Localization of AU-rich Element-containing mRNA in Cytoplasmic Granules Containing Exosome Subunits* [S]

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Eukaryotic mRNAs can be degraded in either decapping/5′-to-3′ or 3′-to-5′ direction after deadenylation. In yeast and mammalian cells, decay factors involved in the 5′-to-3′ decay pathway are concentrated in cytoplasmic processing bodies (P bodies). The mechanistic steps and localization of mammalian mRNA decay are still not completely understood. Here, we investigate functions of human mRNA decay enzymes in AU-rich element (ARE)-mediated mRNA decay (AMD) and find that the deadenylase, poly(A) ribonuclease PARN, and enzymes involved in the 5′-to-3′ and 3′-to-5′ decay pathways are required for AMD. The ARE-containing reporter mRNA accumulates in discrete cytoplasmic granular structures, which are distinct from P bodies and stress granules. These granules consist of poly(A)-specific ribonuclease, exosome subunits, and decay-promoting ARE-binding proteins. Inhibition of AMD increases accumulation of ARE-mRNA in these granules. We refer to these structures as cytoplasmic exosome granules and suggest that some AMD may occur in these granules.

Regulation of mRNA turnover is an important process in determining levels of gene expression. mRNA stability varies considerably from one mRNA species to another and is determined by specific cis-acting elements within the mRNA molecule (1, 2). Two general exonucleolytic mRNA decay pathways exist in Saccharomyces cerevisiae (3). Both pathways initially involve poly(A) shortening that is predominantly mediated by an enzyme complex containing Ccr4p and Caf1p (4). Subsequently, the mRNA is degraded in either a 5′-to-3′ or a 3′-to-5′ direction. In the 5′-to-3′ decay, 7mGDP is removed from the 7mGppN cap by the Dcp1p/Dcp2p decapping complex followed by degradation of the transcript by the 5′-to-3′ exonuclease Xrn1p (3). In the 3′-to-5′ decay, mRNA is degraded by the cytoplasmic exosome (3), a complex containing exoribonucleases (5, 6). Strikingly, 5′-to-3′ decay enzymes and decapping activators are concentrated in discrete cytoplasmic foci called processing bodies (P bodies), which are sites for mRNA decay as well as for mRNA storage (7, 8).

Human homologs of the yeast mRNA decay enzymes have been identified and include the decapping complex, DCP1-DCP2 (9–11), the 5′-to-3′ exonuclease, XRN1 (12), the exosome (13, 14), and the deadenylases CCR4 and PAN2 (15, 16). Human cells also contain an additional deadenylase, poly(A) ribonuclease PARN (17). Several studies have suggested that these human mRNA decay enzymes are involved in mRNA decay. Overexpression of an active site mutant of CCR4 alters the mRNA decay rate mediated by the c-fos major protein-coding determinant of instability (18). PAN2 and CCR4 are the major poly(A) nucleases that act in concert in decay of normal and nonsense-containing mRNAs (19). Furthermore, nonsense-mediated mRNA decay (NMD) requires PARN, components of the human exosome, and DCP2 (20). mRNA decay triggered by tethered SMG7 that mimics NMD requires functions of DCP2 and XRN1 (21). Interestingly, human 5′-to-3′ mRNA decay enzymes and decapping activators are also concentrated in discrete cytoplasmic foci called P bodies or Dcp/GW bodies (22, 23), which are likely mRNA decay sites (24).

A mechanism that rapidly degrades mRNAs containing the AU-rich elements (AREs) present in the 3′-untranslated regions (UTRs) is ARE-mediated mRNA decay (AMD) (25). AMD accounts for degradation of most unstable mRNAs in human cells (26). A number of proteins collectively called ARE-binding proteins (ARE-BPs) regulate AMD and can be divided into two groups by function: decay-promoting/destabilizing ARE-BPs including tristetraprolin (TTP), butyrate response factor 1, AU-rich binding factor 1, and KSRP and stabilizing ARE-BPs, including Hu protein R (27, 28). A current model for AMD is that decay-promoting ARE-BPs recruit mRNA decay machinery involved in the deadenylation, 5′-to-3′ and 3′-to-5′ decay (29–31). Several studies suggest that AMD requires decay enzymes involved in both 5′-to-3′ and 3′-to-5′ directions after deadenylation. Using an in vitro RNA decay system, ARE RNA substrates are preferentially degraded by deadenylation/3′-to-5′ decay pathway that requires PARN and the exosome (14, 32–34). Overexpression of DCP2 facilitates AMD (30). Using an ARE mRNA reporter and RNAi silencing experiments, it was suggested that AMD is inhibited more when

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[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–8.

