3446–3457 *Nucleic Acids Research, 2011, Vol. 39, No. 8* *Published online 6 January 2011*
doi:10.1093/nar/gkq1302

RhoA activation participates in rearrangement of processing bodies and release of nucleated AU-rich mRNAs

Shinya Takahashi[1,2], Kyoko Sakurai[1], Arisa Ebihara[1], Hiroaki Kajiho[1], Kota Saito[1], Kenji Kontani[1], Hiroshi Nishina[2] and Toshiaki Katada[1,*]

[1]Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033 and [2]Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan

Received January 12, 2010; Revised December 4, 2010; Accepted December 6, 2010

ABSTRACT

Cytoplasmic ribonucleoprotein granules, known as processing bodies (P-bodies), contain a common set of conserved RNA-processing enzymes, and mRNAs with AU-rich elements (AREs) are delivered to P-bodies for translational silencing. Although the dynamics of P-bodies is physically linked to cytoskeletal network, it is unclear how small GTPases are involved in the P-body regulation and the ARE-mRNA metabolism. We found here that glucose depletion activates RhoA GTPase and alters the P-body dynamics in HeLa cells. These glucose-depleted effects are reproduced by the overexpression of the RhoA-subfamily GTPases and conversely abolished by the inhibition of RhoA activation. Interestingly, both RhoA activation and glucose depletion inhibit the mRNA accumulation and degradation. These findings indicate that RhoA participates in the stress-induced rearrangement of P-bodies and the release of nucleated ARE-mRNAs for their stabilization.

INTRODUCTION

The regulation of mRNA turnover plays a significant role in controlling gene expression. Recent studies have identified that a number of proteins responsible for mRNA decay are concentrated in cytoplasmic foci, referred to as processing bodies (P-bodies) (1–5). P-bodies are dynamic structures and represent pools of non-translating messenger ribonucleoprotein particles (mRNPs) (4–10) that play important roles not only in mRNA storage (11) but also in mRNA decapping, 5′–3′ decay (4,5) and translational control (12). In addition to mRNAs, P-bodies contain enzymes responsible for decapping, deadenylation and 5′–3′ degradation such as Dcp1/2, Ccr4 and Xrn1, along with decapping enhancers and/or translational repressors including rck/p54 (Dhh1) (1–4,13). Several proteins have been identified as key components in the formation of P-bodies, as their depletion leads to the disappearance of P-bodies in mammalian cells (13,14).

The size and abundance of microscopically visible P-bodies within cells are altered due to mutations that reduce the rate of decapping or degradation of mRNAs (4), suggesting that these structures are actively involved in the regulation of mRNA decay pathways. Additionally, mRNA molecules within P-bodies can return to polysomes for their translation (11). These results suggest that cytoplasmic mRNAs shuttle in and out of P-bodies and that their structural changes may affect the rates of mRNA entry into, its decay within, and its exit from P-bodies. Recent studies have also revealed that P-body dynamics is physically linked to the intracellular microtubule network (15,16). As well as microtubule disruption by chemical reagents such as nocodazole, cell stress induced by glucose depletion or osmotic shock results in a marked increase in P-body abundance (9). However, the alteration in P-body dynamics induces no applicable change in either mRNA decay or global mRNA translation. It has not been elucidated whether the smaller and larger aggregates of P-bodies have compositional and/or functional differences during the process of mRNA metabolism. Furthermore, little is known about the molecular mechanisms by which P-bodies are assembled or disassembled and how they are rearranged under various cell conditions.

In the last decade, specific pathways of mRNA turnover have been identified, and these can be controlled to

*To whom correspondence should be addressed. Tel: +81 3 5841 4750; Fax: +81 3 5841 4751; Email: katada@mol.f.u-tokyo.ac.jp

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

modulate mRNA decay rates. AU-rich elements (AREs), which are repeats of the nucleotide sequence AUUUA, are found in the 3′-untranslated region of many human mRNAs that undergo translational silencing and rapid turnover, a number of which encode interleukins, cytokines and proto-oncogenes (17–22). ARE sequences serve as binding sites for trans-acting factors that regulate the translation and stability of ARE-mRNAs (17–24). ARE-mRNAs appear to be delivered to P-bodies and subjected to translational repression and mRNA decay by proteins including Tristetraprolin (TTP) and BRF-1 (25). However, little is known about the involvement of small GTPases in the P-body dynamics and/or in the effective ARE-mRNA degradation under stress conditions.

In the present study, we have found that glucose depletion alters P-body dynamics through activation of the RhoA subfamily GTPases (RhoA, RhoB and RhoC). These GTPases are influential regulators of signaling pathways that control a wide range of cellular responses including cytoskeletal organization (26–29). We set out to investigate the roles of RhoA activation in regulating P-body dynamics and mRNA metabolism by monitoring TTP protein and the localization and degradation of ARE-mRNAs in mammalian cells. The signaling pathway used under stressed cellular conditions could play an important role in rearranging P-bodies and in preventing rapid degradation of ARE-mRNAs, such as interleukins and cytokines.

