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Comparative Study on Nucleic Acid Binding of the Purified RBF Protein and Its Inhibition of PKR phosphorylation

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Abstract

Column-purified double-stranded RNA binding factor (RBF) protein was tested for its binding affinity for the different forms of nucleic acids structure such as single-stranded(ss) and double-stranded(ds)RNA and ss- and dsDNA. The RBF protein was incubated with each of these nucleic acid structures in separate reactions and its comparative binding affnity was visualized by SDS-polyacrylamide gel electrophoresis. The RBF protein bound to the dsRNA molecule to form a tight RNA:protein complex in agreement with previous studies, but not to the other nucleic acid molecules confirming its distinctive affinity for the dsRNA structure. In phosphorylation assay *in vitro,* the purified RBF protein significantly inhibited the autophosphorylation of the PKR derived from not only human but mouse source in the presence of poly(I):poly(C). It is suggesting that PKR vs. RBF is similarly under a competitive interaction among different eukaryotic organisms during protein synthesis.

Key words : *Nucleic acid binding, PKR phosphorylation inhibition, RBF*

Introduction

RNA : protein interactions mediate many fundamental mechanisms during gene expression[1,2] ; mRNA transcription[3,4], posttranscriptional processing including polyadenylation[5], and translation[6,7]. Many cellular and viral RNA binding proteins that bind unique RNA targets and introduce specificities into the general regulatory mechanisms have been identified[8,9]. In the course of RNA : protein interactions, protein phosphorylation is also concomitant regulatory event critical to gene expression. For example, global regulation of eukaryotic mRNA translation is mediated by protein phosphorylation events, most notably at the translation initiation step, that involve eukaryotic initiation factor 2(eIF-2). The eIF-2 is a heterotrimer(α, β, γ) that forms a complex with the initiator Met-tRNA and GTP and binds to the 40S ribosomal subunit to form 43S species. After mRNA binding, the 60S ribosomal subunit associates with the complex to generate the 80S species concomitant with the hydrolysis of GTP to GDP and release GDP-bound eIF-2. Phosphorylation of the eIF-2 subunits prevents the GTP exchange and eIF-2 recycling and arrests translation[10]. A ribosome-associated protein kinase also called PKR phosphorylates eIF-2α upon activation by double-stranded(ds) RNA[11,12]. PKR is involved in the antiviral and antiproliferative activity and cellular differentiation[13].

A dsRNA binding factor(RBF) is a cellular protein that inhibits phosphorylation of PKR and eIF-2α *in*

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vivo and is implicated as a cellular regulatory protein that binds RNAs to mediate the inhibition of PKR activation and stimulate translation[14]. It was also reported that the RBF protein as a LacZ-fused form produced and purified from *E. coli* inhibited autophosphorylation of PKR *in vitro*[15]. RBF, in electrophoretic mobility shift assay or filter binding assay, displayed affinities for a broad range of RNAs including viral RNAs and synthetic RNAs consisting of stem and loop structures. GC-rich RNA stem helices as short as 11 bp have been suggested to represent the minimal binding motif for RBF. RBF binding to all the natural RNAs tested was reversible by poly(I) : poly(C) addition[16]. Although RBF was characterized for its binding specificity for dsRNA in several studies[14,15,16], little has been done for nucleic acid molecules other than dsRNA. So, in this study, the Lac-Z fused RBF protein was comparatively tested for its affinity for the different forms of nucleic acid structure, RNA and DNA structures in ss- or ds-form, and the results were judged by differential RBF mobility during SDS-polyacrylamide gel electrophoresis. To elucidate a possibly common mechanism by which translation is regulated throughout eukaryotic organisms, RBF was reacted against either the cellular extracts from human or the purified mouse PKR, and the degree of phosphorylation inhibition was comparatively monitored by SDS-PAGE and autoradiography. The results will be discussed.