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[3] The abbreviations used are: PARN, poly(A)-specific ribonuclease; ARE, AU-rich element; ARE-BP, ARE-binding protein; AMD, ARE-mediated mRNA decay; TTP, tristetraprolin; KSRP, KH-type splicing regulatory protein; TIA, T cell intracellular antigen; YFP, yellow fluorescence protein; RFP, red fluorescence protein; GC-CSF, granulocyte/macrocyte colony-stimulating factor; CAT, chloramphenicol acetyltransferase; siRNA, small interference RNA; RNAi, RNA interference; SG, stress granule; 3′-UTR, 3′-untranslated region; Dox, doxycycline; NLS, nuclear localization signal; NMD, nonsense-mediated mRNA decay; GB, β-globin.
decay factors involved in the 5′-to-3′ decay are down-regulated than when exosome subunits are down-regulated (35) and is initiated from both the 5′ and 3′ ends, in which the 5′ and 3′ decay pathways are functionally linked (36).

We investigated mechanisms of AMD and functions of mRNA decay enzymes in this process. We demonstrated that AMD is inhibited by down-regulation of PARN, factors involved in the 5′-to-3′ decay pathway, and exosome subunits. Overexpression of the decapping enzymes or individual exosome subunits enhances AMD. Localization of an ARE-mRNA reporter by indirectly visualizing a tethered yellow fluorescence protein suggests that the mRNA accumulates in discrete cytoplasmic granular structures concentrated with exosome subunits, which are referred to as exosome granules. We further showed that cytoplasmic exosome granules are distinct subcellular structures from P bodies and stress granules. Inhibition of AMD increases accumulation of ARE-mRNA in these granules. Finally, real-time analysis of the dynamics of an exosome focus and ARE-mRNA showed that the mRNA is targeted to and disappears in the focus. These results suggest that one of the pathways for AMD is deadenylation by PARN followed by the 3′-to-5′ decay by exosome subunits, which may occur in cytoplasmic exosome granules.

**Experimental Procedures**

*Plasmids*—A plasmid (TRE-GB) expressing the rabbit β-globin gene under the control of a Tet-responsive promoter containing multiple cloning sites in the 3′-UTR was described (31). The 3′-UTR of GM-CSF was amplified by PCR and subcloned between NotI and Sall sites of TRE-GB. Six copies of MS2 binding site were amplified by PCR and subcloned consecutively into the multiple cloning sites upstream of the GM-CSF 3′-UTR to obtain a construct expressing GB-12bs-ARE mRNA. To express FLAG-tagged proteins, a polylinker encoding the FLAG peptide was subcloned between HindIII and KpnI sites of pcDNA3.1/His vectors. The coding regions of RRP4, RRP46, and CSL4 were amplified by PCR and subcloned into EcoRI and XhoI, KpnI and XhoI, and EcoRV and XhoI sites of pcDNA-FLAGa, -b, and -c, respectively. The coding region of TTP was PCR amplified and subcloned into EcoRV and XhoI sites of pcDNA-FLAGb. An EcoRI-XhoI fragment containing the coding region of KSRP was subcloned into EcoRV and XhoI sites of pcDNA-FLAGb. A DraIII-XhoI fragment containing the entire coding region of KSRP was subcloned into EcoRV and XhoI sites of pcDNA-FLAGb. A EcoRI-XhoI fragment containing the coding region of PARN (17) was subcloned into EcoRI and XhoI sites of pcDNA-FLAGa. Xpress-tagged DCP2 was constructed by subcloning the coding region into pcDNA3.1/His vectors. Red fluorescence protein (RFP)-tagged RFP4 was constructed by subcloning a cDNA, encoding monomeric RFP and amplified by PCR, into XhoI and XbaI of pcDNA-FLAG-RFP4.

*siRNAs*—siRNAs against mRNA decay enzymes were purchased from Qiagen. Sequences of siRNAs against RRP4, RRP46, PM-Sc1100, DCP2, and XRN1 were described previously (31). Sequences of siRNAs used to silence gene expression are PARN#1 (GGAGCUCUGUCUAAUGUAU), PARN#2 (GCAUCUCGAAAGAACAGCCCU), LSM1 (CACAACUAUGUCUACUA), and RRP41 (CUAUGAACUGUCAUAUA).

![FIGURE 1. Down-regulation of PARN inhibits deadenylation step of AMD.](image)

A. HeLa-TO cells were transfected with a construct expressing GB-ARE<sub>MC5</sub> mRNA and a control siRNA (CAT, top panel) or two different siRNAs (middle panel; 1; bottom panel, 2) against PARN. Cytoplasmic RNA was isolated after the addition of Dox following a 2-h transcriptional pulse and subjected to RNase H digestion with a GM-CSF oligonucleotide. Deadenylation was analyzed by using a GM-CSF 3′-UTR probe. RNA treated with both GM-CSF oligonucleotide and oligo(dT) was loaded in lane 6. Ethidium bromide staining of 28 S RNA is shown. Signals of poly(A)<sup>+</sup> 3′ GB-ARE<sub>MC5</sub> mRNA were quantitated by phosphorimaging and normalized to that of 28 S RNA, quantitated by the Gel Doc XR system (Bio-Rad). The levels of poly(A)<sup>+</sup> 3′ GB-ARE<sub>MC5</sub> mRNA during each time point are indicated and represented as the mean values ± S.D. (n = 3). B. Down-regulation of PARN by siRNAs. Extracts of HeLa-TO cells in A were subjected to immunoblot analysis with anti-PARN or anti-KSRP antibodies. Different amounts of CAT siRNA-treated extracts (10, 20, 50, or 100% of the amounts used in lane 7) were loaded to allow for estimation of knockdown efficiency.

