the positive controls (pM3-VP16 alone or combination of pM53 and pVP16-T). DDX1 or RelA alone as well as various negative controls could not activate reporter gene activity.

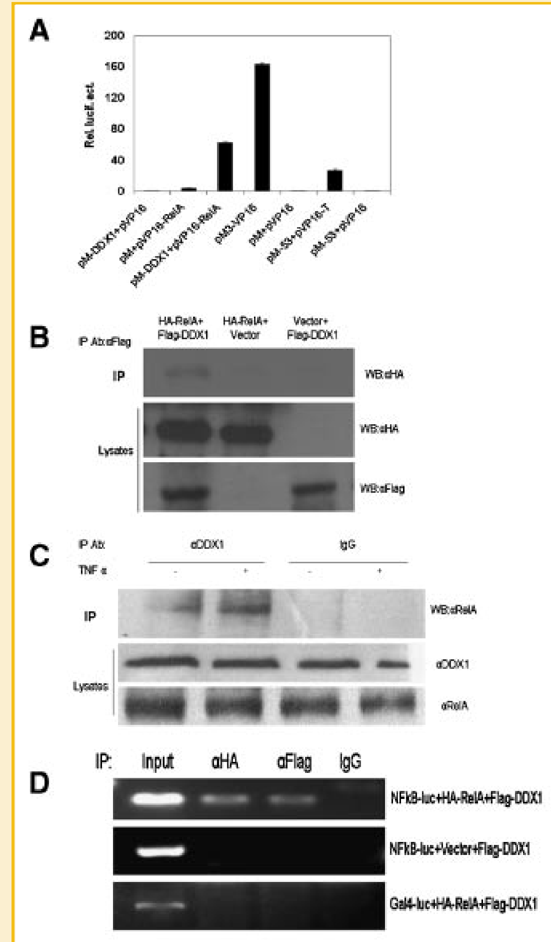

Fig. 1. Binding of DDX1 with NF-κB RelA subunit. A: DDX1 specifically interacts with RelA in mammalian two-hybrid system. DDX1 was fused with Gal4 DNA-binding domain in pM vector and RelA with the VP16 activation domain in pVP16. 293T cells were co-transfected with Gal4-luc reporter plasmid (100 ng) and equal amount of pM-DDX1 and pVP16-RelA (350 ng) or various control plasmids. Luciferase activities were determined 48 h after transfection. These experiments were repeated three times and the results are expressed as the mean ± standard deviation. B: Co-immunoprecipitation of tagged DDX1 and RelA. Lysates of 293T cells transfected with Flag-DDX1 (4 µg) in combination with either HA-RelA (4 µg) or empty vectors (4 µg) were used for immunoprecipitation analysis. Cells were treated with TNFα (10 ng/ml) for 1 h. Anti-Flag antibody was used to pull down the immunocomplex. HA-tagged RelA in the immunoprecipitates was detected by immunoblotting using anti-HA antibody (upper panel). Presence of similar amount of HA-RelA or Flag-DDX1 in the lysates was shown in middle and lower panel. C: Co-immunoprecipitation of endogenous DDX1 and RelA. Lysates from non-transfected 293T cells treated with or without TNFα (10 ng/ml) for 1 h were immunoprecipitated with either a DDX1 antiserum or control IgG. The immunoprecipitates were analyzed with specific anti-RelA antibody (upper panel). Presence of similar amount of DDX1 and RelA in the lysates were shown in middle and lower panels. D: Recruitment of DDX1 to the NF-κB-carrying active gene promoter. 293T cells were co-transfected with pNF-κB-luc or pGal4-luc (2 µg), pFlag-DDX1 (3 µg) in combination with pHA-RelA (3 µg) or empty vector (3 µg) expression plasmids. Transfected cells were induced with TNFα (20 ng/ml) after 36 h of transfection for 30 min. The cross-linked DNA–protein complexes were immunoprecipitated with anti-HA antibody, anti-Flag or control IgG antibody. Total cell lysates was used as positive control. The phenol/chloroform/ethanol purified DNA was used as template for PCR to detect the DNA fragment containing NF-κB binding motifs.

These results suggested that RelA may be an interaction partner for DDX1.

To confirm the interaction of DDX1 with RelA, Flag-tagged DDX1 and HA-tagged RelA were co-expressed and co-immunoprecipitated with monoclonal anti-Flag antibody. As shown in Figure 1B, HA-RelA could be readily detected by anti-HA antibody from the anti-Flag immunoprecipitates but HA-RelA were not detected from the samples, where empty vectors were used as a controls. These data indicate that DDX1 could bind with RelA in transfected cells.

To determine whether DDX1 and RelA interact with each other under physiological conditions, we used specific anti-DDX1 antibody for the in vivo co-immunoprecipitation and anti-RelA antibody to detect RelA from the pull-down immunocomplex (Fig. 1C). The results indicate that endogenous RelA could also be co-precipitated with DDX1, suggesting that endogenous RelA and DDX1 could associate in vivo.

