Xenopus Cold-inducible RNA-binding Protein 2 Interacts with ElrA, the Xenopus Homolog of HuR, and Inhibits Deadenylation of Specific mRNAs*

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Received for publication, July 30, 2003, and in revised form, September 9, 2003
Published, JBC Papers in Press, September 17, 2003, DOI 10.1074/jbc.M308528200

Xenopus cold-inducible RNA-binding protein 2 (xCIRP2) is a major cytoplasmic RNA-binding protein in oocytes. In this study, we identify another RNA-binding protein ElrA, the Xenopus homolog of HuR, as an interacting protein of xCIRP2 by yeast two-hybrid screening. As ElrA stabilizes the RNA body in the in vitro mRNA stability system, we examine the role of xCIRP2 in the stabilization of mRNA and find that xCIRP2 inhibits deadenylation of AU-rich element-containing mRNA. These results suggest that xCIRP2 and ElrA may be involved in the regulation of mRNA stability at different steps. By immunoprecipitation with anti-xCIRP2 antibody, we find that xCIRP2 interacts with several mRNAs including mRNA encoding the centrosomal kinase Nek2B in oocytes. xCIRP2 also inhibits deadenylation of the mRNA substrate containing the 3′-untranslated region of Nek2B mRNA in the in vitro system. Our results suggest that xCIRP2 associates with specific mRNAs and can regulate the length of poly(A) tail in Xenopus oocytes.

In Xenopus, a full-grown stage VI oocyte contains the large quantities of maternal mRNAs for later use, through oocyte maturation to the egg and during the cleavage divisions of early embryogenesis (1, 2). The process of Xenopus oogenesis, from stage I to stage VI, requires at least several months, and the mass of maternal mRNAs found in stage VI oocytes is already accumulated by the end of stage II. All maternal mRNAs are assembled into messenger ribonucleoprotein particles (mRNPs), most of which are stably stored for a long period of time in a translationally repressed state (3).

Elongation or shortening of poly(A) tail length at the 3′ end is an essential mechanism to regulate the translational activity of mRNA (4, 5). In general, polyadenylation of a cytoplasmic mRNA leads to its translational up-regulation, whereas deadenylation reduces translational activity. Several translationally repressed mRNAs in Xenopus oocytes that contain a specific cis-element, the cytoplasmic polyadenylation element (CPE), in their 3′-untranslated region (3′-UTR) have short poly(A) in the oocytes (6, 7). Upon oocyte maturation, these mRNA receives elongated poly(A), which in turn results in translational activation. Molecular mechanisms mediated by specific association of a trans-acting factor CPEB with CPEs to regulate translational repression in oocytes and activation in matured oocytes have been extensively studied (5, 8–10). Some of these mRNAs are again deadenylated in early embryos, also dependent on a specific element, the embryonic deadenylation element, in their 3′-UTR (11, 12). On the other hand, many housekeeping mRNAs that are actively translated in the oocytes become deadenylated upon maturation and possibly thereby translationally repressed (13, 14). Interestingly, it is known that this deadenylation takes place as a default pathway (i.e. a pathway independent of specific cis-elements).

Maintenance of poly(A) tail is important for mRNA stability, since mRNAs in eukaryotes are usually deadenylated before turnover of the body of the transcript. Deadenylation is the rate-limiting step of mRNA decay. It has been shown using in vitro systems that a major degradation pathway of the mRNA body following the deadenylation in higher eukaryotes is mediated by a complex of 3′-5′ exonucleases called the exosome (15–18). As described above for the translational control, it is well established that the lifetime of mRNA can also be specified by specific cis-acting elements within mRNA sequences. The most studied cis-acting elements are the AU-rich elements (AREs), usually found in the 3′-UTR of many short lived mRNAs encoding proto-oncogene products and cytokines (19). These AREs consist of reiterated sequences of the AUUU or oligo(U) sequences in an AU-rich context and are partly responsible for the rapid degradation of the mRNAs that contain them. Among a number of proteins that bind to AREs, HuR has been identified as a trans-acting factor that stabilizes ARE-containing mRNAs (20). Many studies have established that HuR up-regulates gene expression by stabilizing ARE-containing mRNAs in vitro and in vivo (21–24). Xenopus homolog of HuR, ElrA, was identified as an RNA-binding protein that binds to a cis-acting element, the embryonic CPE or eCPE, required for cytoplasmic polyadenylation occurring during early embryogenesis (25).

