Role of cdc2 kinase phosphorylation and conserved N-terminal proteolysis motifs in cytoplasmic polyadenylation-element-binding protein (CPEB) complex dissociation and degradation

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Cytoplasmic polyadenylation-element-binding protein (CPEB) is a well-characterized and important regulator of translation of maternal mRNA in early development in organisms ranging from worms, flies and clams to frogs and mice. Previous studies provided evidence that clam and *Xenopus* CPEB are hyperphosphorylated at germinal vesicle breakdown (GVBD) by cdc2 kinase, and degraded shortly after. To examine the conserved features of CPEB that mediate its modification during meiotic maturation, we microinjected mRNA encoding wild-type and mutated clam CPEB into *Xenopus* oocytes that were subsequently allowed to mature with progesterone. We observed that (i) ectopically expressed clam CPEB is phosphorylated at GVBD and subsequently degraded, mirroring the fate of the endogenous *Xenopus* CPEB protein, (ii) mutation of nine Ser/Thr Pro-directed kinase sites prevents phosphorylation and degradation and (iii) deletion of the PEST box, and to a lesser extent of the putative cyclin destruction box, generates a stable and phosphorylated version of CPEB. We conclude that phosphorylation of both consensus and non-consensus sites by cdc2 kinase targets clam CPEB for PEST-mediated destruction. We also show that phosphorylation of CPEB mediates its dissociation from ribonucleoprotein complexes, prior to degradation. Our findings reinforce results obtained in *Xenopus*, and have implications for CPEB from other invertebrates including *Drosophila*, *Caenorhabditis elegans* and *Aplysia*, which lack PEST boxes.

Key words: maternal mRNA, oocyte maturation, PEST box, *Spisula solidissima*, translational masking.

INTRODUCTION

During meiotic maturation and early embryogenesis, specific maternal mRNAs undergo dramatic activation from translational dormancy in the oocyte, and their products, including c-Mos, cyclins and ribonucleotide reductase, drive or enable completion of meiosis and entry into mitotic cleavages. What distinguishes these mRNAs, and regulates both their quiescence in the oocyte and their activation in the maturing egg, is the presence of one or more cytoplasmic polyadenylation elements (CPEs), typically $U_{4-6}A_{1-2}U$, near to the ubiquitous nuclear polyadenylation signal AAUAAA. These 3′ untranslated region (UTR) elements mediate repression in the oocyte, and polyadenylation and translational activation in the egg, in clams, frogs and mice [1–4]. Increases in poly(A) length may stimulate translation by providing greatly stabilized poly(A)-binding protein-eIF4G-eIF4E contacts, which circularize eukaryotic mRNAs by their association with the 5′ cap structure and 3′ poly(A) tail, leading to mRNA stabilization and enhanced translational reinitiation [5–7].

CPE elements mediate their function by their interaction with CPE-binding protein (CPEB), first cloned and characterized in *Xenopus* [8]. It has two RNA-recognition motifs (RRMs) and an unusual zinc finger in its C-terminus; all three contribute to RNA recognition [9]. *Xenopus* CPEB is the first member to be identified of a growing family of proteins in both vertebrates and invertebrates, e.g. *Drosophila orb* [10], clam *Spisula solidissima* p82 [11] and *Caenorhabditis elegans* CPB-1-4 [12], as well as the more closely related mouse, zebrafish and human homologues [13–15]. *Drosophila*, *C. elegans*, zebrafish and humans possess two or more isoforms. All CPEBs share the C-terminal RNA-binding domains, but the N-termini are more divergent. Interestingly, CPEB functions are not confined to oogenesis or translation: *orb* promotes gurken mRNA localization and translation [16], and none of the CPEB isoforms in *C. elegans* appear to play a role in oogenesis whereas two, CPB-1 and FOG-1, perform critical functions in spermatogenesis [12,17]. Indeed, mouse CPEB, in addition to its functions in oogenesis, is also required for male germ cell development [18].

Several partners of CPEB have been proposed to mediate its role in translational repression and cytoplasmic polyadenylation. In *Xenopus* oocytes, CPEB represses translation of CPE-containing mRNAs by sequestering eIF4E from 5′ caps by its bridging partner maskin (which contains a peptide sequence that is conserved among elF4E-binding proteins), a protein related to TACC3 [19]. The level of maskin is regulated during oogenesis, with significant amounts only detectable during late stages, V/VI, suggesting that repression earlier in oogenesis relies on other mechanisms [20]. During meiotic maturation, the inhibitory maskin–eIF4 link is severed [19].

C. elegans CPB-1 binds FBF (*fem-3* mRNA-binding protein), which controls the germline switch from spermatogenesis to oogenesis by repressing *fem-3* [12]. The *Drosophila* homologue of FBF, Pumilio, interacts with Nanos to repress *hunchback* [21],

Abbreviations used: CPE, cytoplasmic polyadenylation element; CPEB, CPE-binding protein; CPSF, cleavage and polyadenylation specificity factors; DB, destruction box; FBF, *fem-3* mRNA-binding protein; GVBD, germinal vesicle breakdown; HA, influenza haemagglutinin; PAP, poly(A) polymerase; RNP, ribonucleoprotein; RRM, RNA-recognition motif; UTR, untranslated region.

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while in *C. elegans* FBF binds NANOS-3 to control the sperm–oocyte switch [22]. Intriguingly, *Xenopus* Pumilio was shown to associate with both CPEB and Nanos (Xcat-2) in oocytes, although the function of these interactions has yet to be determined [23,24].

Recently we showed that the conserved p47 (Xp54/Me31B/dhh1) DEAD-box RNA helicase associates with CPEB in an RNA-dependent manner in *Spisula* oocytes, and when tethered to non-adenylated luciferase RNA, Xp54 represses its translation in *Xenopus* oocytes [25]. In *Drosophila*, Me31B silences translation of oocyte-localizing RNAs through the formation of a cytoplasmic ribonucleoprotein (RNP) complex during *Drosophila* oogenesis [26]. Interestingly, the yeast homologue dhh1 enhances decapping of mRNA, and interacts with both the decapping and deadenylase complexes [27,28].

Early in meiosis, vertebrate CPEBs are phosphorylated by Eg2/aurora kinase on repeat copies of Leu-Asp-Ser-Arg [29,30], a modification which recruits to CPE-containing mRNAs the cytoplasmic forms of cleavage and polyadenylation specificity factors (CPSF) and thus promotes polyadenylation [31,32]. Invertebrate CPEBs lack aurora kinase consensus sites, and presumably interact with CPSF/poly(A) polymerase (PAP) in maturing eggs in a mechanistically different manner. For example, we reported cell-free experiments which supported a model in which, following fertilization, mitogen-activated protein kinase initially phosphorylates clam CPEB, and cdc2 kinase results in the second wave of phosphorylation of CPEB at germinal vesicle breakdown (GVBD) [33]. Indeed, *Xenopus*, mouse and clam CPEBs are hyperphosphorylated at GVBD by cdc2 kinase prior to degradation [4,11,34]. Here we investigate the role of cdc2 kinase phosphorylation, and of two N-terminal motifs that are conserved between invertebrates and vertebrates, originally noted in *Spisula*, mouse and frog [11], in mediating clam CPEB proteolysis during meiotic maturation.