**Transfection, RNA Isolation, and Northern Blot Analysis**—HeLa-TO cells were transfected using Lipofectamine. After transfection, cells were treated with tetracycline (50 ng/ml). To examine mRNA decay, a 2- or 16-h transcriptional pulse was employed by phosphate-buffered saline washing and incubated with tetracycline-free medium. Cytoplasmic RNA was isolated at different time points after the addition of doxycycline (2
Northern blot analysis using 32P-labeled RNA probes was done as previously described (31).

Antibodies—Rabbit polyclonal antisera against PARN (17), DCP1a (30), RRP4 (5), RRP40, RRP41, RRP46, PM-ScI75, and PM-ScI100 (37, 38) were described. Human antiserum against GW182 was described (23). Mouse monoclonal anti-FLAG and goat anti-TIA-1 were purchased from Sigma and Santa Cruz biotechnology, respectively.

Fluorescence Microscopy and Time-lapse Imaging—Cells were fixed with 2% cold paraformaldehyde for 20 min followed by incubation with 0.5% Triton X-100 for 10 min at room temperature. After blocking by phosphate-buffered saline containing 2% bovine serum albumin and 5% fetal bovine serum, cells were incubated with mouse anti-FLAG (1:5000), human anti-GW182 (1:500), rabbit anti-exosome subunits RRP4, RRP40, RRP41, and PM-ScI75 (1:500), goat anti-TIA-1 (1:500), or rabbit anti-DCP1a (1:500) antibodies. Secondary antibodies conjugated with different Alexa series fluorochromes (Molecular Probes), Texas Red-X (Molecular Probes), or fluorescein isothiocyanate (Jackson Immunoresearch) were used. Cells were visualized using an Olympus IX70 inverted fluorescence microscope. For live cell imaging, cells were plated on 35-mm glass-bottomed tissue culture dishes. YFP- and RFP-positive cells were visualized with an Olympus IX70 inverted fluorescence microscope. Images of cells were acquired with a Photometrics 1400 charge-coupled device camera under the control of IPLab software (Scanalytics, Inc.).

RESULTS

AMD Requires PARN—To investigate mechanisms of AMD, we first sought the deadenylase involved in the decay. RNAi was used to down-regulate known human deadenylases, including PARN, CCR4, and PAN2, and decay of a β-globin (GB) mRNA reporter containing the 3′-UTR of GM-CSF (GB-AREGMCSF) was monitored in a transcriptional pulse-chase (Tet-Off) assay. Down-regulation of PARN, but not CCR4 or PAN2, decreased AMD by 2-fold (data not shown). To investigate whether PARN is involved in deadenylation, we monitored the levels of poly(A)+ GB-AREGMCSF mRNA. To obtain homogenous poly(A) tails, we performed a 2-h transcriptional pulse and isolated cytoplasmic RNA after the addition of doxycycline (Dox), which was then treated with an oligonucleotide complementary to the 5′ end of inserted GM-CSF 3′-UTR and RNase H to resolve adenylated and deadenylated GB-AREGMCSF mRNAs. A smear of truncated 3′ fragments after oligonucleotide-directed RNase H cleavage was detected (Fig. 1A, lane 1). This smear was converted to a single species after additional treatment with oligo(dT)12–18 (Fig. 1A, lane 6), suggesting that the

