Interaction of rat poly(A)-binding protein with poly(A)- and non-poly(A) sequences is preferentially mediated by RNA recognition motifs 3 + 4

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Abstract Vasopressin (VP) mRNA and the non-coding BC200 RNA are sorted to neuronal dendrites. Among proteins interacting specifically with both RNAs is the multifunctional poly(A)-binding protein (PABP) consisting of four RNA recognition motifs (RRMs) and a C-terminal auxiliary domain. The protein/RNA interaction studies presented here reveal that PABPs association with VP- and BC200 RNA is exclusively mediated by RRMs 3 + 4. Quantitative binding studies with PABP deletion mutants demonstrate preferential binding of RRMs 3 + 4 even to poly(A)-homopolymers, while RRMs 1 + 2 exhibit a lower affinity for those sequences. An optimal interaction with both poly(A)- and non-poly(A) sequences is only achieved by full-size PABP.

Keywords: BC200 RNA; PABP; RNA-binding protein; RNA localization; Vasopressin mRNA

1. Introduction

Vasopressin (VP) mRNA and the non-coding BC200 RNA belong to a small group of transcripts that are sorted to neuronal dendrites in vivo [1,2]. Dendritic transport of VP mRNA is mediated by a segment called dendritic localizer sequence (VP-DLS) spanning part of the coding region as well as the 3′-untranslated region [3]. BC200 RNA consists of 200 nucleotides with an Alu-domain at the 5′-end, a central A-rich region and a unique domain at the 3′-end. The function of this small RNA remains to be elucidated [4,5]. Poly(A)-binding protein (PABP) has been identified as *trans*-acting factor that binds specifically to both the VP-DLS [2] and to BC200 RNA [5] implying a role in RNA localization or in the regulation of dendritic protein synthesis. PABP is highly conserved from yeast to human. Its interaction with poly(A) tails of mRNAs is required for translational initiation and mRNA stabilization [6]. More recently, PABP has been shown to associate with regions other than the poly(A) tail of different mRNA species, thereby controlling translation either positively or in a negative manner [7–9]. PABP consists of four functionally non-equivalent RNA-binding domains called RRMs (RNA recognition motif) and a C-terminus lacking RNA-binding activity [6]. In order to define PABP's requirements to associate with VP- and BC200 RNA, protein/RNA interaction studies have been performed with recombinant proteins containing pairwise combinations of RRMs. Binding to the VP-DLS and to BC200 RNA is mediated by RRMs 3 + 4. Deletion mutants consisting of RRMs 1 + 2 and RRMs 2 + 3, respectively, fail to interact. By quantitative studies, we go on and show that PABP preferentially binds even to poly(A)-homopolymers via its C-terminal domains RRMs 3 + 4.

2. Materials and methods

2.1. Cloning procedures

Rat PABP (EMBL/Genbank Accession No. AJ298278) constructs: for expression in human embryonic kidney (HEK) 293 cells, rat PABP cDNA segments were cloned into the *Bam*H1- and *Xho*I-sites of pCMV-Flag-tag 2B (Stratagene) such that the resulting fusion proteins contain an N-terminal Flag-tag sequence (Fig. 1). The constructs encode the following amino acids (aa): PABP (aa 2–636), RRMs 1 + 2 (aa 2–181), RRMs 1 + 2 + C (aa 2–181 and 371–636), RRMs 2 + 3 (aa 82–276), RRMs 2 + 3 + C (aa 82–276 and 371–636), RRMs 3 + 4 (aa 179–370), and RRMs 3 + 4 + C (aa 179–636). For the experiments shown in Fig. 3, a clone encoding a longer version of RRMs 1 + 2 (aa 2–191) was employed. For in vitro transcription/translation, rat PABP cDNA segments were inserted into the *Xba*I- and *Bam*H1-sites of pSP64 poly(A) (Promega): PABP (aa 1–636), RRMs 1 + 2 (aa 1–191), RRMs 2 + 3 (aa 82–276) and RRMs 3 + 4 (aa 178–370). Restriction sites were introduced via primer pairs used for amplification by the polymerase chain reaction of the respective cDNA sequences. All inserts were subjected to DNA sequencing. Cloning of VP- and α-tubulin cDNAs has been described [3]. For generation of human BC200 (EMBL/Genbank Accession No. U01305) transcripts, the following plasmids were used: *Dra*I-linearized pPBC200 [10] for transcription of full-length BC200 RNA; *Mae*III-linearized pPBC200 for generation of BC200 RNA lacking the 3′ unique domain; and *Bsi*I-linearized pPBC200 for transcription of the BC200 RNA 5′ Alu domain.