EXPERIMENTAL

Plasmids and mutagenesis

The open reading frame of clam CPEB [11] was inserted into pS664TEN (a gift from Dr Simon Morley, University of Sussex, Brighton, E. Sussex, U.K.) in which it is flanked by 5′ and 3′ UTRs of β-globin and which also contains in its 3′ terminus the sequence $A_{23}C_{30}$, features that promote expression in oocytes. Fused in-frame at the N-terminus are three copies of an influenza haemagglutinin (HA) epitope tag, to create HAp82.$A_{23}C_{30}$. The N-terminal deletion mutant HAp82ΔBgl was created by restriction digestion of HAp82.$A_{23}C_{30}$ with *Bgl*II, which removes about 220 amino acids from the N-terminal half of p82/CPEB. Site-directed mutagenesis or deletion was performed in accordance with protocols supplied with the QuikChange site-directed mutagenesis kit (Stratagene) using native *Pfu* DNA polymerase and checked by sequencing. All p82 transcription plasmids were linearized with *Sma*I, and transcribed in a capped form with T7 RNA polymerase.

In vitro translation

In vitro-synthesized RNA (final concentration, 10–50 μg/ml) was translated in the micrococcal nuclease-treated reticulocyte lysate system. An equal volume of clam oocyte or egg lysate, which had been pre-cleared by spinning at 9300 ***g*** for 10 min, was then added to the translation mix. Also added was cycloheximide (10 μg/ml, final concentration), to prevent translation of endogenous clam lysate proteins by the reticulocyte lysate [11]. The resulting mixture was incubated at 18 °C for 2 h before adding SDS protein sample buffer, and electrophoresis on a 15% polyacrylamide gel followed by autoradiography.

Microinjection of *Xenopus* oocytes

Ovarian lobes were removed from *Xenopus laevis* females and oocytes were manually staged following collagenase treatment to remove follicle cells. Stage VI oocytes were injected with 15–25 nl of water or 10 nl of 500 ng/ml capped, 3 × HA-tagged clam p82/CPEB RNA and incubated at 20 °C in modified Barth's solution [8.8 mM NaCl, 1 mM KCl, 330 mM $Ca(NO_3)_2$, 410 mM $CaCl_2$, 820 mM $MgSO_4$, 2.4 mM $NaHCO_3$ and 10 mM Hepes/NaOH, pH 7.4] in the presence or absence of progesterone (10 μg/ml) for up to 25 h. They were then washed in maturation-promoting factor buffer (100 mM β-glycerophosphate, 20 mM Hepes/NaOH, pH 7.4, 15 mM $MgCl_2$, 20 mM EGTA, 10 μM PMSF and 3 μg/ml leupeptin) three times and harvested by homogenizing pools of 3–5 oocytes in maturation-promoting factor buffer (5 μl/oocyte). The resulting lysate was spun at 9300 ***g*** in a microfuge for 10 min at 4 °C. An equal volume of 2 × SDS protein loading buffer was added to the supernatants, and samples corresponding to one oocyte equivalent were run on 10, 12.5 or 15% polyacrylamide gels and analysed by Western blotting. Equal protein loading was monitored by Ponceau S staining of the blot and/or over-probing with control antibodies as indicated.

Immunoblotting

Western blotting was performed as described previously [25]. Rabbit anti-clam p82/CPEB antibody [2] was used at a dilution of 1:25000; rabbit anti-Xp54 antibody [35] at 1:5000; rat anti-HA antibody (Boehringer Mannheim) at 1:1000; rabbit anti-GST-*Xenopus* CPEB [8,11] at 1:5000; and guinea-pig anti-FRGY2 at 1:20000 [36]. The anti-XCPEB peptide antibody was obtained by injecting a rabbit with two peptides corresponding to the *Xenopus* CPEB N-terminal 15 amino acids (MAFPLKDDL-GRAKDC) and an internal peptide, located in between the two RRMs (ADSNFVRSPSQRLD), by Abcam (Cambridge, U.K.), and was used at a dilution of 1:4000. Addition of the peptides to the immune serum abolished staining.

Sucrose-gradient analysis

Clam oocyte and egg S10/post-mitochondrial lysates were prepared as described previously [2,11]. *Xenopus* oocytes were crushed in batches of 40 in 400 μl of buffer A (10 mM NaCl, 150 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 10 mM Hepes, pH 7.0, 0.5 mM dithiothreitol and 10 mM β-glycerophosphate) and centrifuged for 10 min at 9300 ***g*** at 4 °C. The supernatants were loaded on to 10–45% sucrose gradients which were centrifuged at 202000 ***g*** (at r_{av}) for 2.5 h in a SW40 rotor, and fractionated manually into 12 fractions of 1 ml. RNA in each fraction was estimated by determining A_{260}. Protein in each fraction was precipitated with trichloroacetic acid and the resulting pellet dissolved in SDS sample buffer, and analysed by SDS/PAGE (15% gels) and Western blotting.

RESULTS

CPEB levels in clam and *Xenopus* oocytes and eggs

To analyse CPEB regulation, we investigated the behaviour of ectopically expressed *Spisula* CPEB in *Xenopus* oocytes, reasoning that such a study would reveal the conserved and presumably important features that govern CPEB modification in meiosis.