To investigate whether the interaction of DDX1 and RelA took place at active promoter, we performed the chromatin immuno-precipitation (ChIP) assays by using the promoter of pNF-κB-luc. As shown in Figure 1D (upper panel), the DNA fragment containing the NF-κB-binding motifs could be detected by PCR in complexes specifically immunoprecipitated by either anti-HA or anti-Flag antibody from lysates of 293T cells transfected with pNF-κB-luc, pFlag-DDX1 and pHA-RelA expression plasmids. The promoter DNA could not be detected from the immunoprecipitates of non-specific IgG or lysates transfected without pHA-RelA (Fig. 1D, middle panel). Since DDX1 has been shown to be recruited to sites containing DNA–RNA structures [Li et al., 2008], we also tested DDX1 and RelA binding at NF-κB non-specific DNA. We used DNA-promoter without RelA binding motifs, pGal4-luc together with pFlag-DDX1 and pHA-RelA. As shown in the Figure 1D (lower panel), there was no DNA fragment. These results indicate that DDX1 was recruited to the NF-κB carrying gene promoter region via association with RelA.

It has been shown previously that DDX1 is mainly localized in nucleus (DDX1/cleavage bodies) although it was also found abundantly in cytoplasm [Godbout et al., 1998; Bleoo et al., 2001]. In an inactive form, NF-κB–IκB complex is localized in cytoplasm, but upon TNFα stimulation, RelA is translocated from cytoplasm to nucleus. 293T cells stimulated with or without TNFα were used to analyze the subcellular localization of the interaction. The confocal immunofluorescence microscopy results showed that DDX1 was co-localized with RelA in nucleus when the nuclear translocation of NF-κB subunit RelA was induced with TNFα (Fig. 2, lower panel). When the cells were not treated with TNFα, the co-localization of DDX1 and RelA was observed mainly in cytoplasm (Fig. 2, upper panel). The observation that DDX1 is co-localized with both activated and non-activated RelA corroborated with the results of co-immunoprecipitation as described above. Taken together, these results indicate that the RNA helicase DDX1 is a cellular interaction partner of NF-κB subunit RelA.

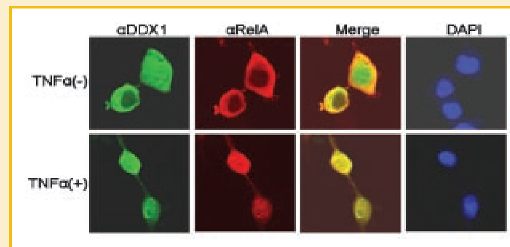

Fig. 2. Co-localization of DDX1 and RelA in cells. Confocal immunofluorescence microscopy was performed on 293T cells treated without (top panel) or with (bottom panel) TNFα (20 ng/ml) for 30 min. The localization of DDX1 and RelA was determined by immunostaining with anti-DDX1 (green) and anti-RelA (red) antibody. Merged images (yellow) of DDX1 (green) and RelA (red) are shown. DAPI (4′,6-diamidine-2′-phenylindole, dihydrochloride) staining was used to visualize the nucleus.

DDX1 STIMULATES NF-κB-INDUCED GENE EXPRESSION

In order to investigate the effect of DDX1 on NF-κB-dependent gene expression, DDX1 was co-transfected into 293T cells with luciferase reporter gene under the control of the promoters containing NF-κB binding sites: 5xNF-κB, IFN-β and E-selectin. NF-κB-responsive genes can be activated by over-expression of RelA and IKKα or induction with TNFα. As shown in Figure 3A, reporter gene activity of 5xNF-κB-luc induced by RelA, TNFα and IKKα increased 2–3 folds when DDX1 was co-expressed. On the other hand, the basal transcriptional level in the absence of induction with RelA, TNFα and IKKα was not affected by DDX1. Similar results were obtained with E-selectin-luc and IFNβ-luc reporter genes induced by RelA (Fig. 3B,C).

To check whether there is specificity for DDX1 to stimulate NF-κB-dependent gene activation, we checked the effect of DDX1 on other cellular transcriptional co-activators such as activated protein 1 (AP-1) that can be activated by mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase-1 (MEKK1) [Xu et al., 2004]. As shown in the Figure 3D, over-expression of MEKK1 could significantly increase the expression level of AP-1-driven luciferase reporter gene (AP-1-luc) in absence of any other stimuli whereas co-expression of DDX1 did not enhance either basal or MEKK1-induced reporter gene activity. In order to determine the effect of DDX1 on the stability of p65 expression, we performed qRT-PCR and Western blot analysis. The results showed that there was no detectable effect of DDX1 on the RNA and protein level of endogenous p65 (Fig. 3E,F). These results concluded that the DDX1 enhanced activity on the NF-κB gene expression is due to the interaction of the two proteins.