Materials and Methods

Assay of RBF binding to nucleic acid molecules

From *E. coli* harboring recombinant plasmid pSKRBF, LacZ-fused RBF protein synthesis was induced by IPTG treatment and purified through procedures such as inclusion body isolation, Guanidine-HCl denaturation/renaturation, and heparin-agarose affinity column chromatography as described[15]. The partially purified protein was stored in -70℃ until use. For its binding to the nucleic acids, synthetic poly(I) : poly(C) was used as a dsRNA source, meanwhile, poly(I) : poly(C) was boiled for 10 minutes and rapidly cooled in ice water to be used as a ssRNA source. Plasmid pSK was cut with EcoRI, extracted with phenol/chloroform, precipitated with ethanol, dissolved in water, and used as a dsDNA source. This preparation was boiled for 10 minutes and rapidly cooled in ice water to be used as a ssDNA source. Each of the different forms of nucleic acid structure was prepared at the concentration of 100 μg/ml and mixed with the RBF protein for the incubation for 1 hour at room temperature in binding buffer(20 mM Tris-HCl, pH 7.5, 0.05 M KCl, 1 mM DTT, 5 mM spermidine, and 2 mg/ml BSA). For RBF binding to the ssRNA or dsRNA, RNasin was added at the concentration of 1000 U/ml. After incubation, the samples were added with 2×SDS-PAGE sample loading buffer[17], boiled for 3 minutes, and run in 8% SDS-polyacrylamide gel.

Phosphorylation inhibition assay

Autophosphorylation activity was assayed using PKR from HeLa cell extracts as human PKR source or purified mouse PKR(150 ng/ml, obtained from Dr. Jacob) in the presence or absence of poly(I) : poly(C) in the reaction mixtures containing 10 mM Tris-HCl, pH 7.8, 5 mM magnesium acetate, 50 mM KCl, and 25 μM [γ-^{32}P]ATP(2 μCi)[15]. Incubation was for 10 minutes at 30℃. PKR inhibition was carried out by the addition of the RBF protein(2 μg/ml) to the same reaction mixtures above. When reactions completed, samples were analysed by SDS/PAGE followed by autoradiography.

Results and Discussion

Distinctive RBF binding to dsRNA

Using a cDNA clone encoding dsRNA binding cellular factor(RBF)[14], RBF protein was produced in IPTG-treated *E. coli* cells and partially purified as described in Material and Methods. To compare binding affinities to

the nucleic acid sturctures, the LacZ-fused 43 kD RBF protein was incubated with ssRNA, dsRNA, ssDNA or dsDNA in separate binding reations. Its binding was analyzed by SDS-PAGE for its retarded mobility due to protein : nucleic acid complex formation. The results are shown in Fig. 1. In Fig. 1, lane 2, the RBF is shown as a 43 kD protein band with three to four neigboring bands. The larger bands are probably due to incomplete protein purification procedures. Smaller-sized bands may represent proteolytic breakdown products of the RBF becuase they reacted to polyclonal antibodies against RBF protein[16]. When the RBF protein was incubated with ssRNA, ssDNA or dsDNA at room temperature (lane3, 5, and 6, respectively), each of the putative protein : nucleic acid complex was resolved at the site in the 8% SDS-polyacylamide gel identical to the sample containing RBF alone(lane 2), suggesting little binding affinity of RBF for these nucleic acid structures. The protein : nucleic acid complex formation, if any, was suggested not to be strong enough to be maintained after boiling in the presence of SDS. In comparison, the 43 kD RBF protein band as well as its proteolytic breakdown products disappeared as represented in lane 4.

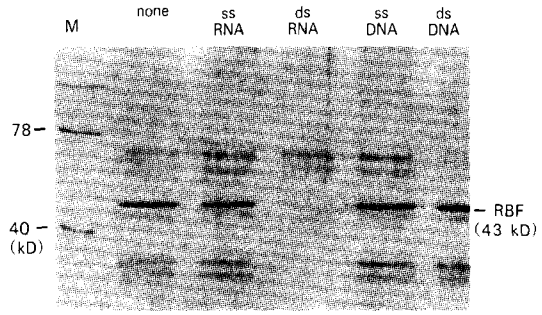

Fig. 1. RBF : nucleic acid complex formation analysed by SDS-PAGE.
M, protein molecular size marker. RBF protein was analysed after incubating without nucleic acid(marked as none), or with different structure of nucleic acids as indicated.

It is indicating that the dsRNA : RBF complex stably forms even after the stringent SDS-boiling treatment and, as a consequence, moves as slow as the larger-sized protein bands as displayed in the figure. This result agrees with the reports of RBF in its binding affinities for dsRNA structures regardless of natural or synthetic sources[16]. RBF has two partially redundant motifs, rich in basic residues between positions 30 and 96, and between 159 and 226 that are homologous to similar motifs of several cellular and viral dsRNA binding proteins [18,19]. Unlike these, RBF has two dsRNA binding motifs instead of one, and this structural feature may explain how RBF maintains strong affinity for dsRNA enough to withstand boiling in the presence of SDS.