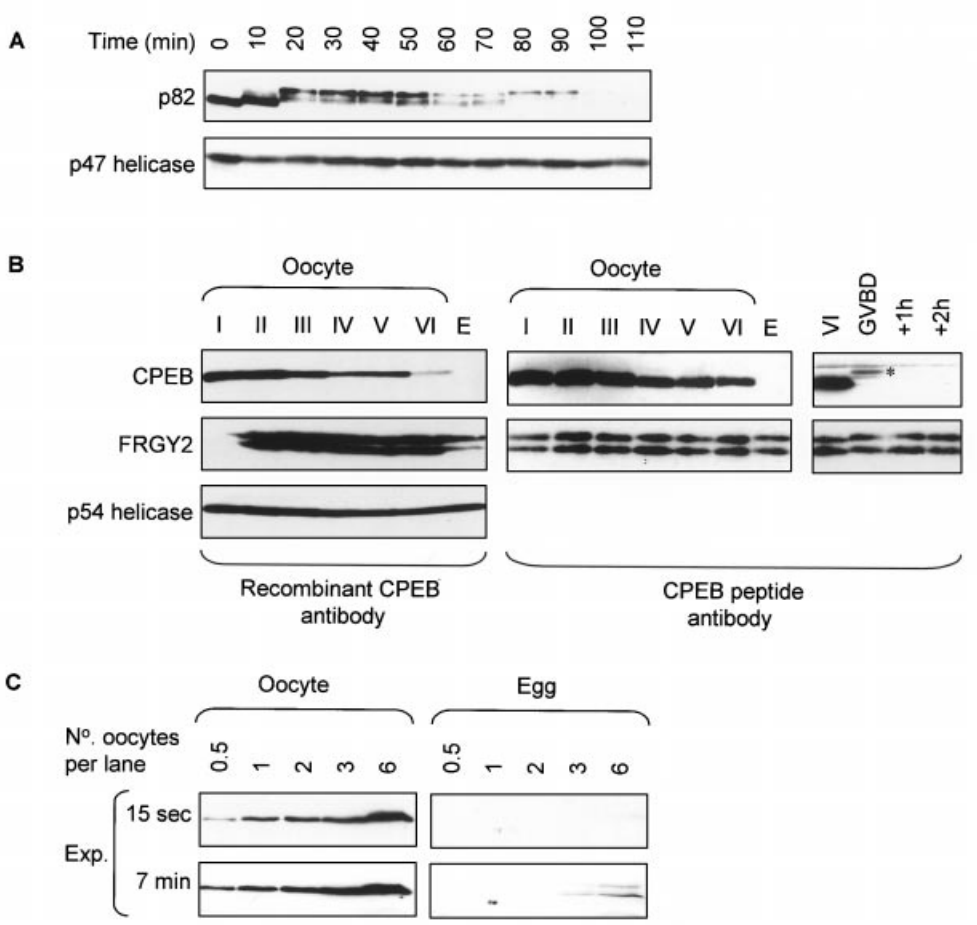

Figure 1 Levels of clam and *Xenopus* CPEB during oogenesis and meiotic maturation

(**A**) A culture of fertilized clam oocytes was sampled at 10 min intervals at 18 °C. Cells were pelleted, and resuspended in SDS protein sample buffer prior to gel electrophoresis and Western blotting. Western blots were developed with p82/CPEB and p47 helicase antibodies, and ECL. (**B**) Left-hand and middle panels: *Xenopus* oocytes were staged as described [49] and lysates were prepared (I–VI). Stage VI oocytes were matured with progesterone, and lysates were made after 16 h incubation (E). Right-hand panel: stage VI oocytes were matured with progesterone, and lysates were prepared from oocytes, at 50% GVBD (4.5 h after progesterone addition), and 1 and 2 h after GVBD. One oocyte equivalent was loaded for each stage. Western blots were developed with anti-XCPEB, -FRGY2 and -Xp54 antibodies as indicated (left-hand panel); and XCPEB peptide and FRGY2 antibodies (middle and right-hand panels), and ECL. The asterisk indicates the phosphorylated forms of CPEB. (**C**) Lysates were prepared from *Xenopus* stage VI oocytes and progesterone-matured eggs (18 h after addition of progesterone) and samples corresponding to 0.5, 1, 2, 3 and 6 cells were compared by Western blotting and developed with *Xenopus* CPEB peptide antibodies and ECL.

First, we compared the expression of the endogenous proteins in clams and frogs.

Spisula CPEB is modified at GVBD, as judged by its mobility shift on SDS/PAGE, about 10 min after fertilization, which initiates meiotic maturation in this organism (Figure 1). Treatment of lysate samples with phosphatase prior to electrophoresis abolishes the mobility shift, demonstrating that it is due to phosphorylation [33,37]. Further phosphorylation is observed at 20 min, and levels begin to decline at 60 min, around the onset of second meiosis. Clam CPEB is phosphorylated at GVBD by cdc2 kinase [11,33,37]. Very low levels persist through the two-cell stage, and thereafter become undetectable. Equal protein loading was controlled by over-probing with an antibody raised against p47 helicase, which is present at constant levels in fertilized oocytes and early embryos (Figure 1A; also see [25]).

We next examined the levels of *Xenopus* CPEB during oogenesis and meiotic maturation using two antibodies, both of which were rabbit polyclonal antibodies, generated against the recombinant protein [8,11] and against two *Xenopus* CPEB peptides (see the Experimental section) respectively. In both cases we noted that relative to *Xenopus* Xp54 helicase and FRGY2, whose levels are constant during oogenesis [35,36], CPEB levels are highest in early stages (I–III) and decline gradually during the later stages of oogenesis (Figure 1B).

CPEB was not detectable in mature frog eggs under standard Western blotting conditions. At GVBD, phosphorylation by cdc2 kinase [8,34] decreases the apparent mobility of CPEB, which is degraded in the following hour or so (Figure 1B). We determined that progesterone-matured eggs could potentially contain up to 3–5% of the stage VI oocyte level of CPEB by Western blotting lysates from 0.5, 1, 2, 3 and 6 oocytes and eggs. With a short exposure we did not detect, even in the six-cell sample, any CPEB in progesterone-matured eggs; whereas with a much longer exposure the amount of CPEB in six eggs corresponded to that present in about 0.2–0.3 oocytes (Figure 1C). As the Western samples were derived from lysate supernatants, we also examined the pelleted material (largely yolk protein in later stages) for CPEB, but none was observed (results

not shown). We should point out that while we could detect a very low level of CPEB in eggs when loading protein from several cell equivalents and with long exposures, experiments reported below were performed in conditions in which egg CPEB was undetectable. In summary, we conclude that CPEB expression is regulated in a very similar fashion in both clams and *Xenopus*: at GVBD it undergoes a mobility shift due to cdc2 kinase phosphorylation, and is subsequently rapidly degraded.

Spisula CPEB constructs

The parent construct HAp82WT contains the entire coding region of clam CPEB [11], fused in-frame with three N-terminal copies of the HA tag, to allow the unambigous detection of all mutation and deletion variants. Surrounding the fused open reading frame are the leader and 3′ UTR sequences, including $A_{23}C_{30}$, derived from pS664TEN of β-globin mRNA, to promote its expression in oocytes [38]. The noteworthy features of clam CPEB include the RNA-binding RRM and zinc-finger regions in the C-terminus, boxes A and B which loosely resemble a cyclin destruction box (DB) and a PEST region respectively, and putative cdc2 kinase and mitogen-activated protein kinase Ser/Thr Pro-directed phosphorylation sites [11,33]. The series of deletion and mutation CPEB variants used in this study are presented in Figure 2. The deletion mutant HAp82ΔBgl was created by restriction digestion of HAp82WT with *Bgl*II, and subsequent re-ligation, which removes about 220 amino acids from the N-terminus, including all Ser/Thr Pro-directed sites and box B. The remaining p82/CPEB constructs were derived by site-directed mutagenesis from the parent vector. Mutagenesis was used to alter Pro-directed Ser or Thr residues to Ala, or to remove short stretches (up to 14 amino acids), or to combine mutagenesis and deletion, as indicated.