MATERIALS AND METHODS

Cell culture, transfection and glucose depletion

HeLa and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 0.16% (w/v) $NaHCO_3$, 0.6 mg/ml L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37°C in 95% air and 5% CO_2. The cells were transfected with 1 μg (35 mm dish) or 3 μg (60 mm dish) of plasmid DNA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) or with 100 pmol (60 mm dish) of siRNA using LipofectAMINE RNAiMAX (Invitrogen). Cells were rinsed three times with phosphate-buffered saline (PBS) and exposed to a glucose-free medium (Invitrogen) containing 10% fetal bovine serum further supplemented with or without glucose (the final concentration of 1 mg/ml).

Construction of plasmids

Open reading frames encoding RhoA, RhoB, RhoC, Rac1, Cdc42, Rhotekin-RBD and TTP were amplified by polymerase chain reaction (PCR) using human whole-brain cDNA (Clontech, Mountain View, CA, USA) and cloned into pCMV5-Myc (described previously) (30), pCMV-FLAG (replaced FLAG tag into Myc tag position) or pGEX-6P (Clonetech). Dcp1a was also isolated by PCR using human whole-brain cDNA and cloned into pEGFP or pDsRed-monomer (Clontech). The point-mutated forms of RhoA (G14V and T19N) were isolated by one-day PCR as previously described (31). pEF-β-globin-ARE was cloned by one-day PCR using pEF-β-globin-MS2 (a generous gift from Paul Anderson). The inserted ARE sequence of the GM-CSF 3′-UTR was previously indicated (32).

RNA interference

HeLa cells were transfected with the Stealth™ siRNA duplex oligonucleotides composed of 5′-CAG GUA GAG UUG GCU UUG UGG GAC A-3′/5′-UGU CCC ACA AAG CCA ACU CUA CCU G-3′ (154–178 nt, AF498970-#7, designed by Block-iT™ RNAi Designer siRNA; Invitrogen) for RhoA knockdown or Stealth RNAi negative control (Invitrogen) as a control dsRNA using Lipofectamine RNAiMAX. After incubation for 48 h, the cells were utilized in experiments.

Antibodies

The antibodies used were anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-rck/p54 (Novus Biologicals, Littleton, CO, USA) polyclonal antibody,
anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Millipore Bedford, MA, USA) and anti-Myc (9E10; Sigma, St Louis, MO, USA) monoclonal antibodies.

Immunofluorescence study and microscopic observation

HeLa cells transiently expressing Myc-tagged proteins were cultured on a poly-L-lysine-coated cover glass (15-mm diameter) and washed three times with PBS, followed by fixation with 4% paraformaldehyde in PBS at 25°C for 15 min. After permeabilization with 0.02% Triton X-100 in PBS for 10 min, the cells were incubated with a blocking solution consisting of 2% bovine serum albumin and 2% fetal calf serum in PBS for 30 min, followed by incubation with the indicated antibodies (1 μg/ml diluted with blocking solution) at 37°C for 2 h (33). The cells were washed three times with PBS and incubated for 1 h with mouse Alexa-488- or Alexa-568-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) diluted in blocking solution. After washing further three times with PBS, the coverglass was mounted onto a glass slide in Permafluor mounting medium (Immunon, Pittsburgh, PA, USA). The images were viewed on a Carl Zeiss confocal microscope with LSM510 software (Carl Zeiss, Jena, Germany) and merged using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA). Cell nuclei were stained with DAPI (Molecular Probes). For the estimation of P-body numbers, the images stained with an anti-rck/p54 antibody were captured using the Axio imager M1 (Carl Zeiss) to count the whole P-bodies in the cells. Approximately 150 transfected cells were blindly scored for the presence or absence of P-bodies by visual examination.

Time-lapse microscopic analysis

The subcellular movement of eGFP-Dcp1a in HeLa cells was obtained by using a confocal system (Yokogawa CSU-10 spinning disk scanhead, Tokyo, Japan) attached

to an Eclipse TE2000-E microscope (Nikon, Tokyo, Japan). The fluorescence was recorded by an iXon Electron Multiplying CCD (Andor Technology, Belfast, Northern Ireland) using NIS element software. A through-focus *z*-stack was recorded from the bottom to the top of the cells. The *z*-stack interval was 0.6 μm and the resulting raw data set was Maximum Intensity Projection reconstructed as a movie. Images were acquired by a 59 ms exposure every 1 min. Cells were rinsed three times with PBS and exposed to the glucose-free medium containing 10% fetal bovine serum at time 0.

