

## Phosphorylation of p68 RNA helicase regulates RNA binding by the C-terminal domain of the protein

Liuqing Yang,<sup>a</sup> Jenny Yang,<sup>b</sup> Youliang Huang,<sup>c</sup> and Zhi-Ren Liu<sup>a,c,\*</sup>

<sup>a</sup> Department of Biology, Georgia State University, Atlanta, GA 30303, USA

<sup>b</sup> Department of Chemistry, Georgia State University, Atlanta, GA 30303, USA

<sup>c</sup> Department of Animal Sciences, Auburn University, Auburn, AL 36849, USA

Received 3 December 2003

### Abstract

We previously reported ATPase, RNA unwinding, and RNA-binding activities of recombinant p68 RNA helicase that was expressed in *Escherichia coli*. Huang et al. [J. Biol. Chem. 277(15) (2002) 12810]. The recombinant protein bound both single-stranded (ss) and double-stranded (ds) RNAs. To further characterize the substrate RNA binding by p68 RNA helicase, we expressed and purified the recombinant N-terminal and C-terminal domains of the protein. RNA-binding property and protein phosphorylation of the recombinant domains of p68 were analyzed. Our data demonstrated that the C-terminal domain of p68 RNA helicase bound ssRNA. More interestingly, the C-terminal domain was a target of protein kinase C (PKC). Phosphorylation of the C-terminal domain of p68 abolished its RNA binding. Based on our observations, we propose that the C-terminal domain is an RNA substrate binding site for p68. The protein phosphorylation by PKC regulates the RNA binding of p68 RNA helicase, which consequently controls the enzymatic activities of the protein.

© 2003 Elsevier Inc. All rights reserved.

**Keywords:** P68 RNA helicase; DEAD-box; RNA-binding; UV crosslinking; Phosphorylation; PKC

The RNA helicases are found in almost all organisms, from bacteria to human. This family of proteins is involved in almost every process of RNA metabolism in cells [1–3]. Members of the RNA helicase family are characterized by a core region of 290–360 amino acids (helicase-core) that consists of eight conserved sequence motifs (Fig. 1A). The principal cellular function of RNA helicases is to unwind dsRNA or RNA–protein complex using the energy derived from NTP (ATP in most cases) hydrolysis. In a number of examples, RNA helicases have been shown to be generic RNA unwinding motors [2,4] that also unwind the RNA–protein complex [5] in vitro.

One key question regarding the mechanism of RNA unwinding is how RNA helicases bind their unwinding substrates during the unwinding process. Mutational analyses and RNA-binding studies with eIF4A reveal a weak RNA binding by the common sequence motif VI

(Fig. 1A) in the helicase core region [6]. However, crystal structure of HCV-NS3 and later mutational analyses with NPH-II imply that this motif may not be the substrate-binding site for the DEAD/DExH boxes of RNA helicases [7,8]. The weak RNA binding property of the motif VI is believed to play an important catalytic role in RNA unwinding [9]. On the other hand, the three-dimensional structure of the HCV-NS3:oligonucleotide complex reveals that the nucleic acid is bound in the second cleft between the domain 1–2 and domain 3 [7,10]. However, this nucleic acid binding cleft is not clearly defined in the assembled three-dimensional structure of translation initiation factor, eIF4A [11–13].

RNA binding motifs outside of the helicase core were suggested for a number of DEAD/DExH-box of RNA helicases [14–16]. However, functional roles of these putative RNA-binding motifs are not yet understood. Further, their roles as RNA binding motifs, in most cases, have not yet been demonstrated. It has been suggested that multiple RNA binding motifs are needed

\* Corresponding author. Fax: 1-404-651-2509.

E-mail address: [biozrl@langate.gsu.edu](mailto:biozrl@langate.gsu.edu) (Z.-R. Liu).

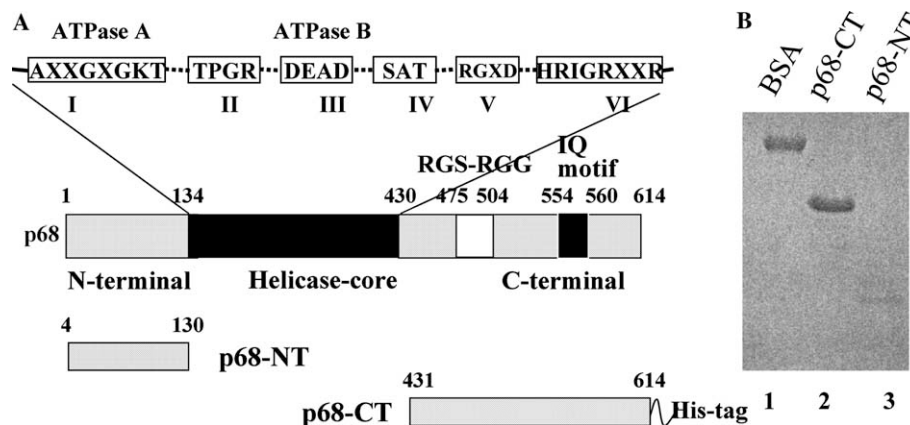


Fig. 1. (A) Schematic illustration of domain structure of p68 RNA helicase. The top panel is the helicase-core region of DEAD-box proteins. The conserved sequence motifs and their putative functions are indicated. The middle panel is the domain structure of p68 RNA helicase. Specific sequence motifs are indicated. The numbers represent amino acid residue numbers. The bottom panel illustrates the sequence region of expressed recombinant C-terminal and N-terminal domains of p68. (B) Coomassie staining the SDS-PAGE of; BSA (lane 1), bacterially expressed and purified p68-CT (lane 2), bacterially expressed and purified p68-NT (lane 3).

for RNA unwinding action, especially for those RNA helicases that act as monomers [17,18]. The translation initiation factor eIF4A represents a very interesting example. The eIF4A alone has very weak RNA affinity and the RNA binding requires ATP [9,19]. Due to the weak RNA binding, eIF4A has very weak RNA unwinding and ATPase activities. The RNA unwinding activity of eIF4A is dramatically enhanced upon the formation of a complex with eIF4B [20,21]. eIF4B functions to stabilize the eIF4A/RNA complex by donating its two RNA-binding domains.