The N-terminus of CPEB promotes phosphorylation and degradation

Previously, we observed the phosphorylation of clam CPEB, labelled with [^{35}S]Met during *in vitro* translation, upon subsequent incubation with clam egg lysates [11]. Here, using this assay, we delineated the portion of clam CPEB that is subject to phosphorylation. mRNA encoding HAp82WT was translated in the rabbit reticulocyte lysate in the presence of [^{35}S]Met before the addition of clam lysate (and cycloheximide to prevent translation of endogenous clam lysate mRNAs). HAp82WT was phosphorylated by egg lysates, as seen by the retardation of the *in vitro*-translated protein, and, as expected, no modification was observed with oocyte lysates. The deletion mutant HAp82ΔBgl was not phosphorylated to any significant extent, suggesting that the major phosphorylation sites, resulting in CPEB mobility shifts, reside in the N-terminus (Figure 3A).

HA-tagged p82/CPEB mRNAs were injected into *Xenopus* oocytes, incubated in the absence or presence of progesterone to induce maturation. In this experiment, 50% GVBD occurred between 5 and 6 h after the addition of progesterone. Lysates were prepared from control and maturing cells, and samples were analysed by Western blotting with anti-HA antibodies. HAp82WT was modified and completely degraded by 8 h after the induction of maturation, but was stable in the absence of progesterone (Figure 3B). The slowly migrating, phosphorylated form of CPEB was not observed in this particular experiment, but see Figure 4(C) for example. (In general, we noted that the phosphorylated species, coincident with GVBD, was transiently expressed, and its detection acutely reflected sample timing). In contrast, the HAp82ΔBgl form of CPEB was neither phosphorylated nor degraded, even after a 20 h incubation period in progesterone (Figure 3B). We concluded that the N-terminal region of clam CPEB contained sites responsible for its hyperphosphorylation, and motifs promoting its instability in maturing oocytes.

Phosphorylation of Ser/Thr Pro-directed kinase sites is required for CPEB degradation

We next investigated the role of cdc2 kinase sites in promoting hyperphosphorylation and instability of clam CPEB. The optimal phosphorylation site for cdc2 kinase, as well as other cyclin-dependent kinases, with the exception of cyclin D-cdk4 kinase, fits the consensus Lys/Arg-Thr/Ser-Pro-Xaa-Lys/Arg [39]. Many cyclin/cyclin-dependent kinase substrates have at least an adjacent Pro in the C-terminal side and a nearby basic residue, although of these two factors the most important appears to be the adjacent Pro. The Pro may be required to introduce a bend in the substrate to fit into the active site, because the crystal structure of cdc2 kinase shows that the cleft of the active site is rather narrow [40].

Five cdc2 kinase sites (amino acids Ser-110, Thr-176, Ser-187, Ser-234 and Ser-269; see Figures 2 and 4A) were identified which fitted the proposed consensus sequence [39]. These phosphorylation sites had their active serine or threonine residues point mutated to alanines. However, phosphorylation *in vitro* was not completely abolished in this mutated protein, following incubation with clam egg extracts (HAp82.1-5A; Figure 4B). Initially, this result was puzzling as the five sites that were mutated were the only ones within the *Bgl*II fragment that resembled the cdc kinase consensus sequence. This implied that other non-canonical cdc2 kinase phosphorylation sites exist or that another kinase may be involved in p82/CPEB phosphorylation.

Colgan et al. [41] showed that phosphorylation of PAP during the M phase of the cell cycle by cdc2 kinase occurs on non-consensus sites in addition to the sites conforming to the consensus sequence. To prevent phosphorylation of PAP and, hence, to repress PAP enzymic activity, all consensus and non-consensus cdc2 kinase sites have to be mutated [41]. The non-consensus sites have the very general sequence Ser/Thr-Pro. Upon re-examination of the p82/CPEB amino acid sequence, four cdc2 kinase non-consensus sites, in very close proximity to each other, were identified in the N-terminus of p82/CPEB (Ser-199, Thr-202, Thr-205 and Ser-212; Figures 2 and 4A). A 14-amino acid stretch (200–213; Figure 2), encompassing all four sites, was removed from constructs HAp82WT and HAp82.1-5A to create HAp82Δ4P and HAp82Δ9P respectively. Phosphorylation of HAp82Δ4P, but not of HAp82Δ9P, was detectable in the mixed reticulocyte/clam lysate assay (Figure 4B), indicating that both consensus and non-consensus sites contribute to CPEB phosphorylation and that eliminination of all nine sites inhibits detectable modification.

Next, HAp82.1-9A was designed to point mutate the four non-consensus sites from Ser/Thr to Ala, rather than deleting the 14-amino acid stretch (Figure 2). Whereas this stretch is small, its removal may still change the structure of p82/CPEB sufficiently to alter its regulation. Therefore, point mutations of these four residues were made in the background of HAp82.1-5A, to give a protein sequence with nine altered Ser/Thr Pro-directed kinase sites, HAp82.1-9A. As expected, this protein was not detectably phosphorylated in the *in vitro* assay (Figure 4B).

The p82/CPEB phosphorylation mutants were then tested in the maturing *Xenopus* egg. In this experiment, GVBD was initiated 5 h after the addition of progesterone. HAp82WT