We previously identified an RNA-binding protein, xCIRP2, which is highly expressed in Xenopus oocytes and eggs (26). xCIRP2 is a relatively small RNA-binding protein consisting of an N-terminal RNA recognition motif (RRM) and a C-terminal glycine-rich region. It is a homolog of mammalian cold-induc-
ible RNA-binding protein (CIRP). The expression level of CIRP in cultured mouse cells increases upon temperature downshift, and CIRP mediates the cold-induced cell growth suppression presumably by prolonging the G1 phase of the cell cycle (27). Although we previously demonstrated the cytoplasmic localization of xCIRP2 in stage VI oocytes and its possible association with ribosomes, its functional roles in oocytes and early embryos are still elusive (26). Recently, we revealed that xCIRP2 shuttles between nucleus and cytoplasm when expressed in cultured cells (28). To gain insight into the molecular mechanisms of how xCIRP2 is involved in RNA metabolism, a search for proteins and RNA interacting with it should be helpful. In this study, we identified ElrA as an xCIRP2-interacting protein. Using an in vitro mRNA stabilization system, we have shown that xCIRP2 inhibits deadenylation of ARE-containing mRNA substrate but not that of nonspecific substrates. By searching for mRNAs that interact with xCIRP2 in Xenopus oocytes, we identified several mRNAs including cyclin B1 and Nek2B mRNAs. Deadenylation of the mRNA substrate containing the Nek2B 3′-UTR sequence was inhibited by the addition of xCIRP2 protein in the in vitro system. Our results suggest that xCIRP2 participates in the regulation of mRNA stability or translational control in the oocytes, eggs, and early embryos.

MATERIALS AND METHODS

Yeast Two-hybrid Assay—Yeast two-hybrid screening for proteins that interact with xCIRP2 was performed as described previously (28).

Recombinant Proteins—Overexpression and purification of the C-terminal His6-tagged xCIRP2 and glutathione S-transferase (GST) fusion xCIRP2 were performed essentially as described previously, except that the purification was performed under native conditions (26, 28). The C-terminal His6-tagged FRGT2 protein was also prepared as described previously (26).

A partial ElrA cDNA fragment was prepared by PCR from Xenopus oocyte total RNA and used as a probe to screen a Xenopus oocyte cDNA library (29). To obtain the C-terminal His6-tagged ElrA (ElrA-His6) protein, the ElrA coding sequence was amplified by PCR from ElrA cDNA with a primer set, CGGGATCCGATGTCTAACGGTTATGAAAGATC and CGACCACGCAGCGTCGACTTTGTGTGACTTGCTGGTTTTG. The PCR fragment was then digested with BamHI and SalI and cloned into pET24b vector (Novagen). Escherichia coli BL21 (DE3) cells (DE3) were transformed with the resulting expression plasmid, and ElrA-His6 protein was overexpressed by the addition of isopropyl β-D-thiogalactopyranoside to the bacterial culture. Bacterial cells were sonicated in buffer A (20 mM Tris-HCl, pH 8.0, and 100 mM NaCl), and the cell lysates were then centrifuged at 12,000 × g for 10 min. The pellet was suspended in buffer B containing 8 M guanidine HCl and protease inhibitor mixture (Complete, EDTA-free; Roche Applied Science), followed by the application to a Ni2+-nitrilotriacetic acid column (Qiagen). ElrA-His6 protein was eluted with buffer B containing 8 M urea and 100 mM imidazole and dialyzed against buffer A containing 0.5 mM phenylmethylsulfonyl fluoride. To obtain GST-ElrA protein, the ElrA coding sequence was amplified by PCR from ElrA cDNA with a primer set, CGGGATCCGATGTCTAACGGTTATGAAAGATC and CGACCACGCAGCGTCGACTTTGTGTGACTTGCTGGTTTTG. The PCR fragment was then digested with BamHI and Sull and cloned into pGEX 4T-3 (Amersham Biosciences). GST-ElrA protein was expressed in E. coli as above and purified using glutathione-Sepharose column chromatography (Amersham Biosciences) as recommended by the manufacturer.

