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OLIGOMERIZATION OF EDEN-BP IS REQUIRED FOR SPECIFIC mRNA DEADENYLATION AND BINDING

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Abbreviations ARE, AU-Rich Element; EDEN, Embryo Deadenylation Element; EDEN-BP, EDEN-Binding Protein; 3'UTR, 3' Untranslated Region; IPTG, Isopropyl-Thio-Galactoside; GST, Glutathion-S-Transferase; EMSA, Electrophoretic Mobility Shift Assay; XEF1 α , *Xenopus* Elongation Factor 1 α ; RRM, RNA Recognition Motif.

Abstract.

Background. mRNA deadenylation (shortening of the poly (A) tail) is often triggered by specific sequence elements present within mRNA 3' untranslated regions and generally causes rapid degradation of the mRNA. In vertebrates, many of these deadenylation elements are called AU-Rich Elements (ARE). The EDEN (Embryo Deadenylation Element) sequence is a *Xenopus* "class III" ARE. The EDEN acts by binding a specific factor, EDEN-BP (EDEN-Binding Protein) that in turn stimulates deadenylation.

Results. We showed here that EDEN-BP is able to oligomerize. A 27 amino acids region of EDEN-BP was identified as a key domain for oligomerization. A mutant of EDEN-BP deleted of this region was unable to oligomerize, and a peptide corresponding to this region competitively inhibited the oligomerization of full-length EDEN-BP. Impairing oligomerization by either of these two methods specifically abolished EDEN-dependent deadenylation. Furthermore, impairing oligomerization inhibited the binding of EDEN-BP to its target RNA, demonstrating a strong coupling between EDEN-BP oligomerization and RNA binding.

Conclusions. These data showing that the oligomerization of EDEN-BP is required for binding of the protein on its target RNA and for EDEN-dependent deadenylation in *Xenopus* embryos will be important for the identification of cofactors required for the deadenylation process.

Introduction.

The post-transcriptional regulation of mRNAs in the cytoplasm is an important process implicated in controlling the dynamic changes in the expression of a number of regulatory proteins. Furthermore, in the early embryos of many, if not all, metazoans, gene transcription is essentially silent and the post-transcriptional controls exerted on the inherited maternal mRNAs are of paramount importance for the ensuing development. The post-transcriptional regulation affecting maternal mRNAs has been largely studied in *Xenopus* (*Xenopus laevis*) early embryos that are particularly suited to these studies due to their large size and availability. Studies in a number of laboratories have shown that the translation of maternal mRNAs is tightly correlated with the adenylation status; mRNAs that are polyadenylated are in general recruited into polysomes and translated whereas deadenylated mRNAs are released from polysomes (reviewed in Paillard and Osborne, 2003). In the large majority of cases in eukaryotes, deadenylation (shortening of the poly (A) tail) is the rate-limiting event of mRNA degradation. In *Xenopus* embryos, this is not the case as mRNA degradation does not immediately follow deadenylation. Although deadenylated mRNAs are not translated, they are stable until the blastula stage, several hours after fertilization (Audic et al., 1997; Voeltz and Steitz, 1998). Therefore, mRNA deadenylation and degradation, although causally coupled, are temporally separated. Targeting of a mRNA for deadenylation is generally achieved via specific cis sequence element(s) that reside within mRNA 3' untranslated regions (3'UTR). These cis elements bind specific proteins that in turn influence mRNA deadenylation. The rapid deadenylation of unstable mRNAs such as those encoding mammalian cytokines or proto-oncogenes is mediated by AU-rich elements (AREs) that have been divided into three classes, depending on the presence and the distribution of the core motif AUUUA (Chen and Shyu, 1995). A number of ARE-binding proteins have been characterized, and a role, either stimulatory or inhibitory, in mRNA deadenylation established for some of them (for a recent review see Barreau et al., 2005).

We have shown previously that a sequence termed EDEN (Embryo Deadenylation ElemeNt), and an associated RNA-binding protein (EDEN-BP) are responsible for the deadenylation of a subclass of mRNAs after fertilization of

Xenopus eggs (Paillard et al., 1998). Immunodepletion of EDEN-BP from egg extracts abolishes EDEN-dependent deadenylation activity (Paillard et al., 1998), that is restored by the addition of recombinant protein (Paillard et al., 2003). When EDEN-BP was depleted in developing embryos by the injection of specific antibodies or morpholino antisense oligonucleotides, an important disruption of somite segmentation was observed (Gautier-Courteille et al., 2004). Interestingly, a reporter RNA containing human c-jun ARE, a "class III" (non-AUUUA containing) ARE (Chen and Shyu, 1995), is rapidly deadenylated after injection in Xenopus embryos. This deadenylation is also abrogated by immunoneutralizing EDEN-BP, demonstrating that it depends on active EDEN-BP (Paillard et al., 2002). This similarity between human c-jun ARE and Xenopus EDEN sequences led us to propose that EDEN sequences define a subgroup of class III AREs (Paillard et al., 2002). EDEN-BP is one member of the CELF (CUG-binding and Etr3 like factors) family of RNA-binding proteins (for a recent review, Barreau et al., 2006) and human CUG-BP1/CELF1 can act as a deadenylation factor in HeLa cell extracts (Moraes et al., 2006).

As for most ARE/ARE-binding proteins, the molecular mechanism linking EDEN-BP binding to a RNA and rapid deadenylation remains essentially unknown. A first step toward understanding this mechanism is to identify the partners of EDEN-BP. Here, we have demonstrated using a biochemical assay that EDEN-BP can oligomerize, and we have mapped a domain required for this oligomerization. Moreover, using deletion mutants and a peptide derived from the interaction domain, we demonstrated the functional importance of EDEN-BP oligomerization for EDEN deadenylation and RNA binding. This is therefore an important step toward understanding the molecular composition of the protein complex that is targeted to mRNAs by the EDEN and is responsible for rapid deadenylation.

RESULTS

Identification of a 27 amino acid domain of EDEN-BP required for oligomerization

We have previously observed that EDEN-BP is able to interact with itself in a yeast two-hybrid assay (Bonnet-Corven et al., 2002). However, the yeast system can not be taken as a definitive proof that such interactions occur. Therefore, to more

clearly demonstrate an EDEN-BP/EDEN-BP interaction, a "GST pull-down" assay was developed. Radiolabelled, untagged EDEN-BP was incubated with GSH beads previously loaded with GST protein, or a fusion of GST with EDEN-BP. The eluate was analyzed by electrophoresis and autoradiography (Figure 1A). A significant percentage of the loaded protein was detected in the eluate of the GST-EDEN-BP column (lane 3), as compared with the eluate from the GST column (lane 2). Only a weak signal was detected when the unrelated protein *Xenopus* Elongation Factor 1 α (XEF1 α) was chromatographed on the GST-EDEN-BP column (lane 6), and this signal was not significantly above the background signal obtained after chromatography on the GST column (lane 5).

To ensure that the binding of EDEN-BP to the GST EDEN-BP fusion protein could occur in the context of the embryo, *Xenopus* eggs were injected with a mRNA encoding a GST-EDEN-BP fusion protein and incubated to allow synthesis of this protein. Undiluted cytoplasmic extracts containing the GST EDEN-BP fusion protein were made from the injected eggs and these extracts were complemented with ³⁵S labelled EDEN-BP or Firefly luciferase in a minimal amount of rabbit reticulocyte lysate. After incubation with GSH beads, the retained proteins were eluted and analysed by electrophoresis and after transfer to a nitrocellulose membrane. The ³⁵S labelled proteins were revealed by autoradiography and quantified (Figure 1B, upper panel). To ensure that equivalent amounts of the fusion protein had been retained on the GSH beads the membrane was then probed with an anti GST antibody (Figure 1B, lower panel). These analyses showed that the ³⁵S labelled EDEN-BP was retained on the beads (lane 2) whereas the unrelated ³⁵S labelled Firefly luciferase was not (lane 6). In these experiments, the ³⁵S labelled EDEN-BP and the endogenous EDEN-BP can both bind to the GST-EDEN-BP, hence the amount of input radiolabelled protein retained on the beads is less than when only recombinant protein is used as in Figure 1A. Importantly, addition of RNase A to the extract before incubation with the beads did not significantly decrease the amount of labelled EDEN-BP retained by the fusion protein on the GSH beads (in Figure 2B, upper panel, compare lanes 2 and 4).