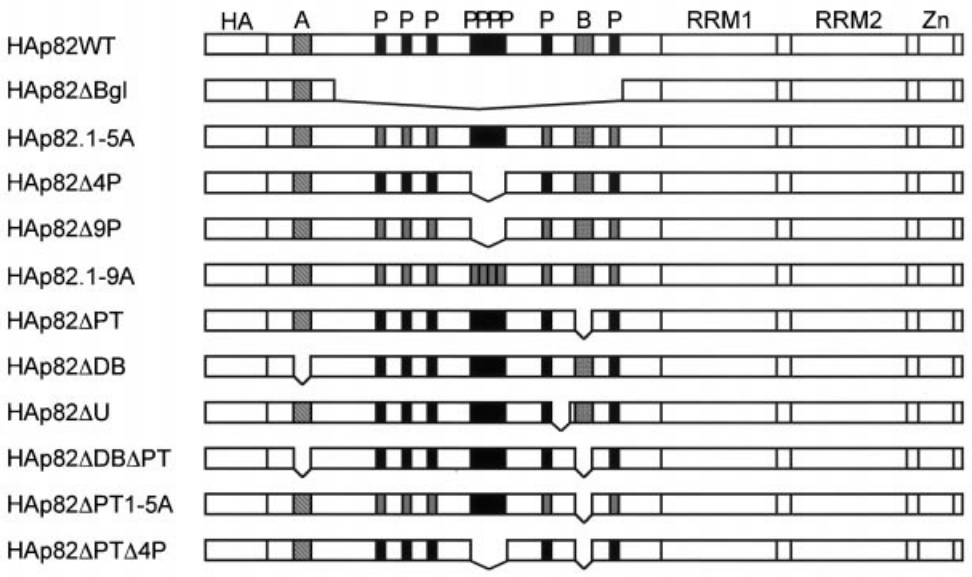

Figure 2 Clam CPEB constructs

Schematic diagram of clam CPEB constructs used in this study. Mutation of Pro-directed Ser/Thr kinase sites (P; black) to Ala (P; grey) is indicated, as are deletions of motif A (A; putative cyclin DB), motif B (B; putative PEST box) and U (uninteresting) peptides.

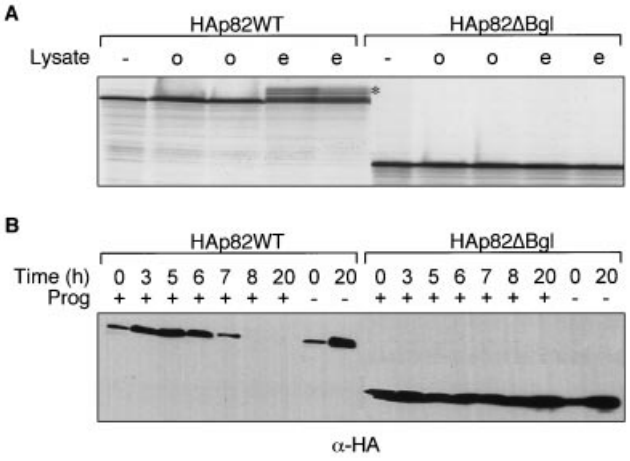

Figure 3 The N-terminus of clam CPEB promotes phosphorylation and degradation

(**A**) mRNAs encoding full-length HAp82WT, or an N-terminally deleted construct HAp82ΔBgl, were translated in the reticulocyte lysate (—). The reticulocyte lysate was mixed with two independent batches of clam oocyte (o) or egg (e) lysates and incubated for a further 2 h at 18 °C, prior to SDS/PAGE and autoradiography. (**B**) Stage VI *Xenopus* oocytes were injected with 10 ng of capped mRNA encoding HA-tagged p82/CPEBs as indicated. Translation of these mRNAs was allowed to proceed for 3 h before the addition of progesterone (Prog; 10 μg/ml) to induce oocyte maturation. Batches of three oocytes were harvested at time points (0–20 h) after the addition of progesterone (indicated above each lane). Lysates were made and added to SDS protein sample buffer before loading one oocyte equivalent on to a 15% polyacrylamide gel. Western analysis was performed with anti-HA antibodies and ECL. The asterisk in (**A**) indicates the phosphorylated forms of CPEB.

was phosphorylated at 7 h, and undetectable at 25 h after the addition of progesterone, although it accumulated stably in control oocytes. HAp82.1-5A was also phosphorylated, and partially degraded subsequently. Both the *in vitro* assay and this experiment agree that Ser/Thr sites in addition to those mutated in this construct are kinase and proteolysis targets. Loss of the four non-consensus cdc2 kinase sites in HAp82Δ4P has no discernible effects on its degradation at 25 h after progesterone addition, although in contrast to the HAp82WT and HAp82.1-5A versions no phosphorylated species was observed at 7 h. However, this difference may not be significant, since, as noted above, detection of phosphorylated CPEB at GVBD is thwarted by the transience of the modified protein. Interestingly, when the two sets of mutations are combined, in HAp82Δ9P or HAp82.1-9A, neither phosphorylation nor proteolysis are observed (Figure 4C). In other words, mutation of nine putative cdc2 kinase sites, from Ser/Thr to Ala, abolishes CPEB phosphorylation, and results in its complete stabilization. The Western blots were over-probed with anti-Xp54 antibodies, demonstrating that approximately equal protein loading occurred for each sample. We also

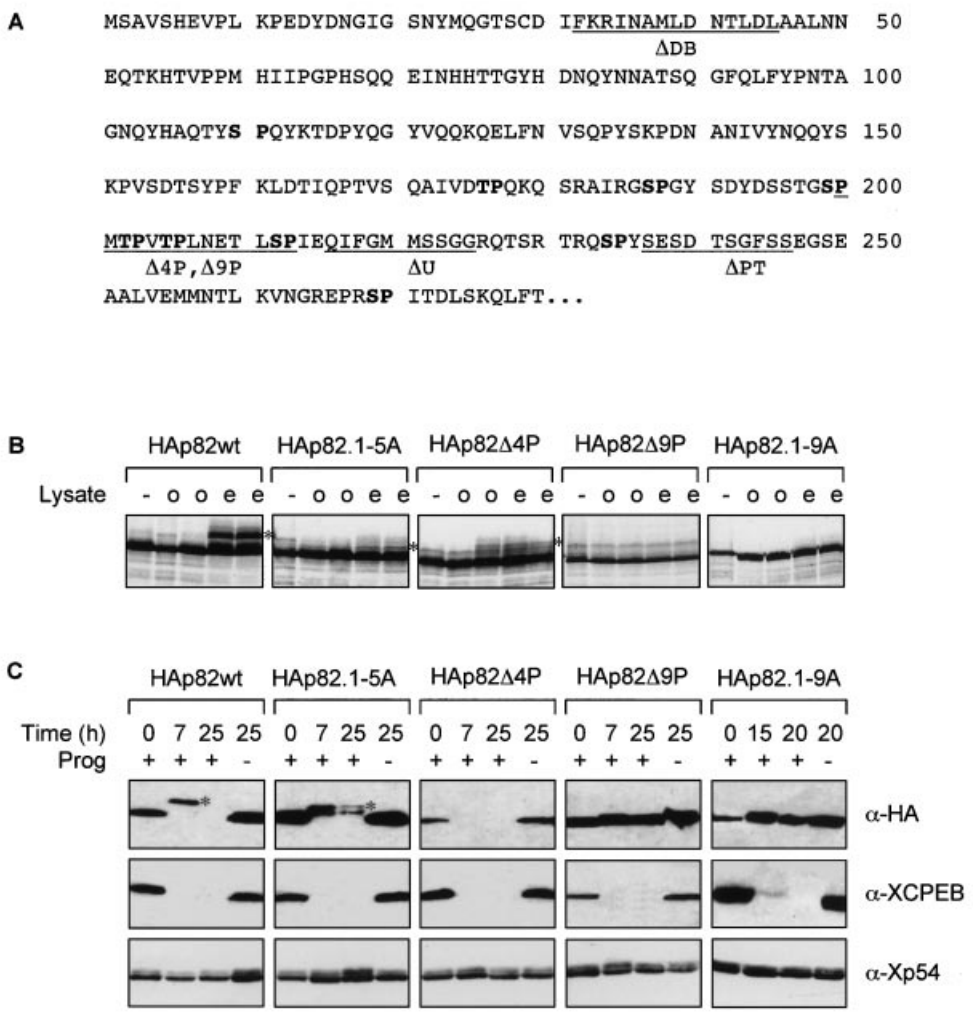

Figure 4 Mutation of nine putative cdc2 kinase sites prevents p82/CPEB phosphorylation and degradation