Analogy of RBF activities between different organisms

The LacZ-fused RBF was examined whether its competition for binding to the dsRNA substrate occured against mouse PKR in a mode similarly against human PKR[14,15]. First, human PKR as a standard for comparison was prepared as crude samples extracted from NP-40 treated HeLa monolayer cell cultures[14]. The crude extracts were incubated with [γ-^{32}P]ATP in the presence of poly(I) : poly(C) as a dsRNA substrate at the concentration of 0, 0.1 and 1.0 μg/ml, respectively. The results of PKR phosphorylation are presented in Fig. 2. Human PKR as a 68 kD protein band was shown to be moderately phosphorylated in the absence of the dsRNA substrate(Fig. 2-A, lane 1). By the presence of poly(I) : poly(C) at the concentration of 0.1 μg/ml(lane 2), PKR was highly autophosphorylated to the almost maximum level which was still maintained by even more of poly(I) : poly(C) (1.0 μg/ml) (lane 3). To the same reaction mixture as above, RBF(2 μg/ml) was added and its effect on PKR phosphorylation was observed. The results are shown in Fig. 2-B. The phosphorylation level of PKR in a reaction without dsRNA(lane 1) was almost similar to the reaction with poly(I) : (C) (0.1 μg/ml, lane 2) indicating a competition of RBF for the

dsRNA substrate against PKR. This is consistent with the results *in vitro* using PKR from interferon-induced cell culture system[14,15] and surely agrees with the fact that dsRNA activation of the PKR kinase appears to require RNA binding to the bi-partite RNA binding motif[9,20,21].

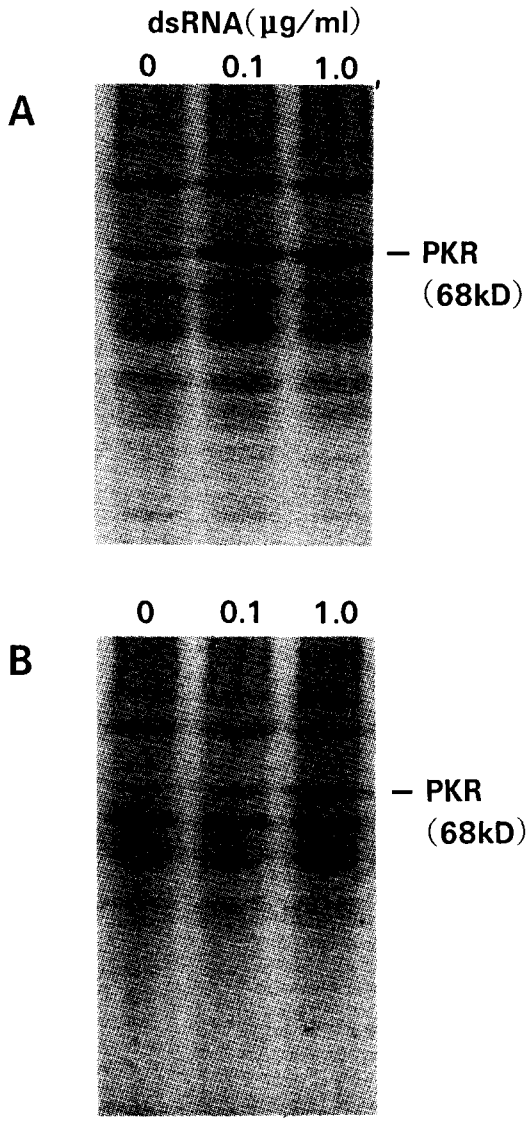

Fig. 2. PKR phosphorylation and its inhibition analysed by SDS-PAGE and autoradiography.
In the presence of dsRNA as indicated, RBF was omitted(A) or added(B) to the PKR reaction mixtures.