2.2. Transient transfections and preparation of cell lysates

HEK 293 cells were grown in 10 cm petri dishes in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μg/ml streptomycin and 100 μg/ml penicillin. Transient transfections were

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Abbreviations: BC, brain cytosolic; DLS, dendritic localizer sequence; HEK, human embryonic kidney; PABP, poly(A)-binding protein; RRM, RNA recognition motif; UV, ultraviolet; VP, vasopressin

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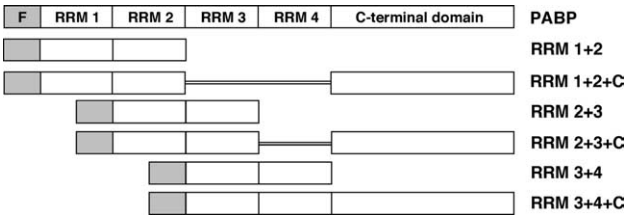

Fig. 1. Schematic representation of constructs encoding full-size rat PABP and PABP deletion mutants lacking individual domains. C, C-terminal domain; F, vector-encoded Flag-tag sequence; RRM, RNA recognition motif.

performed using PolyFect transfection reagent (Qiagen) and 8 μg DNA according to the manufacturer's instructions. Cells were scraped off in media 48 h post-transfection, centrifuged (1500 × *g*, 10 min) and washed once with phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, and 150 mM NaCl). Following centrifugation, cells were homogenized in 100 μl/10 cm dish of 10 mM HEPES, pH 7.8, 5 mM KCl, 2 mM DTT, 0.02% (v/v) Triton X-100 and Complete™ protease inhibitor (Roche). Lysates were centrifuged for 30 min and 4 °C at 24 000 × *g*. Supernatants were snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. In vitro transcription/translation

Recombinant proteins were synthesized in the presence of L-[^{35}S]methionine (Amersham Biosciences) using the TNT® Quick Coupled Transcription/Translation system (Promega) and quantified exactly according to the manufacturer's instructions. Equimolar amounts of proteins were used for protein/RNA interaction analyses (see below).

2.4. Protein/RNA interaction analyses

Interaction of recombinant proteins with biotinylated RNA immobilized on streptavidin-coated paramagnetic particles and ultraviolet (UV)-crosslinking analyses: synthesis of biotinylated in vitro transcripts, coupling to paramagnetic particles (Promega) and affinity purification of RNA-binding proteins as well as UV-crosslinking analyses with ^{32}P-labeled UTP transcripts and cytosolic proteins have been described elsewhere [2,11,12].

Interaction of recombinant proteins with poly(A)-Sepharose: 0.5 g of poly(A)-Sepharose 4B (Amersham Biosciences) was reconstituted according to the manufacturer's instructions and equilibrated in 250 μl binding buffer (10 mM Tris–HCl, pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mg/ml heparin, 0.5% (w/v) Triton X-100, and Complete™ protease inhibitor). Equimolar amounts of ^{35}S-labeled methionine proteins were incubated with poly(A)-Sepharose 4B (20 μl each of the suspension) in a final volume of 100 μl binding buffer for 10 min at room temperature. Following brief centrifugation and removal of supernatants, poly(A)-Sepharose was washed four times (5 min each) with 250 μl of binding buffer. Proteins were eluted with 20 μl of 0.4% (w/v) SDS in H_2O for 10 min at 80 °C and subjected to liquid scintillation counting. For each binding study, an assay containing the same amount of [^{35}S]methionine (in cpm) instead of the labeled protein was run in parallel to allow for correction of background binding.