These data together indicate that DDX1 could specifically stimulate NF-κB-mediated gene expression.

THE C-TERMINAL TRANSCRIPTIONAL ACTIVATION DOMAIN OF RelA IS INVOLVED IN DDX1-MEDIATED UP-REGULATION OF NF-κB-DEPENDENT GENE EXPRESSION

In order to dissect the domains that are involved in DDX1-stimulated transcriptional activity of NF-κB, we adopted the GAL4-luc reporter system in which the gene expression is under the control of Gal4-RelA fusion protein. When DDX1 was co-expressed with Gal4-RelA (full length) fusion protein, it enhanced the gene expression from the Gal4-dependent promoter, thus mimicking the situation where DDX1 stimulated RelA-dependent transcriptional activation.

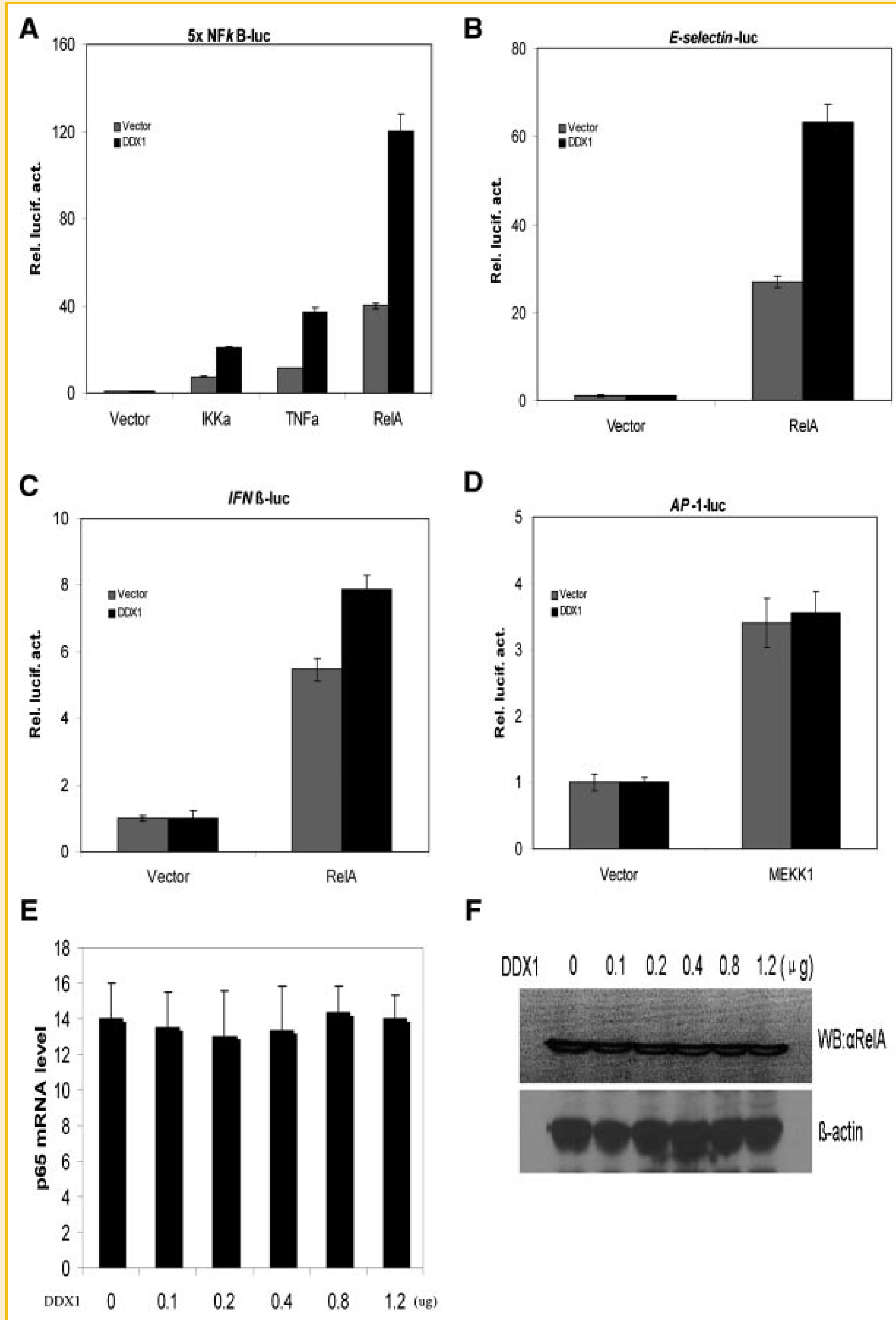

Fig. 3. Enhancement of NF-κB-mediated gene expression by DDX1. 293T cells were co-transfected with a reporter plasmid (50 ng), Renilla luciferase expression plasmid pRL-TK as an internal control (25 ng) and activating plasmid (100 ng) together with pFLAG-DDX1 or the corresponding empty vector pCMV2A (250 ng). After 24 h of transfection, firefly and renilla luciferase activities were determined. A: 5xNF-κB binding site-containing promoter-driven luciferase reporter (5xNF-κB-luc) assay activated by IKKα, TNFα (10 ng/ml) and RelA. B: E-Selectin promoter-driven luciferase reporter (E-selectin-luc) assays activated by RelA. C: IFNβ promoter-driven luciferase reporter (IFNβ-luc) assays activated by RelA. D: AP-1-driven luciferase reporter (AP-1-luc) assays activated MEKK1. E: DDX1 does not affect the p65 (RelA) RNA level. 293T cells were co-transfected with different concentration of Flag-DDX1. Total RNA was used for reverse transcription and real-time PCR was performed to determine p65 level. β-actin was used to normalize the p65 values. The results shown are from one of the two experiments. F: DDX1 does not affect the p65 (RelA) protein expression. 293T cells were transfected with different amount of Flag-DDX1 expression plasmid. Cells were harvested after 48 h of transfection. Western blot analysis of endogenous p65 level was done to confirm that DDX1 over-expression does not influence the p65 expression level. Mouse anti-p65 antibody was used for immunoblotting. β-actin antibody was used as an internal control.

To identify the functional interacting domains of RelA with DDX1, we generated a series of deletion mutants of RelA in fusion with Gal4 DNA-binding domain (Fig. 4A) and analyzed their transcriptional activities by co-expression of DDX1. The results showed that the RelA mutant (286–551) that contains the C-terminal nuclear localization signal (NLS) and transactivation (TA) domains was most responsive to the stimulation of DDX1 (Fig. 4B).