(**A**) The N-terminus of clam CPEB, with the nine potential cdc2 kinase sites, Ser/Thr Pro-directed, in bold. Also indicated are deletions (underlined) of four Ser/Thr Pro-directed sites in the Δ4P and Δ9P constructs, and deletions of DB (box A), PT (box B) and U (uninteresting) peptides. (**B**) A mixed reticulocyte/clam lysate assay of indicated CPEB variants. See Figure 3 for experimental details. (**C**) Clam CPEB variant mRNAs were injected into stage VI oocytes, which were incubated in the presence (+) or absence (−) of progesterone (Prog; 10 μg/ml), for up to 25 h. Lysates were prepared from pools of five oocytes at indicated times, and one oocyte equivalent of protein was analysed by SDS/PAGE (15% gel) and Western blotting. The blots were developed with anti-HA, -XCPEB and -Xp54 antibodies, and ECL.

monitored the levels of endogenous *Xenopus* CPEB with anti-XCPEB antibodies. *Xenopus* CPEB was not detectable in progesterone-matured eggs, irrespective of whether the clam protein was wild-type and degraded, or mutated and stable. Thus ectopically expressed stable clam CPEB did not delay the proteolysis of endogenous CPEB, implying that the components of the CPEB-degradation machinery were not limiting. Furthermore, expression of stable clam CPEB did not affect the onset or kinetics of GVBD (results not shown). We concluded that phosphorylation by cdc2 kinase targeted clam CPEB for degradation, by factors which are not limiting in the maturing oocyte.

Role of box A/DB and box B/PEST motifs in clam CPEB proteolysis

Previously, we noted the conservation of two short regions in the N-terminal halves of clam, frog and mouse CPEBs, which we called motifs A and B. We noted the possible homology of motif A to cyclin DBs or D-boxes and of motif B to PEST regions (Figure 5A [11]). D-boxes, present within 40 or so residues of the N-terminus of cyclins A and B, have the consensus sequence Arg-Xaa$_2$-Leu-Xaa$_2$-Ile-Xaa-Asn. The Arg is invariant and critical, and the first Leu residue, present in almost all cyclins, is also critical for proteolysis. Mutation or deletion of the D-box prevents cyclin degradation by ubiquitin-mediated delivery to the proteasome, which requires a dedicated ubiquitin ligase called the anaphase-promoting complex/cyclosome (APC/C; reviewed in [42,43]). Regions rich in Pro, Glu, Ser and Thr residues also promote proteolysis by ubiquitination, in this case by the 26 S proteasome [44]. A PEST region is defined as a hydrophilic stretch of amino acids greater than or equal to 12 residues in length which contains at least one Pro (P), one Glu (E) or Asp and one Ser (S) or Thr (T). PEST sequences are flanked by lysine, arginine or histidine residues; positively charged residues are excluded from the PEST sequence [44].

