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Biochemical characterization of the *Caenorhabditis elegans* FBF•CPB-1 translational regulation complex identifies conserved protein interaction hotspots

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Abstract

Caenorhabditis elegans CPB-1 (cytoplasmic polyadenylation element binding protein) and FBF (*fem-3* mRNA binding factor) are evolutionary conserved regulators of mRNA translation that belong to the CPEB (cytoplasmic polyadenylation element binding) and PUF (Pumilio and FBF) protein families, respectively. In hermaphrodite worms CPB-1 and FBF control key steps during germline development, including stem cell maintenance and sex determination. While CPB-1 and FBF are known to interact, the molecular basis and function of the CPB-1•FBF complex are not known. The surface of CPB-1 that interacts with FBF was localized using *in vivo* and *in vitro* methods to a ten-residue region at the N-terminus of the protein and these residues are present in the FBF-binding protein GLD-3 (germline development defective). PUF proteins are characterized by the presence of eight α -helical repeats (PUF repeats) arranged side by side in an elongated structure. Critical residues for CPB-1 binding are found in the extended loop that connects PUF repeats seven and eight. The same FBF residues also mediate binding to GLD-3, indicating a conserved binding mode between different protein partners. CPB-1 binding was competitive with GLD-3, suggestive of mutual exclusivity *in vivo*. RNA binding measurements demonstrated that CPB-1 alters the affinity of FBF for specific RNA sequences, implying a functional model where the coregulatory protein CPB-1 modulates FBF target selection.

Keywords

Pumilio; PUF protein; FBF; protein-protein interactions; germline development

Introduction

Post-transcriptional regulation of gene expression is vital for many diverse biological processes, including stem cell maintenance and differentiation^{1, 2}, neuronal synaptic plasticity³ and cellular senescence⁴. The fate of the mRNA is often determined by the length

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of the poly(A) tail, which controls its localization, stability and translational efficiency. Many mRNAs are stored with a short poly(A) tail and their translation is activated by polyadenylation⁵. Shortening of the poly(A) tail triggers mRNA degradation⁶. The regulation of poly(A) tail length is often mediated by specific proteins that bind to elements located in the 3' untranslated region (UTR) of the mRNA and nucleate formation of large multi-protein complexes.

Caenorhabditis elegans CPB-1 (cytoplasmic polyadenylation element binding protein homolog)⁷ and FBF (*fem-3* mRNA binding factor)⁷ are evolutionary conserved 3' UTR regulatory proteins that control key steps in germline development. CPB-1 is required for spermatogonia to progress from first to second meiosis⁷. CPB-1 belongs to the cytoplasmic polyadenylation element binding (CPEB) family of proteins, that is found in mammals and invertebrates⁴. CPEB proteins contain two RNA recognition motifs (RRMs) followed by a zinc finger domain (Figure 1A), that are required for the interaction with the cytoplasmic polyadenylation element (CPE) consensus sequence in the 3'UTR of target mRNAs⁸. In *Xenopus* oocytes, CPEB regulates both mRNA activation and repression^{5; 9}. Both processes require the dynamic assembly of a complex of proteins at the CPE. During oocyte maturation, cytoplasmic polyadenylation involves, in addition to CPEB, the scaffolding protein symplekin, the poly(A) polymerase GLD-2 (germline development factor-2) and the multisubunit cleavage and polyadenylation specificity factor CSPF¹⁰. CPEB-mediated translational repression involves the poly(A) ribonuclease, PARN¹¹.

In many species, including *Xenopus* and *C. elegans*, CPEB proteins interact with relatives of *Drosophila* PUM and *C. elegans* FBF, termed PUF proteins^{12,13}. PUF proteins recruit the conserved deadenylase complex CCR4–Pop2–Not^{14; 15}, Argonaute (Ago)¹⁶, and the translational repressor Nanos^{5; 17}. In *C. elegans* FBF controls sex determination by interacting with GLD-3 (germline development defective)¹⁸.

FBF belongs to the Pumilio and FBF (PUF) family of RNA-binding proteins found in *Drosophila*, *C. elegans*, humans and yeast. FBF plays an important role in germline stem cell maintenance², spermatogenesis⁷ and in the spermatogenesis to oogenesis switch¹⁹. PUF proteins are characterized by the presence of eight adjacent repeats (PUF repeats) arranged side by side in an elongated structure^{20; 21}. Each repeat consists of three α -helices and recognizes a single RNA base^{20; 22; 23}. In addition to binding RNA, the PUF repeats also mediate the interaction with protein partners^{16; 23; 24}. PUF proteins repress² or activate mRNA translation^{1; 3} depending on the mRNA target and the repertoire of associated proteins. PUF protein–RNA interactions have been extensively characterized in recent years^{20; 21; 23; 25; 26; 27; 28; 29}, but less is known about mechanisms of molecular recognition of protein partners. An additional open question about PUF mediated regulation of gene expression is if PUF proteins simply function as a scaffold for the recruitment of protein cofactors to the mRNA or if the interacting partners modulate their RNA binding activity^{24; 30; 31}.

In this study the specificity of the interaction between CPB-1 and FBF was investigated, as well as cooperative binding of the multiprotein complex to RNA. Using a combination of *in vitro* studies with recombinant proteins and the yeast two-hybrid approach, the FBF-binding region of CPB-1 was narrowed down to a short stretch of amino acids and key residues were identified, whose mutation had a deleterious effect on the interaction. Residues in the FBF loop between repeats seven and eight were also found to be important for the interaction. Residues in the CPB-1/FBF interface are also found in the GLD-3•FBF complex³¹, indicating a conserved mode of interaction between different protein partners. Finally, the FBF-binding region of CPB-1 enhances binding of FBF to a putative mRNA target of the

CPB-1•FBF complex both *in vitro* and *in vivo*, supporting a mechanistic model where cooperative RNA-protein complex formation can modulate FBF's target selection.