To examine whether PKR phosphorylation among mammalian species is under a regulation in common by RBF, the RBF fusion protein was added to the reaction mixtures containing the purified mouse PKR instead of human's. Poly(I) : poly(C) was added at the concentration ranging from 0 to 100.0 μg/ml per reaction. The RBF protein(2 μg/ml) was omitted in one set of experiment and added to the other. In the absence of RBF, mouse PKR showed an increase in the level of phosphorylation due to the presence of dsRNA in a dose-dependent manner(0 - 1.0 μg/ml, Fig. 3-A, lane 1, 2, 3, and 4). Poly(I) : poly(C) at higher concentrations(>1.0 μg/ml, lane 5 and 6), however, inhibited PKR autophosphorylation to the level as low as in the reaction without dsRNA(lane 1). This is consistent with the observation resulted from the reaction of human PKR vs. human RBF[15]. In a RBF-added set of reactions(Fig. 3-B), PKR phosphorylation was maintained at the basal level in reactions without(lane 1) or with various amounts of dsRNA(0.01 to 100 μg/ml, lane 2 to lane 6), suggesting an analogous mechanism in the mouse system for the regulation of PKR phosphorylation by RBF during protein synthesis. That is, putative RBF from mouse may be operated similarly to human's during translation initiation. In this experiment, two features of RBF were studied using the partially purified LacZ-fused RBF protein. The first set of experiment aimed to clarify for RBF in respect of its differential affinities for binding to the nucleic acid structures. Becuase RBF has been known to display strong binding affinity for various types of dsRNA, synthetic or natural ones and to inhibit dsRNA-dependent PKR activation which occurs in cytoplasmic environment during mRNA/rRNA-involved translation initiation, RBF, in natural assumption, has been regarded only to have a distinctive affinity for the dsRNA structures. In this study, RBF showed binding to poly(I) :

poly(C) as dsRNA but not to dsDNA, ssDNA or ssRNA judged from mobility retardation of the protein : nucleic acid complex during electrophoresis. This observation may confirm RBF as a protein factor specifically requiring dsRNA structures during the regulation for protein synthesis in the cytoplasm of eukaryotes. The second set of experiment was performed to see that an analogy during the regulation of translation initiation may exist in different mammalian species. PKR has been characterized from human[21] and mouse[22] species and its dependence on dsRNA for autophosphorylation has been well understood. Meanwhile, its inhibition by RBF due to competition for dsRNA as a substrate has been characterized only in human[14,15]. Antagonistic interaction between human RBF and human PKR in nature occurs in the presence of dsRNA. Meanwhile, as was seen above, mouse PKR vs. human RBF was shown to be in a interaction similar to human PKR vs. human RBF, displaying the excess amount of dsRNA to be enough for proper PKR activation. This implies that putative mouse RBF may inhibit dsRNA-dependent PKR activation and participate in the regulation of protein synthesis in a similar way as detected in human system.

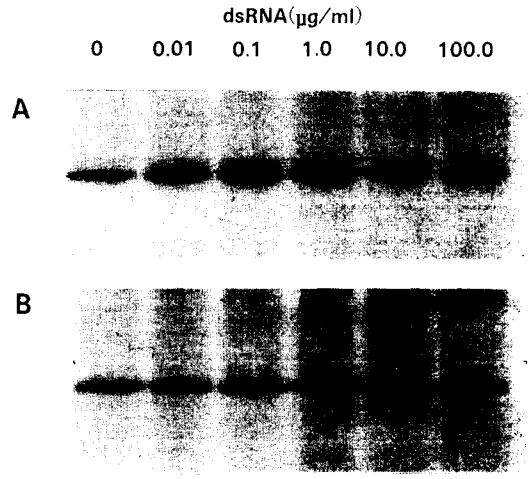

Fig. 3. Mouse PKR phosphorylation and its inhibition analysed by SDS-PAGE and autoradiography. In the presence of dsRNA as indicated, RBF was omitted(A) or added(B) to the mouse PKR reaction mixture.

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초록 : RBF정제단백질의 핵산결합도 및 PKR효소의 인산화억제효과의 비교에 관한 연구

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dsRNA결합인자인 RBF단백질을 정제하여 이의 단일 또는 이중선의 RNA 또는 DNA와의 결합도를 측정하였다. RBF단백질은 이들과 각각 반응시켜 그 결합도는 SDS-PAGE에 의하여 비교관찰하였다. RBF단백질은 dsRNA와는 강한 결합력을 나타낸 반면 기타의 핵산구조에 대해서는 이러한 결과를 나타내지 못하였다. 인산화 실험의 결과, RBF단백질은 poly(I) : poly(C)의 존재하에서 사람 또는 쥐 모두로 부터의 PKR효소의 자가인산화를 유사한 방식으로 억제하였다. 이는 다른 종류의 진핵세포생물에서 단백질합성조절을 위한 PKR과 RBF가 유사한 경쟁적 관련성을 유지하면서 존재함을 시사하고 있다.