The nuclear p68 RNA helicase was first identified by cross-reaction with a monoclonal antibody PAb204 that was originally raised against SV40 large-T antigen two decades ago [22,23]. The protein is a prototypical DEAD box family of RNA helicases. As an early example of cellular RNA helicase, the ATPase and the RNA unwinding activities of p68 RNA helicase were documented with the protein purified from human 293 cells [17,24,25]. The helicase plays very important roles in cell proliferation and organ maturation [23,26,27]. However, the biological functions of the helicase on a molecular level are not well understood [28]. It has been suggested that p68 RNA helicase might be involved in transcription regulation [29–31] and DNA damage–repair pathways [32]. Most recently, the experiments carried out in our laboratory demonstrated that p68 RNA helicase is an essential human splicing factor *in vitro* that plays a role in unwinding the transient U1:5' splice site duplex [33]. Consistently, experiments in other research laboratories also suggested that p68 RNA helicase has a functional role in the pre-mRNA splicing process [34–38].

We previously reported the expression and purification of recombinant p68 RNA helicase in *Escherichia coli* [39]. The purified recombinant protein binds both

single-stranded and double-stranded RNAs. In this report, we further characterize the RNA binding by domains of the protein. Our data demonstrate that the C-terminal domain of p68 RNA helicase binds ssRNA. More interestingly, the C-terminal domain is a target of protein kinase C. Phosphorylation of the C-terminal domain of p68 abolishes its RNA binding. Based on our observations, we propose that protein phosphorylation regulates the RNA binding of p68 RNA helicase, which consequently controls the enzymatic activities of the protein.

## Materials and methods

### Protein expression and purification.

The reading frames that encode N-terminal and C-terminal domains of p68 RNA helicase were amplified from a p68 RNA helicase clone [39] using two pairs of PCR primers. The PCR products were cloned into an expression vector pET-32a by *Bam*HI/*Hind*III sites. For expression of the C-terminal domain of p68 (referred to as p68-CT), the vector pET-32a was modified so that the DNA sequences that encode the thioredoxin and other fusion tags flanking the 5' site of the p68-CT insertion were removed. The procedures for induction of recombinant protein expression in *E. coli* and subsequent disruption of bacterial cells were similar to those described in a previous report [39]. After disruption of bacterial cells and centrifugation, the expressed recombinant p68-CT was precipitated in bacterial inclusion bodies. The precipitates were washed twice with wash-buffer (50 mM Tris-HCl, pH 7.5, 800 mM urea, 250 mM NaCl, and 1 mM DTT). The precipitates were then dissolved in denaturing buffer containing 7 M urea, 50 mM Tris-HCl, 0.2% Triton X-100, and 250 mM NaCl, pH 7.5. The protein was purified through a Ni-NTA column and the recombinant proteins were eluted with 200 mM imidazole in protein elution buffer (7 M urea, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mM DTT, 10% glycerol, and 0.2% Triton X-100). The obtained protein solution was diluted drop-wise (1:10) to a refolding buffer containing 200 mM arginine, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mM DTT, 10% glycerol, and 0.2% Triton X-100. Finally, the

protein was dialyzed three times against the same refolding buffer to remove urea residues. The N-terminal domain was expressed as thioredoxin and his-tag fusion protein in bacterial cells. After purification over a Ni-NTA column, the fusion protein was cleaved by enterokinase (Invitrogen). The thioredoxin and other fusions were further removed by a new round of Ni-NTA column. Enterokinase was then removed using a commercially available enterokinase-away resin (Invitrogen). The purified N-terminal domain of p68 (referred to as p68-NT) was finally dialyzed twice against a buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mM DTT, 10% glycerol, and 0.2% Triton X-100.

#### DNA/RNA substrates and transcriptions

RNAs were synthesized by run-off transcriptions of linearized vectors using T7/SP6 RNA polymerase. Following linearized vectors were used for RNA syntheses, pGC + DX/*XhoI*/SP6, pGEM-3Z/*PvuII*/T7 (polylinker region of pGEM-3Z was removed). The RNAs were uniformly labeled with [ $\alpha$ - $^{32}$ P]UTP. The RNA for UV-crosslinking was labeled to high specificity activity (~27,550 CPM/ng). The RNA used for gel-mobility shift assay was labeled to low specificity activity (~2170 CPM/ng). The dsRNA substrate for RNA-binding assays is the hybridization of equal molar amounts of two complementary strands transcribed from both directions of polylinker of pSP72 (pSP72/*BgIII*/SP6 and pSP72/*XhoI*/T7). Annealing solution contained 30 mM Tris-HCl, pH 7.5, 100 mM NaCl. The RNA annealing mixture was heated to 85 °C for 10 min and was then slowly cooled down to room temperature. A DNA oligonucleotide, 5'-GGCCGCTCACTTTTCGAATTCTGGG TGGGACCAAATGAGTCAAGCTTG-3', was used for gel-mobility shift assays. The oligonucleotide was end-labeled with  $^{32}$ P by polynucleotide kinase under standard kinase reaction conditions.

#### RNA-binding analyses

*Gel-mobility shift assays* were performed by following the same experimental protocols as described previously [39].

*UV-crosslinking.* About 60 ng of proteins was mixed with 4–6 ng  $^{32}$ P-labeled RNA in RNA-binding buffer (20 mM Hepes, pH 8.0, 5 mM MgCl<sub>2</sub>, 50 mM NaCl, and 2 mM DTT) in a total volume of 10  $\mu$ l. After 10 min incubation at 30 °C, heparin was added to a final concentration of 0.5 mg/ml. The mixtures were incubated for an additional 5 min. The samples were photolyzed in a crosslinker mounted with 4  $\times$  8 W 312<sub>max</sub> nm UV light bulbs for 15 min. During photolyses, the samples were placed on ice and 4–6 cm below the light source. After photolyses, 1  $\mu$ l of RNase cocktail containing 2 mg/ml RNase A and 0.3 U/ $\mu$ l RNase T1 (USB) was added to the samples. After 25 min of RNase digestion, the samples were analyzed by 12% SDS-PAGE followed by auto-radiography.

#### Protein phosphorylation/dephosphorylation

Proteins were phosphorylated by protein kinase C (PKC) (Promega) under optimal conditions at 30 °C for 30 min. In a typical 50  $\mu$ l phosphorylation reaction, the mixture contains 25 ng/ $\mu$ l protein, 1 ng/ $\mu$ l protein kinase C, and 1  $\times$  PKC buffer (2 mM CaCl<sub>2</sub> and 10 mM MgCl<sub>2</sub>, and 4 mM ATP or 5  $\mu$ Ci [ $^{32}$ P- $\gamma$ ]ATP). After incubation, the phosphorylation reaction was immediately used for further RNA-binding assays or SDS-PAGE analyses.