GST Pull-down Assay—For the GST pull-down assay with purified recombinant proteins, ElrA-His6 protein was incubated with GST or GST-xCIRP2 bound to glutathione-Sepharose in buffer B (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM dithiothreitol) containing 1% bovine serum albumin at 4°C for 1 h. After extensive washes with buffer B, the bound materials were eluted with 10 mM reduced glutathione.

To determine the domains of ElrA interacting with xCIRP2, full-length and deletion constructs of ElrA were generated by PCR amplification of the respective cDNA sequences and subcloning them into pTNT vector (Promega). ElrA proteins were expressed in the TNT-T7 quick coupled in vitro transcription-translation system (Promega) using each construct in the presence of [35S]methionine. GST pull-down assay using aliquots of the reactions was performed as above.

Antibody Production, Immunoprecipitation, and Immunoblotting—Polyclonal antiseraum against ElrA was produced following standard procedures by injecting a rabbit with the purified ElrA-His6, which had been renatured by dialysis against phosphate buffered-saline. Preparation of rabbit polyclonal antiseraum containing antibodies against the recombinant xCIRP2 protein has been described (26).

For immunoprecipitation, Xenopus oocytes were homogenized in buffer C (20 mM Heps, pH 7.5, 2 mM MgCl2, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) containing 100 mM KCI and the lysates were cleared by centrifugation at 12,000 × g for 10 min. The oocyte lysates were incubated with anti-xCIRP2 antibody bound to protein A-agarose in buffer C containing 100 mM KCI at 4°C for 30 min. After extensive washing with buffer C containing 175 mM KCI, the bound materials were analyzed in SDS-polyacrylamide gel. Immunoblotting was performed as described previously (28). Rabbit polyclonal antiseraum against the recombinant xCIRP2 protein, mouse anti-HuR monoclonal antibodies (3A2; Santa Cruz), and anti-His6 monoclonal antibodies (Clontech) were used as primary antibodies.

Identification of xCIRP2 Targets—Immunoprecipitation of oocyte lysates with anti-xCIRP2 or anti-ElrA antibodies was performed as...
**FIG. 2.** RRM3 of ElrA is involved in interaction with xCIRP2. A, schematic diagrams of ElrA deletion mutants used for mapping of the domain involved in interaction with xCIRP2. B, ElrA deletion mutants were synthesized in rabbit reticulocyte lysate in the presence of \[^{35}\text{S}\]methionine. Each reaction mixture was incubated with GST or GST-xCIRP2 bound to glutathione-Sepharose. The bound materials were analyzed by SDS-PAGE (lanes 1–8). An aliquot of each reaction was electrophoresed in parallel (lanes 9–12).

above, and the coprecipitated RNA was purified. Double-stranded cDNA was synthesized using SuperScript Plasmid System (Invitrogen) and ligated to SalI- and NotI-digested pGEM-11Zf(+) vector (Promega). Individual clones were identified by DNA sequencing. The binding specificity of each mRNA to xCIRP2 or ElrA was determined by reverse transcriptase-mediated PCR (RT-PCR) using specific primer sets.