Assay for GTP-bound RhoA GTPase

The GTP-bound form of RhoA was detected by a pull-down assay with the glutathione *S*-transferase (GST)-conjugated RhoA-binding domain of Rhotekin (RBD) (34). Briefly, the cells were washed with PBS and lysed in a buffer consisting of 50 mM Tris–HCl, (pH 7.5), 1% (w/v) Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl-flonyl-flouride (Pefabloc SC, Roche, Indianapolis, IN, USA), 1.7 μg/ml aprotinin and 10% (w/v) glycerol. After centrifugation at 15 000 rpm for 5 min at 4°C, the supernatants were incubated at 4°C for 30 min with glutathione-Sepharose beads coupled to GST-RBD. The beads were washed three times with PBS containing 0.1% (w/v) Triton X-100, 5 mM $MgCl_2$ and 1 mM DTT. The amount of total and GTP-bound Rho GTPases was detected by Western blotting with a polyclonal antibody against RhoA (1:500 dilution). Immunoblotting was performed as described previously (33).

In situ-hybridization assay

In situ hybridization experiments were performed as follows. The cells were transfected with reporter mRNA expression plasmid and pDsRed-monomer-Dcp1a. Twenty-four hours after transfection, cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min and incubated in 0.1 M triethanolamine with acetic anhydrid. The cells were permeabilized in 0.2 M HCl, and incubated overnight at 37°C in a hybridization buffer (containing 50% formamide, 5× SSC, 0.1% Tween-20, 5 mg/ml total yeast RNA and 50 μg/ml heparin). To detect the localization of the β-globin-ARE mRNA, FITC-labeled 48-nt DNA oligo probes (Invitrogen) complementary to sequences in the β-globin ORF were also added to the hybridization buffer at a concentration of 200 ng/ml and incubated overnight. The cells were washed twice for 10 min at 37°C in 50% formamide diluted in 2× SSC prior to visualization.

Immunoprecipitation

HeLa cells were lysed in a homogenization buffer consisting of 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1% Triton-X, 1 mM EDTA, 1 mM DTT, 500 μM 4-(2-aminoethyl)-benzenesulfonyl-flonyl-flouride (Pefabloc SC, Roche) and 2 μg/ml aprotinin. Cell lysates were precleared with Protein G Sepharose (GE healthcare, Buckinghamshire, UK) and incubated with anti-FLAG M2 Agarose (Sigma) at 4°C for 2 h. The beads were washed three times with a wash buffer consisting of 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.7% Triton-X, 1 mM EDTA and 1 mM DTT and eluted with FLAG peptide (Sigma) in the wash buffer for 30 min. Supernatants containing immunoprecipitated FLAG-tagged proteins were boiled and fractionated (along with samples of the original lysate) by SDS–PAGE. Proteins of interest were identified by immunoblotting with the appropriate antibodies. Immunoblotting was performed as described previously (33).

Northern blot analysis

NIH3T3 cells were transiently transfected with pEF-β-globin-ARE. After 24 h, the cells were treated with 5 μg/ml Actinomycin D to inhibit transcription and harvested at the indicated times. Total RNA was isolated with Sepasol(R) (Nacalai Tesque, Kyoto, Japan) and separated on a 1.2% (w/v) agarose gel by electrophoresis using a NorthernMax Kit (Ambion, Huntingdon, Cambridgeshire, UK). β-Globin-ARE mRNA was detected by northern blotting using [^{32}P]-labeled β-globin ORF as a probe. The labeled probes for β-globin-ARE mRNA were prepared using the cDNAs of the coding sequences for β-globin and Rediprime II Random Prime Labelling System (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Hybridization was carried out in the ULTRAHyb (Ambion) in the presence of ^{32}P-cDNA probes. The membrane was washed twice with 2× SSC (1× SSC consists of 0.15 M NaCl, 0.015 M sodium citrate) at 25°C, and twice with 1× SSC at 37°C. The membrane was visualized by autoradiography. GAPDH mRNA was used as an internal loading control.

All experiments were repeated at least three times with different batches of cell samples and all the results were fully reproducible.

RESULTS

Glucose depletion not only alters P-body dynamics but also activates RhoA GTPase

Recent studies have revealed that glucose depletion leads to a marked increase in P-body numbers in yeast cells (11). We confirmed such an increase, when HeLa cells were incubated in a glucose-depleted medium (Figure 1A). The stimulatory effect of glucose depletion is concomitant with the formation of small P-bodies. We evaluated the dynamics of P-bodies by first monitoring an endogenous marker protein, rck/p54, which is known to be required for the formation of P-bodies (13). Since the target of rapamycin (TOR) and its downstream GTPase, RhoA, appear to be responsible for limiting protein synthesis and cell growth during cell stress such as glucose depletion (35), we examined the relationship between the effect of glucose depletion on P-body dynamics and the activation states of the Rho-subfamily GTPase in detail. HeLa cells were incubated without glucose, and GTP-bound RhoA in the cells was measured by its ability to bind to the RhoA