Results

Mapping the FBF-binding site within CPB-1 indicates that the interaction is mediated by a short stretch of amino acids

Yeast two-hybrid experiments previously showed that CPB-1 binds to FBF-1 through the first 80 N-terminal amino acids⁷. This interaction was confirmed *in vitro* with a pulldown assay with the RNA-binding domain of FBF-1 and additionally with the same domain of the protein FBF-2¹⁹ (not shown). Since these two proteins are almost identical in sequence, have overlapping function *in vivo*¹⁹ and yielded the same results in the binding assays with CPB-1, they will collectively be referred to as FBF.

Analysis of the CPB-1(1-80) peptide by circular dichroism (CD) showed that the FBF-binding region of CPB-1 is largely unordered (Supplementary Figure 2A), in agreement with results from secondary structure prediction algorithms (<http://bioinf.cs.ucl.ac.uk/psipred/>). Moreover, the observation that CPB-1 was sensitive to low concentrations of protease further supported the absence of a compact structure (see below).

Many protein interactions are mediated by short peptide segments that bind to a globular domain^{32, 33}. Such peptide motifs often lie within disordered regions³³. The observation that CPB-1(1-80) is mostly unfolded led us to investigate whether a shorter motif within this segment was sufficient to bind FBF. Two approaches were used in parallel to accurately define the FBF-binding region: limited proteolysis experiments with recombinant proteins and yeast two-hybrid assays with CPB-1 deletion mutants. Limited proteolysis with trypsin was performed on free CPB-1 and CPB-1 that had been chemically cross-linked to FBF. Comparison of the digestion pattern of CPB-1 alone and CPB-1 cross-linked to FBF identified regions of the protein that were no longer accessible to the protease upon binding to FBF. FBF was refractory to mild proteolysis with trypsin, which facilitated the analysis of the CPB-1 fragments. This crude approach identified CPB-1 residues 26–63 as the shortest fragment protected from proteolysis when bound to FBF (Supplementary Figure 1). At the same time, a complex of FBF with the longest recombinant CPB-1 construct that could be expressed and purified (residues 1–363) was digested with trypsin. The CPB-1 fragments bound to FBF were isolated and identified by mass spectrometry (not shown). The CPB-1 fragments were then produced recombinantly as hexahistidine (His)-tagged proteins and tested for binding to FBF with a pulldown binding assay (Figure 1B). In the pulldown assay the His-tagged protein is immobilized on NiNTA resin and eluted with buffer containing high concentration of imidazole. The second protein, the putative interacting partner, is produced without the affinity tag and is therefore not retained by the NiNTA resin. When protein-protein interaction occurs both proteins are retained on the resin and co-elute with high imidazole concentration. With this approach, the FBF-binding region within CPB-1 was narrowed to amino acid residues 32–60 (lower panel in Figure 1B), although CPB-1 deletion fragments that did not contain residues 32–47 did not bind to FBF. In parallel, a number of CPB-1 deletion mutants were tested for binding with the yeast two-hybrid method. The smallest CPB-1 fragment that still showed high β -galactosidase activity consisted of residues 40–70, in good agreement with the *in vitro* data (Figure 1C). Removal of the 40–50 stretch of amino acids abolished binding. A summary of all the constructs tested for binding *in vitro* and with the yeast two-hybrid method is shown in Supplementary Figure 2B. Taken together, the data indicate that the minimal FBF-binding region is contained within the 40–60 stretch of amino acids.

Upon narrowing down the FBF-binding region of CPB-1 we proceeded to investigate the binding mode of the CPB-1/FBF system. Upon binding to their specific target some disordered protein segments may transition to regular secondary or tertiary structures, inducing long-range structural rearrangements, while others remain ordered coils in their bound form. To gain insights into the binding mode of CPB-1, heteronuclear single quantum coherence [^{15}N , ^1H]-HSQC spectra were acquired using ^{15}N -labeled CPB-1(32-80). The spectra were recorded in the presence and absence of the FBF•RNA complex. In the absence of RNA, FBF tended to precipitate out of solution under NMR conditions at high concentration for an extended period of time at room temperature. CPB-1 and RNA form a stable ternary complex on FBF as discussed below. Free CPB-1(32-80) is flexibly disordered in solution, as indicated by the low chemical shift dispersion in the [^{15}N , ^1H]-HSQC spectrum shown in Supplementary Figure 2C. The spectrum is characterized by a crowded set of backbone amide moieties located between the 7.5 to 8.5 ppm proton-frequency range, which is typically populated by residues found in flexibly disordered polypeptide segments. In the presence of FBF the [^{15}N , ^1H]-HSQC spectrum of CPB-1 exhibits a similar pattern with a reduced number of peaks. The conserved pattern indicates that no major structural rearrangement occurs for the residues observed in these conditions (Supplementary Figure 2C). Such residues can be confidently mapped in the protein sequence to the regions 32 to 37 and 64 to 80 using the characteristic backbone HN Gly, and the side-chain H_2N Gln and Asn peaks as guides. The missing peaks in the [^{15}N , ^1H]-HSQC of the CPB-1•FBF complex most likely correspond to residues bound to FBF or proximal to the binding site, mapped to residues 38 to 63. Although the current data do not allow us to rule out a structural rearrangement in the segment 38 to 63, they indicate that FBF does not induce long-range conformational changes in other regions of the CPB-1 sequence employed.