**In Vitro mRNA Stabilization Assay**—To create pGEM-pA+ plasmid, oligonucleotides AGCTTAAGTTACCGAGCACTC and GAGTGTTTTTTGTTTCTGTGGGTAAC and GCTCTAGAAGCTTTCTCGAGACGTCCGCCCTCCTCCCTCCCTGGACCATCGATCAGGA, and AGCTTTGACGCTCTTCCGAGCT were annealed, digested with NspI, and inserted into HindIII- and NsiI-digested pGEM-11Zf(+) vector (Promega). A DNA fragment containing Nek2B 3'-UTR was amplified by PCR from Nek2B cDNA using a primer set, GCTCTAGAAGCTTTCTCGAGACGTCCGCCCTCCTCCCTCCCTGGACCATCGATCAGGA, and AGCTTTGACGCTCTTCCGAGCT, was annealed, digested with NspI, and inserted into HindIII- and NsiI-digested pGEM-pA+ in the sense orientation. A template fragment containing Nek2B 3'-UTR was amplified by PCR from Nek2B cDNA using a primer set, GCTCTAGAAGCTTTCTCGAGACGTCCGCCCTCCTCCCTCCCTGGACCATCGATCAGGA, and AGCTTTGACGCTCTTCCGAGCT.

**Gel Retardation Assay**—Fifty fmol of mRNA was incubated with the recombinant xCIRP2 and ElrA at 30 °C for 10 min under the same conditions that were used for the in vitro mRNA stabilization assay, except that HeLa S100 fraction was omitted. The mixture was then electrophoresed in a 5% polyacrylamide gel in 0.5× Tris borate/EDTA buffer at room temperature.

**RESULTS**

**Interaction of xCIRP2 with ElrA**—To explore cellular functions of xCIRP2, we tried to identify proteins interacting with it by yeast two-hybrid screening. As we reported previously, one of the positive clones encodes Xenopus protein arginine methyltransferase 1, which methylates the arginine- and glycine-rich region of xCIRP2 and thereby regulates its nucleocytoplasmic distribution (28). Another clone, 1.6.3, which was confirmed to interact with xCIRP2 bait, encoded an RNA-binding protein, ElrA (Fig. 1A). ElrA was originally isolated as one of cDNAs encoding Xenopus ELAV-like RNA-binding proteins (30). ElrA shows highest homology with mammalian HuR protein, the ubiquitously expressed member among ELAV-like proteins. The ElrA cDNA clone that we isolated by the two-
A. ARE-A RNA was incubated in the in vitro mRNA stabilization system in the presence of 0.5 μM xCIRP2 and ElrA for the times indicated. RNA products were purified and analyzed in a 5% polyacrylamide gel containing 7 M urea. The positions of ARE-A<sub>60</sub> and ARE-A<sub>0</sub> RNA are indicated. Radioactivity of RNA in the range between ARE-A<sub>60</sub> and the ARE-A<sub>0</sub> indicated by a bracket on the left, was quantified. Relative values normalized by the value of time 0 (lanes 1, 4, 7, and 10) as 100% are shown at the bottom. B, ARE-A<sub>0</sub> RNA was incubated in the in vitro system in the presence of increasing amounts of xCIRP2 and 0.5 μM ElrA for 0 h (lane 1) or 3 h (lanes 2–6). RNA was analyzed as above. ARE-A<sub>0</sub> RNA was prepared and electrophoresed in parallel (lane 7).

To confirm the results of the two-hybrid screening, we examined whether ElrA could directly interact with xCIRP2 by an in vitro binding assay. We prepared recombinant ElrA protein and performed a GST pull-down assay with GST and GST-xCIRP2 (Fig. 1B). In agreement with the results of the two-hybrid assay, ElrA interacted with GST-xCIRP2 but not with GST. To determine whether xCIRP2 is indeed associated with ElrA in the oocytes, we conducted immunoprecipitation experiments (Fig. 1C). Previous experiments revealed that both xCIRP2 and ElrA proteins are present predominantly in the oocyte cytoplasm (25, 26). Xenopus oocyte lysates were prepared and subjected to immunoprecipitation with anti-xCIRP2 antibodies. The presence of ElrA was examined by subsequent immunoblotting with anti-HuR antibodies. ElrA was communoprecipitated when anti-xCIRP2 antibody was used but not when preimmune serum was used for immunoprecipitation. We therefore concluded that ElrA could interact with xCIRP2 both in vitro and in vivo.