To carry out protein dephosphorylations, the ATP in the above protein phosphorylation reactions was removed by buffer exchange with dephosphorylation buffer (for PPase 2B, 50 mM Tris-HCl, pH 7.4, 0.5 mg/ml BSA, 5  $\mu$ g/ml calmodulin, and 1 mM NiCl<sub>2</sub>) as suggested by the manufacturer. Two microliters (0.5 U/ $\mu$ l) of protein phosphatase 2B was added to a 50  $\mu$ l reaction. After 60 min incubation at 30 °C, the dephosphorylated proteins were analyzed by SDS-PAGE or directly used for RNA-binding assays without further treatment.

## Results

### *Expression and purification of N-terminal and C-terminal domains of p68 RNA helicase*

In the previous report [39], we demonstrated that p68 RNA helicase bound both single-stranded (ss) and double-stranded (ds) RNAs. The dsRNA substrate is more efficient in stimulating the ATPase activity of p68. Due to the unique amino-acid sequence structure at the C-terminal domain of the protein, we believed that the sequence motif(s) at the C-terminal domain might constitute an RNA-binding site(s) for p68 RNA helicase. Thus, in an effort to understand whether the sequence domains of p68 outside the helicase core possess RNA-binding property, the coding regions for N-terminal (aa residue 4–130) and C-terminal (aa residue 431–614) (Fig. 1A) were sub-cloned and expressed in *E. coli*. Unlike the expression of full-length recombinant p68 RNA helicase described in our previous report, in which significant amounts of recombinant proteins were purified as soluble form, the p68-CT appeared to precipitate completely in bacterial inclusion body. Thus, the recombinant p68-CT was purified under denaturing conditions (7 M urea). The protein was refolded by diluting 10 times in the refold buffer and subsequently dialyzed to remove the urea completely. We have observed that 200 mM of arginine is necessary to maintain the protein in soluble form. It was also essential to slowly add the denatured p68-CT to the refolding buffer. After purification and refolding, we were able to obtain ~1.5 mg/ml of recombinant p68-CT (Fig. 1B, lane 2). The N-terminal of p68 (p68-NT) was first expressed and purified as his-tag and thioredoxin fusion protein. After cleavage of the thioredoxin and his-tag fusions by enterokinase, the recombinant p68-NT was purified as ~0.4 mg/ml of soluble protein (Fig. 1B, lane 3).

### *The C-terminal domain of p68 RNA helicase binds ssRNA but not dsRNA and DNA*

To test the RNA-binding property of the bacterially expressed N-terminal and C-terminal domains of p68 RNA helicase, the gel-mobility shift assay was carried out. We used a 124 nt RNA transcribed from pGC + DX/*XhoI* [40]. It was evident that the p68-CT bound the RNA substrate (Fig. 2A, lane 3). However, the p68-NT did not bind (Fig. 2A, lane 4). As a negative control, BSA did not bind the RNA substrate (Fig. 2A, lane 2). The substrate RNA G + DX/*XhoI* is an RNA derived from the exon 2 of  $\alpha$ -tropomyosin gene with purine-rich sequences. To test whether the binding of the p68-CT to this RNA is due to recognition of this specific RNA, we employed another ~186 nt RNA substrate that was transcribed from pGEM-3Z/*PvuII*. The polylinker region of the vector (*EcoRI*-*HindIII*) was

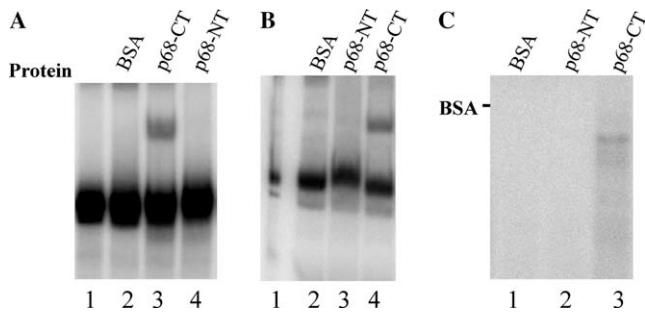


Fig. 2. RNA binding analyzed by gel-mobility shift assays (A,B) and UV crosslinking assays (C). (A) The gel mobility shift assays of 6 ng GC + DX/*XhoI* RNA with; 400 ng BSA (lane 2), 60 ng p68-CT (lane 3), and 30 ng p68-NT (lane 4). Lane 1 is the GC + DX/*XhoI* RNA alone. (B) The gel mobility shift assays of 4 ng pGEM-3Z/*PvuII* RNA with; 400 ng BSA (lane 2), 60 ng p68-CT (lane 3), and 30 ng p68-NT (lane 4). Lane 1 is the pGEM-3Z/*PvuII* RNA alone. (C) UV crosslinking of the mixture of recombinant p68 truncates and 4 ng pGEM-3Z/*PvuII* RNA. The crosslinking reactions were carried out with; 4 ng pGEM-3Z/*PvuII* RNA and 400 ng BSA (lane 1), 4 ng pGEM-3Z/*PvuII* RNA and 30 ng p68-NT (lane 2), and 4 ng pGEM-3Z/*PvuII* RNA and 60 ng p68-CT (lane 3).

removed. A slower migration band was observed in the gel-mobility shift assays with the recombinant p68-CT and pGEM-3Z/*PvuII* RNA (Fig. 2B, lane 4). No band shift was observed with p68-NT. To verify the RNA-binding results obtained from the gel-mobility shift assays, we carried out UV-induced crosslinking experiments with the RNA substrate pGEM-3Z/*PvuII*. Consistent with the gel-mobility shift assays, we observed that the p68-CT crosslinks to this RNA substrate (Fig. 2C, lane 3). The p68-NT did not crosslink to this RNA (Fig. 2C, lane 2). Thus, both gel-mobility shift and UV-crosslinking experiments confirmed the RNA-binding property of the C-terminal domain of p68 RNA helicase.