Molecular underpinnings of the CPB-1•FBF complex

FBF interacts with GLD-3²⁴³¹. Previous experiments demonstrated that loss of the FBF loop (residues 479–485) joining PUF repeats seven and eight disrupts binding to both GLD-3 and CPB-1, suggesting that the two proteins share a common FBF-binding site³⁴³¹. If CPB-1 and GLD-3 bind to the same site on FBF this would prevent formation of a ternary complex and binding of the two proteins may be competitive. In order to test this hypothesis, CPB-1 and GLD-3 were bound to FBF varying the order of addition. Upon complex formation the proteins were covalently cross-linked in order to allow their identification by denaturing protein gel electrophoresis (SDS PAGE). The chemically cross-linked GLD-3•FBF and CPB-1•FBF complexes showed different migration patterns on the gel (Figure 2A). GLD-3 bound to free FBF but not to FBF pre-incubated with CPB-1. When CPB-1 was added to FBF a complex with a 1:1 stoichiometry was formed. When added to the GLD-3•FBF complex, which also has a 1:1 stoichiometry, CPB-1 replaced GLD-3 on FBF. No ternary complex formation was observed, independent of the order of protein addition. CPB-1 and GLD-3 may therefore bind to the same site, with CPB-1 binding with a greater affinity.

The sequence alignment of the FBF-binding region of GLD-3 with CPB-1 indicated that the most important residues in the GLD-3 FBF-binding region are also present in CPB-1³¹ and are found within the newly identified FBF-binding region (Figure 2B). Mapping of the residues in CPB-1 involved in binding to FBF was done by substituting pairs of amino acids with alanines. The CPB-1 double alanine mutants were produced recombinantly as His-tagged proteins, and subjected to a pulldown assay with FBF. The residues with the greatest effect on binding to FBF were located in the 40–47 region (Figure 2C). The effect of each residue in this region was then quantified by systematic single alanine scanning mutagenesis. The relative affinity of the CPB-1 alanine mutants for FBF was measured by competition titration experiments using fluorescence polarization assay (Figure 3A). In the

competition-binding assay FBF was bound to fluorescently labeled wild-type CPB-1(19-80) (fl-CPB-1) and increasing concentrations of unlabeled competitor CPB-1 (wild-type or mutant) were titrated into the complex. The plot of the normalized polarization as a function of competitor CPB-1 concentration was used to derive the half-maximal inhibitory concentration (IC_{50}) (Figure 3B). The IC_{50} of self-competitor (wild-type) CPB-1 was $1.15 \pm 0.05 \mu M$. The reduction in competition efficiency relatively to wild-type CPB-1 ranged from approximately three- to forty-fold for the most significant single alanine mutants. Some double alanine mutants were also tested for loss of binding and the effects were additive (not shown). Similar effects were observed when the alanine-scanning strategy was used in the yeast two-hybrid assay (Figure 3C). Residues whose mutations caused the greatest loss of binding were Leu 40, Lys 44, Thr 45, Leu 47 and Ile 49.

To test if additional amino acid substitutions at key sites in CPB-1 compromised binding to FBF, additional mutagenesis was conducted. Random mutants were introduced into four key sites in CPB-1 using mutagenesis primers containing three random oligonucleotides (Figure 3D). The resulting mutants were transformed into yeast and their relative interactions quantified using a yeast-two hybrid assay. The identity of each mutant was then inferred based on the DNA composition obtained by Sanger sequencing³⁵. The majority of mutations at positions 40, 45, and 47 reduce binding to FBF. However, multiple mutations of Ile 49 were permissible. A small gain in apparent binding activity was observed for a conservative Ile to Leu mutation. We conclude that the majority of mutations at key sites in CPB-1 are deleterious for binding of FBF.

Identification of key FBF residues for CPB-1 binding

Removal of the long loop connecting PUF repeats seven and eight of FBF disrupts binding to CPB-1³⁶. In order to investigate the contribution of each residue in the loop to CPB-1 binding, a mutational analysis was performed *in vitro* with recombinant proteins (Figure 4A). To ensure that the reduced affinity between CPB-1 and mutant FBF was due to genuine loss of binding contacts at the protein-protein interface rather than being caused by protein misfolding, the FBF mutants were tested for folding defects using RNA binding as a readout. Electrophoretic mobility shift assay (EMSA) experiments showed the mutants were properly folded and active in binding RNA (not shown). Pulldown assays indicated that mutation of Tyr 479 to Ala completely disrupted binding to CPB-1 (Figure 4A). This qualitative study also indicated that Ile 480 and Thr 485 play an important role in binding (Figure 4A). The effects of the alanine mutations were quantified with the fluorescence polarization assay by direct titration into fl-CPB-1 (Figure 4B). Fl-CPB-1 bound to FBF with a $K_d=49 \pm 3$ nM, residues that most significantly affected binding to CPB-1 when mutated to alanines were Tyr 479 (which showed > 60 fold increase in K_d), Ile 480, with a $K_d=778 \pm 49$ nM, and Thr 485, with a $K_d=1261 \pm 146$ nM, in agreement with the pulldown assay. Important residues for the interaction with CPB-1 are mapped onto the three-dimensional structure of FBF-2 in complex with the protein binding element of *fem-3* (UGUGUCAUU)²⁹ in Figure 4C (PDB accession code=3K64).

CPB-1 alters the affinity of FBF for *cyb-1* mRNA

EMSA experiments were performed to investigate binding of FBF to RNA in the presence of CPB-1. The mRNA target for the CPB-1•FBF complex is unknown. However, in *Xenopus*, CPEB and PUF families act together to regulate expression of cyclin B⁹. Since cyclin B mRNA is a putative conserved target of FBF³⁷, we used cyclin B of *C. elegans* (*cyb-1*) in our *in vitro* studies. FBF bound to *cyb-1* RNA with a $K_d=24$ nM (Hill=1.5) but in the presence of CPB-1 binding was about 4 times tighter ($K_d=5.8$ nM, Hill=1.0). (Figure 5A and 5B). The effect of CPB-1 on FBF binding to *cyb-1* RNA *in vitro* is modest but suggestive that CPB-1 binding and RNA binding to FBF may be cooperative.