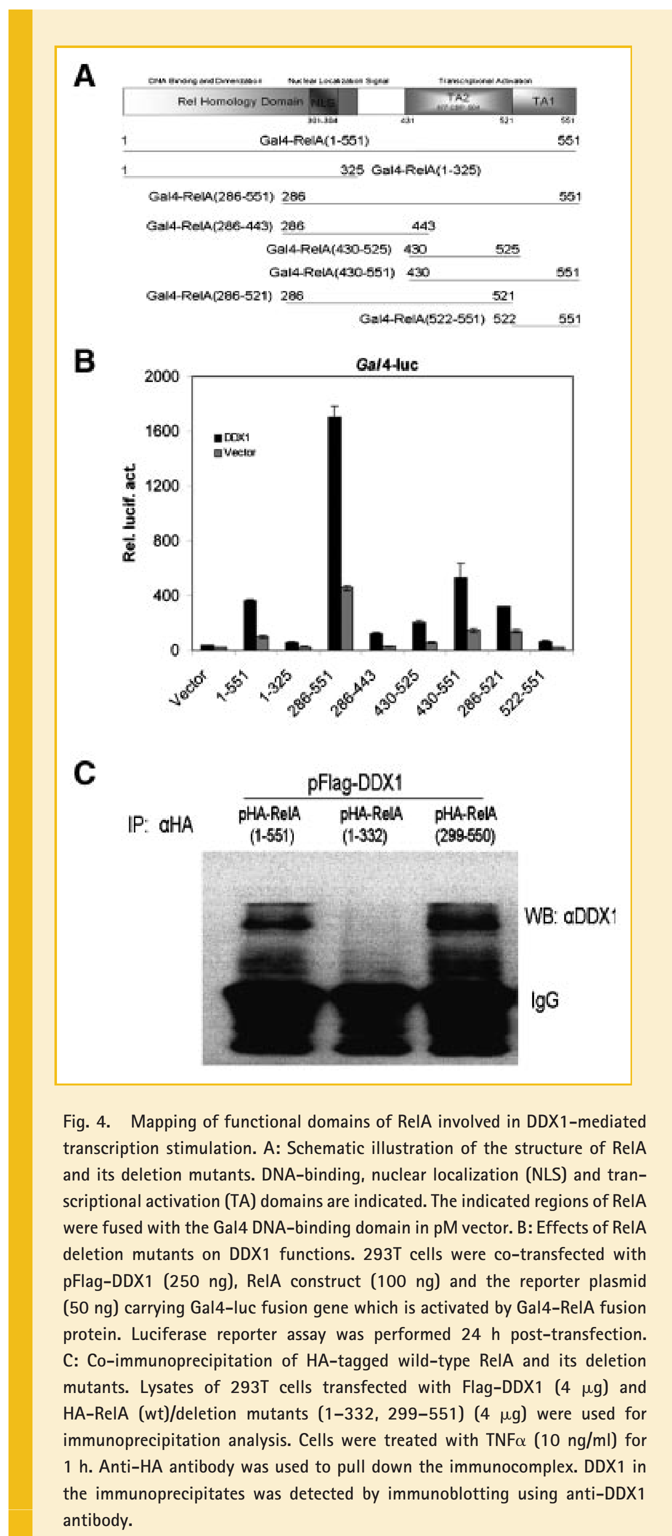

Fig. 4. Mapping of functional domains of RelA involved in DDX1-mediated transcription stimulation. A: Schematic illustration of the structure of RelA and its deletion mutants. DNA-binding, nuclear localization (NLS) and transcriptional activation (TA) domains are indicated. The indicated regions of RelA were fused with the Gal4 DNA-binding domain in pM vector. B: Effects of RelA deletion mutants on DDX1 functions. 293T cells were co-transfected with pFlag-DDX1 (250 ng), RelA construct (100 ng) and the reporter plasmid (50 ng) carrying Gal4-luc fusion gene which is activated by Gal4-RelA fusion protein. Luciferase reporter assay was performed 24 h post-transfection. C: Co-immunoprecipitation of HA-tagged wild-type RelA and its deletion mutants. Lysates of 293T cells transfected with Flag-DDX1 (4 μg) and HA-RelA (wt)/deletion mutants (1–332, 299–551) (4 μg) were used for immunoprecipitation analysis. Cells were treated with TNFα (10 ng/ml) for 1 h. Anti-HA antibody was used to pull down the immunocomplex. DDX1 in the immunoprecipitates was detected by immunoblotting using anti-DDX1 antibody.

As the RelA itself is a transcription factor, any deletion related to the activation domain would lead to the change of transcription activity of the reporter system. Therefore, we next used co-immunoprecipitation to confirm the interaction domain of RelA with DDX1. The HA-tagged RelA (full length), N-terminus domain (1–332) and C-terminus domain (299–551) were co-expressed with Flag-tagged DDX1 (full length). Mouse monoclonal HA-antibody was utilized to capture the immunocomplex and rabbit polyclonal anti-DDX1 antibody was used in Western blotting to detect precipitated DDX1. As demonstrated in Figure 4C, the full-length RelA and the C-terminus domain were readily co-precipitated with DDX1 but the N-terminus domain was not. Collectively, these data suggest that the C-terminus transcription-modulating domain of RelA is important for the stimulation effect of DDX1.

THE INTACT HELICASE DOMAIN OF DDX1 IS REQUIRED FOR ITS TRANSCRIPTIONAL CO-ACTIVATION ACTIVITY IN RelA-DEPENDENT GENE EXPRESSION

