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RNA Binding Specificities of Double-Stranded RNA Binding Protein (RBF) as an Inhibitor of PKR Kinase

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Abstract

A double-stranded RNA binding factor (RBF), characterized as an inhibitor of PKR kinase in our previous study, was evaluated for its RNA binding specificities by RNA gel electrophoretic mobility shift analysis and membrane filter binding assay. RBF 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 are 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, but *E. coli* 5S RNA was inefficient.

Key words : *Double-stranded RNA binding factor, RNA binding specificities*

Introduction

In many fundamental biological regulatory mechanisms governed by RNA : protein interactions[1,2] in the processes such as transcription[3,4], posttranscription[5,6], and translation[7,8], protein phosphorylation has been considered to mediate many RNA : protein interactions, as notably found during the eukaryotic mRNA translation initiation step. A ribosome-associated protein kinase, PKR, phosphorylates eukaryotic translation initiation factor (eIF-2) upon activation by double-stranded (ds) RNA[9,10,11]. Phosphorylation of the eIF-2 prevents the GTP exchange and eIF-2 recycling and arrests translation[12,13]. PKR mediates the antiviral and antiproliferative effects of interferon and has been known to modulate cellular differentiation and stress response[14,15,16]. Some viral RNAs and proteins that regulate PKR activation have been identified. For instance, a 15 kDa protein, dRF, is thought to induce a reversible inhibition of PKR autophosphorylation in undifferentiated cells by complexing with PKR and preventing dsRNA binding[17]. PKR activation was inhibited in *v-ras* transformed cells by a thermolabile, diffusible factor that was destroyed by phenol/chloroform extraction, but was not bound by dsRNA. A 58 kDa cellular protein that inhibits the eIF-2α phosphorylation by activated PKR has been identified in influenza virus infected cells[18]. In uninfected cells, the 58 kDa inhibitor remains complexed with another protein in an inactive state.

In a recent report by Park *et al.*[19], a cDNA encoding a HeLa cellular protein isolated on the basis of a selective reactivity to HIV-1 Rev-responsive element RNA has been described to show that the protein complemented the inhibition of protein synthesis upon the locali-

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zed activation of PKR *in vivo* and inhibited PKR phosphorylation upon activation by double stranded RNA *in vitro*[20], suggesting its role for mediating the inhibition of PKR activation and stimulating translation in a localized manner. In this manuscript, we have characterized the RNA binding properties of the RBF which displayed affinities for a broad range of highly structured RNAs with a 11 bp GC-rich double-stranded RNA consitituting the minimal binding motif.

Materials and Methods

In vitro transcription

DNA templates for natural RNA transcripts HIV-1 Rev responsive element RNA (RRE), antisense RRE[21], adenovirus-2 VAI[22], HTLV-1 Rex responsive element RNA (RexRE)[23] and MS2 operator[24] or synthetic short RNAs in the reaction mixture containing the T7 promoter-tagged primer were amplified by polymerase chain reaction (30 cycles of reaction, each cycle constituting 95℃ for 30 sec, 55℃ for 30 sec and 72℃ for 90 sec). PCR products were electrophoresed in 2% agarose gel and harvested by Gene-Clean method (Bio101, USA). *In vitro* transcription was by use of a commercial T7 transcription kit (Stratagene, USA). [α-^{32}P]UMP labelled transcription products were extracted with phenol : chloroform and with chloroform : isoamylalcohol and purified by Sephadex G−50 chromatography. RNA purity was determined by polyacrylamide gel electrophoresis (PAGE) in 8 M urea, and the transcripts were purified from gels as necessary[21]. The gel-purified RNAs were denatured by boiling and self-annealed by slow cooling to 50℃ in 0.2 M NaCl over a 60 min period.

RNA Gel Electrophoretic Mobility Shift Analysis (EMSA)

A cDNA encoding a double-stranded RNA binding protein (RBF) was isolated[19] and the lacZ-fused RBF protein produced from *E. coli* XL1-Blue upon IPTG induction was purified by affinity column chromatography as described elsewhere[20]. Reaction mixture containing RBF protein sample and heparin (5 μg/25 μl of total reaction) in binding buffer (20 mM HEPES-KOH, pH 7.9, 62 mM KCl, 0.15 mM DTT, 6% glycerol, and RNAsin at 1000 U/ml) was incubated at 30℃ for 10 min followed by the addition of labeled RNA probe (0.1 μM) and further incubation for 10 min at 30℃. Samples were electrophoresed through non-denaturing 5% polyacrylamide gels in 0.5×TBE at 30 mA at 4℃. The gel was routinely prerun at 30 mA for 30 min at 4℃. The radioactivity was visualized by dry gel autoradiography.