To corroborate these results and examine the effects of CPB-1 binding on other RNA targets, we utilized a modified yeast-three hybrid assay (Figure 5C and D). In these experiments, binding of FBF to candidate RNAs is measured in the presence or absence of CPB-1 fused to an SV40 NLS. Thus, the effects of complex formation on the affinity of FBF for a given RNA can be determined in a cell-based assay. CPB-1 appears to stimulate binding to the NRE (nanos response element), *cyb-1* and *fog-1a* (feminization of germline) RNAs. Based on comparison of the various RNAs, we conclude that there is a sequence dependence to the observed cooperativity. Intriguingly, expression of CPB-1 appeared to consistently enhance binding to the target RNA consistent with previous results on synthetic targets^{34,38}.

Discussion

CPEB and PUF proteins are key regulators of post-transcriptional gene expression and are conserved from mammals to invertebrates^{4, 39}. Generally, post-transcriptional regulators control translation of multiple mRNA targets by nucleating the assembly of dynamic protein complexes. In this study the specificity determinants of the interaction between the *C. elegans* member of the CPEB family CPB-1 and PUF protein FBF were determined. CPEB proteins contain an N-terminal sequence of no conserved structural motif and two RRM and zinc finger motives that mediate their binding to mRNA⁴⁰. PUF proteins bind target mRNAs as well as protein partners through their PUF repeats. In recent years a wealth of biochemical and structural information on the mRNA binding specificity of PUF proteins has been published²⁷, but less is known about how they interact with their protein partners. Biochemical analysis of the CPB-1 and FBF surface allowed the identification of key residues for the interaction. CPB-1 binds FBF through a short stretch of amino acids located at the N-terminus of the protein that has no regular secondary structure free in solution, and undergoes no major structural rearrangement upon binding to FBF. *In vitro* analysis of recombinant proteins and yeast two-hybrid assays showed that CPB-1 residues important for the interaction with FBF are found within the 10-mer peptide LSTDKTNLDI encompassing residues 40–49. Amino acids whose mutation to alanine mostly disrupted binding to FBF were Leu 40, Lys 44, Thr 45, Leu 47 and Ile 49. In particular, the Leu 47 to Ala mutation had a deleterious effect on the interaction.

This newly identified FBF-binding region of CPB-1 is similar in sequence to the FBF-binding region of GLD-3³¹. In GLD-3 residues important for binding are also contained in a short stretch of amino acids and include Lys 864, Thr 865 and most importantly Leu 867, whose mutation to Ala completely disrupts binding to FBF³¹. The conserved KTXL (X= any residue) motif plays a key role in both the GLD-3 and CPB-1 interaction with FBF. Although a KTXL sequence is important for both proteins to bind to FBF, CPB-1 and GLD-3 have different affinities for FBF, with CPB-1 binding with a K_d in the low nanomolar range and GLD-3 showing a K_d about 20 times higher³¹. Binding of both proteins to FBF is mediated by the loop between PUF repeats seven and eight and mutation of Tyr 479, Ile 480 and Thr 485 to Ala significantly impacts the affinity of FBF for both proteins³¹, showing a conserved mode of binding.

In vitro, CPB-1 and GLD-3 compete with one another for binding to the same region of FBF. The *in vivo* consequences of their binding to the same site in FBF are unclear, and a key area for future analysis. CPB-1 and GLD-3 might antagonize one another in controlling FBF activity *in vivo*; alternatively, they could act additively on FBF, or in different cells and times. CPB-1 and GLD-3 are both expressed in early stage spermatocytes⁷⁴¹, but their co-localization with one another, and with FBF, has not been analyzed. Similarly, loss of either CPB-1 or GLD-3 leads to the same spermatogenesis defect, arrest as primary spermatocytes^{7, 18}, but further genetic analysis is required to understand their regulatory

relationships and the mechanisms leading to that arrest. In particular, similar mutant phenotypes could arise from triggering a common checkpoint, or from additional, as yet unexplored roles of the two proteins. Use of the mutations described here as *C. elegans* transgenes should provide precise probes for the biological roles of the interactions between FBF and its partners. The CPB-1/FBF interaction is also disrupted or considerably reduced by point mutations in FBF (Tyr 479 to Ala and Thr 485 to Ala), although these mutations will certainly affect FBF's interaction with multiple proteins^{34,31} and therefore will not be the ideal candidate for *in vivo* studies.

FBF physically associates with ~7% of *C. elegans* transcriptome³⁷, interacts with a number of proteins including CPB-1, GLD-3, NOS-3¹⁷ (Nanos-related), CCF-1 (the *C. elegans* CAF-1)¹ and forms a ternary complex with EF1A and CSR-1 (a translation initiation factor and Ago family member, respectively)¹⁶. The key FBF residues for the interaction with CPB-1 are contained within the loop connecting PUF repeats seven and eight. These results indicate that relatively small surfaces are involved in binding, consistent with the fact these are transient interactions. Mutation of single residues on either one of the protein interaction surfaces has a deleterious effect on their interaction. Protruding loops on the concave surface of PUF proteins mediate a plethora of interactions^{34,31}. For instance *Drosophila* Brain Tumor (Brat) protein is recruited by Pumilio to repress translation of hunchback mRNA⁴². The structural model of the Pumilio•Brat complex showed that an extended loop in PUF repeat eight fits in the entrance to the central channel of the β -propeller formed by Brat's NHL domain²³. Flexible loops on the outer concave surface of PUF proteins bind specifically to both structured proteins like Brat and disordered proteins such as CPB-1 or GLD-3²³.