2.5. Immunoprecipitation and Western blot analysis

UV-crosslinking, using 15 fmol of ^{32}P-labeled in vitro transcribed VP RNA and 120 μg protein in a total volume of 100 μl, was performed as described [12]. Flag-tagged proteins were immunoprecipitated using 20 μl each of EZ View Red ANTI-FLAG M2 affinity gel (Sigma) and washed according to the manufacturer's instructions. Immunoprecipitates were dissolved by boiling beads for 10 min in Laemmli sample buffer [13]. They were separated by SDS–gel electrophoresis and blotted onto Protran-*S* nitrocellulose membranes (Schleicher and Schuell). Protein/RNA complexes were detected by autoradiography. Aliquots of cell lysates (20 μg of protein each) were separated by SDS–gel electrophoresis and blotted onto nitrocellulose to detect Flag-tagged proteins with a monoclonal mouse anti-FLAG-tag M2 antibody (1:1000, Sigma) and horseradish peroxidase-conjugated goat anti-mouse IgG (1:10000, Jackson Immunoresearch) as secondary antibody. Antibodies were visualized by enhanced chemiluminescence using Lumi-Light Western Blotting Substrate (Roche) and blot exposure to X-ray film (Cronex 5, Agfa).

3. Results

3.1. PABP binds to VP RNA via RRMs 3 + 4

To assess which of PABP's RNA-binding domains are responsible for VP-DLS interaction different combinations of Flag-tagged PABP domains (Fig. 1) expressed in HEK 293 cells have been employed for UV-crosslinking analyses combined with immunoprecipitations of the resulting protein/VP RNA complexes. As shown in Fig. 2A, only those recombinant

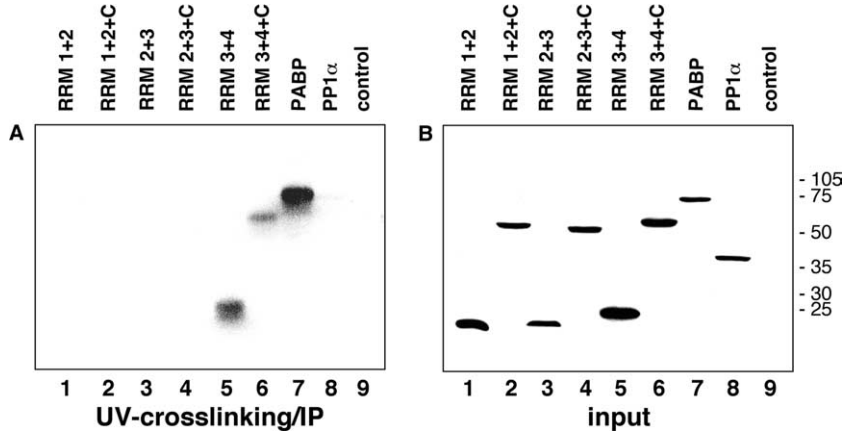

Fig. 2. Interaction of rat PABP with the dendritic localizer sequence of VP RNA is mediated by RRM 3 + 4. (A) Autoradiograph of UV-crosslinking analyses performed with ^{32}P-labeled VP transcripts and protein from HEK-293 cells expressing Flag-tagged full-size PABP (lane 7) or deletion mutants lacking individual domains of the molecule (lanes 1–6). As negative controls, HEK-293 cell lysates expressing Flag-tagged rat protein phosphatase 1α (PP1α, lane 8) and lysate from non-transfected cells (control, lane 9) were included. Following UV-crosslinking, recombinant proteins were precipitated with immobilized mouse anti-Flag-tag M2 antibody. Eluted immunoprecipitates were resolved by SDS–gel electrophoresis and transferred to nitrocellulose. (B) Aliquots of lysates employed for UV-crosslinking assays were electrophoretically separated, blotted onto a nitrocellulose membrane that was sequentially stained with mouse anti-Flag-tag M2 antibody and goat anti-mouse IgG-horseradish peroxidase conjugate. Secondary antibody was detected by enhanced chemiluminescence. Positions of molecular weight markers (in kDa) are indicated on the right side. C, C-terminal domain; RRM, RNA recognition motif.