FIGURE 2. Down-regulation of XRN1, LSM1, and some exosome subunits inhibits AMD. A, decay of GB-AREGMCSF mRNA was analyzed in HeLa-TO cells transfected with siRNAs against CAT, DCP2, XRN1, or LSM1 after the addition of Dox after transcriptional pulse. Signals of GB mRNA were quantitated and normalized to that of 28 S rRNA. The calculated half-lives (t1/2) of GB-ARE mRNA are shown. Numbers in the parentheses indicate fold changes in t1/2 (n = 2) and are represented as the mean values ± S.D. B, decay of GB-AREGMCSF mRNA was analyzed in cells transfected with siRNAs against CAT, RRP4, RRP41, RRP46, PM-ScI100, RRP4+RRP46, XRN1+RRP46, or LSM1+RRP46. The calculated t1/2 (n = 3) of GB-AREGMCSF mRNA and fold changes in t1/2 are shown. C, down-regulation of DCP2, XRN1, and LSM1 by RNAi. Extracts of HeLa-TO cells transfected with siRNAs as indicated were subjected to immunoblot analysis with anti-DCP2 or anti-α-tubulin antibodies (top panel). RNA was prepared from cells transfected with siRNAs as indicated and subjected to Northern blot analysis with probes against XRN1, LSM1, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; middle and bottom panels). D, down-regulation of exosome subunits by RNAi. Extracts from transfected cells in B were subjected to immunoblot analysis using antisera against exosome subunits.
heterogeneity is due to poly(A) length variation. Although the poly(A) tail and the mRNA body were rapidly removed in control cells after Dox addition (Fig. 1A, top panel), the poly(A) tail was removed slower in cells treated with two different PARN siRNAs (Fig. 1A, middle and bottom panels). Quantitation of the levels of poly(A)\(^{+}\) GB-ARE\(^{GMCSF}\) mRNA indicated that down-regulation of PARN by the first (#1) siRNA and the second (#2) siRNA decreased deadenylation by 2.5- and 1.6-fold, respectively (Fig. 1A). The differential reduction in deadenylation by PARN siRNAs might be due to knockdown efficiency, in which the first siRNA down-regulated PARN by \(\sim 80\%\) and the second siRNA down-regulated PARN by \(\sim 40\%\) (Fig. 1B). In a similar assay down-regulation of PARN did not significantly alter deadenylation of a GB mRNA without an ARE, although its deadenylation was rather inefficient (see supplemental Fig. S1). These results suggest that PARN is involved in the deadenylation step of AMD.

**FIGURE 3. Overexpression of the decapping enzymes DCP2 or DCP1a and individual exosome subunits RRP4 or CSL4 enhance AMD.** A, decay of GB-ARE\(^{GMCSF}\) mRNA was analyzed in cells transfected with siRNAs against CAT or RRP46 and constructs expressing decay enzymes DCP2, DCP1a, RRP4, or CSL4. The calculated \(t_{1/2}\) \((n = 3)\) are shown. B, immunoblot analysis shows down-regulation of RRP46 by RNAi in A. C, expression of DCP2 and expression of DCP1a, RRP4, and CSL4 in A were analyzed by anti-Xpress and anti-FLAG, respectively.

Down-regulation of mRNA Decay Factors Involved in the 5'-to-3' and 3'-to-5' Decay Pathways Inhibits AMD—We next examined effects of down-regulation of mRNA decay enzymes/factors involved in the 5'-to-3' and 3'-to-5' decay on AMD. Although down-regulation of DCP2 did not significantly alter mRNA decay rate, down-regulation of XRN1 or LSM1, which is one of subunits of the LSM1–7 complex required for decapping, moderately (1.5-fold) decreased the decay rate of GB-ARE\(^{GMCSF}\) mRNA (Fig. 2A). Down-regulation of each factor by RNAi was between 80 and 90% analyzed by immunoblot or Northern blot (Fig. 2C). Down-regulation of individual exosome subunits, such as RRP4, RRP41, and RRP46, inhibited AMD by 1.5–2-fold depending on the subunits subjected to RNAi (Fig. 2B). In contrast, down-regulation of PM-Scl100 did not alter the mRNA decay rate (Fig. 2B). The varied inhibition of AMD by down-regulation of individual exosome subunits might reflect the efficiency of down-regulation (Fig. 2D). Simultaneous down-regulation of two exosome subunits, RRP4 and RRP46, or down-regulation of both XRN1 and RRP46 or both LSM1 and RRP46 did not further inhibit AMD (Fig. 2B). Altogether, these results suggest that mRNA decay enzymes involved in the 5'-to-3' and 3'-to-5' decay are required for AMD.

**Overexpression of the Decapping Enzymes or Individual Exosome Subunits Enhances AMD**—To further demonstrate that the mRNA decay enzymes tested above are involved in AMD, we overexpressed the decapping enzymes, DCP2 and DCP1a, and individual exosome subunits, RRP4 and CSL4, and examined their effects on AMD. Overexpression of these mRNA decay enzymes only minimally enhanced AMD (data not shown), suggesting that they are not limiting for AMD. We next examined whether overexpression of these enzymes enhances AMD in cells depleted of an exosome subunit RRP46. Although down-regulation of RRP46 resulted in a 2-fold inhibition of AMD (Fig. 3A), overexpression of the decapping enzymes, DCP2 or DCP1a, or individual exosome subunits, RRP4 or CSL4, restored mRNA decay in RRP46 siRNA-treated cells (Fig. 3A). Under these conditions, RRP46 was equally down-regulated (Fig. 3B). Immunoblot analysis using antibodies against DCP1a, DCP2, and RRP4 suggested that 2–5-fold overexpression of epitope-tagged proteins was achieved as compared with the endogenous levels (data not shown). In a similar assay, overexpression of these enzymes did not significantly enhance decay rate of a globin mRNA lacking an ARE (see supplemental Fig. S2). These results suggest that activation of either the decapping or the 3'-to-5' decay pathway can enhance AMD when decay efficiency is decreased.