The direct, physical interaction of CPEB and PUF proteins is broadly conserved from humans to *C. elegans*^{12,36}. On many 3' UTRs *in vivo*, PUF and CPEB proteins are likely to be bound to their own elements, and physically interact with one another as well. Our data show that CPEB also can influence FBF specificity directly, independent of binding RNA. Two key observations support this hypothesis. First, the affinity of a PUF protein, FBF-2, for specific RNA sequences is influenced by a fragment of CPEB that lacks RNA recognition domains and possesses no specificity for RNA itself³⁶. Second, different RNAs vary in their sensitivity to CPEB's effects on PUF specificity: for example, CPB-1 increases the affinity of FBF for the binding element in *fog-1* but not *gld-1* RNAs. In a parallel study, we examined the global specificity for RNA of FBF-2 alone, CPB-1 alone, and the protein complex³⁰. In those studies, the short segment of CPB-1 broadened the specificity of FBF-2. We suggest that CPEB facilitates binding of PUF proteins to RNAs that lack the highest affinity PUF binding elements. Indeed, sub-optimal sites appear to be common among the targets of FBF-2 and human Pumilio^{43,44}. We propose that on some mRNAs, CPEB acts and influences PUF protein activity without that CPEB molecule binding to the RNA. Such mRNA targets would lack CPEs, but nonetheless respond to the protein.

PUF proteins bind RNA through a conserved mechanism. The PUF domain forms a curved, elongated structure where each of the 8 PUF repeats recognizes a single nucleotide^{20; 21; 29} (Figure 4C). Despite the conserved binding mode, the length of the consensus sequence is protein specific. For instance, human Pumilio binds 8 nucleotide RNA sequences, while the target recognized by FBF is 9 nucleotides in length. The flatter curvature of FBF relative to human Pumilio requires an additional spacer nucleotide, whose base flips out and points away from the protein. The RNA specificity is therefore obtained through perturbations of the curvature of the PUF domain⁴⁵. By binding to the outer surface of FBF, opposite the concave RNA binding surface, CPB-1 may alter the curvature of the protein, causing a tighter binding to specific RNA sequences. This result suggests a functional model where the FBF target selection is modulated by the coregulatory factor CPB-1, consistent with the observation that the FBF-binding region of CPB-1 is sufficient for promoting translational

repression in the presence of FBF³⁴. Structural analysis of the FBF•CPB-1•RNA complex is needed to understand how CPB-1 modulates FBF's RNA binding activity, and is now an important goal.

Materials and Methods

Protein expression and purification

CPB-1(1-80) and CPB-1(19-80) constructs were cloned into pET-22b(+) (Novagen) between restriction sites NdeI-XhoI in order to introduce a C-terminal heahistidine (His) tag. Strains used for cloning and expression of recombinant proteins were *E. coli* DH5α (Invitrogen) and *E. coli* BL21-Gold(DE3) (Stratagene) respectively. Cells were grown at 37°C in Luria Bertani medium supplemented with 50 µg/ml ampicillin (LB/Amp) and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 hours. Cell pellets were re-suspended in lysis buffer [20 mM Tris-HCl (pH 7.0), 300 mM NaCl, 20 mM imidazole, 5% glycerol) supplemented with Complete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche) and subjected to mild sonication followed by 30 minute incubation at 4°C with 0.5X FastBreak Cell Lysis Reagent (Promega). The cleared lysate was loaded onto TALON Metal Affinity Resin (Clontech). The resin was washed with lysis buffer supplemented with 1 M NaCl and the protein was eluted with lysis buffer containing 150 mM imidazole (pH 7.0). Only fractions where protein purity was >95% were pooled and concentrated in Amicon Ultra-15 Centrifugal Filter Units (Millipore).

The CPB-1(19-80) construct used in the EMSA experiments and the CPB-1(1-363) construct contained an N-terminal maltose binding protein (MBP) tag. The proteins were cloned into a modified pMAL-c2x vector (New England Biolabs) in which a tobacco etch virus (TEV) cleavage site had been introduced between the MBP tag and the multiple cloning site. A C-terminal His tag was introduced by PCR. Protein expression was carried out in *E. coli* BL21-Gold(DE3) cells as described for the previous proteins. Cell pellets were resuspended in lysis buffer [0.5M NaCl, 20 mM Tris HCl (pH 8.5), 20 mM imidazole, 5 mM betamercaptoethanol (βME)] supplemented with Complete, EDTA-free Protease Inhibitor Cocktail Tablets and lysed by sonication. The cleared lysate was mixed with NiNTA Superflow resin (Qiagen) and the protein was purified with the batch-binding procedure. The resin was washed with lysis buffer containing 1 M NaCl, equilibrated again with lysis buffer and finally eluted with 20 mM Tris HCl (pH 8.5), 300 mM imidazole, 250 mM NaCl, 10 mM βME. The eluate was applied to amylose resin (New England Biolabs) equilibrated with lysis buffer. The resin was washed with 20 mM Tris HCl (pH 8.5), 200 mM NaCl, 10 mM βME and the protein was eluted with the same buffer supplemented with 10 mM maltose. The buffer was exchanged to 20 mM Tris HCl (pH 8.5), 100 mM NaCl, 5 mM DTT by dialysis and the protein was stored at -80°C.

Double and single alanine mutations were introduced in the CPB-1(19-80) construct by PCR mutagenesis and proteins were purified as wild-type CPB-1(19-80).