To characterize the region of DDX1 that bind to RelA, we constructed the DDX1 truncation mutants fused to the Gal4 DNA-binding domain in pM vector as shown in the Figure 5A. Wild-type and all DDX1 mutants were co-transfected together with pVP16-p65 and Gal4-luciferase reporter gene. Luciferase activity was assayed as a measure of DDX1 interaction with RelA. Mammalian two hybrid assay results demonstrated that DDX1 mutant pM-DDX1 (1–525) showed stronger binding to RelA than that of wild-type DDX1 (1–740). The mutants pM-DDX1 (1–185), pM-DDX1 (1–295), and pM-DDX1 (525–740) do not bind or weakly bind to RelA. The mutant pM-DDX1 (185–525) showed weak but detectable binding to RelA (Fig. 5B).

We further mapped the region within DDX1 that is responsible for stimulation of the transcriptional activity of RelA. For this purpose, we used a set of DDX1 deletion mutants fused with Flag tag (Fig. 5A) and determined the ability of these mutant proteins to up-regulate the transcriptional activity of Gal4 reporter plasmid induced by Gal4-RelA. The results demonstrate that the DDX1 mutant (amino acid 1–525) which contains the ATPase/helicase domain could enhance the Gal4-RelA-mediated reporter gene expression, so was the full-length DDX1 (Fig. 5C). In contrast, all the DDX1 mutants which had deletion in the conserved helicase domain lost the stimulation activity and manifested an inhibitory effect probably in a dominant-negative manner (Fig. 5C). All the DDX1 mutants could be readily detected by Western blot (data not shown); suggesting that lack of stimulation activity was not due to the instability of mutant proteins. These data indicate that the intact helicase domain of DDX1 may be involved in the stimulation of RelA activity.

To further analyze whether enzymatic activity of the ATPase/helicase of DDX1 is required, we introduced a Glu → Gly mutation in the conserved DEAD-box motif to produce a dominant-negative DDX1 mutant (DDX1-DGAD) in which the ATPase/helicase activity was abolished [Tetsuka et al., 2004]. As shown in Figure 5C, the DDX1-DGAD mutant exerted a strong inhibitory effect on RelA activity, suggesting the importance of DDX1 ATPase/helicase activity in its stimulation activity.