In previous studies, we demonstrated that p68 RNA helicase has strong affinity for dsRNA substrate [39]. We suspected that the C-terminal domain of p68 may contribute to the dsRNA-binding property of the protein. To examine the dsRNA binding by the C-terminal domain of p68, we employed the gel-mobility shift assay and MB crosslinking method similar to those described in our previous studies [39,41,42] to probe the dsRNA–protein interactions. The dsRNA substrate was obtained by annealing two transcripts that were transcribed from polylinker region of pPSP72 in both directions by T7/SP6 RNA polymerases. The dsRNA contains 91 base-pairs of RNA duplex flanked by a 7 and a 4 nt of 5' overhang on each side. Under our experimental conditions, no dsRNA bindings by both p68-CT and p68-NT were observed in the gel-mobility shift assays (Fig. 3A, lanes 3 and 4). In addition, no crosslinking signals were detected in our MB crosslinking experiments (data not shown). Both gel-mobility shift assays and MB crosslinking experiments

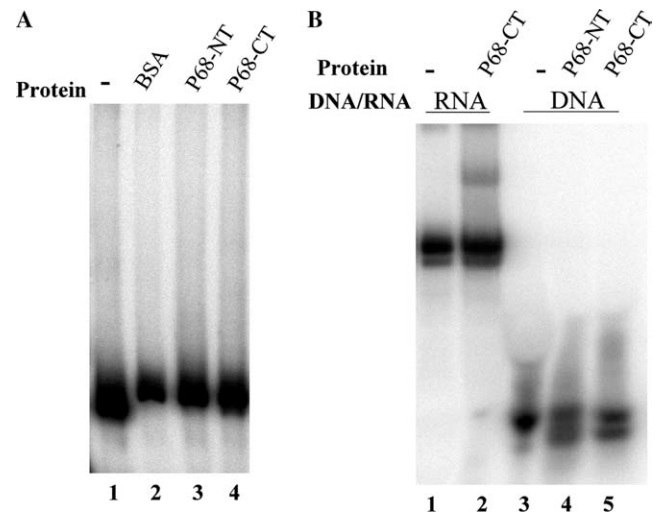


Fig. 3. Gel-mobility shift assays of p68 truncates with dsRNA and DNA. (A) Gel-mobility shift assays of 6 ng dsRNA with; 400 ng BSA (lane 2), 30 ng p68-NT (lane 3), and 60 ng p68-CT (lane 4). Lane 1 is dsRNA alone. The dsRNAs were hybridization of equal molar of SP6-pSP72/*BglII* vs T7-pSP72/*XhoI*. (B) Gel-mobility shift assays of p68 truncates with ssRNA and DNA oligonucleotides. The gel-mobility assays were carried out with; 60 ng p68-CT + 6 ng pGC + DX/*XhoI* RNA (lane 2), 30 ng p68-NT + 15 ng of <sup>32</sup>P-labeled DNA oligonucleotides (lane 4), and 60 ng p68-CT + 15 ng <sup>32</sup>P-labeled DNA oligonucleotides (lane 5). Lane 1 is the pGC + DX/*XhoI* RNA alone. Lane 3 is the <sup>32</sup>P-labeled DNA oligonucleotides.

suggested that the N-terminal and C-terminal domains of p68 do not bind dsRNA.

It is noted that a number of positively charged amino acid residues are scattered in both N-terminal and C-terminal domains of p68. Since the RNA binding by p68-CT was relatively weak, as detected by both gel-mobility shift assays and crosslinking assays, we suspect that the p68-CT:RNA interactions may be due to non-specific electrostatic interactions between the negatively charged phosphate backbone of RNA and positively charged amino acid residues. One test for this possibility is to examine the interactions of the protein with the same negatively charged DNA. Thus, we used the same gel-mobility shift assays to examine whether the N-terminal and C-terminal domains of p68 would bind DNA oligonucleotides. A synthetic 48 nt DNA oligonucleotide was used as the binding substrate. Under the same binding conditions, neither recombinant p68-CT nor p68-NT interacted with the DNA oligonucleotides (Fig. 3B, lanes 4 and 5). We also employed a dsDNA substrate, pGEM-3Z/*HindIII*, to perform the binding analyses. No bindings were detected with dsDNA (data not shown). The results suggested that the recombinant p68-CT:RNA interaction is not simply due to non-specific electrostatic interactions between the negatively charged phosphate backbone of RNA and positively charged amino acid residues of the protein.

*The C-terminal domain of p68 RNA helicase can be phosphorylated by protein kinase C and phosphorylation abolishes the RNA binding property*

Buelt and colleagues reported that p68 RNA helicase purified from PC12 cells was a target by protein kinase C and Ca-calmodulin binding. The consequence of phosphorylation by PKC or Ca-calmodulin binding was the inhibition of ATPase activity of p68. It is noted that a conserved IQ motif locates in the C-terminal (Fig. 1A). Thus, the authors suggested that the IQ motif was the phosphorylation and Ca-calmodulin binding site of p68 [43]. It is, however, not known how the phosphorylation or Ca-calmodulin binding affects the enzymatic activities of the protein. Since the bacterially expressed C-terminal domain of p68 harbors the IQ motif of the protein, we were interested in examining whether the p68-CT would be phosphorylated by protein kinase C. To this end, we used a commercially available protein kinase C (Promega) to test the phosphorylation of the recombinant p68-CT and p68-NT. It was evident that the p68-CT was phosphorylated by protein kinase C under our experimental conditions (Fig. 4A, lane 2). The p68-NT was not phosphorylated (Fig. 4A, lane 3) nor was a control BSA phosphorylated under the same phosphorylation conditions (Fig. 3A, lane 1). We were able to achieve phosphorylation of >70% of the protein by using significantly low substrate protein concentra-

tion in the reaction (15 ng/μl) and longer phosphorylation time (2.5 h). No detectable increase in the phosphorylation after 2.5 h was observed.