CPB-1 deletion mutants shorter than the CPB-1(19-80) construct were also expressed as N-terminal, TEV cleavable, MBP fusions and with a C-terminal His tag. The proteins were purified over NiNTA and amylose resins, all the buffers were supplemented with 10% glycerol. The MBP tag was removed by overnight digestion at 4°C with recombinant His-tagged TEV protease. The MBP tag was separated from CPB-1 by centrifugation in Amicon Ultra-15 Centrifugal Filter Units with a molecular weight cut off of 30kDa (Millipore).

FBF-1 (164-566) and FBF-2 (164-566) were cloned into a modified pETDuet-1 expression plasmid (Novagen) that encoded for an N-terminal, TEV cleavable, His tag. Protein expression was carried out in *E. coli* BL21(DE3) cells (Stratagene) for 12 hours at 18°C

upon induction with 0.5 mM IPTG. Cell pellets were resuspended in lysis buffer [20 mM Tris HCl (pH 8.6), 200 mM NaCl, 10% glycerol, 10 mM β ME, 5 mM imidazole, 0.5 M UREA, Complete, EDTA-free Protease Inhibitor Cocktail Tablets] and disrupted by sonication. The proteins were purified by NiNTA affinity chromatography and the His tag removed overnight at 4°C with recombinant His tagged TEV protease. A second NiNTA purification step was performed to remove the released tag and the TEV protease, followed by size exclusion chromatography (Superdex 200, GE Healthcare) in storage buffer [20 mM Tris HCl (pH 8.6), 200 mM NaCl, 5 mM DTT, 10% glycerol]. FBF-2 construct encompassing residues 164–575 was overexpressed and purified as described in Wu et al³¹.

Qualitative protein binding assay (NiNTA pulldown)

Protein complexes were reconstituted as follows: 10 to 20 μ M FBF was incubated for 3 hours with 1.5–2.0 molar excess CPB-1 in reconstitution buffer [20 mM Tris HCl (pH 7.5 or pH 8.6), 100 mM NaCl, 10 mM β ME, 5% glycerol]. Pulldown assays were performed by applying the reconstituted protein-protein complexes or the individual proteins as controls, to His SpinTrap columns (GE Healthcare) pre-equilibrated in reconstitution buffer. Columns were washed three times with 0.6 ml of buffer and the proteins were eluted with 0.4 ml of buffer containing increasing concentrations of imidazole (up to 300 mM). The content of each elution fraction was visualized by SDS PAGE. Wild-type FBF-1 and FBF-2 (residues 164–566) were used for pulldown experiments with the His tagged CPB-1 deletion mutants. Wild-type FBF-2 (residues 164–575) was used for pulldown assays with the single and double alanine mutant CPB-1 constructs. Single alanine mutations were introduced in the FBF-2 (164–575) construct. The reason for using slightly different FBF constructs is that for initial experiments we designed the FBF-1 (164–566) and FBF-2 (164–566) constructs based on sequence alignments with other PUF proteins. Since in parallel we were also pursuing crystallization attempts, when the crystal structure of FBF-2 (164–575) was published²⁹ we switched to this slightly longer construct and for consistency we also used it for the biochemical experiments. It is worth noticing that the last 8 amino acids (residues 568–575) are not included in the crystal structure because of poor electron density²⁹ and do not affect binding to CPB-1.

Yeast two and three-hybrid assays

Point mutants were generated using site directed mutagenesis (Invitrogen). Mixed base oligonucleotides were obtained by machine mixed random codons (IDT). In the yeast two-hybrid assays, CPB-1(1–80) was cloned into pBTM116 and FBF-2 (121–C-term) into pACT2 and expressed in strain L40U[–]. Modified yeast three-hybrid experiments were conducted as described^(34; 38). Yeast strain YBZ-1 was transformed with an additional plasmid encoding p414TEF CPB-1 (40–80) fused to an SV40 nuclear localization signal. Measurements were determined in triplicate from three individual yeast transformants. Luminescence data were collected using the β -Glo reagent (Promega).

Tryptic digestion of CPB-1

11 μ M His tagged FBF-1(164–566) was incubated on ice for 2 hours with 15 μ M CPB-1(1–80) in binding buffer [20 mM Na-phosphate (pH 8.6), 100 mM NaCl, 10 mM β ME]. A freshly prepared solution of ethylene glycol bis[succinimidylsuccinate] (EGS) in DMSO was added to a final concentration of 1.25 mM to half of the reaction mix and the sample was incubated on ice for 5 minutes. The crosslinking reaction was quenched by adding 50 mM Tris HCl pH 8.6. Trypsin was added to both samples to a final concentration of 0.5 ng/ μ l and the reactions were incubated at room temperature for 50 minutes. The tryptic digestion was stopped by adding 3% formic acid. The trypsinised fragments were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry. The CPB-1 fragments that were protected upon binding to FBF were

identified by comparison of the digestion pattern of the crosslinked FBF•CPB-1 sample with the non cross-linked sample.

Protein cross-linking

Cross-linking experiments were performed in parallel with both FBF-1(164-566) and FBF-2(164-566) and yielded the same results regardless of the protein used. A constant concentration of FBF (7.5 μ M) was equilibrated with a 2 fold molar excess of GLD-3(860-949) in 20 mM sodium phosphate (pH 8.5), 200 mM NaCl, 10 mM β ME in 20 μ l total volume. Increasing concentrations of CPB-1(1-80) were added to a final concentration of 3.8 μ M, 15.0 μ M and 30.0 μ M. 0.5 μ l of freshly prepared 40 mM solution of EGS in DMSO was added and the cross-linking reaction was allowed to proceed for 1 minute at room temperature. The reaction was quenched by adding 1 μ l of 100 mM Tris HCl (pH 8.6) and the samples were analyzed by SDS PAGE. The same type of experiment was repeated with FBF incubated with constant amount of CPB-1 and where GLD-3 was added in a 0.5, 2 and 4 fold molar excess prior to EGS crosslinking.