To determine the role of endogenous DDX1 in transcriptional activity, we tested the effect of the dominant-negative mutant

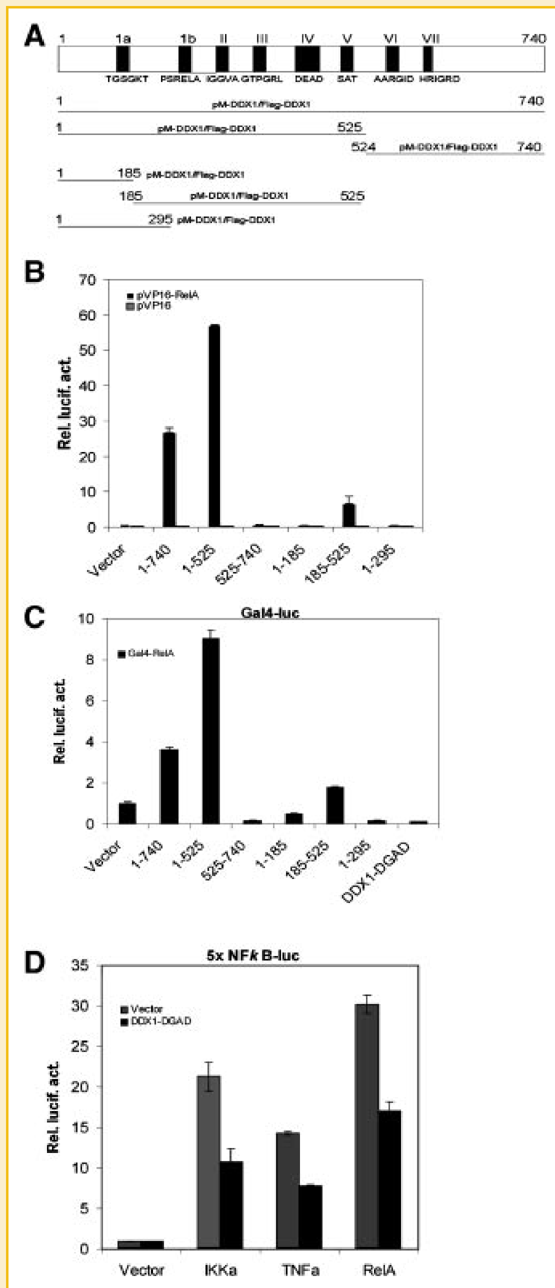

Fig. 5. Identification of functional domains of DDX1 that stimulates RelA activity. The assays for Gal4-RelA mediated reporter gene activities were conducted as described in Figure 4B. A: Schematic representation of the structure of DDX1 and its deletion mutants. The eight conserved RNA helicase motifs including the DEAD box (motif IV) are shown. B: DDX1 interact with RelA. Mammalian two-hybrid assay was performed to detect the interaction of DDX1 specific region with RelA. pM-DDX1 (Wt/mutants) and pVP16-RelA plasmids were co-transfected along with the Gal4-luc reporter plasmid. Luciferase activities were determined 48 h after transfection. These experiments were repeated three times and the results are expressed as the mean ± standard deviation. C: The effects of DDX1 deletion mutants and the DEAD-box mutant (DDX1-DGAD) on Gal4-RelA mediated gene activation. The mutant DDX1-DGAD contains an E > G mutation in the DEAD box and should abolish its ATP hydrolysis and helicase activity. D: Suppression of RelA activity by DDX1 DEAD-box mutant (DDX1-DGAD). The 5xNF-κB-luc reporter gene was activated by IKKα, TNFα, and RelA, respectively, or co-transfected with empty vector for basal level expression.

DDX1-DGAD on NF-κB-dependent gene expression induced by RelA, IKKα and TNFα, respectively. As shown in Figure 5D, the mutant DDX1-DGAD inhibited the activity of endogenous DDX1 and attenuated the NF-κB-mediated gene expression. Taken together, these results suggest that the conserved ATPase/helicase domain of DDX1 mediates the transcriptional activity of NF-κB RelA.

KNOCKDOWN OF ENDOGENOUS DDX1 REDUCED NF-κB-MEDIATED TRANSCRIPTIONAL CO-ACTIVATION

To determine the physiological role of endogenous DDX1 in NF-κB-dependent transcriptional activity, we adopted RNA interference (RNAi) to knockdown DDX1 expression. The Western blot analysis indicated that the protein level of endogenous DDX1 was significantly reduced upon transfection with the effective siRNA against DDX1 (Fig. 6A). Similarly at RNA level, anti-DDX1 siRNA considerably reduced the DDX1 mRNAs level (data not shown). As shown in Figure 6B, the depletion of endogenous DDX1 reduced NF-κB-mediated transcriptional activation induced by IKKα, TNFα, and RelA, respectively (Fig. 6B). In contrast, knockdown of DDX1 did not affect the AP-1-mediated reporter gene activity induced by MEKK1 (Fig. 6C). These data indicate that DDX1 plays a role in NF-κB activation pathway in physiological conditions.