Mapping of the Domain of ElrA Involved in Interaction with xCIRP2—ElrA/HuR contains three RRMs and the hinge region located between its second and third RRMs (Fig. 2A). To determine the xCIRP2-binding region of ElrA, we performed a GST pull-down assay using several deletion mutants of ElrA. Full-length ElrA and ElrA<sub>3N</sub> containing the hinge region and RRM3 bound to GST-xCIRP2 (Fig. 2B). In contrast, ElrA<sub>ΔC</sub> and ElrA<sub>ARRM3</sub> did not bind to GST-xCIRP2. These results suggest that RRM3 of ElrA is required for the complex formation with xCIRP2. These findings are consistent with the results of yeast two-hybrid screening in which the ElrA cDNA clone containing the hinge region and RRM3 was isolated.

**xCIRP2 Inhibits Deadenylation of ARE-containing mRNA in the In Vitro Stabilization System**—ElrA shows high homology to Drosophila ELAV and mammalian Hu proteins. It has been established that binding of mammalian Hu proteins to ARE of mRNA results in the RNA stabilization both in vivo and in vitro (21, 24). Therefore, identification of ElrA as a protein interacting with xCIRP2 prompted us to test whether xCIRP2 could be involved in the turnover of ARE-containing mRNAs. To test this possibility, we employed an in vitro mRNA stabilization system using HeLa cell S100 extract, which was developed by Ford et al. (23). In this system, the general pathways and many of the regulatory aspects of mRNA degradation can be reproduced (18, 31). The rate of both deadenylation and degradation of exogenous capped and polyadenylated RNA substrates is up-regulated by the presence of ARE in the body of the RNA substrates (23).

First we examined the effects of xCIRP2 protein on the stability of an artificial RNA substrate containing ARE, which is derived from tumor necrosis factor α mRNA, with a poly(A)<sub>60</sub> tail (Fig. 3). By incubating in S100 extract supplemented with ATP, the ARE-A<sub>60</sub> RNA was first deadenylated, and then the body of the RNA substrate was degraded (Fig. 3A). The addition of xCIRP2 in this system resulted in the inhibition of deadenylation in a dose-dependent manner (e.g., compare lanes 2, 4, and 6 in Fig. 3A). As shown previously with Hu proteins (23), ElrA failed to affect deadenylation of the ARE-A<sub>60</sub> RNA but stabilized the deadenylated RNA (Fig. 3B; also see Fig. 4). Another RNA-binding protein, FRGY2, did not affect either deadenylation or degradation of the ARE-A<sub>60</sub> RNA. Although FRGY2 belongs to the Y-box protein family, whose members are involved in general and specific mRNA stabilization, we did not observe any protective roles of FRGY2 in this system. These results indicate that xCIRP2 stabilizes mRNA by inhibiting deadenylation.

To test the sequence requirement of the RNA substrate for...
the inhibitory effect of xCIRP2, we compared degradation of ARE-A60 RNA with that of nonspecific transcripts that lack AREs but possess the 5'-cap and 3'-poly(A)_60 tail (Fig. 3C). During a 60-min incubation, ARE and nonspecific RNAs were similarly degraded, and the deadenylated intermediate was detected. Remarkably, inhibition of deadenylation by xCIRP2 was only observed with ARE-A60 RNA. xCIRP2 barely affected the stability and deadenylation of nonspecific RNAs. These data suggest that xCIRP2 specifically inhibited deadenylation of ARE-containing mRNA in this in vitro system.