Nitrocellulose filter binding assay

Filter binding assays were done essentially as before [21]. Typically, 200−500 fmol of the labeled probe RNA was incubated at 25℃ for 10 min in 10 μl of binding buffer (20 mM Tris-HCl, pH 7.5, 0.05 M KCl, 1 mM DTT, 5 mM spermidine, 20 g bovine serum albumin, 1 μg yeast tRNA, and RNAsin at 1000 U/ml) with increasing amounts of purified RBF. The samples were filtered through prewetted 25 mm nitrocellulose filters (0.45 μm pore size) at a flow-rate of 5 μl/sec. Filters were rinsed twice with 0.75 ml of binding buffer without bovine serum albumin, spermidine, tRNA and RNAsin, air-dried and the bound radioactivity was determined by liquid scintillation counter.

Results

RNA binding specificities of RBF

The *in vitro* RNA binding properties of bacterially expressed RBF proteins were analyzed by several means. Initially, crude extracts of RBF expressing cells were tested for RRE RNA binding by EMSA. The mobility of RRE RNA was retarded only when incubated with the RBF extracts, but not with the control *E. coli* XL1-Blue cell extracts (not shown). However, with crude lysates, there was substantial RNA degradation. Therefore, a partial purification scheme finally through heparin agarose

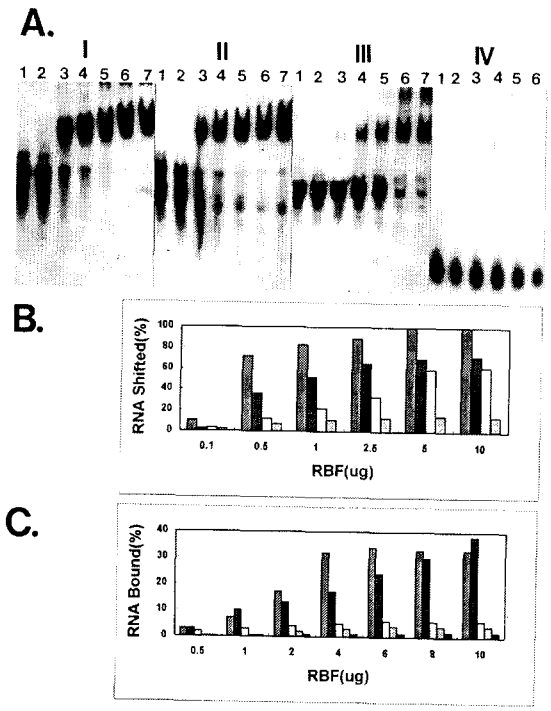

Fig. 1. RNA binding specificities of RBF.

A. RNA mobility shift in 5% non-denaturing PAGE. (I) RRE, (II) antisense RRE, (III) VAI, (IV) MS2 RNA. Free RNAs (lane 1) were mixed with RBF at the concentrations of 0.1 (lane 2), 0.5 (lane 3), 1 (lane 4), 2.5 (lane 5), 5 (lane 6) or 10 μg (lane 7).

B. Results from (A), scanned and expressed as percent of total free RNA, are plotted as a function of RBF concentration. Bars presented for each of RBF concentration are in the order of I, II, III and IV as specified in (A).

C. RNA filter binding assay. Filter bound RNA radioactivity complexed with RBF was determined by scintillation counting. The results are plotted as percentages of bound RNA as a function of RBF concentration. Bars displayed for each of RBF concentration represent VAI RNA, RexRE RNA, antisense RRE, RRE and MS2 in the order.