DISCUSSION

DEAD-box proteins belong to a large family of nucleic acid helicases which function in many aspects of RNA metabolism, including ribosome biogenesis, pre-mRNA splicing, RNA transport, transcription and RNA degradation [Lorsch, 2002; Rocak and Linder, 2004]. DDX1, a typical member of DEAD-box RNA helicase family, contains the conserved DEAD-box, ATP-binding, helicase and ATPase domains. It has been reported previously that DDX1 plays important roles in pre-mRNA processing of nuclear genes [Bleoo et al., 2001; Chen et al., 2002] and Rev-RRE-mediated nuclear export of unspliced and singly spliced HIV-1 RNAs [Fang et al., 2004]. In this study, we showed that DDX1 could associate with the NF-κB subunit RelA and hence enhance NF-κB-mediated transcriptional activation, suggesting that the multifunctional RNA helicase DDX1 also plays an active role in RNA transcriptional regulation in nucleus. Very recently, DDX1 has been shown to be involved in promoter transactivation of JC virus, indicating that DDX1 may also participate in the transcriptional regulation of invading viruses [Sunden et al., 2007a,b].

It has been known that the ATPase/helicase activity of RNA helicases plays important roles in transcriptional pre-initiation and initiation [Tanner and Linder, 2001; von Hippel and Delagoutte, 2001]. For example, ATPase/helicase activity is found to associate with the general transcription factor TFIIH and chromatin remodeling complexes. Several DEAD-box helicases and related DEAH-box helicases have been shown to be actively involved in assembly of basal transcription machinery and RNA transcription. The most studied RNA helicases of the DExD/H-box family are RHA (DHX9) and p68 for their roles in RNA transcription [Fuller-Pace, 2006]. RHA was shown to act as a bridging factor between the CREB-

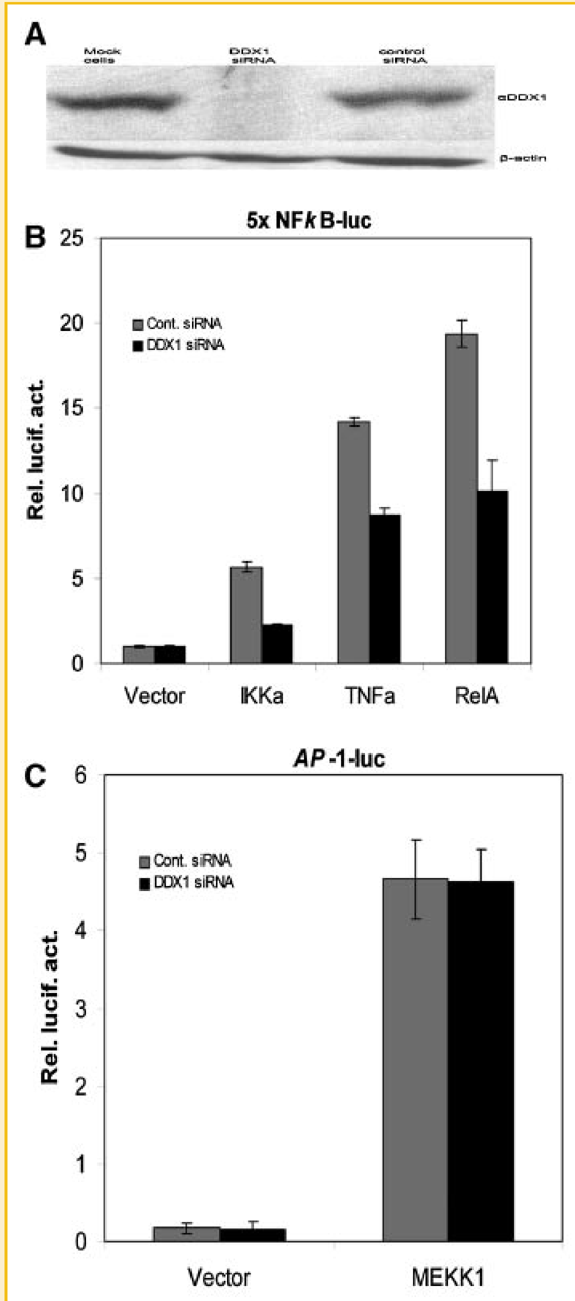

Fig. 6. Silencing of endogenous DDX1 by RNAi results in the depression of NF-κB-mediated transcription activation. A: Inhibition of endogenous DDX1 protein expression by DDX1-specific siRNA. A GFP-specific siRNA or mock treatment were used as controls. DDX1 was detected by anti-DDX1 antiserum (upper panel). β-actin was used as an internal control to show that equal amounts of total protein were loaded (lower panel). B: Knockdown of endogenous DDX1 inhibits NF-κB-mediated gene activation induced by IKKα, TNFα, or RelA. C: Inhibition of DDX1 by siRNA has no significant effect on the AP-1-driven gene expression (AP-1-luc) induced by MEKK1.