Labeling of CPB-1 with Alexa Fluor 488

A cysteine residue was introduced by PCR mutagenesis between residues Ser 35 and Phe 36 in the CPB-1(19-80) construct. Protein expression was performed as for wild-type protein. The cell pellet was re-suspended in lysis buffer supplemented with 2 mM DTT and lysed as described for wild-type CPB-1. The cleared lysate was loaded onto NiNTA resin. The resin was washed with labeling buffer [20 mM Hepes (pH 7.0), 150 mM NaCl, 2% glycerol] to remove the reducing agent. Precautions were taken to avoid cysteine oxidation (all the buffers were thoroughly degassed and the resin was flushed with nitrogen). The protein was incubated with a 5-fold molar excess of AlexaFluor 488 C₅ maleimide (Invitrogen) while on the resin. Labeled CPB-1 was eluted and dialysed in 20 mM Tris HCl (pH 7.0), 50 mM NaCl, 10% glycerol, 2 mM DTT. The protein was loaded onto Q column (GE Healthcare) equilibrated with the same buffer. CPB-1(19-80) did not bind to the column in these conditions but was present in the flow through >95% free of contaminants.

Quantitative analysis of protein-protein interactions by fluorescence polarization

Fluorescence polarization experiments were carried out in 96-well opaque floutrak 200 μ l plates (Greiner), the polarization was determined using an Envision plate reader (Perkin Elmer). In the direct titration experiments 6 nM fl-CPB-1 was incubated for at least 1 hour at room temperature with increasing concentrations of wild-type or mutant FBF-2(164-575) in a total volume of 100 μ l. The equilibration buffer contained 20 mM Tris HCl (pH 8.6), 100 mM NaCl, 2 mM DTT, 0.10 mM EDTA, 0.10 mg/ml tRNA, 0.05 mg/ml BSA, 5% glycerol. The experiments were performed at least in triplicate and data were fitted with the Hill equation using IGOR (Wavemetric) to determine the K_d. For the binding-competition experiments 6 nM fl-CPB-1 was mixed with 200 nM wild-type FBF-2(164-575) in the same buffer as for the direct titration experiments and increasing concentrations of unlabelled competitor CPB-1 were added. IC₅₀ values were calculated by fitting the data to the Hill equation.

RNA binding assay

A constant concentration of 75 pM ³²P labeled *cyb-1* RNA (5' CGAAAUAAACAUUUUGUACCAUUCAGUC 3') was incubated for 1 hour at room temperature with increasing concentrations of FBF-2(164-575) alone or pre-incubated for 2 hours with 6 μ M MBP-CPB-1(1-80) in 10 mM Tris HCl (pH 7.5), 0.5 mM EDTA, 50 mM NaCl, 2 mM DTT, 0.1 mg/ml BSA, 0.02% Tween, 0.2 mg/ml tRNA. Samples were loaded onto native polyacrylamide gel, visualized and analyzed essentially as described in Wu et

al³¹. The FBF Y479A point mutant and the FBF Δ (479-485) deletion mutant were tested for binding to 32P labeled FBE RNA in the same conditions.

NMR spectroscopy

¹⁵N labeled CPB-1(32-80) was expressed in M9 minimal medium containing 1 g/l (¹⁵NH₄)₂SO₄ (Cambridge Isotope Laboratories) as the source of nitrogen. The protein was purified as described above and buffer exchanged into 10 mM Tris HCl (pH 6.0), 50 mM NaCl, 5 mM DTT. For complex reconstitution ¹⁵N labeled CPB-1(32-80) was mixed with 1.5 molar excess of FBF-2(164-575)/FBE RNA (5'-UGUGCCAUA-3') and the samples were concentrated to approximately 0.7 mM. NMR samples were prepared by adding 15% (v/v) D₂O. The 2D [¹⁵N, ¹H]-HSQC spectra were recorded at 298K and 700 MHz on a Bruker DRX spectrometer.

Circular dichroism measurements

The CD spectrum of CPB-1(1-80) was recorded on a JASCO J-815 CD spectropolarimeter at protein concentration of 47 μ M in 20 mM sodium phosphate (pH 8.0), 20 mM NaCl at 20°C in a 0.1 cm cuvette.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PUF	Pumilio and FBF
FBF	<i>fem-3</i> binding factor
CPEB	cytoplasmic polyadenylation element binding
CPB-1	cytoplasmic polyadenylation element binding protein homolog-1
UTR	untranslated region
GLD-3	germline development defective-3

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Highlights

- *C. elegans* proteins CPB-1 and FBF act together to regulate mRNA translation.
- The protein/protein interface is conserved with the FBF interacting partner GLD-3.
- CPB-1 competes with GLD-3 for binding to FBF.
- CPB-1 modulates the affinity of FBF for its putative mRNA target.