column chromatography was devised for RBF protein enrichment as described elsewhere[20]. Increasing amounts of heparin agarose RBF fraction (0.1 to 10 μg, approximately $0.5-5.0\times10^{-6}$ M total protein) was mixed with fixed molar amounts ($\sim10^{-7}$ M) of the indicated labeled RNAs and the resulting RBF : RNA complexes were separated from unbound RNAs by gel electrophoresis. The distribution of radioactivity across the gel was scanned to determine the fraction of the retarded RNA. As shown in Figure 1A-I, between 2.5 and 5 μg of RBF completely converted the free RRE to the RRE-RBF complex. Essentially, similar results were obtained with antisense RRE (Figure 1A-II). Adenovirus-2 VAI RNA were slightly less efficient for RBF binding under the same conditions (Figure 1A-III). In contrast, the 22 nt MS2 phage translational operator RNA was a poor candidate for RBF binding (Figure 1A-IV). The mean values for RNA binding by the partially purified RBF protein were determined from the EMSA experiments and plotted as a funtion of RBF input (Figure 1B). With 5 μg of RBF, RRE, antisense RRE and VA1 RNA binding were saturable at binding efficiencies approaching 90, 70, and 60% respectively. MS2 operator RNA was notably poor binder, saturable to 10 to 20% binding with 2 μg of RBF. The RNA binding affinities of RBF were somewhat different when evaluated by filter binding assay. VA1 RNA bound most efficiently under these conditions and was saturable to 35% binding with 10 g of RBF (Figure 1C). In contrast, RRE and antisense RRE RNAs were saturable only to 5 to 15%.

A 11 bp GC-rich dsRNA is the minimal motif for RBF binding

The VAI RNA secondary structure that has been experimentally verified consists of a long apical stem/loop joined to a shorter basal stem through a complex central domain that folds into a bulge and a branched stem-loop structure[22]. Although the apical stem-loop of VAI RNA alone can bind to PKR kinase, interaction with the central domain is required for inhibiting PKR activation.

In EMSA, the RBF binding potential of the VAI apical stem/loop was about 70% as compared with the complete VAI RNA (Figure 2A−1). Excising the apical loop in this fragment had no effect on RBF binding (Figure 2A−2), but unpaired bubble, if introduced in the apical stem structure, weakened RBF binding (Figure 2A-4 and 5). Using this information, short stem or stem/loop GC-rich generic RNAs were synthesized *in vitro* and analyzed for RBF binding by EMSA. The GC-rich RNAs bound RBF at least as well as VAI apical stem (Figure 2, GC1 to GC6 RNAs).

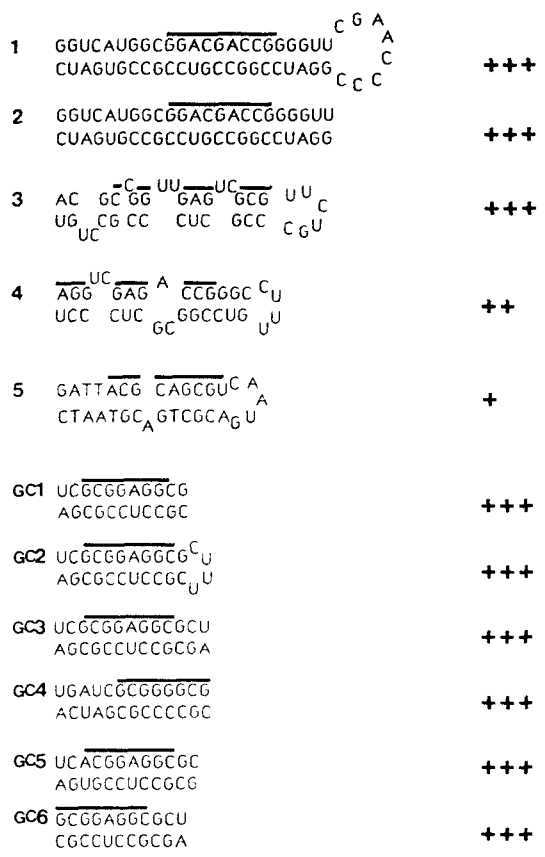

Fig. 2. RBF binding affinities for various RNA structures. 1 : VAI apical stem. 2, 3, 4 and 5 : mutated VAI apical stem structures. GC1 to GC6 : Synthetic GC-rich RNA structures. The degree of RNA affinity is expressed as the number of + (+ to +++).

Competitive inhibition of RBF binding to RNAs by poly(I) : poly(C)

To examine whether RBF binding to individual viral RNAs was sequence specific, we carried out RNA competition assay using either unlabeled poly(I) : poly(C) or RRE RNA. When increasing amounts of unlabeled RRE RNA was premixed with labeled RRE RNA, there was a progressive decrease in the subsequent RBF binding to the labeled RNA (Figure 3I). RRE binding was completely blocked by the addition of 1 μg of unlabeled RRE RNA. With 5 g of poly(I) : poly(C) as the cold competitor, RRE was almost completely dissociated from RBF (Figure 3II). RexRE and VAI RNA, in comparison, were more resistant to dissociation by the same amount of poly(I) : poly(C) (Figure 3III and 3IV). RBF : RRE RNA complex formation, however, was not affected by up to 5 μg of *E. coli* 5S RNA.