An ARE-mRNA Reporter Accumulates in Discrete Cytoplasmic Exosome Granules Distinct from P Bodies and Stress Granules—To investigate localization of ARE-mRNA in the cytoplasm, we expressed a GB mRNA containing 12 copies of the bacteriophage MS2 coat protein binding site and the 3'-UTR of GM-CSF (\(GB-12bs-ARE\); Fig. 4A) under the control of the T7-regulatory promoter in HeLa-TO cells and indirectly visualized the mRNA
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by a coexpressed MS2-YFP fusion protein containing the nuclear localization signal (NLS). Expression of GB-12bs-ARE mRNA with coexpressed MS2-YFP-NLS was analyzed by Northern blot after a 6-h transcriptional pulse. The major mRNA species are the full-length transcripts whose expression is repressed by Dox (see supplemental Fig. S3A). Furthermore, mRNA decay assays showed that GB-12bs-ARE mRNA was rapidly degraded in the presence of coexpressed MS2-YFP-NLS (supplemental Fig. S7 and data not shown), suggesting that this mRNA is degraded through the AMD pathway. Under this condition, we detected weaker diffuse cytoplasmic YFP signals and a few stronger YFP signals in discrete cytoplasmic granules (Fig. 4).

To demonstrate that the observed granules indeed result from localization of exosome subunits, we expressed FLAG-tagged RRP46 and examined its cytoplasmic localization. FLAG-RRP46 was observed in cytoplasmic granules, which expressed (see supplemental Fig. S3, B and C). Furthermore, these YFP granules almost completely, if not all, overlapped with RNA granules detected by in situ hybridization using a GB probe (see supplemental Fig. S3D). These results strongly suggest that cytoplasmic YFP-containing granules represent GB-12bs-ARE mRNA tethered with MS2-YFP-NLS, which accumulates in discrete cytoplasmic structures called ARE-mRNA granules.

We next investigated colocalization of ARE-mRNA granules with known cytoplasmic structures implicated in RNA metabolism; that is, P bodies and stress granules (SGs). Cells expressing GB-12bs-ARE mRNA and MS2-YFP-NLS were stained with an antibody to DCP1a, a P body component. Surprisingly, ARE-mRNA granules did not overlap with DCP1a (Fig. 4A). To examine whether ARE-mRNA is only transiently associated with P bodies (24), we down-regulated XRN1 and visualized the mRNA. ARE-mRNA granules largely did not colocalize with P bodies in XRN1 siRNA-treated cells (Fig. 4A). Occasionally, few RNA granules containing DCP1a were detected in cells treated with XRN1 siRNA (Fig. 4A, indicated by an arrowhead). We next stained cells with an antibody against TIA-1, a SG maker (39). TIA-1-containing granules were not detected in cells grown in non-stressed condition, whereas ARE-mRNA granules were observed (Fig. 4B). When treated with arsenite, SGs were formed, and the majority, if not all, of ARE-mRNA granules did not overlap with SGs (Fig. 4B). These results suggest that ARE-mRNA granules are distinct from P bodies and SGs.

Cytoplasmic Exosome Subunits Are Enriched in Discrete Granules—It was suggested that Drosophila exosome subunits localized in discrete cytoplasmic foci (40). We examined whether human exosome subunits are also enriched in cytoplasmic foci. HeLa cells were stained with rabbit antisera against exosome subunits including RRP4, RRP40, RRP41, and PM-Sc175. These anti-exosome sera, which primarily recognized their corresponding proteins in immunoblot analysis (see supplemental Fig. S4), displayed strong nucleolar and nuclear staining as well as weak cytoplasmic staining, which is readily observable upon longer exposure (Fig. 5). Strikingly, in addition to weak diffuse localization, staining of these exosome subunits exhibited a number of small granular appearance (granules) with small as well as large evenly defined bright punctate spots (foci) in the cytoplasm (Fig. 5). Because these granules and foci vary in size and number from cell to cell, we examined 100–200 cells for cytoplasmic localization of exosome subunits including RRP4, RRP40, and PM-Sc175. Cytoplasmic staining was categorized into two major patterns, 1) weak-diffused cytoplasmic staining with a number of small granules as well as small or large well defined foci and 2) diffuse cytoplasmic staining with a number of small granules but without apparent well defined foci (Fig. 5). These exosome subunits exhibited similar cytoplasmic localization. ~50% of cells examined showed that these subunits are localized to small granules as well as bright well defined foci, and only small granules concentrated with exosome subunits were observed in another ~50% of cells (Fig. 5). To demonstrate that the observed granules indeed result from localization of exosome subunits, we expressed FLAG-tagged RRP46 and examined its cytoplasmic localization. FLAG-RRP46 was observed in cytoplasmic granules, which