These data confirm the yeast two-hybrid data that EDEN-BP interacts with itself. Furthermore, they demonstrate that this interaction is not dependent on the presence of RNA. Finally, this interaction is observed both in the context of the embryo cytoplasm and *in vitro* using proteins expressed in bacteria. We will refer to

the self association of EDEN-BP as oligomerization, as it can not be determined from these experiments if EDEN-BP forms a dimer or a higher oligomer.

We next used the GST-pull-down assay to identify the domain of EDEN-BP that is required for oligomerization. In preliminary experiments, fragments of EDEN-BP that were successively shortened from the COOH-terminal extremity were synthesised in reticulocyte lysates and tested for their capacity to be retained on GST-EDEN-BP beads. We observed that a fragment of EDEN-BP covering amino acids 1 to 238 (see Figure 2 for a schematic diagram of EDEN-BP) was efficiently retained on the GST-EDEN-BP, whereas this was not the case for the fragment covering amino acids 1 to 155 (data not shown). These preliminary results indicated that the 84 amino acids region of EDEN-BP between residues 155 and 238 is a key determinant in EDEN-BP oligomerization. This was tested by constructing a deletion mutant of EDEN-BP that was devoid of amino acids 155 to 238 (EDEN-BP Δ 155-238). As indicated in the diagram in Figure 2, amino acid 155 borders the second RNA Recognition Motifs (RRM), therefore this deletion covers part of the linker region between RRMs 2 and 3. The ability of this deletion mutant to associate with EDEN-BP was assayed in a GST-pull-down assay in parallel with the full length protein (Figure 2). As previously shown, full-length EDEN-BP was retained (lane 6). Relative to the amount of full length protein retained on the beads, about 3 times less of this mutant EDEN-BP was retained on the GST-EDEN-BP beads (lane 7). To further delimit the region of EDEN-BP required for oligomerization, a second mutant protein was made in which the central one third (amino acids 184 to 210) of this 84 amino acid region was deleted. When analysed in the GST-pull-down assay with full length GST EDEN-BP, the EDEN-BP Δ 184-210 mutant was significantly less well retained on the column (Figure 2, lane 8). As a control, the capacity of GST EDEN-BP to capture a 52kDa portion of an unrelated protein (*Xenopus* CAP-D2, previously referred to as pEg7 (Cubizolles et al., 1998)) was tested. Although some of this N-terminal fragment of XCAP-D2 protein was retained, the amount was about 4 times less than that for the full length EDEN-BP and about half of that for the EDEN-BP Δ 184-210 mutant. These results identify a region of 27 amino acids, which is necessary for the oligomerization of EDEN-BP; they do not imply that this region is sufficient for oligomerization. Furthermore, we can not formally exclude that EDEN-BP molecules do not interact via other regions of the protein.

Mutant EDEN-BP deleted of the 27 amino acids required for oligomerization does not support rapid deadenylation

We have previously shown that EDEN-BP provokes the rapid deadenylation of EDEN-containing RNAs (Paillard et al., 1998). To test if the oligomerization of EDEN-BP is required for deadenylation we used cytoplasmic egg extracts that are proficient for EDEN-dependent deadenylation (Legagneux et al., 1995) and an EDEN-BP immunodepletion/rescue protocol. EDEN-dependent deadenylation is characterised by the appearance of completely deadenylated (A^-) transcript containing an EDEN ("EDEN", Gb-Eg2-410 (Legagneux et al., 1995)) upon incubation in these extracts (see Figure 3, lanes 1-3). Transcripts devoid of an EDEN or other regulatory sequence elements are also deadenylated in these extracts, albeit much less rapidly, by a "default" process (see the gradual shortening of the "Default" transcript (Gb-Eg2-410A (Legagneux et al., 1995))). For the default transcript no completely deadenylated molecules are formed even after 3 hours of incubation (see Figure 3, lanes 1-3). We have previously shown (Paillard et al., 1998) that immunodepletion of EDEN-BP from egg extracts abolishes EDEN-dependent deadenylation and that the addition of recombinant, full-length EDEN-BP, can restore EDEN-dependent deadenylation (Paillard et al., 2003). Default deadenylation is not affected by these changes in EDEN-BP. Therefore to analyse the effect of EDEN-BP oligomerisation on EDEN-dependent deadenylation the above mentioned transcripts (radiolabelled and capped) were incubated in extracts depleted or not of EDEN-BP (Figure 3, lanes 4–12 and 1-3 respectively). The EDEN-BP depleted extracts were complemented with wheat germ lysate that had been either left unprogrammed by a mRNA (lanes 4–6) or programmed with mRNAs encoding full length EDEN-BP (lanes 7–9) or the EDEN-BP Δ 184-210 mutant (lanes 10-12). As expected the immunodepletion of EDEN-BP abrogated the EDEN-dependent deadenylation (lanes 4–6) and this activity was restored by the addition of full length EDEN-BP (lanes 7–9). In contrast, the EDEN-BP Δ 184-210 mutant was unable to restore EDEN-dependent deadenylation (lanes 10–12). Full-length and mutant EDEN-BP were produced in the presence of trace amounts of ^{35}S -aminoacids, allowing a verification that similar concentrations of these two proteins were added (Figure 3, right panel). Hence, the 27 amino acids deletion mutant that is unable to oligomerize (Figure 2) does not support EDEN-dependent deadenylation.

A 28 amino acids peptide inhibits both oligomerization and EDEN-dependent deadenylation

The above data strongly suggest that the oligomerization of EDEN-BP is required for EDEN-dependent deadenylation. However, the incapacity of mutant EDEN-BP to support EDEN deadenylation could also be an indirect consequence of the deletion of the domain required for oligomerization, for instance if this deletion modified some structural aspects of EDEN-BP. To confirm that oligomerization is required for EDEN deadenylation, we designed another tool to inhibit EDEN-BP oligomerization, namely, a peptide corresponding to the sequence of the amino acids of EDEN-BP required for oligomerization that should compete with full-length EDEN-BP, and thereby inhibit oligomerization by site occupation.

A peptide corresponding to the region 183-210 of EDEN-BP was chemically synthesized. As a control, another peptide, containing the same amino acids as peptide 183-210 but in a different order ("scrambled" peptide) was also synthesized. The effects of these two peptides on oligomerization were tested in a GST-Pull-Down assay (Figure 4A). As above, a strong signal corresponding to EDEN-BP was detected in the eluate of the GST-EDEN-BP beads (0 peptide, lane 2). This signal was reduced about 3 fold when the incubation was performed in the presence of 100 or 400 μ M of peptide 183-210 (lanes 3 and 4). In contrast, the same concentration of the Scrambled peptide had no effect on the capacity of the GST-EDEN-BP beads to retain radiolabelled EDEN-BP (lanes 5 and 6).