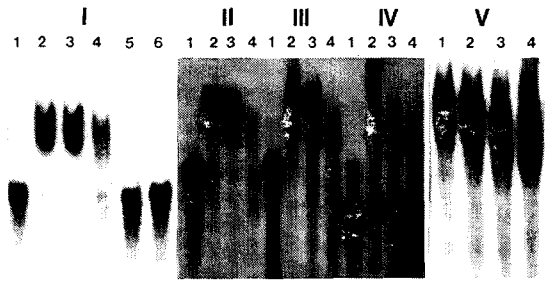

Fig. 3. RNA inhibition of RBF binding to RRE analyzed by RNA gel elecrophoretic mobility shift. (I) : 1, RRE alone ; 2, RRE : RBF complex ; 3 to 6 represent the complex premixed with cold RRE (0.1, 0.5, 1 and 5 μg of RRE, respectively). (II), (III) and (IV) : RBF bindings (lane 2) to RRE (II, lane 1), RexRE(III, lane 1) and VAI(IV, lane 1) were inhibited by the addition of 0.5 (lane 3) to 5 μg (lane 4) of poly(I) : poly(C). (V) : RRE : RBF complex formation was inhibited by *E. coli* 5S RNA at the concentrations 0.01 (lane 1), 0.1 (lane 2), 1 (lane 3) and 5.0 g (lane 4).

Discussion

The double-stranded (ds)RNA binding cellular factor (RBF)[19] had two partially redundant motifs, rich in basic residues between positions 30 and 96, and between 159 and 226 that were homologous to similar motifs of several cellular and viral dsRNA binding proteins, and especially dsRNA dependent PKR kinase[25,26], and the vaccinia virus encoded E3L protein that inhibits the dsRNA binding and activation of PKR kinase[19]. Elsewhere, we have shown that RBF was a competitive inhibitor of dsRNA activation of PKR kinase *in vitro* and *in vivo*. RBF over-expression prevented localized activation of PKR kinase. RBF also complemented the E3L defect of vaccinia virus[19]. The purified lacZ-fused RBF protein competitively bound to poly(I) : poly(C) required for PKR phosphorylation and inhibited PKR activation[20]. These results, however, did not address any particular RNA species specifically required for RBF binding for PKR inhibition. So, in this study, the relative binding specificities of purified RBF for the various RNAs were determined by quantitative EMSA and filter binding studies. We have shown that RBF displayed binding affinities for a very broad range of RNAs. Collective information of RNA binding with several different natural and synthetic RNAs suggested the following order of affinity of RBF : RexRE≈ VAI〉RRE〉antisense RRE〉small GC-rich RNA stem〉〉MS2. Although, of all the natural or synthetic RNAs, VAI RNA was a consistently good RBF binder in different assays, these results led us to conclude that RBF bound to a broad spectrum of RNA structures, rather than to any particular shared sequences and that 11 bp G : C rich stem helix constituted the minimal RBF recognition motif. Short AU-rich stems, in contrast to GC-rich stems, displayed poor RBF binding, probably reflecting the lower thermodynamic stability of A : U base pairing rather than any intrinsic affinity of RBF for G : C base pairs. However, RBF appeared to prefer dsRNA containing alternating G : C base-pairs over GC-rich base pairs with random G : C base pairs or oligo (G) : oligo(C) (Park, unpublished data), raising the possibility that RBF binding may require a novel RNA configuration, such as the Z-DNA or GC-DNA repeats, rather than the usual A-form configuration of dsRNA.

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초록 : PKR인산화효소 억제인자인 이중선RNA결합단백질 (RBF)의 RNA결합특이성

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PKR인산화효소의 억제인자로서 밝혀진 이중선RNA결합단백질 (RBF)의 RNA결합특이성을 전기영동에 의한 RNA이동변화실험과 여과막결합도실험에 의해 측정하였다. RBF는 바이러스RNA나 stem/loop구조를 지니는 합성RNA들에 대한 다양한 친화력을 지니는 것으로 나타났으며 충분한 GC가 포함된 11염기쌍으로 이루어진 RNA stem helix는 RBF가 결합하기 위한 최소한의 RNA구조로 제시되고 있다. 자연적 RNA구조에 대한 RBF의 결합은 poly(I) : poly(C)의 첨가에 의해 반전되었으며 *E. coli* 5S RNA경우는 효과를 거의 나타내지 않았다.