proteins containing RRMs 3 + 4 (lanes 5–7) are bound to the probe, while proteins lacking one or both of these domains fail to interact (lanes 1–4). Apparently, the C-terminus is dispensible for this interaction, since proteins RRMs 3 + 4 and RRMs 3 + 4 + C bind equally well to VP RNA. Western blot analysis with aliquots of lysates used for UV-crosslinking verified that all of the recombinant proteins were appropriately expressed in HEK 293 cells (Fig. 2B). Further experiments revealed that both RRM 3 and RRM 4 are necessary for this interaction, since the individual domains RRM 3 or RRM 4 (in the absence or presence of the C-terminal domain) lack VP RNA binding competence (data not shown).

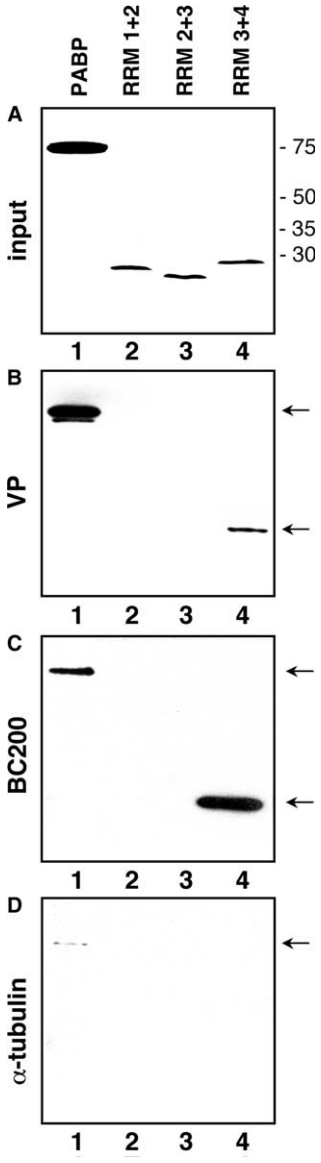

Fig. 3. PABP RRMs 3 + 4 bind specifically to VP- and BC200 RNA. Flag-tagged full-size PABP and deletion mutants encompassing RRM 1 + 2, RRM 2 + 3 and RRM 3 + 4, respectively, were incubated with immobilized biotinylated RNAs. Proteins bound to the target RNAs were eluted, separated by denaturing gel electrophoresis and blotted onto nitrocellulose membranes. Recombinant proteins were detected with a mouse anti-Flag M2 antibody (for details see legend to Fig. 2B). (A) 10 μg/lane of HEK-293 cell lysates containing the recombinant proteins (input). Positions of molecular weight markers (in kDa) are indicated on the right side. Recombinant proteins (arrows) eluted from immobilized VP- (B), BC200- (C) and α-tubulin- (D) RNA, respectively. It should be noted that a somewhat longer version of RRMs 1 + 2 (see Section 2) was used in these experiments.

3.2. Binding specificity of RRMs 3 + 4 to VP- and BC200 RNA

PABP binds with high specificity to the VP-DLS [2,12]. To further investigate the binding properties of combinations of individual rat PABP domains, affinity purifications were conducted with biotinylated in vitro transcripts immobilized on streptavidin-coated paramagnetic particles. BC200 RNA was included in these studies because it is also associated with PABP [5]. The assays shown in Fig. 3 confirmed binding of full-size PABP and RRMs 3 + 4 to VP RNA (Fig. 3A and B). Interestingly, recombinant proteins containing RRMs 3 + 4 also bound to BC200 RNA while RRMs 1 + 2 and RRMs 2 + 3, respectively, did not (Fig. 3C). Negligible binding of full-size PABP to α-tubulin RNA was observed, while none of the deletion mutants exhibited detectable interactions (Fig. 3D). This confirms earlier observations, namely a lack of PABP interaction with this transcript [12]. Hence, binding of PABP to both VP- and BC200 RNA via RRMs 3 + 4 is specific.