We next tested the effects of the 183-210 and scrambled peptides on deadenylation in egg extracts. Various concentrations of the scrambled peptide were added to a deadenylation-proficient egg extract, and two test transcripts were incubated in these supplemented extracts (Figure 4B). Scrambled peptide concentrations up to 400 μ M (upper panel) had no detectable effect on either EDEN-dependent deadenylation, as measured with the "EDEN" transcript, or on default deadenylation ("Default" transcript). In the same experiment, 400 μ M of peptide 183-210 (lower panel, lanes 13-16) almost completely inhibited EDEN-deadenylation, but had no effect on the default deadenylation. 160 or even 64 μ M of peptide 183-210 also hampered EDEN-deadenylation though less efficiently and still had no effect on default deadenylation (lanes 5-12). Quantification of these data (Figure 4C, left panel), showed that the percentage of completely deadenylated EDEN-containing transcript increased with the time of incubation, but less and less rapidly as

increasing amounts of peptide 183-210 were added. The rate at which the deadenylated transcript accumulated is a direct indication of the deadenylation efficiency (right panel). Comparison of these efficiencies for the various samples confirmed that the Scrambled peptide had no effect on EDEN-dependent deadenylation, whereas this deadenylation was decreased by peptide 183-210 in a dose-dependent manner, reaching 10% of the control with 400 μ M of peptide. Therefore, peptide 183-210, but not a scrambled version of this peptide, specifically inhibits EDEN-dependent deadenylation.

Three supplementary controls for the specificity of the peptide 183-210 were made. First, we verified that the effect that was previously observed in cell-free extracts was the same *in vivo*. The capped, radiolabelled, EDEN-containing transcript was injected into *Xenopus* embryos, together with water (Figure 5 upper panel, lanes 1-4), or 200 ng of peptide 183-210 (lanes 9-12). Assuming a homogenous distribution of the injected peptide inside the cell, this would correspond to a peptide concentration of about 300 μ M. The adenylation behaviour of the reporter transcript was analyzed by denaturing electrophoresis and autoradiography of RNAs extracted at different times after injection (Figure 5, upper panel). As in extracts (Figure 4B), when injected with water, the reporter RNA was very rapidly shortened to the position of the completely deadenylated transcript (lanes 1-4), whereas this deadenylation was significantly reduced by the 183-210 peptide (lanes 9-12). Second, we verified that this inhibition of the EDEN-dependent deadenylation was not observed with any peptide derived from the sequence of EDEN-BP. A peptide of the same length as the 183-210, and corresponding to the sequence of EDEN-BP immediately adjacent (155-182) was chemically synthesized. When injected into *Xenopus* embryos (200 ng/embryo), this peptide had virtually no effect on the rapid deadenylation of the EDEN-containing transcript (Figure 5 upper panel, lanes 5-8).

Third, we verified that the inhibitory effect of the peptide 183-210 on EDEN-dependent deadenylation was observed with substrates other than the Eg2-derived EDEN-containing transcript used in previous figures. Accordingly, two additional transcripts were used, the synthetic globin ORF (Gb ORF, (Audic et al., 1997)) and this same mRNA containing the c-jun ARE in the 3'UTR (GbORFjun (Paillard et al., 2002)). The GbORF mRNA is a substrate for default deadenylation whereas the GbORFjun mRNA is deadenylated by the EDEN-dependent process (Audic et al., 1997; Paillard et al., 2002). As shown in Figure 5 (middle panel) the slow

deadenylation of the GbORF mRNA injected into embryos was not affected by the co-injection of 200 ng of the 183-210 peptide. In contrast the deadenylation of the GbORFjun transcript was strongly inhibited by the 183-210 peptide (Figure 5 lower panel, compare lanes 4-6 with 1-3). We have shown above that this peptide has no effect on the slow, default-type deadenylation (see Figure 4B). We also observed that it did not inhibit the AUUUA-dependent deadenylation, a rapid mechanism in *Xenopus* embryos that does not require active EDEN-BP (Paillard et al., 2002; Voeltz and Steitz, 1998) (data not shown). Hence, peptide 183-210 specifically inhibits the deadenylation of EDEN-containing transcripts in *Xenopus* embryos or extracts, but not other types of deadenylation.

Oligomerization of EDEN-BP is required for RNA binding

We previously observed that binding of EDEN-BP to the EDEN motif required sequence information proximal to amino acid 320 (Bonnet-Corven et al., 2002). This suggested to us that RNA binding may require EDEN-BP oligomerization. To test this, an Electrophoretic Mobility Shifts Assay (EMSA) was used. Increasing concentrations of purified recombinant, His-tagged EDEN-BP were incubated with radiolabelled s3'Eg5wt RNA. This RNA was chosen as an EMSA probe since it contains all the necessary sequence information to be a target of the EDEN-dependent mechanism when injected in *Xenopus* embryos, but is reasonably short (120 nucleotides) (Paillard et al., 1998). Two radiolabelled complexes were detected (Figure 6A). As the reaction mix only contains purified recombinant EDEN-BP and s3'Eg5wt RNA, they probably correspond to increasing states of oligomerization of EDEN-BP on this target RNA. Based on three independent experiments, the concentration of EDEN-BP required to achieve 50% of bound s3'Eg5wt RNA is 15 +/- 5 nM. To verify the specificity of these complexes, the same experiment was reproduced using s3'Eg5C6 as an EMSA probe. This RNA contains a 6 nucleotides mutation (C6) within the EDEN sequence that is sufficient to strongly decrease EDEN-BP binding in UV-cross-linking or affinity chromatography assays (Paillard et al., 1998). The EMSA pattern obtained with this probe was very different, and a small amount of bound probe was only detected with the highest concentrations of EDEN-BP tested (Figure 6B); the concentration of EDEN-BP required to achieve 50% of binding of this mutant probe is therefore far more than 200 nM.

When an EMSA was made in the presence of 0.3 mM of peptide 183-210, binding to s3'Eg5wt RNA was strongly impaired (Figure 6C). Virtually no bound probe was detected even with the highest concentrations of EDEN-BP tested. Finally, the peptide "Scrambled" did not alter the EMSA pattern (Compare Figure 6D to 6A) nor the concentration of EDEN-BP required to achieve 50% binding of the probe (14 +/- 4 nM, three independent experiments). Therefore, EDEN-BP requires oligomerization to bind the RNA substrate.

DISCUSSION

In this article, we demonstrated by a biochemical assay that EDEN-BP is able to interact with itself, and a 27 amino acids domain was mapped that is necessary for the interaction. A self-interaction of EDEN-BP was previously suggested based on yeast two-hybrid data (Bonnet-Corven et al., 2002). Two complementary tools were developed to analyze the requirement of this interaction for the functions of EDEN-BP. First, a mutant of EDEN-BP devoid of the 28 amino acids domain did not oligomerize and did not support EDEN-dependent deadenylation in a depletion/rescue assay. Second, a peptide corresponding to this 28 amino acids domain impaired EDEN-BP oligomerization, and inhibited EDEN-dependent deadenylation both *in vitro* (extract) and *in vivo* (embryo). These results show that the oligomerization of EDEN-BP is required for rapid, EDEN-type deadenylation.