Figure 1. Mapping of the FBF-binding site within CPB-1

A) Domain organization of CPB-1 and FBF. CPB-1 contains two RRM domains and a zinc finger domain. The previously identified FBF-binding region (amino acids 1–80) is indicated. FBF contains 8 PUF repeats (R1 to R8) flanked by conserved regions Csp1a and Csp2 indicated as R1' and R8'. The bar represents 100 amino acids. **B)** *In vitro* binding assay of CPB-1 deletion fragments to FBF. His-tagged CPB-1 fragments were mixed with FBF and applied onto NiNTA resin. The resin was washed with buffer containing increasing concentrations of imidazole and the eluate was analyzed by SDS PAGE. Load indicates the samples that were used in the binding assay. FBF was eluted from the NiNTA resin with low imidazole concentration (upper panel) and was retained on the resin only when bound to the His-tagged CPB-1. **C)** Yeast two-hybrid analysis of the interaction of CPB-1 fragments with FBF. N- and C- terminal CPB-1 deletion mutants fused to the LexA DNA-binding domain were tested with FBF fused to the Gal4 transcriptional activation domain. CPB-1 (1–560) indicates full-length protein, vector indicates no CPB-1. The shortest CPB-1 fragment that strongly activated the *lacZ* reporter consisted of residues 40–70. Taken together, the *in vitro* and *in vivo* experiments indicate that the FBF-binding region of CPB-1 is contained within residues 40–60. Statistical analysis was performed using a two-tailed Student's t-test, P-values were computed relative to the no CPB-1 control. P-values less than 0.05 were considered statistically significant and are indicated with an asterisk (* P-value < 0.05). Results are representative of three independent experiments, all the P-values are reported in Supplementary Table 1.

Figure 2. Sequence alignment with GLD-3 shows sequence conservation between the FBF-binding region of GLD-3 and CPB-1. CPB-1 and GLD-3 do not co-exist on FBF

(A) CPB-1 and GLD-3 do not form a ternary complex with FBF. Protein complexes were covalently linked before analysis by SDS PAGE. The in-gel mobility of proteins alone and in complex is indicated on the left. The first panel from the left shows GLD-3 titration into a constant amount of FBF (increasing GLD-3 concentration is indicated with a triangle above the gel). The second panel shows GLD-3 titration to FBF pre-incubated with CPB-1 (constant amounts of protein are indicated with a rectangle). In the last two panels the order of addition of CPB-1 and GLD-3 to FBF was reversed. Increasing amounts of CPB-1 were added to FBF (third panel) and finally increasing amounts of CPB-1 were added to FBF pre-incubated with GLD-3. In the last experiment CPB-1 replaced GLD-3 on FBF. (B) Sequence alignment of CPB-1 with the FBF-binding region of GLD-3 (residues 864–874). Conserved residues are highlighted. The alignment was performed with ClustalW⁴⁶, asterisks indicate positions that have a fully conserved residue, colons indicate conservation between groups of strongly similar properties and periods indicate conservation between groups of weakly similar properties. (C) Pulldown assay with FBF and CPB-1 double alanine mutants. Residues that most affected binding to FBF were located in the conserved 40–47 region.

Figure 3. Alanine scan of the minimal FBF-binding region of CPB-1 identifies key residues
(A) Determination of CPB-1/FBF binding specificity by competition-binding assay. The relative affinity of CPB-1 alanine mutants for FBF was measured by competition-binding assay using fluorescently-labeled CPB-1 (fl-CPB-1). Fl-CPB-1 was bound to FBF in order to achieve 90% complex formation and was competed off with increasing concentrations of competitor CPB-1. The plot of the normalized polarization as a function of competitor CPB-1 concentration is shown for wild-type CPB-1 (wt) self-competition and representative CPB-1 alanine mutants. **(B)** Fluorescence polarization data were fit to a Hill equation to determine the apparent IC_{50} . The relative IC_{50} of each mutant relative to wild-type CPB-1 is shown. Experiments were performed at least in triplicate, asterisks indicate statistically significant P-values computed versus wt CPB-1 (* P-value <0.05). **(C)** The same alanine mutants were also tested for binding to FBF in the yeast two-hybrid system. **(D)** Additional mutations of the key CPB-1 residues were screened in the yeast-two hybrid assay for interaction with FBF. Asterisks indicate statistically significant P-values (* P-value <0.05) computed versus the vector control (no CPB-1).

Figure 4. Identification of FBF-2 amino acids that are necessary for the interaction with CPB-1
(A) Single alanine FBF mutants were tested for binding to CPB-1 with the NiNTA pulldown assay. The first two panels show the controls with CPB-1 or FBF only, mutation of FBF Tyr 479 to Ala completely disrupted the interaction with CPB-1 (fourth panel). **(B)** The affinity of fl-CPB-1 for FBF wild-type and FBF alanine mutants was determined by fluorescence polarization assays. Fluorescence polarization is plotted as a function of FBF concentration and fitted to the Hill equation to determine the apparent equilibrium dissociation constant K_d (shown in the table; the Hill coefficient was set to 1). The significance (* P-value < 0.05) was determined by Student-t-test versus wild type FBF. A graphical summary of the effect of single alanine mutations in the Y479-T485 loop is shown. **(C)** FBF residues that affect binding to CPB-1 are highlighted in the crystal structure of FBF-2 in complex with PME RNA²⁹. The 8 PUF repeats (R1–R8) are colored blue and grey, the loop connecting repeats seven and eight encompassing residues 479–485 is in red, key residues for the interaction with CPB-1 are shown as spheres.

Figure 5. CPB-1 alters the affinity of FBF for specific RNAs

(A) Representative EMSA gel determining FBF association with *cyb-1* RNA in the absence (left) or presence of CPB-1 (right). In gel migration of free RNA, binary and ternary complexes is indicated on the left with a black line, purple diamond and blue square respectively. **(B)** Quantification of gel shifts shown on the left. The fraction of bound RNA was plotted as a function of FBF concentration and fitted to the Hill equation to determine K_d . **(C)** Schematic representation of the modified yeast three-hybrid assay. **(D)** Cell based assays confirm the cooperative effects observed for the *cyb-1* RNA. Four additional RNAs were examined for stimulated binding in the presence of CPB-1. The “none” construct lacks an insert, an additional negative control, PME U9C, is included. The RNA sequences are shown in the table as is the fold activation determined by dividing binding activity in the presence of CPB-1 by the corresponding negative control experiment conducted in the absence of CPB-1. All of the values are normalized to cell density. Asterisks indicate P-values < 0.05.