3.3. PABP interacts with the A-rich domain of BC200 RNA

The DLS of VP mRNA contains two PABP binding sites as suggested by UV-crosslinking assays [12]. Notably, this sequence lacks long stretches of A-residues. BC200 RNA, in contrast, exhibits a three domain structure (Fig. 4A) with an A-rich central region of 40 nucleotides. To map the contact site of PABP within BC200 RNA, interaction studies were performed with Flag-tagged RRMs 3 + 4 and partial sequences encompassing the Alu-domain alone (which contains at most two consecutive A-residues), the combined Alu-domain and oligo(A)-stretch as well as full length BC200 RNA. Strong binding to the transcripts containing the oligo(A) domain is observed as opposed to a lack of interaction with the isolated Alu-domain (Fig. 4B). Thus, PABP recognizes the A-rich region within BC200 RNA. Furthermore, these data reveal that the unique region of BC200 RNA is not necessary for a PABP association.

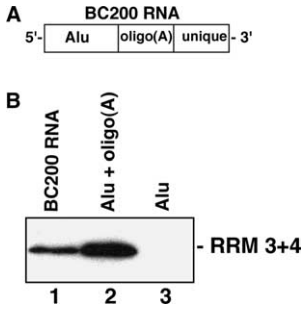

Fig. 4. PABP binds to the A-rich central domain of BC200 RNA. (A) Schematic representation of BC200 RNA consisting of an Alu-like domain at the 5′-end, an A-rich central region (oligo(A)) and a unique domain at the 3′-end. (B) HEK-293 cell lysate expressing Flag-tagged RRM 3 + 4 was incubated with immobilized full-size BC200 RNA (lane 1), the Alu-domain combined with the oligo(A) region (lane 2) or the Alu-domain alone (lane 3). Proteins were eluted, separated by denaturing polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Recombinant proteins were detected with a mouse anti-Flag M2 antibody (see legend to Fig. 2B).

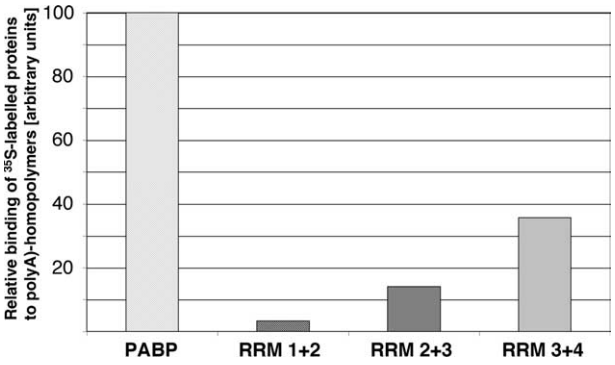

Fig. 5. Binding properties of PABP and PABP deletion mutants to poly(A)-ribohomopolymers. Full-size PABP and deletion mutants consisting of RRM 1 + 2, RRM 2 + 3 and RRM 3 + 4, respectively, were synthesized in vitro in the presence of [^{35}S]methionine. Equimolar amounts of recombinant proteins were incubated with poly(A)-agarose. Bound proteins were eluted and subjected to liquid scintillation counting. The diagram indicates relative binding (arbitrary units) of recombinant proteins to poly(A)-ribohomopolymers and are mean values from experiments performed in duplicate. RRM, RNA recognition motif.