The 28 amino acids EDEN-BP derived peptide that inhibited oligomerization and EDEN-dependent deadenylation also inhibited the binding of EDEN-BP to a substrate RNA, demonstrating that oligomerization is required for RNA binding. Examples of RNA-binding proteins that are able to oligomerize have been reported, and, in some cases, a reciprocal dependence of oligomerization and RNA binding was analysed. AUF1 was shown to be present as dimers in the absence of RNA. Upon RNA binding, AUF1 dimers are sequentially recruited to the host RNA, forming tetramers and higher polymers of dimers (Wilson et al., 1999). U1A is able to homodimerize even in mutants that have lost their capacity to interact with RNA; however, in the wild-type protein, homodimerization permits cooperative binding (Klein Gunnewiek et al., 2000). A mutant FMR1 (FMR1 I304N) that is unable to oligomerize can still interact with RNA (Laggerbauer et al., 2001). In these three

examples, RNA binding and oligomerization could be uncoupled, though oligomerization could reinforce binding. In contrast, it was shown by mutating key residues involved in homodimerization (as predicted from structural data) that yeast She2p dimerization is required for RNA binding (Niessing et al., 2004). This last situation is evocative of the strong relationship between RNA-binding and oligomerization that we describe here for EDEN-BP.

The *Drosophila* protein Bruno is closely related to *Xenopus* EDEN-BP and acts as a translational repressor in oocytes (Castagnetti et al., 2000; Lie and Macdonald, 1999). Recently, Chekulaeva et al. (2006) showed that in *Drosophila* oocytes the binding of Bruno to oskar mRNA caused a oligomerisation of the mRNA in a silencing particle. This particle contains Bruno, Cup an eIF4E binding protein and Me31B that is a homolog of the yeast P-body component and translational repressor Dhh1P. To our knowledge it has not been determined whether Bruno alone can oligomerize but our data suggests that this may be the case and that the oligomerisation of oskar mRNA is driven by interactions between Bruno monomers.

EDEN-BP is one member of the CELF/Bruno like family of RNA-BPs. Alignment of the 184 - 210 region of EDEN-BP with the corresponding region of the other CELF proteins (Figure 7) showed that this region is highly conserved between the CELF1 (EDEN-BP and CUG-BP1) and CELF2 (ETR-3 and CUG-BP2) proteins. These two proteins also exhibit the highest degree of overall similarity (see Barreau et al, 2006). The conservation of this region is less for CELF 3, 4 and 5 and the least for CELF 6. However, it should be noted that a certain number of the amino acid changes are conservative changes (eg K for R at positions 191, 193 and 196; E for D at position 192). This observation suggests that most if not all of the different CELF proteins are also able to oligomerise and that they could form homo- or hetero-oligomers depending on the presence or absence of the different CELF proteins in the same cell. This potential flexibility in the RNA binding partner of a CELF protein may be at the base of the large variety of functions described for these proteins (see Barreau et al., 2006).

Several consequences can be envisaged to result from the oligomerisation of an RNA-BP. First, the binding site of the oligomerized proteins may not match a strict linear "consensus". The sequence could be either direct or an inverted repeat and each individual "motif" could be separated by a variable spacer that could be looped out when the RNA /protein complex formed. Second, the oligomerization could create

new surfaces with which partners can interact and these surfaces could evolve with the degree of oligomerisation. In this context it is interesting that Chekulaeva et al. (2006) observed that the protein Cup was present in the heavy but not the light RNP complexes. This would mean that oligomerization of a RNA-BP on a RNA gives added value to binding specificity. Only those mRNAs that can bind two or more molecules of the RNA-binding protein will be a site for complex formation that is required to fulfil the specific function.

EXPERIMENTAL PROCEDURES

Recombinant protein production

EDEN-BP open reading frame was PCR amplified and cloned into the pTRC-His (Invitrogen) and pGEX2T (Amersham Pharmacia) plasmids to yield pTRC-His-EDEN-BP and pGEX2T-EDEN-BP. His-EDEN-BP and GST-EDEN-BP were produced from these respective plasmids in TOP10 bacteria by overnight induction at 25°C with 5 mM Isopropyl-Thiogalactoside (IPTG). They were purified on Ni-NTA agarose (Qiagen) or GST-Sepharose 4B (Amersham Pharmacia) columns following the instructions of the manufacturers.

To produce GST-EDEN-BP in vivo unfertilised *Xenopus* eggs were injected with 5 ng of in vitro synthesised GST-EDEN-BP mRNA and incubated for 4h at 20°C before making deadenylation proficient (Legagneux et al., 1995).

Radiolabelled EDEN-BP was produced by in vitro transcription and translation (ProteinScript II, Ambion) in the presence of ³⁵S-aminoacids using the pT7TS-EDEN-BP plasmid (Paillard et al., 2002) as a matrix. Radiolabelled COOH-shortened fragments of EDEN-BP were produced similarly, but the transcription matrices used were PCR products containing the fragments of EDEN-BP previously described (Bonnet-Corven et al., 2002) amplified with the following primers #82, **GCGTAATACGACTCACTATAGGGCTATGGCTGGGATCCGGTACCG** (contains a promoter for T7 RNA polymerase, bold), and #81, GTGACACTATAGAACCAGATCG. EDEN-BP Δ155-238 was produced as follows. First, the fragment corresponding to the amino acids 1 to 154 was PCR amplified with the following primers: #608, GAAGATCTGCC**ATGA**ATGGCACAATGGACC (contains a BglII site, italicized, and EDEN-BP translation initiation codon, bold) and #549,

CCACTTAGGGAGTTGAGGTTTCGTAACGAATGCACATCCTC. The fragment corresponding to the amino acids 239 to 489 was PCR amplified with the following primers: #550, GAGGATGTGCATTCGTTACGAACCTCAACTCCCTAAGTGG and #609, GCTCTAGAT**C**AGTAGGGTTTGCTGTCATTC (contains a XbaI site, italicized and EDEN-BP translation termination codon, bold). Primers #549 and #550 are complementary. Second, the two PCR products were mixed and used for a second round of PCR with primers #608 and #609. The resulting amplicon was digested with BglII and XbaI and cloned in the BglII and SpeI sites of the pT7TS vector (Cleaver et al., 1996). EDEN-BP Δ 184-210 was similarly produced, except that the first rounds of PCR used primers #543, CCAGTCAGGTTACCCACATTGAGGCCACTATTGGTGAGGAACAGCCC (instead of #549), and #544, GGGCTGTTCTCACC AATAGTGGCCTCAATGTGGGGTAACCTGACTGG (instead of #550). These internal mutant deletions were produced either as radiolabelled proteins with the ProteinScriptII kit as above or, for rescue experiments, by in vitro transcription (Message Machine, Ambion) and translation in nuclease-treated Wheat Germ Extract (Promega) in the presence of trace amounts of 35 S-aminoacids. The control radiolabelled Xenopus Elongation Factor 1 α was produced from the pTRI-XEF plasmid provided by Ambion. The portion of Xenopus CAP-D2 (XCAPD2 previously named pEg7) (Cubizolles et al., 1998) cDNA corresponding to the 465 N-terminal amino acids was cloned into the pT7TS vector. The corresponding radiolabelled protein (52.2 kDa) was produced with ProteinScript kit as above.

Peptides

Peptides used were synthesized and HPLC-purified by the Service Protéomique de l'Est du Québec, Québec, Canada. They were dissolved in water at 10 mg/ml (about 3 mM), aliquoted and stored at -70°C. Their sequences are:

155-182: FTTRSMAQMAIKSMHQAQTMEGCSSPIV

183-210: VKFADTQKDKEQKRMTQQLQQQMQLNA

Scrambled of 183-210: ATQLQEDQVQANQQQMQRMKKDKFTQL.