We next tested the effects of phosphorylation on the RNA-binding property of the recombinant p68-CT. The protein was phosphorylated by PKC under the manufacturer suggested conditions (see the paragraph above). The phosphorylated proteins were tested for RNA binding by gel-mobility shift assays without further treatment. If the phosphorylation reaction was carried out almost to completion (>70% of proteins were phosphorylated) by PKC (see the paragraph above), the RNA binding activity was almost abolished (Fig. 4C, lane 3). However, if the phosphorylations were carried out to less completion (~20% of proteins were phosphorylated), the RNA binding was significantly reduced (Fig. 4C, lane 4). To further confirm the effects of protein phosphorylation on the RNA-binding property of p68-CT, we examined whether the RNA-binding property can be restored by dephosphorylation of the phosphorylated protein. We used commercially available protein phosphatase 2B. As indicated in the Materials and methods section, the protein phosphatases were directly added to the phosphorylation reactions after removing ATP by buffer exchange. The phosphoprotein of the p68-CT was successfully dephosphorylated by

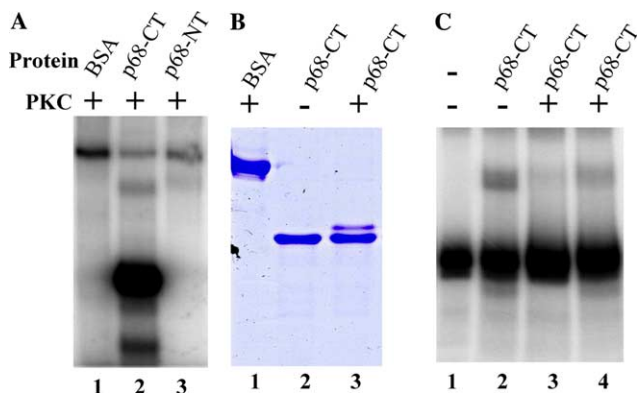


Fig. 4. Phosphorylation of p68 truncates with PKC and the effects on the RNA binding. (A) Autoradiography of SDS-PAGE of phosphoproteins; 20 ng/μl BSA (lane 1), 25 ng/μl p68-CT (lane 2), and 10 ng/μl p68-NT (lane 3). Proteins were phosphorylated by 1 ng/μl protein kinase C in the presence of 150 μM ATP including 5 μCi [ $\gamma$ - $^{32}$ P]ATP. (B) Coomassie staining the SDS-PAGE of; BSA (lane 1), p68-CT (lane 2), and phosphoprotein p68-CT (lane 3). Twenty-five nanograms of p68-CT was phosphorylated by 1 ng/μl protein kinase C at 30 °C for 30 min. It is estimated by density scan that about 20% of p68-CT were phosphorylated under the conditions. (C) Gel-mobility shift RNA-binding assays of 6 ng pGC + DX/XhoI RNA with; 60 ng p68-CT (lane 2), 60 ng phosphorylated p68-CT. The phosphorylation reactions were carried out with 15 ng/μl p68-CT in the presence of 2 ng/μl PKC for 2.5 h (lane 3) and 60 ng of phosphorylated p68-CT. The phosphorylation reactions were carried out with 25 ng/μl p68-CT in the presence of 1 ng/μl PKC for 0.5 h (lane 4). The lane 1 is the pGC + DX/XhoI RNA alone.

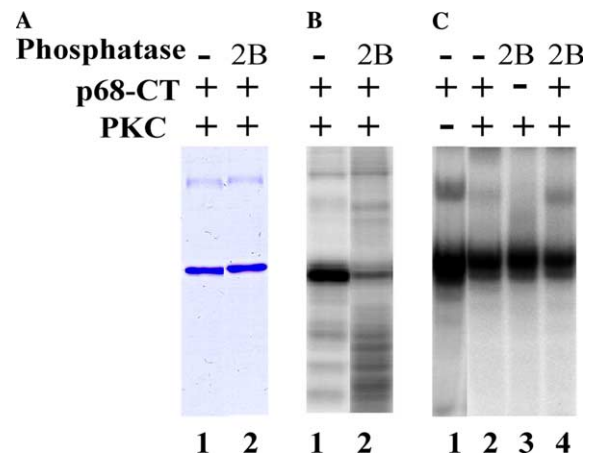


Fig. 5. (A) Coomassie staining and (B) autoradiography of the SDS-PAGE of; p68-CT phosphorylated by PKC (lane 1) and phosphorylated p68-CT treated with 2 μl protein phosphatase 2B for 60 min at 30 °C (lane 2). The phosphorylation reactions were carried out in the presence of 5 μCi [ $\gamma$ - $^{32}$ P]ATP without cold ATP. The dephosphorylation reactions were carried out by directly adding protein phosphatases to 50 μl of phosphorylation reactions after buffer exchange and removal of ATP. (C) Gel-mobility shift assays of 6 ng pGC + DX/XhoI RNA with; p68-CT (lane 1), p68-CT phosphorylated by PKC (lane 2), protein kinase C + protein phosphatase 2B (lane 3), and phospho-p68-CT dephosphorylated by protein phosphatase 2B (lane 4). The phosphorylation reactions were catalyzed by 2 ng/μl PKC in the presence of 2 mM ATP at 30 °C for 2.5 h. The dephosphorylation reactions were carried out by directly adding 4 μl protein phosphatases to 50 μl of phosphorylation reactions at 30 °C for 2 h after buffer exchange and removal of ATP.

protein phosphatase 2B (Figs. 5A and B, lane 2). The dephosphorylated proteins were used for the RNA-binding test. It was evident that the RNA-binding property of the p68-CT was restored by dephosphorylation of the phosphoprotein (Fig. 5C, lane 4). RNA binding was somewhat decreased after phosphorylation and dephosphorylation cycle. We reason that the decreased RNA binding may be due to incomplete dephosphorylation by phosphatase 2B (Fig. 5B, lane 2). The results strongly suggest that phosphorylation at the C-terminal domain of p68 by PKC abolishes the RNA-binding property.

## Discussion

In this paper, we report expression and purification of recombinant N-terminal and C-terminal domains of p68 RNA helicase. RNA-binding analyses indicate that the C-terminal domain of p68 binds ssRNA but not dsRNA nor DNA. Phosphorylation experiments suggest that the C-terminal domain of p68 could be phosphorylated by protein kinase C. More importantly, our data demonstrate that phosphorylation of the recombinant C-terminal domain of p68 RNA helicase abolishes the RNA binding property of the protein. Effects of protein phosphorylation on RNA binding suggest a possible mechanism by which phosphorylation pathways regulate the functions of p68 RNA helicase by controlling the interactions of the protein with its substrate.