FIGURE 4. ARE-mRNA accumulates in discrete cytoplasmic granules distinct from P bodies and stress granules. A, HeLa-TO cells expressing GB-12bs-ARE mRNA and MS2-YFP-NLS were transfected with a control CAT siRNA (top panel) or an XRN1 siRNA (bottom panel) and stained with anti-DCP1a. The mRNA was visualized by YFP signal, and mRNA granules are indicated (arrows). Anti-DCP1a was also visualized (red). A schematic on the top of the panels shows the mRNA bound by MS2-YFP-NLS. An MS2 site interacts with a dimer of MS2-YFP-NLS. B, HeLa-TO cells expressing GB-12bs-ARE mRNA and MS2-YFP-NLS were untreated (top panel) or treated (bottom panel) with arsenite (ARS, 0.5 mM) for 30 min and stained with anti-TIA-1. The mRNA granules were indicated (arrows). Anti-TIA-1 was also visualized (red).
overlapped with endogenous RRP40 (see supplemental Fig. S5). Furthermore, RFP-tagged RRP4 was also localized in cytoplasmic granules, which colocalized with endogenous PM-Scl75 (see supplemental Fig. S8). Altogether, these results suggest that cytoplasmic exosome subunits are localized to granules as well as well defined discrete foci, whose size and number vary from cell to cell.

**Exosome Subunit-containing Granules Are Distinct from P Bodies and SGs**—We further characterized cytoplasmic exosome subunit-containing granules. HeLa-TO cells were co-stained with antisera against exosome subunits, RRP4, RRP40, and PM-Scl75, and human serum against GW182, a key P body component (23). GW182 completely colocalized with DCP1a (Fig. 6A), indicating that the staining by anti-GW182 represents P bodies. Small and well defined cytoplasmic granules containing RRP4, RRP40, or PM-Scl75 largely did not overlap with P bodies (Fig. 6B). The overlapped signal in the nucleus was because the human anti-GW182 serum also displays addi-
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from mRNA decay enzymes found in P bodies, we named them cytoplasmic exosome granules.

Localization of ARE-mRNA Reporter in Cytoplasmic Exosome Granules—Examination of ARE-mRNA granules and exosome subunit-containing granules showed significant colocalization (Fig. 7A, indicated by arrows). Few ARE-mRNA granules did not colocalize with the exosome subunits tested (Fig. 7A). FLAG-tagged RRP46, which overlapped with endogenous RRP40, colocalized with ARE-mRNA granules (Fig. 7B, indicated by arrows). We next examined whether PARN colocalized with ARE-mRNA granules. Cells expressing GB-12bs-ARE mRNA, MS2-YFP-NLS, and FLAG-PARN were stained with anti-FLAG and anti-exosome subunits, RRP40 or RRP41. ARE-mRNA granules significantly colocalized with FLAG-PARN and exosome subunits (Fig. 7C, indicated by arrows). We coexpressed GB-12bs-ARE mRNA and MS2-YFP-NLS with FLAG-KSRP or FLAG-TTP, both of which promote AMD. In cells coexpressing FLAG-KSRP, ARE-mRNA granules contained FLAG-KSRP, which significantly overlapped with RRP40 but not with DCP1a (see supplemental Fig. S6A). In cells coexpressing FLAG-TTP, ARE-mRNA granules consisted of FLAG-TTP, which overlapped either with DCP1a or with RRP40 (see supplemental Fig. S6B). Altogether, these results suggest that an ARE-mRNA reporter accumulates in discrete cytoplasmic granules composed of mRNA decay enzymes involved in deadenylation (PARN) and 3′-to-5′ decay pathway (exosome subunits) and that distinct ARE-BPs colocalize with ARE-mRNA in different cytoplasmic granules.

Down-regulation of RRP46 Increases the Number of ARE-mRNA Granules, and Inhibition of AMD Increases the Accumulation of ARE-mRNA in Exosome Granules—We investigated whether inhibition of AMD would alter the feature of ARE-mRNA granules. We down-regulated RRP46 in cells expressing GB-12bs-ARE mRNA and MS2-YFP-NLS and examined ~100 cells for ARE-mRNA granules (Fig. 8A). The majority of cells treated with a control siRNA contained

tional nuclear-envelope staining that does not represent GW182 (41). Occasionally, few granules containing exosome subunits and GW182 were detected (Fig. 6B, indicated by arrows). In cells treated with arsenite, exosome subunits largely did not overlap with SGs, and occasionally exosome subunits embedded in a few large SGs were observed (Fig. 6C, indicated by arrows). Altogether, these results suggest that exosome subunit-containing granules are distinct from P bodies and SGs. Because these structures contain exosome subunits distinct with ARE-mRNA in different cytoplasmic granules.