Biochemical methods

For each GST-pull down assay, 2 μ g of proteins (GST or GST-EDEN-BP) were incubated with 40 μ l of GSH-Sepharose beads (Amersham Pharmacia), rinsed

and equilibrated in H buffer (Tris 50 mM pH 7.8; NaCl 50 mM; EDTA 1mM; DTT 1 mM; NP-40 1% supplemented with 10 µg/ml (each) of leupeptin, pepstatin and chymostatin). The protein beads were then incubated (2h, 4°C) with 5 µl of radiolabelled protein diluted in 5 µl of H buffer and containing the appropriate peptides when required. Beads were rinsed 3 times by 1 ml of H buffer and eluted by boiling in SDS buffer. The eluted proteins were separated by denaturing electrophoresis and transferred to a nitrocellulose membrane. The ³⁵S labelled EDEN-BP was revealed by autoradiography using phosphoimager screen. The amount of bound protein was quantified using a Phosphoimager (STORM 840, Molecular Dynamics). Quantifications were made using the ImageQuant software (Version 5.4, Molecular Dynamics).

For GST-pull down assays using in vivo translated GST-EDEN-BP, 25 µl of extract from embryos injected with GST-EDEN-BP mRNA were mixed with 5µl of Rabbit reticulocyte lysate previously programmed with mRNA encoding either EDEN-BP or Firefly Luciferase (Promega) and supplemented with ³⁵S-aminoacids. The resulting solution was incubated for 1h at 20°C to facilitate integration of the ³⁵S labelled EDEN-BP into pre-formed complexes and then processed as above. Where indicated RNase A (1ng/µl) was added to the solution of GST-EDEN-BP and ³⁵S labelled EDEN-BP that was then incubated (1h, 20°C) before addition of the GSH beads.

For Electrophoretic Mobility Shift Assays (EMSA), serial dilutions of His-EDEN-BP were made in the following buffer: HEPES 70mM (pH=7.9); KCl 150 mM; EDTA 0.08 mM; MgCl₂ 2 mM; DTT 0.2 mM; Glycerol 8%; Yeast tRNA 2µg/µl; Heparin 6.6 µg/µl; BSA 6.6 µg/µl; IGEPAL-CA30 0.06%, and peptide when required. Radiolabelled probes were made by in vitro transcription with α³²P-UTP and the Promega Riboprobe products using BamH1-linearized s3'Eg5wt and s3'Eg5C6 plasmids (Paillard et al., 1998). 3 nM of heat-denatured radiolabelled transcript were added to the diluted proteins and incubated for 1h on ice. Complexes were separated by electrophoresis on 5% polyacrylamide gel in 0.5X TBE and 5% glycerol. The dried gels were submitted to Phosphoimager (STORM 840, Molecular Dynamics) analysis. Quantifications were made using the ImageQuant software (Version 5.4, Molecular Dynamics).

Deadenylation analyses

For deadenylation analyses, capped, polyadenylated, uniformly radiolabelled GbEg2-410, GbEg2-410A, GbORF and GbORFjun transcripts were obtained by *in vitro* transcription in the presence of $\alpha^{32}\text{P}$ -UTP using the Promega Riboprobe products and the corresponding plasmids (Audic et al., 1997; Legagneux et al., 1995; Paillard et al., 2002) previously linearized with EcoRV. These transcripts were injected, alone or with peptides, into two-cell embryos. Alternatively, RNAs were incubated in 5 μl of deadenylation-proficient activated egg extracts (Legagneux et al., 1995). In some experiments, these extracts were mock depleted or immunodepleted of EDEN-BP as previously described (Paillard et al., 1998). Complementation with full-length or mutant EDEN-BP was made by mixing one volume of depleted extract with one volume of programmed wheat germ extract (Paillard et al., 2003). At the indicated times, pools of 5 embryos, or 5 μl of extract, were crushed in Tri-reagent (Euromedex), and RNAs were extracted following the instructions of the manufacturer. They were resolved on 4% polyacrylamide-urea gels before phosphoimager analysis (STORM 840, ImageQuant software). Quantifications were made using the ImageQuant software (Version 5.4, Molecular Dynamics).

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REFERENCES

- Audic, Y., Omilli, F. and Osborne, H.B. (1997) Postfertilization deadenylation of mRNAs in *Xenopus laevis* embryos is sufficient to cause their degradation at the blastula stage. *Mol. Cell. Biol.* **17**, 209-218.
- Barreau, C., Paillard, L., Mereau, A. and Osborne, H.B. (2006) Mammalian CELF/Bruno-like RNA-binding proteins: molecular characteristics and biological functions. *Biochimie* **88**, 515-525.
- Barreau, C., Paillard, L. and Osborne, H.B. (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res.* **33**, 7138-7150.
- Bonnet-Corven, S., Audic, Y., Omilli, F. and Osborne, H.B. (2002) An analysis of the sequence requirements of EDEN-BP for specific RNA binding. *Nucleic Acids Res.* **30**, 4667-4674.
- Castagnetti, S., Hentze, M.W., Ephrussi, A. and Gebauer, F. (2000) Control of oskar mRNA translation by Bruno in a novel cell-free system from *Drosophila* ovaries. *Development* **127**, 1063-1068.
- Chekulaeva, M., Hentze, M.W. and Ephrussi, A. (2006) Bruno acts as a dual repressor of oskar translation, promoting mRNA oligomerization and formation of silencing particles. *Cell* **124**, 521-533.
- Chen, C.-Y.A. and Shyu, A.-B. (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci.* **20**, 465-470.
- Cleaver, O.B., Patterson, K.D. and Krieg, P.A. (1996) Overexpression of the tinman-related genes *XNkx-2.5* and *XNkx-2.3* in *Xenopus* embryos results in myocardial hyperplasia. *Development* **122**, 3549-3556.
- Cubizolles, F., Legagneux, V., Le Guellec, R., Chartrain, I., Uzbekov, R., Ford, C. and Le Guellec, K. (1998) pEg7, a new *Xenopus* protein required for mitotic chromosome condensation in egg extracts. *J Cell Biol.* **143**, 1437-46.
- Gautier-Courteille, C., Le Clainche, C., Barreau, C., Audic, Y., Graindorge, A., Maniey, D., Osborne, H.B. and Paillard, L. (2004) EDEN-BP-dependent post-transcriptional regulation of gene expression in *Xenopus* somitic segmentation. *Development* **131**, 6107-6117.
- Klein Gunnewiek, J.M., Hussein, R.I., van Aarssen, Y., Palacios, D., de Jong, R., van Venrooij, W.J. and Gunderson, S.I. (2000) Fourteen residues of the U1

- snRNP-specific U1A protein are required for homodimerization, cooperative RNA binding, and inhibition of polyadenylation. *Mol. Cell. Biol.* **20**, 2209-2217.
- Laggerbauer, B., Ostareck, D., Keidel, E.M., Ostareck-Lederer, A. and Fischer, U. (2001) Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum. Mol. Genet.* **10**, 329-338.
- Legagneux, V., Omilli, F. and Osborne, H.B. (1995) Substrate-specific regulation of RNA deadenylation in *Xenopus* embryo and activated egg extracts. *RNA* **1**, 1001-1008.
- Lie, Y.S. and Macdonald, P.M. (1999) Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in *Drosophila* ovarian extracts. *Development* **126**, 4989-4996.
- Moraes, K.C., Wilusz, C.J. and Wilusz, J. (2006) CUG-BP binds to RNA substrates and recruits PARN deadenylase. *RNA* **12**, 1084-1091.
- Niessing, D., Huttelmaier, S., Zenklusen, D., Singer, R.H. and Burley, S.K. (2004) She2p is a novel RNA binding protein with a basic helical hairpin motif. *Cell* **119**, 491-502.
- Paillard, L., Legagneux, V., Maniey, D. and Osborne, H.B. (2002) c-Jun ARE targets mRNA deadenylation by an EDEN-BP (embryo deadenylation element-binding protein)-dependent pathway. *J. Biol. Chem.* **277**, 3232-3235.
- Paillard, L., Legagneux, V. and Osborne, H.B. (2003) A functional deadenylation assay identifies human CUG-BP as a deadenylation factor. *Biol. Cell.* **95**, 107-113.
- Paillard, L., Omilli, F., Legagneux, V., Bassez, T., Maniey, D. and Osborne, H.B. (1998) EDEN and EDEN-BP, a cis element and an associated factor that mediates sequence-specific mRNA deadenylation in *Xenopus* embryos. *EMBO J.* **17**, 278-287.
- Paillard, L. and Osborne, H.B. (2003) East of EDEN was a poly(A) tail. *Biol. Cell.* **95**, 211-219.
- Voeltz, G.K. and Steitz, J.A. (1998) AUUUA sequences direct mRNA deadenylation uncoupled from decay during *Xenopus* early development. *Mol. Cell. Biol.* **18**, 7537-7545.
- Wilson, G.M., Sun, Y., Lu, H. and Brewer, G. (1999) Assembly of AUF1 oligomers on U-rich RNA targets by sequential dimer association. *J. Biol. Chem.* **274**, 33374-33381.