ElrA and xCIRP2 Cooperate to Stabilize mRNA—The results with ARE-A60 RNA imply that xCIRP2 inhibited deadenylation, whereas ElrA stabilized the deadenylated RNA (Fig. 3B), suggesting that these proteins are involved in stabilization of ARE-RNA at distinct steps. We next examined RNA stabilization by the addition of both xCIRP2 and ElrA into the in vitro system (Fig. 4). For this set of experiments, we used another batch of HeLa S100 extract than that used in the previous experiments, in order to clarify the effect of ElrA on stabilization of deadenylated RNA. This extract shows relatively slow deadenylation activity, but no accumulation of deadenylated RNA was observed without exogenous ElrA protein (e.g., compare Fig. 4A, lane 3, with Fig. 3A, lane 3). Consistent with the data shown above, xCIRP2 inhibited the deadenylation in this extract as well. When radioactivity of RNA in the range between ARE-A60 and the deadenylated ARE (ARE-A54) was quantified to measure the stability of the mRNA, xCIRP2 seemed to modestly affect the RNA stability. Our results suggest that RNA that had once escaped from the inhibitory effect of xCIRP2 on deadenylation could be degraded in the absence of exogenous ElrA. When ElrA was added to the system, deadenylated RNA was accumulated, and thereby degradation of the RNA body was almost completely inhibited (Fig. 4A). Further, it is clear that xCIRP2 and ElrA act additively in stabilization of RNA possessing the poly(A) tail. Titration of xCIRP2 in the presence of ElrA confirmed the inhibition of deadenylation by xCIRP2 (Fig. 4B). We concluded that xCIRP2 and ElrA could stabilize ARE-containing RNA cooperatively.

Identification of mRNAs Interacting with xCIRP2 in the Oocytes—Given that polyadenylation and deadenylation have a significant impact on the stability and translational activity of mRNA, the data shown above suggest that xCIRP2 affects the fate of specific mRNA by regulating the length of the poly(A) tail. We then sought to isolate mRNAs that interact with xCIRP2 in Xenopus oocytes. We performed immunoprecipitation with anti-xCIRP2 antibody from the oocyte lysate. RNA was then recovered from the immunoprecipitates and reverse-transcribed with oligo(dT) primer, and the resulting cDNA was cloned into a plasmid vector. Sequences of isolated clones were compared with the GenBank™/EMBL/DDJB data bases through the BLAST search engine. The 18 S ribosomal RNA was most frequently identified (11 clones) among 75 clones whose sequences were determined, consistent with our previous results showing that xCIRP2 is possibly associated with ribosomes (26). Yang and Carrier (32) have also reported the association of human CIRP/A18 heterogeneous nuclear ribonucleoprotein with 18 S ribosomal RNA. Some of the mRNAs interacting with xCIRP2 were verified by RT-PCR using the primers specific for each mRNA. To examine whether these mRNAs are associated with ElrA as well, RT-PCR was performed using RNA recovered from immunoprecipitates with anti-xCIRP2 or anti-ElrA antibodies (Fig. 5). As xCIRP2-interacting mRNAs, we identified mRNAs that encode Xenopus cyclin B1, chromatin assembly factor 1 p150 subunit, and the centrosomal kinase Nek2B and those that show high homologies with human cytochrome c oxidase peptide VIb and bixin mRNAs. Among these, cyclin B1 and Nek2B mRNAs were also detected in the immunoprecipitates with anti-ElrA antibody. These results suggest that there exist MRNPs containing specific mRNAs together with xCIRP2 and ElrA proteins in Xenopus oocytes.