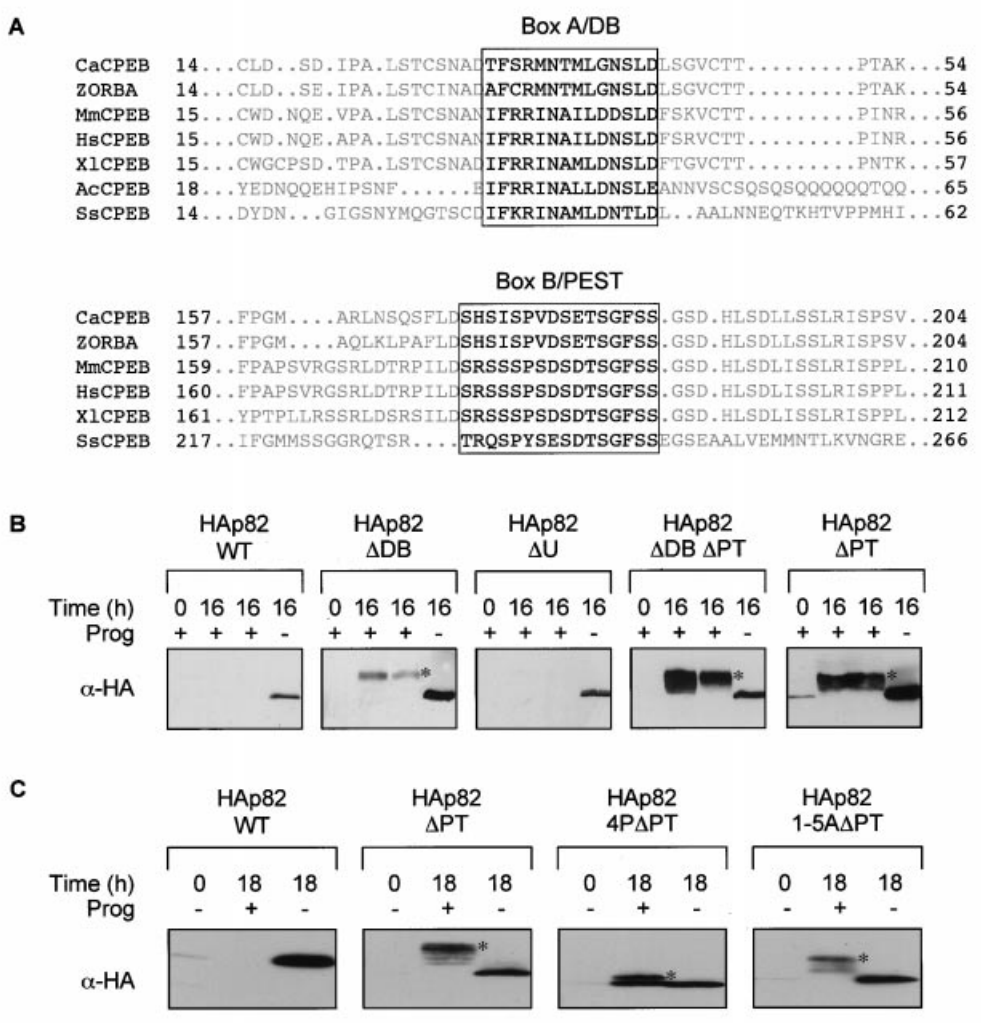

Figure 5 Both box A/DB and box B/PEST motifs mediate CPEB degradation

(**A**) Sequence alignments of the conserved A and B N-terminal motifs of CPEB from goldfish *Carassius auratus* (accession no. AB044534), zebrafish ZORBA [14], mouse [13], human [15], *Xenopus laevis* [8], the sea hare *Aplysia californica* (AAK52834.1) and the clam *S. solidissima* [11]. Conserved motifs are in black and boxed, the remaining residues are shaded grey. (**B**) Clam CPEB variant mRNAs, as indicated, were injected into stage VI oocytes, which were incubated in the presence (+) or absence (−) of progesterone (Prog; 10 μg/ml), for up to 16 h. Lysates were prepared from pools of five oocytes at the indicated times, and one oocyte equivalent of protein was analysed by SDS/PAGE (15% gel) and Western blotting. The blots were developed with anti-HA and -Xp54 antibodies, and ECL. (**C**) Clam CPEB variant mRNAs, as indicated, were injected into stage VI oocytes, which were incubated in the presence (+) or absence (−) of 10 μg/ml progesterone (Prog), for up to 18 h. Other details are the same as for (**B**). Asterisks indicate phosphorylated forms of CPEB.

According to these criteria, the putative PEST region in *Spisula* CPEB has a low score (+2.49), but the equivalent regions from vertebrates score very highly (approx. +17.0; http://at.embnet.org/embnet/tools/bio/PESTfind/). In Figure 5(A) we aligned all CPEBs which contain sequences homologous to the putative PEST region, including clam CPEB. We would like to take this opportunity to suggest a correction to the interpretation according to which *Spisula* CPEB does not have a motif homologous to vertebrate PEST motifs [45]; the alignment shown in Figure 5(A) indicates that the sequence SESDTSGFSS, part of the *Xenopus* PEST region [45,46], is present in clam and five vertebrates CPEBs, with only minor variations.

CPEBs have been identified in several organisms in addition to frog, mouse and clam, including *Drosophila* [10], *C. elegans* [12], zebrafish [14], humans [15], goldfish (*Carassius auratus*; accession no. AB044534) and the sea hare *Aplysia californica* [47,48]. Both A and B motifs are present in all vertebrates, except in the short alternatively spliced form of human CPEB which lacks motif A [15]. However, their presence in invertebrates seems variable. Thus neither motif is discernible in *Drosophila*, nor in *C. elegans* CPEBs. Interestingly though, the marine snail *A. californica*'s CPEB, the closest homologue of *Spisula* CPEB [48], contains a highly conserved A motif, though it lacks the equivalent of a PEST region (Figure 5A).

We investigated their respective contributions to CPEB degradation by making deletion mutants which lacked the putative DB (HAp82ΔDB) or the putative PEST box (HAp82ΔPT) or a control 10-amino acid region, lying between the two boxes (HAp82ΔU; see Figures 2 and 4A). mRNAs encoding wild-type and deleted forms of CPEB were injected into oocytes, some of which were matured with progesterone for 16 h. The wild-type and control deletion CPEBs were undetectable in maturing eggs, though they both accumulated in the absence of progesterone. Loss of the DB weakly stabilized CPEB in its phosphorylated

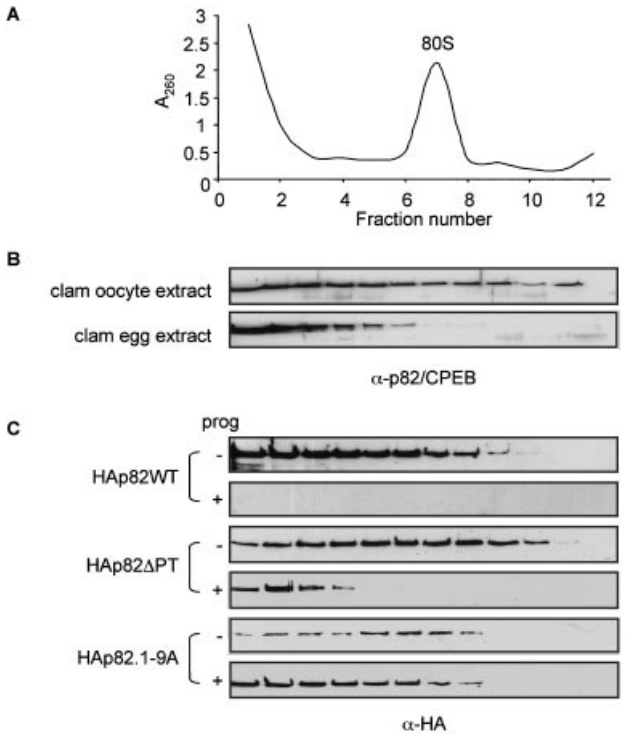

Figure 6 Phosphorylation of CPEB promotes its dissociation from RNP complexes

(**A**) Lysates from clam and frog oocytes and eggs were centrifuged though 10–45% sucrose gradients, and the A_{260} of each fraction determined. A typical trace is shown; the 80 S monosome peak is located in fractions 7 and 8. (**B**) The sucrose-gradient fractions obtained from clam oocytes and eggs were analysed by Western blotting using anti-p82/CPEB antibodies and ECL. (**C**) The sucrose-gradient fractions obtained from *Xenopus* oocytes and eggs microinjected with indicated clam p82/CPEB variant mRNAs were analysed by Western blotting using anti-HA antibodies and ECL.

form, whereas the absence of the PEST box substantially increased the stability of CPEB in maturing eggs. Interestingly, deletion of both regions led to even greater stabilization, relative to the stabilization seen in either single region deletion variant (Figure 5B). Our data thus implicate both regions in CPEB degradation, with the PEST region playing the principal, albeit not the sole, role.

The ability to stabilize CPEB in eggs by deleting the PEST region allowed us to examine the relative contribution of consensus and non-consensus cdc2 kinase sites to CPEB hyperphosphorylation. Indeed, as the double CPEB variants HAp82ΔPTΔ4P and HAp82Δ4P.1-5A were clearly phosphorylated (to different extents; Figure 5C), we concluded that both types of cdc2 kinase site were modified in CPEB during meiotic maturation.