The nuclear p68 RNA helicase was first identified by cross-reaction with a monoclonal antibody PAb204 that was originally raised against SV40 large-T antigen two decades ago [22,23]. The protein shares a common sequence domain of DEAD-box (or helicase core) with a family of proteins, so-called DEAD box of RNA helicases. In addition to the conserved helicase core, an RGS–RGG–RGG sequence motif (simplified as RGS–RGG) between amino acid residues 477–504 in the C-terminal of the protein has been noted (Fig. 1A). It was speculated that the RGS–RGG motif in p68 may function as an RNA-binding site of p68 [17,25]. This RGS–RGG motif may resemble a conserved protein sequence motif of RGG repeats. The RGG repeats are commonly found in many RNA-binding proteins [44,45], such as hnRNP A1 [46], nucleolin [47], hnRNP U&K [48], FMRP [49], poly(A) binding protein [50], and viral RNA-binding protein [51]. One characteristic of these RNA-binding RGG repeats motifs is that a number of positively charged residues, Arg and Lys, and aromatic residues are usually scattered among the RGG repeats. It is believed that the positively charged and aromatic residues may also provide additional binding force for protein–RNA interactions [48]. Sequence analyses reveal that a number of positively charged and aromatic residues are also scattered among the RGS–RGG motif

of p68, which provides additional indication that the RGS–RGG motif of p68 may resemble many RNA-binding RGG repeats motifs functioning as an RNA binding site. We demonstrate here that the C-terminal domain of p68 RNA helicase binds ssRNA. Our observation provides experimental evidence to support the long-time speculation that the RGS–RGG motif may function as an RNA-binding site of p68. Interestingly, the RGG repeats outside of the helicase-core are also found in a number of DExD/H box putative RNA helicases [14,52–54]. It is, however, not known whether the RGG repeats motif in those proteins possesses RNA binding property.

How does the RNA binding by the C-terminal domain of p68 contribute to the overall substrate RNA binding during the unwinding process is an open question. It is believed that multiple contacts, including dsRNA binding, between the RNA helicase and the unwinding substrate are necessary for an active unwinding process [14,18]. It was suggested, in a number of examples, that putative RNA-binding motif(s) outside of the helicase-core region often represent one of the substrate binding sites during RNA unwinding [55,56]. For p68 RNA helicase, the C-terminal domain may constitute a ssRNA-binding site. This view explains our previous observations that p68 RNA helicase binds both dsRNA and ssRNA [39]. In addition, we observed that both full length of p68 RNA helicase [39] and the C-terminal domain bind ssRNA with very similar affinity (data not shown). Alternatively, the C-terminal domain may function to stabilize the RNA binding by the helicase core region or other RNA binding sites. In this case, the C-terminal domain itself may not be the constitutive substrate RNA-binding site for unwinding. The RNA binding by C-terminal domain may cooperate with the substrate RNA binding to enhance the unwinding efficiency of p68 helicase. The notion that the RGS–RGG motif cooperates with the substrate RNA binding, to some extent, resembles the case of eIF4A/eIF4B. The eIF4A has very weak RNA unwinding and ATPase activities. The RNA unwinding activity of eIF4A is greatly enhanced by eIF4B due to that eIF4B provides RNA binding that strengthen the weak eIF4A:RNA interactions. It is conceivable that p68 resembles the case in eIF4A/eIF4B but the extra RNA binding is provided in *cis* from the C-terminal rather than in *trans* from another protein. Although the dsRNA binding was not examined, it should be noted that eIF4A unwinds a dsRNA with both sides blunt-ended suggesting the dsRNA binding of the protein [57]. Consistent with this view, the RGG repeats motifs are indeed often found associated with other RNA-binding sequence motifs, such as RRM or RBDs, in many RNA-binding proteins. This arrangement provides the proteins with high affinity and specificity of RNA binding [44,45,51].

Phosphorylation of p68 RNA helicase was first observed by Buelt et al. [43] with p68 RNA helicase purified from PC12 cell line. Phosphorylation of the recombinant p68-CT by PKC supports the previous observations. Both our experimental data and previous observations strongly suggest the role of the PKC signal transduction pathway in regulating the cellular functions of p68 RNA helicase. This link may provide a partial explanation for the critical role(s) of p68 RNA helicase in cellular differentiation and organ maturation [3,26]. Furthermore, we showed that phosphorylation on the C-terminal domain of p68 abolishes the RNA-binding property of the protein and dephosphorylation restores the RNA binding. We believe that our experimental data indicate that the RNA binding by p68 RNA helicase could be regulated by this phosphorylation/dephosphorylation cycle. Consequently, regulation of RNA-binding by the phosphorylation/dephosphorylation cycle will control the functions and the enzymatic activities of p68 RNA helicase in cells. The relationships between phosphorylation and RNA binding and between phosphorylation and ATPase activity have been demonstrated with purified/recombinant p68. It certainly will be interesting to investigate this regulatory mechanism in a particular biological process. We have previously demonstrated the essential function of p68 RNA helicase in the pre-mRNA splicing process [33]. We showed that p68 interacted with the transient U1:5' splice site duplex in the early spliceosome complexes [40]. The function of p68 in the spliceosome may provide an excellent system to test the role of phosphorylation on p68 RNA helicase in controlling its enzymology. It will be very interesting to examine whether the phosphorylation on p68 RNA helicase affects the functions of the protein in the spliceosome and the interactions between p68 and the U1:5' splice site duplex.

RNA binding property is also an intriguing question. One possibility is that phosphorylation introduces a negative charge at the phosphorylation site. The consequent electrostatic interactions may disrupt the protein–RNA interactions. However, given the sequence characteristics and possible phosphorylation site (the IQ motif) and the putative RNA binding site (possibly RGS–RGG motif), it is more likely that phosphorylation may lead to a conformational change that may cause subsequent changes in the RNA-binding site. It is noteworthy that the putative phosphorylation site, IQ domain, locates within a long 85 amino acid (aa 528–614) segment in which none of the amino acid residue are charged under physiological conditions. Introduction of one or multiple charged phosphate group(s) into this long non-charged segment may change its structure. We are currently attempting to obtain the structural information before and after the

phosphorylation of p68-CT. We hope the structural information will shed light on how the phosphorylation on the C-terminal domain of p68 affects its RNA-binding property.

## Acknowledgments

This work was supported by a grant from the American Heart Association to Z-R.L. (0265043B). We thank Werner Bergen and Dan Adams for detailed critical comments on the manuscript.