xCIRP2 Inhibits Deadenylation of Nek2B mRNA—Having identified several mRNAs associating with xCIRP2 in Xenopus oocytes, we tested whether xCIRP2 affects the stability of these mRNAs. We chose Nek2b mRNA for analysis in the in vitro mRNA stability system, since this mRNA contains a relatively long U-rich region in its 3'-UTR, although there is no AUUUA pentamer in the 3'-UTR (Fig. 6A). In addition, Nek2b mRNA was shown to interact both with xCIRP2 and ElrA, suggesting that it might be subjected to regulation of the poly(A) tail length. We prepared a RNA substrate (Nek2b-A60) containing the full length of the Nek2b mRNA 3'-UTR followed by a poly(A)_60 tail. A gel retardation assay revealed that xCIRP2 and ElrA could bind to Nek2b-A60 RNA in vitro (Fig. 6B). When Nek2b-A60 RNA was added to the in vitro mRNA stability system, deadenylation was observed in a 30-min incubation, albeit the rate and extent of the deadenylation was lower than those of ARE-A60 RNA (Fig. 6C). By adding the increasing amounts of xCIRP2, deadenylation of Nek2b-A60 RNA was almost completely inhibited. As shown above for ARE-A60 RNA (Fig. 4), ElrA inhibited deadenylation of the RNA body of Nek2b-A60 RNA (Fig. 6D). Our data demonstrate that xCIRP2 could regulate the lengths of the poly(A) tail of specific mRNAs with which it associates in Xenopus oocytes.
DISCUSSION

To date, three Xenopus CIRP homologs, XCIRP, XCIRP-1, and xCIRP2, have been reported (26, 33, 34). xCIRP2 protein is accumulated during oogenesis and is abundant in full-grown oocytes (26). Suppression of XCIRP-1 produced tailbuds with deformations of the brain and internal organs and also disrupted morphogenetic migration of specific blastomeres (34). Therefore, although it has been clarified that Xenopus CIRPs play important roles in differentiation and morphogenesis during early development, the functional roles played by xCIRP2 in the oocytes remain unclear. In this study, we found that ElrA interacts with xCIRP2. Since the mammalian homolog of ElrA, HuR, plays an important role in stabilization of ARE-containing mRNAs, we tested whether xCIRP2 would be involved in mRNA stabilization. Using the in vitro mRNA stabilization system, we revealed that xCIRP2 and ElrA are involved in the regulation of mRNA stability at different steps.

ELAV proteins contain three RRMs with a less conserved hinge region between RRM2 and RRM3. Previous studies showed that RRM1 and RRM2 are sufficient for binding to the ARE (35, 36), whereas RRM3 is capable of binding to poly(A) in a filter binding assay (37). By deletion mutant analysis, it has been shown that RRM3 of HuR is necessary for stabilization (21) and that the hinge region contains the nucleocytoplasmic shuttling signal (38). More recent study revealed that any combination of two of the three RRMs is sufficient for the ARE-binding activity and mRNA stabilization activity of HuR (39). Here we delineated that RRM3 of ElrA is involved in the interaction with xCIRP2. In the in vitro system, xCIRP2 and ElrA could stabilize ARE-containing mRNA cooperatively. Thus, we propose that the role of RRM3 of ElrA/HuR in mRNA stabilization is in part carried out by the complex formation with xCIRP2.

Studies on subcellular localization of ElrA/HuR have provided clues to the elucidation of its functions in RNA metabolism, and this might be applicable to xCIRP2/CIRP. First, both shuttle between the nucleus and the cytoplasm in cultured cells, although they are found predominantly in the nucleus (28, 38). HuR has been proposed to associate with its target mRNAs in the nucleus and accompany them into the cytoplasm, where it functions as an mRNA stabilizer (20). It is possible that the shuttling of xCIRP2 and HuR is required for efficient export of the target mRNAs or for protection of them prior to the nuclear export. Second, localization and expression levels of HuR and CIRP are regulated by cellular stresses (24, 27, 32, 40–42). Among them, upon UV irradiation, cytoplasmic fractions of both proteins are increased and are involved in stabilization of specific mRNAs (24, 32). Third, both ElrA and xCIRP2 proteins are predominantly cytoplasmic in Xenopus oocytes (25, 26). As a large stockpile of maternal mRNA is accumulated in the oocyte cytoplasm, ElrA and xCIRP2 may play roles in the control of poly(A) lengths of these mRNAs.