FIGURES LEGENDS

Fig. 1 EDEN-BP oligomerizes in vitro and in vivo.

A, 5 μ l of Rabbit reticulocyte lysate programmed to synthesize radiolabelled Xenopus EDEN-BP, MW 51.8 kDa (lane 2, 3) or Elongation Factor 1 α XEF1 α , MW 50.2 kDa (lanes 5, 6) were mixed with GSH-Sepharose beads that had been previously incubated with bacterially produced GST (lanes 2, 5) or GST-EDEN-BP (G-E, lanes 3, 6). After incubation (90 min) and then extensive washing, the bound proteins were eluted by boiling in SDS buffer, and separated by denaturing electrophoresis. The radiolabelled proteins were revealed and quantified using a phosphoimager and ImageQuant software. Input corresponding to 1 μ l of reticulocyte lysate expressing EDEN-BP (lane 1) or XEF1 α (lane 4) were loaded on the gel as input controls. The percentages of input protein retained on the beads are indicated below the lanes. **B**, Upper panel, 25 μ l of Xenopus egg cytosol extract made from eggs previously injected with GST-EDEN-BP mRNA was mixed with 5 μ l of either EDEN-BP (lanes 2 and 4) or Firefly Luciferase (lane 6) produced in reticulocyte lysates supplemented with ^{35}S labelled amino acids and then incubated for 60 min. In lane 4, RNase A was added to the protein solution and the incubation continued for another 60 min. The samples were then processed and separated as in **A**. After electrophoresis the proteins were transferred to nitrocellulose membrane and the radiolabelled proteins were revealed and quantified using a phosphoimager and ImageQuant software. Inputs correspond to 3 μ l of the mixed protein solution. The percentages of input protein retained on the beads are indicated below the lanes. Lower panel, After exposure to phosphoimager screens the membrane was probed with anti-GST antibodies that were revealed with alkaline phosphatase coupled to the secondary antibody and ECF using a Storm 840.

Fig. 2 Mapping of the domain of oligomerization of EDEN-BP.

Overall structure of EDEN-BP is shown at the top. The positions of the three RNA Recognition Motifs (RRM) are indicated. GST pull down assays were performed as in Figure 1A. 5 μ l of reticulocyte lysate programmed to synthesize full-length (1-489, lane 6), the indicated mutants of EDEN-BP (lanes 7 and 8) or a portion of the unrelated XCAP-D2 protein (lane 5) were mixed with GSH-Sepharose beads previously incubated with GST-EDEN-BP. 1 μ l of reticulocyte lysate expressing the

same fragments of EDEN-BP were loaded on the gel as input controls (Input, lanes 1 - 4). For the different recombinant proteins proportions of the input proteins retained on the beads are indicated below the lanes.

Fig. 3 A domain of EDEN-BP required for oligomerisation is required for EDEN-dependent deadenylation.

Left panel. A deadenylation proficient egg extract was either untreated (lanes 1-3), or immunodepleted of EDEN-BP (lanes 4-12) and then supplemented with unprogrammed Wheat Germ extract (lanes 4-6) or Wheat Germ extract programmed to synthesize full length EDEN-BP (lanes 7-9) or EDEN-BP harbouring an internal deletion of the amino acids 184 to 210 (lanes 10-12). Capped, polyadenylated, radiolabelled Gb-Eg2-410 (EDEN) and Gb-Eg2-410A (Default) transcripts (Legagneux et al., 1995) were incubated in these extracts for the indicated times. RNAs were extracted and the adenylation behaviours of the transcripts were analyzed by denaturing electrophoresis. The radiolabelled RNAs were revealed and quantified using a phosphoimager and ImageQuant software. The positions of the polyadenylated (A^+) and deadenylated (A^-) RNAs are indicated on the left.

Right panel. Autoradiogram of the radiolabelled full length (lane 2) and $\Delta 184-210$ (lane 2) EDEN-BPs after separation from the unincorporated ^{35}S -labelled amino acid by SDS-PAGE.

Fig. 4. Peptide 183-210 inhibits EDEN-BP oligomerization and EDEN-dependent deadenylation.

A, 5 μl of reticulocyte lysate programmed to synthesize radiolabelled EDEN-BP were mixed with GSH-Sepharose beads previously incubated with GST-EDEN-BP (lanes 2-6), in the presence of the indicated concentration of peptide 183-210 (lanes 3 and 4) or Scrambled (Scrambled, lanes 5 and 6). Bound proteins were analyzed as described in the legend to Figure 1A. Lane 1 is an input control corresponding to 1 μl of reticulocyte lysate. The amount of EDEN-BP retained on the beads in the presence of the peptides, relative to that retained in the absence of peptides (lane 2) is indicated below the lanes. **B**, Capped, radiolabelled Gb-Eg2-410 (EDEN) and Gb-Eg2-410A (Default) transcripts were incubated in *Xenopus* activated eggs extracts, together with the indicated concentrations of peptide 183-210 (lower panel) or scrambled (upper panel). RNAs were extracted at the indicated times, and their

adenylation behaviour was analyzed as described in the legend to Figure 3. **C**, Quantification of the data shown in **B**. The left panel shows the percentage of the completely deadenylated form of the EDEN-containing transcript during the time of experiment. No peptide, black line, Scrambled peptide; 64 μ M purple squares, 160 μ M blue disc, 400 μ M red triangle; 183-210 peptide; 64 μ M open purple square, 160 μ M open blue triangle, 400 μ M red circle. Right panel, deadenylation efficiencies were estimated from the slopes of these lines, normalized to 100% for the control (no peptide) experiment, and are shown for the indicated concentrations of peptide 183-210 (grey) or scrambled (black).

Fig. 5. Specificity of the inhibitory activity of peptide 183-210.

Upper panel, A capped, radiolabelled Gb-Eg2-410 (EDEN) transcript was injected into *Xenopus* embryos, together with water (lanes 1-4), or 200 ng of the indicated peptides (lanes 5-12). RNAs were extracted at the times indicated above each lane. The adenylation behaviour of the injected transcript was analyzed by denaturing electrophoresis and autoradiography as described in the legend to Figure 3. The positions of the polyadenylated (A^+) and deadenylated (A^-) RNA are indicated on the left.

Middle panel, A capped, radiolabelled GbORF transcript was injected into *Xenopus* embryos, together with water (lanes 1-5), or 200 ng of peptides 183-210 (lanes 6-10). RNAs were extracted at the times indicated above each lane and analyzed as above. Lower panel, A capped, radiolabelled GbORFjun transcript was injected into *Xenopus* embryos, together with water (lanes 1-3), or 200 ng of peptides 183-210 (lanes 4-6). RNAs were extracted at the times indicated above each lane and analyzed as above.