3.4. *RRMs 3 + 4 are the main mediators of binding to poly(A)-homopolymers*

The high affinity binding of *Xenopus* and yeast PABP to poly(A) has been reported to be mainly mediated by RRMs 1 + 2, while RRMs 3 + 4 exhibit a 10-fold lower affinity for those sequences [14–16]. Hence, PABP's interaction via RRMs 3 + 4 with the VP-DLS (lacking poly(A)-stretches) and the A-rich region within BC200 RNA was surprising. Therefore, binding of full-size PABP and deletion mutants encompassing pairwise combinations of RRMs to poly(A)-homopolymers was quantitatively analyzed using equimolar amounts of in vitro translated ^{35}S-labeled proteins. As shown in Fig. 5, full-size PABP bound much better than any of the deletion mutants. Among those, the order of interaction strength is RRMs 3 + 4 > RRMs 2 + 3 > RRMs 1 + 2. Obviously, the whole protein sequence is required for an optimal binding to target sequences. Similar results have been obtained when comparing the binding behavior of radiolabeled PABP and RRMs 3 + 4 to VP transcripts and to BC200 RNA (data not shown).

4. Discussion

PABP regulates mRNA translation and stability through an association with the poly(A) tail of eukaryotic mRNAs [6]. Earlier data indicate that high affinity binding of *Xenopus* and yeast PABP to poly(A) is mediated by the aminoterminal RRMs 1 + 2. Furthermore, these domains were shown to exhibit binding properties nearly identical to that of full-size PABP [14–16]. However, a crucial contribution of RRMs 3 + 4 rather than RRMs 1 + 2 to PABP's poly(A)-binding activity was substantiated by several findings: (i) A PABP mutant lacking RRM 4 and the C-terminal domain is not associated with polysomes in *Xenopus* oocytes underscoring that in vivo RRMs 1–3 alone do not possess binding competence to poly(A) tails of endogenous mRNAs [17]. (ii) PABP is essential for yeast cell viability [18]. The activity sufficient to rescue lethality of a PABP-deleted strain is in the carboxyl-terminal RNA-binding domains [15]. (iii) High affinity binding of RRMs 3 + 4 of human PABP to A_{25}-homopolymers with a dissociation constant a little lower than that of RRMs 1 + 2 has recently been shown [19].

Rat PABP binds specifically to VP mRNA and BC200 RNA. In both cases, as shown here, this interaction is exclusively mediated by RRMs 3 + 4. Remarkably, the VP-DLS represents a non-poly(A)-sequence, whereas the PABP binding site within BC200 RNA consists of A-stretches interrupted by a few non-A-nucleotides such that the longest sequence encompasses 11 A-residues [1]. This length is not sufficient to allow for binding of RRMs 1 + 2 to BC200 RNA. Furthermore, a major contribution of domains RRMs 3 + 4 with respect to binding to poly(A)-homopolymers was observed in our studies. Hence, RRMs 1 + 2 (and RRMs 2 + 3) interact selectively with poly(A), even though less efficiently when compared to poly(A)-binding of RRMs 3 + 4, while RRMs 3 + 4 are able to bind to both poly(A)- and non-poly(A) sequences. Similar observations have been reported recently for the human PABP deletion mutants RRMs 1 + 2 and RRMs 3 + 4, respectively [19]. Furthermore, we observed that full-size PABP is considerably more competent for associating with diverse target sequences than any of the deletion mutants consisting of various combinations of RNA-binding domains.

The discrepancies to earlier findings, namely the lower affinity of RRMs 3 + 4 for A_{23} homoplymers in comparison to the high affinity of RRMs 1 + 2 for this sequence [14,16], may be due to several reasons: In particular, we have used equimolar amounts of radiolabeled PABP and its deletion mutants for the binding studies. Concentration determinations of bacterially expressed proteins with dyes such as Coomassie G-250 are not strictly quantitative and may vary from protein to protein. This could have an impact on determinations of dissociation constants. Moreover, while in earlier studies [14,16] an excess of protein and very low amounts of radiolabeled poly(A) have been employed, the experiments described here have been done with a much higher quantity of binding sites which might better mimic the situation within the cell.

In conclusion, the results presented here provide evidence for PABP's RRMs 3 + 4 playing a more important role in RNA binding than previously acknowledged.

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