Role of cdc2 kinase phosphorylation in CPEB/RNP complex disassembly

Previously we documented that clam CPEB migrates as a component of a large RNP complex in oocytes and that it dissociates from this complex in eggs, as analysed in sucrose gradients (Figure 6B and [11]). In these experiments, lysates were prepared within 45 min of fertilization, between the two meiotic divisions, at a time when CPEB is hyperphosphorylated, but before it begins to be degraded (Figure 1A). We confirmed that the ectopically expressed wild-type clam protein had a very similar distribution in *Xenopus* oocytes to that of the endogenous protein in clam lysates (Figure 6C), implying its participation in large complexes. In progesterone-matured *Xenopus* eggs, the wild-type clam protein was undetectable, as seen previously (Figures 3, 4 and 5). Next, we exploited the ability to synthesize stable and unphosphorylated, or stable and phosphorylated, forms of clam CPEB in *Xenopus* eggs to ask whether phosphorylation was responsible for this alteration in complex association. Indeed, as shown in Figure 6(C), we noted that the distribution of HAp82ΔPT, the stable phosphorylated form, in oocytes and eggs mirrored the distribution of clam lysate CPEB, whereas HAp82.1-9A, the stable but unphosphorylated form, remained in large complexes in eggs, strongly suggesting that phosphorylation by cdc2 kinase normally mediates CPEB dissociation from RNP upon maturation, prior to degradation.

DISCUSSION

Levels of endogenous CPEB proteins

To investigate the conserved features of CPEB that mediate its destruction during meiotic maturation, we examined the behaviour of clam CPEB and variants in maturing *Xenopus* oocytes. First we confirmed that the endogenous clam and frog proteins undergo degradation, following GVBD (Figure 1). Indeed, *Xenopus* CPEB levels appear to begin to decline during oogenesis. More striking however, is the very rapid and near-total destruction of CPEB observed within 1 h of GVBD (Figure 1). We estimate that at most 3–5% of oocyte levels of CPEB remain in the mature egg. For reasons that are not entirely clear, other investigators found constant [20] or increasing levels of CPEB [46] during oogenesis, and much higher levels of CPEB (10–30% of oocyte levels) in the egg and early embryos [8,45,46]. Because of these discrepancies, we compared two different antibodies and carefully monitored protein loading. In addition, we sought to establish whether our lower levels were due to CPEB pelleted during lysate preparation, but this was not the case. However we note that zebrafish and mouse CPEB are also degraded during oocyte maturation and are undetectable in 1 and 2 cell embryos [4,14]. We surmise that the absolute levels observed depend critically on the exact conditions used in lysate preparation and Western blotting.

Role of Ser/Thr Pro-directed phosphorylation sites in CPEB proteolysis

Mutation of nine putative cdc2 kinase Ser/Thr Pro-directed phosphorylation sites in clam CPEB prevented its degradation during oocyte maturation (Figure 4). Alongside previous evidence including timing and the use of specific inhibitors [11,33,37], these data clearly implicate cdc2 kinase in targeting CPEB for destruction. Similar results were reported recently with *Xenopus* CPEB [45], suggesting that such targeting is a conserved feature of CPEB regulation. It is of interest therefore to examine whether the location and context of the cdc2 kinase sites are conserved between clam and frog, and indeed other members of the CPEB family. In fact, apart from their location in the N-terminal portion of the protein and the requirement for multiple phosphorylation sites, there is very little sequence conservation between the clam and frog proteins. In *Spisula*, we know that both consensus and non-consensus cdc2 kinase sites have to be modified for degradation, although we have not established the precise identity of all sites. Mass spectrophotometric analysis of CPEB immunopurified from clam eggs established that Ser-269 (Figure 4A) is phosphorylated *in vivo* (results not shown).

Interestingly, based on its location relative to the PEST box, this is most likely to be the equivalent of Ser-210 which was shown to be one of the principal cdc2 kinase sites in *Xenopus* CPEB [45].

Role of conserved A and B motifs in CPEB proteolysis

In agreement with [45] and [46], deletion of a putative PEST box region dramatically stabilizes CPEB during oocyte maturation (Figure 5). The role of this region in normally targeting CPEB for degradation is now therefore extended to invertebrates. Indeed, so far, clam CPEB is the only invertebrate with the conserved PEST box as *Drosophila*, *C. elegans* or *Aplysia* CPEBs appear to lack such a sequence. Little is known yet of whether and how CPEB levels are regulated in *Drosophila* or *Aplysia*, but, at least in *C. elegans*, there are indications that it undergoes stage-specific proteolysis. Two CPEB homologues, CPB-1 and FOG-1, have key functions in spermatogenesis, although they are dispensable for oogenesis. CPB-1 is essential for progression through meiosis, and the protein is present in the germ line just prior to spermatogenesis, but is degraded once sperm differentiation begins [12]. Presumably, a mechanism other than a PEST-motif-mediated one operates in this case. Our work with clam CPEB indicates an additional role in proteolysis of the conserved motif A or DB-like motif, which appears to enhance the function of the PEST box, and may even replace it. Thus we note that *Aplysia* CPEB that functions in sensory neurons contains the DB-type motif, but not the PEST box motif (Figure 5A) and that the long form of alternatively spliced human CPEB, which is enriched in the brain, contains both motifs, whereas the shorter ubiquitously expressed form lacks the DB [15]. It remains to be determined whether the DB has a role in regulating CPEB levels in these organisms.

Phosphorylation by cdc2 kinase of *Xenopus* and clam CPEB containing a PEST box results in its degradation ([45,46] and this study), involving ubiquitylation and the proteasome pathway [46]. CPEB levels are tightly regulated to allow translational activation of dormant mRNAs. We also show that, prior to degradation, phosphorylation is required to dissociate CPEB from large RNP complexes. A stable, non-phosphorylated version of the protein remains in the large complexes (Figure 6). CPEB dissociation from a complex is probably unrelated to RNA binding, as phosphorylated and unphosphorylated CPEB cross-link RNA with similar efficiency [8] and may rather reflect the loss of protein–protein interactions, perhaps those important for translational masking. Indeed, it was recently found that *Xenopus* Pumilio, with a potential role in the timing of cyclin B1 mRNA translational activation, is bound to CPEB in oocytes, but not to its cdc2 kinase-phosphorylated form in eggs (M. Yamashita, personal communication). Phosphorylation of *Xenopus* CPEB on Ser-210 by cdc2 kinase, which occurs about the time of cyclin B1 translation, is sufficient for selective translational activation of cyclin B1 mRNA [45]. While this modification may solely determine critical levels of CPEB for activation, via degradation [45], it is also possible that the dissociation of phosphorylated CPEB from *Xenopus* Pumilio plays a role in translational activation (M. Yamashita, personal communication). We conclude that cdc2 kinase phosphorylation of CPEB has two functions, one influencing RNP complex stability and the other targeting the protein for destruction.

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