FIGURE 7. ARE-mRNA accumulates in cytoplasmic exosome granules containing PARN. A, HeLa-TO cells expressing GB-12bs-ARE mRNA and MS2-YFP-NLS were stained with antisera against exosome subunits RRP4 or RRP40. The mRNA granules (green) and anti-RRP4 and -RRP40 (red) were visualized. Colocalization of ARE-mRNA granules with exosome subunits is indicated by arrows. B, HeLa-TO cells expressing GB-12bs-ARE mRNA, MS2-YFP-NLS, and FLAG-RRP46 were stained with anti-FLAG and anti-RRP40. Arrows indicate colocalization of ARE-mRNA granules with FLAG-RRP46 and endogenous RRP40. C, HeLa-TO cells expressing GB-12bs-ARE mRNA, MS2-YFP-NLS, and FLAG-PARN were stained with anti-FLAG and anti-RRP40 or anti-FLAG and anti-RRP41. Arrows indicate colocalization of ARE-mRNA granules with FLAG-PARN and exosome subunits.
1–5 ARE-mRNA granules. In contrast, more ARE-mRNA granules (6–15 granules) were observed in cells treated with an RRP46 siRNA, although the number varied from cell to cell (Fig. 8B). We next examined whether activation of the p38 signaling pathway by treatment with anisomycin, which inhibits AMD (42, 43), could interfere with localization of ARE-mRNA in exosome granules. No decay of GB-12bs-ARE mRNA was detected in cells treated with anisomycin after the 2-h addition of Dox (see supplemental Fig. S7). Cells were examined for ARE-mRNA granules (Fig. 8C). Anisomycin treatment increased the number of ARE-mRNA granules as compared with untreated cells (10–20 granules versus 1–5 granules).
granules; Fig. 8D). These granules significantly colocalized with RRP40 (Fig. 8C). These results suggest that inhibition of mRNA decay by decreasing the levels of RRP46 or by activation of the p38 pathway increases the number of ARE-mRNA granules.

To provide support that ARE-mRNA is targeted to and may be degraded in exosome granules, we investigated the dynamics of exosome granules and ARE-mRNA in living cells. We expressed a RFP-tagged RRP4 (RRP4-RFP) with GB-12bs-ARE mRNA and MS2-YFP-NLS. Analysis of fixed cells showed that most RRP4-RFP colocalized with PM-ScI75, suggesting that tagged RRP4 was incorporated into exosome complex, and that RRP4-RFP colocalized well with ARE-mRNA granules (see supplemental Fig. S8). RRP4-RFP and GB-12-ARE mRNA tethered by MS2-YFP-NLS were viewed by live cell imaging and RFP and YFP signals displayed in separate images. Although signal of an RRP4-RFP focus was relatively constant over time (Fig. 8E, indicated by an arrow), the YFP signal (indicated by an arrowhead) representing targeted mRNA tethered with MS2-YFP-NLS in the same focus disappeared rapidly. These results suggest that the targeted mRNA is degraded in the RRP4-RFP focus or released from that as soon as it is targeted.

**DISCUSSION**

**AMD Requires Function of PARN**—In the present study we investigated the deadenylase involved in ARE-mediated mRNA decay. Using RNAi knockdown analysis, we found that PARN is involved in AMD and is required for rapid removal of the poly(A) tail triggered by the GM-CSF ARE. Although the removal of poly(A) tail is impaired by down-regulation of PARN, poly(A) shortening still takes place. Although residual PARN activity may still be sufficient for the deadenylation, our results could also suggest that additional deadenylases may take over the deadenylation step in the absence of PARN but with a slower deadenylation rate. Alternatively, AMD may occur in a two-phase mechanism by two distinct deadenylases as recently elucidated for normal mRNA decay and NMD (19).

**AMD Requires Factors Involved in the 5′-to-3′ Decay and Exosome Subunits**—We investigated the mRNA decay enzymes involved in AMD after deadenylation. We observed that down-regulation of decay enzymes and factors involved in the 5′-to-3′ decay, including XRN1 and LSM1, had moderate effects on AMD and down-regulation of some exosome subunits, including RRP4, RRP41, and RRP46 involved in the 3′-to-5′ decay, also moderately inhibited AMD. Furthermore, overexpression of the decapping enzymes DCP1a or DCP2 and individual exosome subunits RRP4 or CSL4 restored mRNA decay when AMD efficiency is decreased. These results suggest that both the 5′-to-3′ and 3′-to-5′ decay pathways are involved in AMD after deadenylation and that activation of either the 5′-to-3′ decay or the 3′-to-5′ decay increases AMD. The moderate inhibition of AMD by down-regulation of either the 5′-to-3′ or the 3′-to-5′ decay enzymes suggests that these decay pathways are redundant for AMD. It is surprising that down-regulation of DCP2 does not inhibit AMD since it is the catalytic subunit of the decapping complex (9–11), and decapping is required for XRN1 activity (3). Nevertheless, our results are consistent with a previous finding in which down-regulation of DCP2 did not inhibit AMD (35) and may be explained by incomplete depletion of DCP2 activity by RNAi.