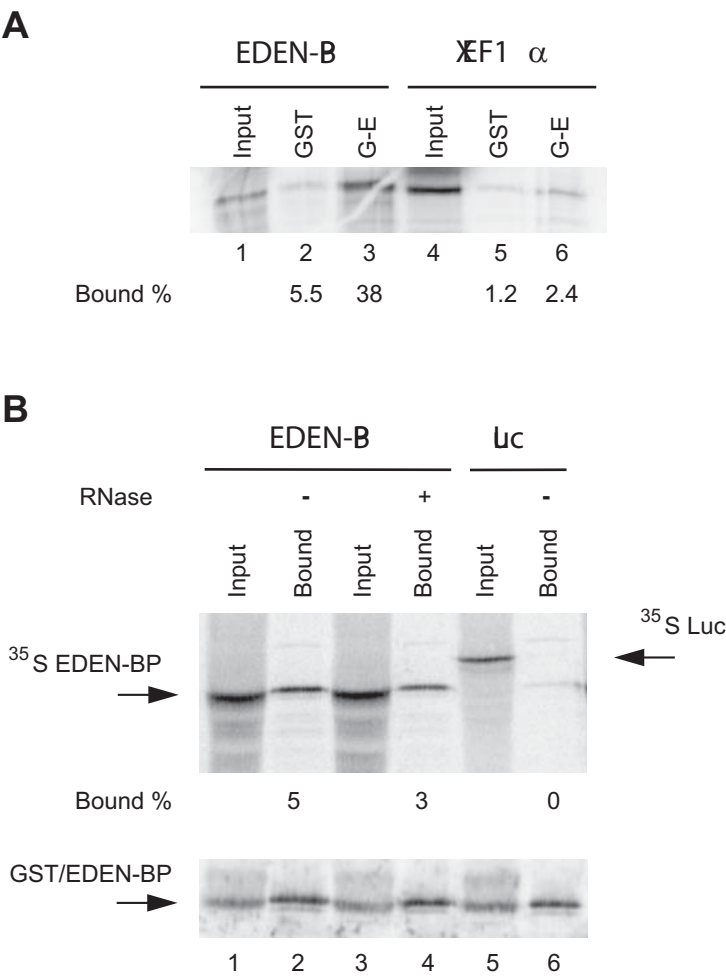
Fig. 6. Peptide 183-210 inhibits EDEN-BP binding to an EDEN.

3 nM of radiolabelled s3'Eg5wt (**A**, **C**, **D**) or s3'Eg5C6 (**B**) RNA was incubated with increasing concentrations of recombinant EDEN-BP (lane 1, 0; lane 2, 0.7 nM; lane 3, 3 nM; lane 4, 12 nM; lane 5, 50 nM; lane 6, 200 nM). In **C** and **D**, incubations were performed in the presence of 0.3 mM of peptide 183-210 and Scrambled respectively. RNA-protein complexes were resolved by native electrophoresis and the radiolabelled RNAs were revealed and quantified using a phosphorimager and ImageQuant software. The positions of the free and bound probes are indicated.

Fig. 7. Alignment of amino acids 184 to 210 of EDEN-BP with the corresponding regions of other CELF proteins.

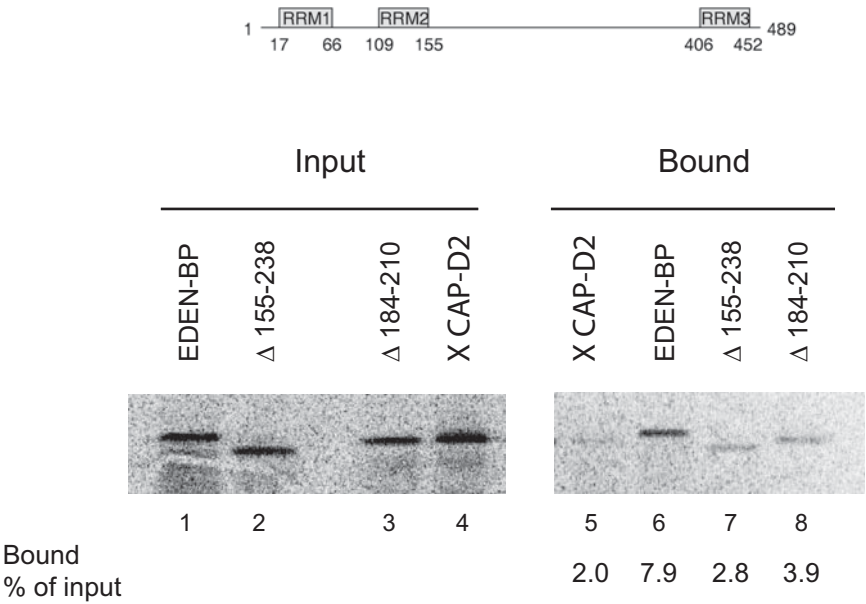
Xenopus laevis EDEN-BP and human CUG-BP1 (hsCUG-BP1) are orthologous CELF1 proteins. Xenopus laevis Etr-3 (Xl Etr-3) and human CUG-BP2 (hsCUG-BP2) are the corresponding CELF2 proteins. The human sequences for the other CELF proteins are shown. (*) indicates a conserved amino acids; (:) indicates a conservative replacement. Amino acids identical to the Xenopus EDEN-BP sequence are on a grey background.

Figure 1 Cosson et al.



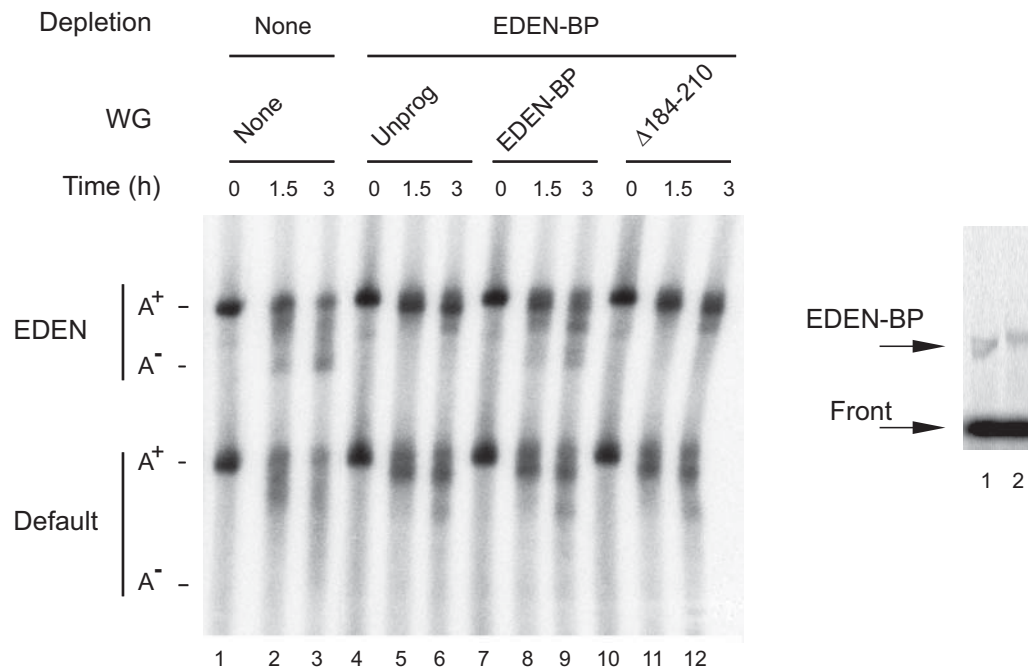
The final Version of Record is available at www.biolcell.org