Previous experiments revealed that xCIRP2 and FRGY2 are abundant RNA-binding proteins in the full-grown oocytes (26). Whereas xCIRP2 is a homolog of CIRP that is overexpressed upon cold shock in mammalian cells, FRGY2 is a member of the Y-box protein family that shares an RNA binding domain conserved to those of bacterial cold shock proteins (43, 44). Considering the translation and stabilization of mRNAs in the context of mRNP, it is conceivable that a packaging role of
Y-box proteins, as the major core component of mRNPs, is required for the assembly of translationally repressed mRNPs in the oocytes (43, 45). It is established that the association with Y-box proteins protects mRNAs from degradation (46–48). However, in this study, we did not observe any mRNA stabilization effect of FRGY2. One possibility for this discrepancy is that the ARE-Rho RNA template is too short to be protected by Y-box proteins, given that Y-box proteins form homomultimers and that a single Y-box protein molecule associates with 35–40 nucleotides of RNA (48).

As an inhibitor of deadenylation of ARE-containing mRNAs, Xenopus embryonic poly(A)-binding protein was identified in an in vitro system using egg extract (49). It was suggested that embryonic poly(A)-binding protein inhibits deadenylation through associating with the poly(A) tail of the RNA substrate. However, it is not likely that xCIRP2 protects poly(A) tail of the substrate by associating with it, because in the in vitro system using HeLa S100 extract, poly(A) competitor RNA was included to sequester the poly(A)-binding protein. Another possible mechanism for inhibition of deadenylation is to inhibit the enzymatic activity or the recruitment of a deadenylase(s). The enzyme responsible for the default deadenylation upon Xenopus oocyte maturation is a deadenylase PARN/DAN (50). The human homolog of PARN has been shown to be also functional in the in vitro system that we used here (31). Recent work revealed that another deadenylase, Xenopus nocturnin, is involved in posttranscriptional control of circadian rhythm-related mRNAs (51). Nocturnin mRNA shows circadian expression in the Xenopus retina with a peak in the early night. In this context, it is worth pointing out that mouse CIRP mRNA is expressed in the suprachiasmatic nucleus and the cerebral cortex with a peak during daytime (52). In contrast, CIRP mRNA increases in the Xenopus brain during the night (53). CIRP mRNA might maintain poly(A) tail of specific mRNAs by regulating the activity of these deadenylases.

The amount of xCIRP2 protein remains essentially the same in a full-grown oocyte and in an egg, yet it might have different roles in modulating RNA metabolism in oocytes, eggs, and early embryos. When eCPE-binding proteins were identified by UV cross-linking, ElrA is the only protein in oocyte cytoplasmic extract that binds to eCPEs in C12 and activin receptor mRNAs, whereas in egg extract, an additional 45-kDa protein is UV-cross-linked to eCPEs (54). Therefore, different proteins appear to associate with ElrA and regulate the changes in poly(A) length in different stages of development. Among xCIRP2-interacting mRNAs in the oocytes that we identified here, cyclin B1 mRNA has short poly(A) in the oocytes and is polyadenylated upon oocyte maturation (55). Although the poly(A) length of Nek2B mRNA in Xenopus development has not been examined, Nek2B protein is synthesized late in oogenesis and increased during meiosis II, suggesting that Nek2B mRNA is translated both in the oocytes and eggs (56, 57). Therefore, it is likely that Nek2B mRNA is not deadenylated in matured oocytes by the default pathway. Our finding that xCIRP2 inhibits deadenylation of specific mRNAs, such as those containing ARE and Nek2B 3′-UTR adds an interesting possibility that xCIRP2 is involved in translational control of mRNA by regulating the poly(A) length in the oocytes and eggs.

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