We found that AMD is differentially inhibited by down-regulation of different exosome subunits. Our results are consistent with the finding by other groups that down-regulation of individual exosome subunits has differential effects on NMD and AMD (20, 35). Based on the studies of the yeast exosome, it was suggested that it functions in all the processes of RNA metabolism as an intact exosome complex containing 10 core subunits (44, 45). Consistent with this, each yeast exosome subunit is an essential protein, and inactivation of any subunit gives similar defects (44, 45). In addition, there does not appear to be a substantial free pool of exosome subunits (13). Along this line, the differential effects on AMD by down-regulation of different exosome subunits may be attributed to differences in depletion efficiency by RNAi. However, we could not exclude that different exosome subunits may play different roles in the complex. For example, we observed that down-regulation of RRP46 inhibits AMD, although recent in vitro constitution of the human exosome suggested that the enzymatic activity of the exosome complex is attributed to the RRP41/RRP45 heterodimer (6). RRP46 subunit may be required for recognition of RNA substrates by the exosome complex or for interaction with exosome cofactors.

We found that overexpression of RRP4, CSL4, DCP1a, or DCP2 enhances AMD under the conditions of RRP46 down-regulation. RRP4 and CSL4, which are not suggested to have enzymatic activity (6), may enhance AMD by forming partial complexes with other subunits and/or increasing substrate recognition by the exosome complex. Overexpression of the decapping enzymes may facilitate targeting of ARE-mRNA to P bodies for degradation as we found that exogenously expressed DCP1a and DCP2 colocalized with ARE-mRNA granules (data not shown). We did not detect further inhibition of AMD by simultaneous down-regulation of mRNA decay enzymes involved in both decay pathways, such as XRN1+RRP46 or LSM1+RRP46. A simple explanation for this is that the residual enzymatic activity in siRNA-treated cells is still sufficient to function in AMD.

**ARE-mRNA Accumulates in Cytoplasmic Granules Concentrated with Exosome Subunits**—By indirectly visualizing an ARE-mRNA reporter with a tethered YFP, we demonstrated that the mRNA accumulates in discrete cytoplasmic granules consisting of mRNA decay enzymes, including PARN and exosome subunits. Previous studies have shown that human exosome subunits are localized both in the nucleus and particularly enriched in the nucleolus and in the cytoplasm (37, 38). However, it was not reported that cytoplasmic exosome subunits are localized to discrete granules. Because the nuclear/nucleolar staining of exosome subunits is usually stronger than that of cytoplasmic signals (37, 38), the appearance of cytoplasmic exosome granules might have been overlooked. Our finding of human exosome subunits concentrated in cytoplasmic granules is consistent with a
recent study suggesting that several Drosophila exosome subunits are enriched in cytoplasmic foci (40). We demonstrated that several exosome subunits are enriched in a number of granules as well as small and large well defined foci, which vary from cell to cell. The majority of exosome granules do not overlap with P bodies and SGs, suggesting that they are distinct subcellular structures. Localization of exosome subunits in cytoplasmic granules may depend on the cell growth state, such as cell cycle, and/or the overall levels of mRNA decay.

Despite all our efforts to trap mRNA decay intermediates by insertion of a variety of secondary structures into an ARE-mRNA reporter, we have not been able to do so. Nevertheless, our studies provide several important observations. First, an ARE-mRNA reporter accumulates in cytoplasmic granules consisting of mRNA decay enzymes, PARN, and exosome subunits involved in AMD. Second, down-regulation of an exosome subunit, RRP46, increases the number and size of ARE-mRNA granules. Third, activation of PARN, and exosome subunits involved in AMD. Second, further assemble into granules, which is followed by mRNA decay. Further studies of functions of diffuse cytoplasmic exosome complexes, and these mRNA-exosome complexes may be ARE-mRNA storage sites. It is also possible that diffuse cytoplasmic exosome complexes are responsible for mRNA decay, and localization of ARE-mRNA to granules is the consequence rather than the cause of mRNA decay. Alternatively, ARE-containing mRNA may be first targeted to the diffuse exosome complexes, and these mRNA-exosome complexes further assemble into granules, which is followed by mRNA decay. Further studies of functions of diffuse cytoplasmic exosome subunits and exosome granules in mRNA decay are warranted.

Our observation that ARE-mRNA localizes to exosome granules somewhat contradicts the report by Franks and Lykke-Andersen (46), who showed that ARE-mRNA localized to P bodies by using DCP1a-GFP as a P body marker. Nevertheless, our results are actually complementary, but not contradictory, to their results. We also found that ectopically expressed DCP1a or DCP2 colocalized with GB-12bs-ARE mRNA (data not shown) and colocalization of ARE-mRNA with coexpressed KSRP in exosome granules and with coexpressed TTP in exosome granules or P bodies (supplemental Fig. S6). Although colocalization of ARE-BPs with ARE-mRNA in these granules may be correlative, whether these ARE-BPs regulate localization of the mRNA in distinct cytoplasmic granules and perhaps the sites for AMD are currently unknown. Based on these results, we suggest that ARE-mRNA is localized to both P bodies and exosome granules and that its localization can be regulated depending on the expression levels or activities of distinct ARE-BPs and/or mRNA decay enzymes localized in distinct cytoplasmic foci.

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