binding protein (CBP) and RNA polymerase II [Nakajima et al., 1997; Aratani et al., 2001] and between tumor suppressor BRCA1 and RNA polymerase II [Anderson et al., 1998] and as a component of the STAT6-dependent enhanceosome [Valineva et al., 2006] and to interact with RelA and enhance NF-κB-dependent transcription [Tetsuka et al., 2004]. It was shown that the helicase p68 acts as transcriptional co-activator for the nuclear receptor estrogen receptor alpha [Endo et al., 1999] and the tumor suppressor p53 [Bates et al., 2005] and interacts with the transcriptional co-activators Smad3, CBP/p300, and RNA polymerase II [Rossow and Janknecht, 2003; Warner et al., 2004]. The current study adds DDX1 to the list of RNA helicases that regulate RNA transcription.

The finding of this study that DDX1 could stimulate NF-κB-mediated RNA transcription may have multifold implications as NF-κB is a key cellular transcriptional factor involved in a wide variety of cellular events such as immune and inflammatory responses, cell survival, proliferation and apoptosis [Baldwin, 1996; Silverman and Maniatis, 2001; Tak and Firestein, 2001; Ghosh and Karin, 2002; Karin and Lin, 2002]. In this study, we found that DDX1 synergizes with NF-κB RelA to augment the NF-κB-dependent gene expression. RelA is one of the five mammalian NF-κB family members (c-Rel, RelA [p65], RelB, NF-κB1 [p50/p105], and NF-κB2 [p52/p100]) that can form hetero- and homodimers with distinct specificities for transcription activation [Baldwin, 1996]. Association of RNA helicases with individual NF-κB subunits such as the binding of DDX1 to RelA revealed in this study may contribute to differential effects of different NF-κB combinations. The heterodimer of RelA and p50 is the most common form of NF-κB which exists widely in various cell types and the results of this study provide new insight into the regulation mechanisms of this important transcription activator.

NF-κB subunit RelA contains an amino-terminal REL homology domain (RHD) that is required for dimerization and DNA-binding, and transcription-modulating region including two transcription activation (TA) domains (TA1 and TA2) at its carboxyl terminus [Schmitz et al., 1995a,b]. It has been shown that the TA domains are responsible for binding to transcriptional co-activators, such as p300/CREB-binding protein [Gerritsen et al., 1997; Perkins, 2004], A07 [Asamitsu et al., 2003], FUS/TLS [Uranishi et al., 2001], RHA [Tetsuka et al., 2004], general transcriptional factors including TBP [Xu et al., 1993], TFIIB [Schmitz et al., 1995b], RNA polymerase II, and TAF 105 [Sheppard et al., 1999]. We showed in this study that it is also the transcriptional activation domain of RelA that associates with DDX1. Therefore, it could be envisaged that the TA domains in RelA act as a platform for interactions with various transcriptional co-activators and thus facilitate the assembly of transactivation complex and modulate its transcriptional activity.

NF-κB exists in the cytoplasm in an inactive form associated with a class of inhibitory protein kinases (IκBs) in resting cells. Stimulation with inducers such as cytokines, infectious agents or radiation-induced DNA double-strand breaks results in phosphorylation, ubiquitination and degradation of the IκB proteins, leading to the nuclear translocation of NF-κB from cytoplasm [Schmitz et al., 1995a,b; Asamitsu et al., 2003; Perkins, 2004]. In this study, we showed that DDX1 could associate with RelA in either stimulated or non-stimulated cells (Figs. 1 and 2). DDX1 is distributed abundantly both in nucleus and cytoplasm (Fig. 2) [Godbout et al., 1998; Bleoo et al., 2001]. This observation is consistent with its role in transcription regulation in nucleus.

RNA helicase is a molecular motor that unwinds RNA or modulates RNA–protein structures in an energy-dependent manner.

For DDX1, the conserved ATPase/helicase domain is required for its stimulation effect on NF-κB-mediated transcription activation and the mutation in DEAD-box abolished its activities (Fig. 5), strongly suggesting that the enzymatic activities of DDX1 are needed for its involvement in transcription co-activation. Similar results have been reported for the DExH-box RNA helicase RHA [Tetsuka et al., 2004]. Although the exact mechanism for its helicase activity in transcription is not clear, it is most likely involved in the initiation stage of NF-κB-related transcription because in our experimental systems, we used different promoters but the same reporter gene; it should not make difference in transcription elongation, RNA processing and down-stream processes. In the future work, the exact role of DDX1 in NF-κB-mediated transcription activation should be revealed. Furthermore, as DDX1 is also involved in several RNA metabolic processes, it is interesting to address how DDX1 coordinates these diversified functions and couples different processes like transcription, pre-mRNA processing and mRNA nuclear export in the future.

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