Cosson et al. Figure 2 revised

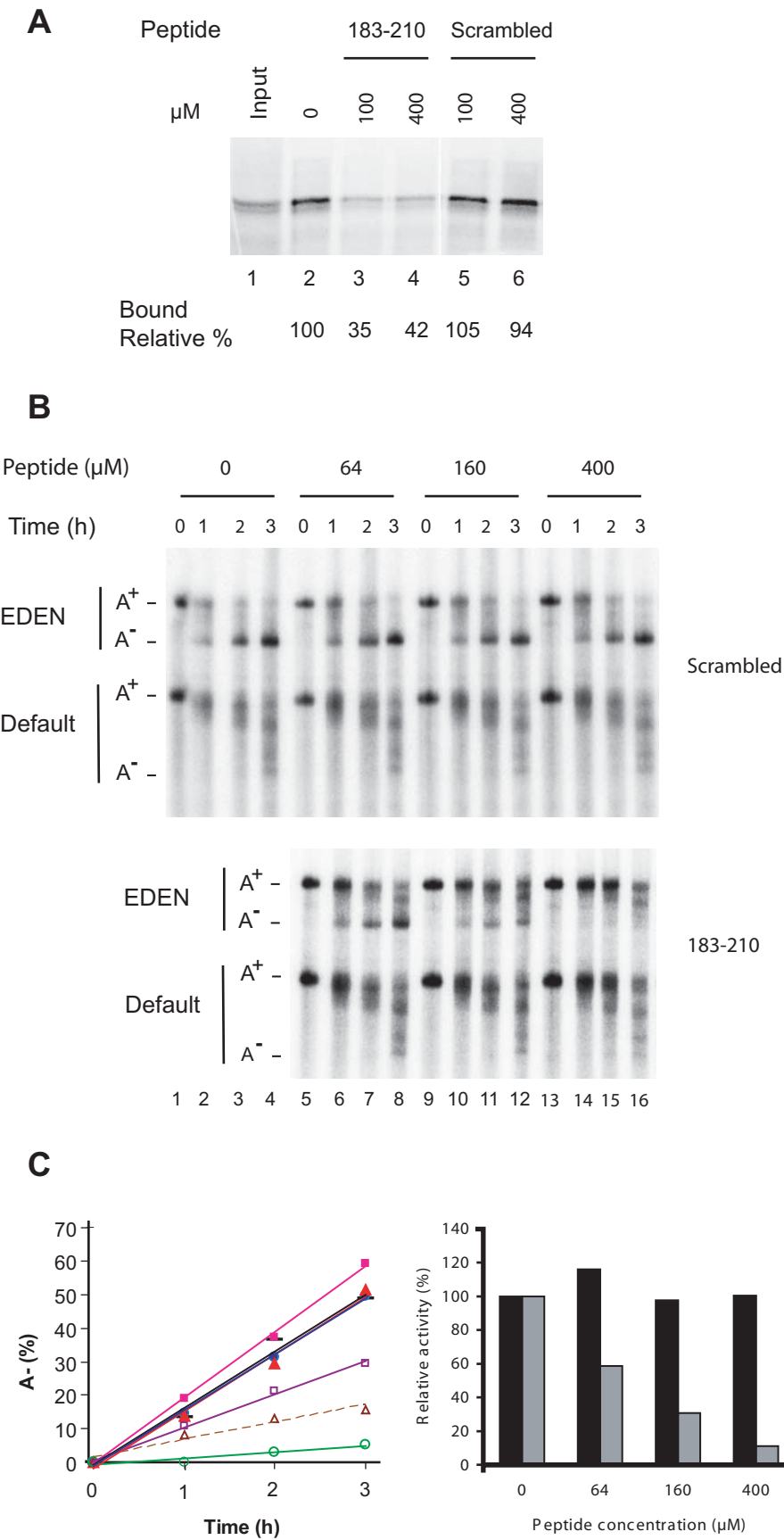


The final Version of Record is available at www.biolcell.org

Cosson et al. Figure 3

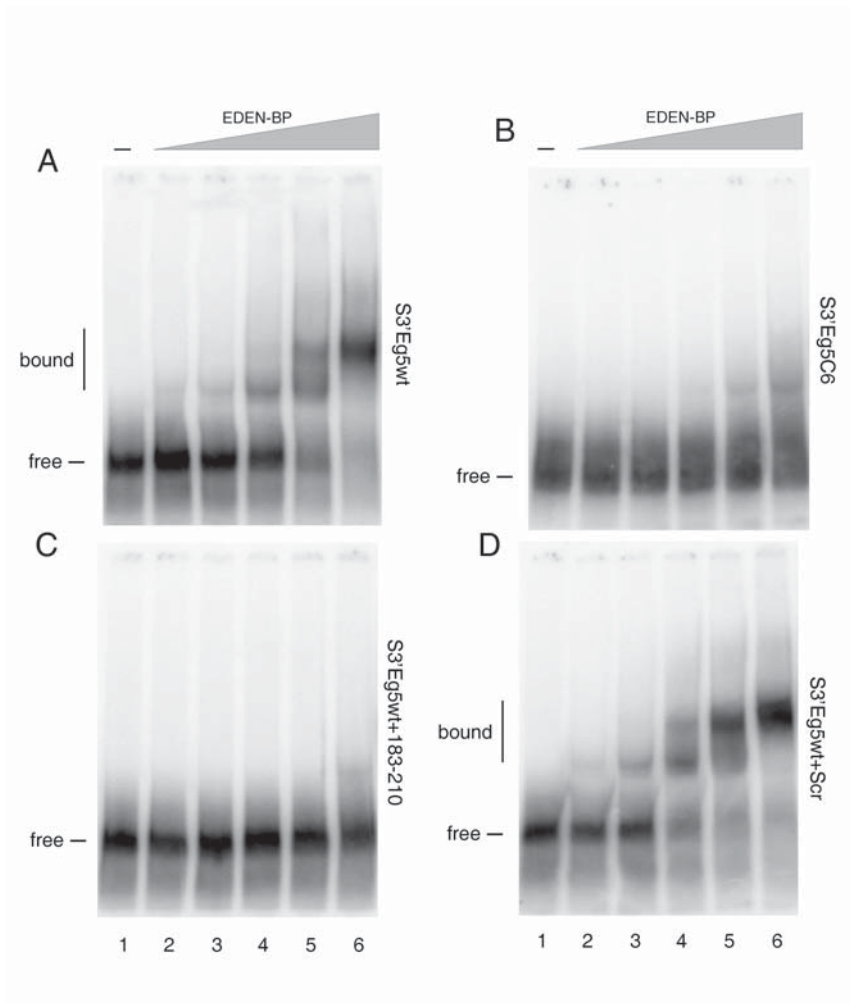


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Cosson et al. Figure 4



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Cosson et al. Figure 6



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Cosson et al BoC Figure 7

Xl	EDEN-BP	VKFADTQKDKEQKRMT	QQLQQQM	QQLNA
Xl	ETR-3	VKFADTQKDKEQRRLO	QQLAQQM	QQLNT
hs	CUG-BP1	VKFADTQKDKEQKRMA	QQLQQQM	QQLISA
hs	CUG-BP2	VKFADTQKDKEQRRLO	QQLAQQM	QQLNT
hs	CELF3	VKFADTEKERGLRRM	QQVATQL	GMFSP
hs	CELF4	VKFADTDKERTMRRM	QQMAGQM	GMFNP
hs	CELF5	VKFADTDKERTLRRM	QQMVGQL	GILTP
hs	CELF6	VKLADTDRERALRRM	QQMAGHL	GAFHP
		**	***	::: :: **