## References

- [1] A. Luking, U. Stahl, U. Schmidt, The protein family of RNA helicases, *Crit. Rev. Biochem. Mol. Biol.* 33 (1998) 259–296.
- [2] J. de la Cruz, D. Kressler, P. Linder, Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families, *Trends Biochem. Sci.* 24 (1999) 192–198.
- [3] U.A. Heinlein, Dead box for the living [editorial], *J. Pathol.* 184 (1998) 345–347.
- [4] N.K. Tanner, P. Linder, DEXD/H box RNA helicases: from generic motors to specific dissociation functions, *Mol. Cell* 8 (2001) 251–262.
- [5] J.P. Staley, C. Guthrie, Mechanical devices of the spliceosome: motors, clocks, springs, and things, *Cell* 92 (1998) 315–326.
- [6] A. Pause, N. Sonenberg, Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A, *EMBO J.* 11 (1992) 2643–2654.
- [7] J.L. Kim, K.A. Morgenstern, J.P. Griffith, M.D. Dwyer, J.A. Thomson, M.A. Murcko, C. Lin, P.R. Caron, Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding, *Structure* 6 (1998) 89–100.
- [8] C.H. Gross, S. Shuman, The QRxGRxGRxxxG motif of the vaccinia virus DEXH box RNA helicase NPH- II is required for ATP hydrolysis and RNA unwinding but not for RNA binding, *J. Virol.* 70 (1996) 1706–1713.
- [9] A. Pause, N. Methot, N. Sonenberg, The HRIGRXXXR region of the DEAD box RNA helicase eukaryotic translation initiation factor 4A is required for RNA binding and ATP hydrolysis, *Mol. Cell. Biol.* 13 (1993) 6789–6798.
- [10] N. Yao, T. Hesson, M. Cable, Z. Hong, A.D. Kwong, H.V. Le, P.C. Weber, Structure of the hepatitis C virus RNA helicase domain, *Nat. Struct. Biol.* 4 (1997) 463–467.
- [11] E.R. Johnson, D.B. McKay, Crystallographic structure of the amino terminal domain of yeast initiation factor 4A, a representative DEAD-box RNA helicase, *RNA* 5 (1999) 1526–1534.
- [12] J. Benz, H. Trachsel, U. Baumann, Crystal structure of the ATPase domain of translation initiation factor 4A from *Saccharomyces cerevisiae*—the prototype of the DEAD box protein family, *Struct. Fold Des.* 7 (1999) 671–679.
- [13] J.M. Caruthers, E.R. Johnson, D.B. McKay, Crystal structure of yeast initiation factor 4A, a DEAD-box RNA helicase, *Proc. Natl. Acad. Sci. USA* 97 (2000) 13080–13085.
- [14] Z.J. Lorkovic, R.G. Herrmann, R. Oelmüller, PRH75, a new nucleus-localized member of the DEAD-box protein family from higher plants, *Mol. Cell. Biol.* 17 (1997) 2257–2265.
- [15] F.V. Fuller-Pace, S.M. Nicol, A.D. Reid, D.P. Lane, DbpA: a DEAD box protein specifically activated by 23s rRNA, *EMBO J.* 12 (1993) 3619–3626.

- [16] O.G. Rossler, A. Straka, H. Stahl, Rearrangement of structured RNA via branch migration structures catalysed by the highly related DEAD-box proteins p68 and p72, *Nucleic Acids Res.* 29 (2001) 2088–2096.
- [17] H. Hirling, M. Scheffner, T. Restle, H. Stahl, RNA helicase activity associated with the human p68 protein, *Nature* 339 (1989) 562–564.
- [18] T.J. Gibson, J.D. Thompson, Detection of dsRNA-binding domains in RNA helicase A and *Drosophila* maleless: implications for monomeric RNA helicases, *Nucleic Acids Res.* 22 (1994) 2552–2556.
- [19] B.K. Ray, T.G. Lawson, J.C. Kramer, M.H. Cladaras, J.A. Grifo, R.D. Abramson, W.C. Merrick, R.E. Thach, ATP-dependent unwinding of messenger RNA structure by eukaryotic initiation factors, *J. Biol. Chem.* 260 (1985) 7651–7658.
- [20] M. Jaramillo, T.E. Dever, W.C. Merrick, N. Sonenberg, RNA unwinding in translation: assembly of helicase complex intermediates comprising eukaryotic initiation factors eIF-4F and eIF-4B, *Mol. Cell. Biol.* 11 (1991) 5992–5997.
- [21] M. Altmann, B. Wittmer, N. Methot, N. Sonenberg, H. Trachsel, The *Saccharomyces cerevisiae* translation initiation factor Tif3 and its mammalian homologue, eIF-4B, have RNA annealing activity, *EMBO J.* 14 (1995) 3820–3827.
- [22] L. Crawford, K. Leppard, D. Lane, E. Harlow, Cellular proteins reactive with monoclonal antibodies directed against simian virus 40 T-antigen, *J. Virol.* 42 (1982) 612–620.
- [23] D.P. Lane, W.K. Hoeffler, SV40 large T shares an antigenic determinant with a cellular protein of molecular weight 68,000, *Nature* 288 (1980) 167–170.
- [24] R.D. Iggo, D.P. Lane, Nuclear protein p68 is an RNA-dependent ATPase, *EMBO J.* 8 (1989) 1827–1831.
- [25] M.J. Ford, I.A. Anton, D.P. Lane, Nuclear protein with sequence homology to translation initiation factor eIF-4A, *Nature* 332 (1988) 736–738.
- [26] R.J. Stevenson, S.J. Hamilton, D.E. MacCallum, P.A. Hall, F.V. Fuller-Pace, Expression of the ‘dead box’ RNA helicase p68 is differentially and growth regulated and correlates with organ differentiation/maturation in the fetus, *J. Pathol.* 184 (1998) 351–359.
- [27] B. Mahal, W. Nellen, Developmental regulation of DEAD box proteins and cloning of putative RNA helicase genes from *Dictyostelium discoideum*, *Biol. Chem. Hoppe Seyler* 375 (1994) 759–763.
- [28] S.R. Schmid, P. Linder, D-E-A-D protein family of putative RNA helicases, *Mol. Microbiol.* 6 (1992) 283–291.
- [29] H. Endoh, K. Maruyama, Y. Masuhiro, Y. Kobayashi, M. Goto, H. Tai, J. Yanagisawa, D. Metzger, S. Hashimoto, S. Kato, Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha, *Mol. Cell. Biol.* 19 (1999) 5363–5372.
- [30] T. Fujita, Y. Kobayashi, O. Wada, Y. Tateishi, L. Kitada, Y. Yamamoto, H. Takashima, A. Murayama, T. Yano, T. Baba, S. Kato, Y. Kawabe, J. Yanagisawa, Full activation of estrogen receptor alpha activation function-1 induces proliferation of breast cancer cells, *J. Biol. Chem.* 278 (2003) 26704–26714.
- [31] M. Watanabe, J. Yanagisawa, H. Kitagawa, K. Takeyama, S. Ogawa, Y. Arai, M. Suzawa, Y. Kobayashi, T. Yano, H. Yoshikawa, Y. Masuhiro, S. Kato, A subfamily of RNA-binding DEAD-box proteins acts as an estrogen receptor alpha coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA, *EMBO J.* 20 (2001) 1341–1352.
- [32] J.P. Jost, S. Schwarz, D. Hess, H. Angliker, F.V. Fuller-Pace, H. Stahl, S. Thiry, M. Siegmann, A chicken embryo protein related to the mammalian DEAD box protein p68 is tightly associated with the highly purified protein–RNA complex of 5-MeC-DNA glycosylase, *Nucleic Acids Res.* 27 (1999) 3245–3252.
- [33] Z.R. Liu, p68 RNA helicase is an essential human splicing factor that acts at the U1 snRNA-5′ splice site duplex, *Mol. Cell. Biol.* 22 (2002) 5443–5450.
- [34] M.S. Jurica, L.J. Licklider, S.R. Gygi, N. Grigorieff, M.J. Moore, Purification and characterization of native spliceosomes suitable for three-dimensional structural analysis, *RNA* 8 (2002) 426–439.
- [35] G. Neubauer, A. King, J. Rappsilber, C. Calvio, M. Watson, P. Ajuh, J. Sleeman, A. Lamond, M. Mann, Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex [see comments], *Nat. Genet.* 20 (1998) 46–50.
- [36] K. Hartmuth, H. Urlaub, H.P. Vornlocher, C.L. Will, M. Gentzel, M. Wilm, R. Luhrmann, Protein composition of human pre-spliceosomes isolated by a tobramycin affinity-selection method, *Proc. Natl. Acad. Sci. USA* 99 (2002) 16719–16724.
- [37] A. Honig, D. Auboeuf, M.M. Parker, B.W. O’Malley, S.M. Berget, Regulation of alternative splicing by the ATP-dependent DEAD-box RNA helicase p72, *Mol. Cell. Biol.* 22 (2002) 5698–5707.
- [38] S. Guil, R. Gattoni, M. Carrascal, J. Abian, J. Stevenin, M. Bach-Elias, Roles of hnRNP A1, SR proteins, and p68 helicase in c-Ha-ras alternative splicing regulation, *Mol. Cell. Biol.* 23 (2003) 2927–2941.
- [39] Y. Huang, Z.R. Liu, The ATPase, RNA unwinding, and RNA binding activities of recombinant p68 RNA helicase, *J. Biol. Chem.* 277 (2002) 12810–12815.
- [40] Z.R. Liu, B. Sargueil, C.W. Smith, Detection of a novel ATP-dependent cross-linked protein at the 5′ splice site-U1 small nuclear RNA duplex by methylene blue-mediated photo-cross-linking, *Mol. Cell. Biol.* 18 (1998) 6910–6920.
- [41] Z.R. Liu, A.M. Wilkie, M.J. Clemens, C.W. Smith, Detection of double-stranded RNA–protein interactions by methylene blue-mediated photo-crosslinking, *RNA* 2 (1996) 611–621.
- [42] Z.R. Liu, B. Lagerbauer, R. Luhrmann, C.W. Smith, Crosslinking of the U5 snRNP-specific 116-kDa protein to RNA hairpins that block step 2 of splicing, *RNA* 3 (1997) 1207–1219.
- [43] M.K. Buelt, B.J. Glidden, D.R. Storm, Regulation of p68 RNA helicase by calmodulin and protein kinase C, *J. Biol. Chem.* 269 (1994) 29367–29370.
- [44] I.W. Mattaj, RNA recognition: a family matter?, *Cell* 73 (1993) 837–840.
- [45] C.G. Burd, G. Dreyfuss, Conserved structures and diversity of functions of RNA-binding proteins, *Science* 265 (1994) 615–621.
- [46] Q. Liu, G. Dreyfuss, In vivo and in vitro arginine methylation of RNA-binding proteins, *Mol. Cell. Biol.* 15 (1995) 2800–2808.
- [47] L. Ghisolfi, A. Kharrat, G. Joseph, F. Amalric, M. Erard, Concerted activities of the RNA recognition and the glycine-rich C-terminal domains of nucleolin are required for efficient complex formation with pre-ribosomal RNA, *Eur. J. Biochem.* 209 (1992) 541–548.
- [48] M. Kiledjian, G. Dreyfuss, Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box, *EMBO J.* 11 (1992) 2655–2664.
- [49] E.W. Khandjian, Biology of the fragile X mental retardation protein, an RNA-binding protein, *Biochem. Cell Biol.* 77 (1999) 331–342.
- [50] J.T. Anderson, S.M. Wilson, K.V. Datar, M.S. Swanson, NAB2: a yeast nuclear polyadenylated RNA-binding protein essential for cell viability, *Mol. Cell. Biol.* 13 (1993) 2730–2741.
- [51] W.E. Mears, S.A. Rice, The RGG box motif of the herpes simplex virus ICP27 protein mediates an RNA-binding activity and determines in vivo methylation, *J. Virol.* 70 (1996) 7445–7453.

- [52] S. Zhang, F. Grosse, Domain structure of human nuclear DNA helicase II (RNA helicase A), *J. Biol. Chem.* 272 (1997) 11487–11494.
- [53] M. Okanami, T. Meshi, M. Iwabuchi, Characterization of a DEAD box ATPase/RNA helicase protein of *Arabidopsis thaliana*, *Nucleic Acids Res.* 26 (1998) 2638–2643.
- [54] M. Costa, A. Ochem, A. Staub, A. Falaschi, Human DNA helicase VIII: a DNA and RNA helicase corresponding to the G3BP protein, an element of the ras transduction pathway, *Nucleic Acids Res.* 27 (1999) 817–821.
- [55] M. Company, J. Arenas, J. Abelson, Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from spliceosomes, *Nature* 349 (1991) 487–493.
- [56] G.E. Pugh, S.M. Nicol, F.V. Fuller-Pace, Interaction of the *Escherichia coli* DEAD box protein DbpA with 23 S ribosomal RNA, *J. Mol. Biol.* 292 (1999) 771–778.
- [57] G.W. Rogers Jr., W.F. Lima, W.C. Merrick, Further characterization of the helicase activity of eIF4A. Substrate specificity, *J. Biol. Chem.* 276 (2001) 12598–12608.