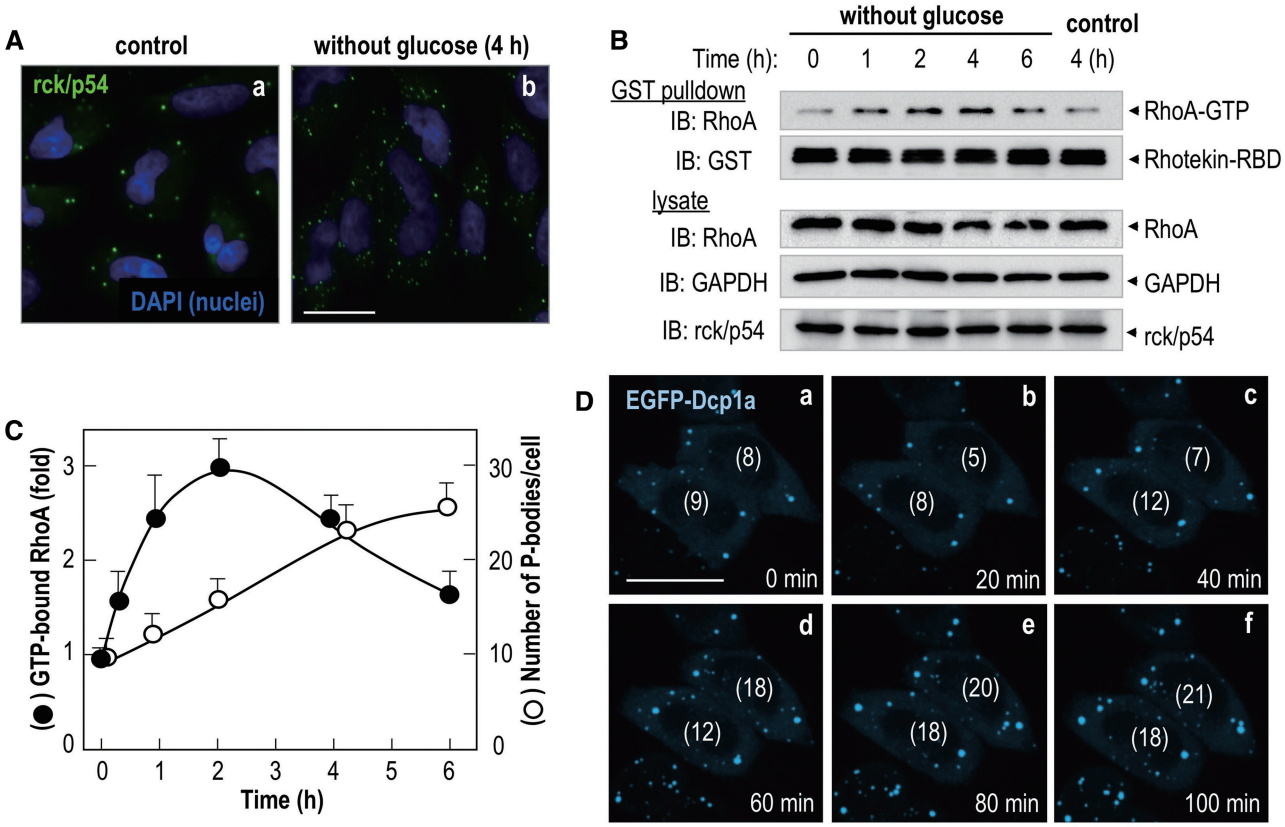

Figure 1. Glucose depletion alters P-body dynamics and activates RhoA. HeLa cells were incubated with a glucose-depleted medium for 4 h (**A**) or the indicated times (**B–D**). (A) The cells were stained with an anti-rck/p54 antibody to detect the localization of endogenous rck/p54 (green), and cell nuclei were stained with DAPI (blue). Merged images are also represented. (B) HeLa cells that had been incubated without glucose for the indicated times were harvested, and the GTP-bound form of endogenous RhoA was quantified by a pulldown assay using the GST-conjugated RhoA-binding domain of Rhotekin (GST-RBD). RhoA in the total cell lysates (input) was analysed by immunoblotting with an anti-RhoA antibody. Protein amounts of GAPDH and rck/p54 in the cells were also quantified by immunoblotting with their specific antibodies (input). (C) The intensity of the GTP-bound and total RhoA in Figure 1B was densitometrically analyzed, and the relative ratio of GTP-bound RhoA is shown by closed circles as the fold stimulation of the 0-time value. The number of rck/p54-positive P-bodies per cell, which was counted in approximately 150 cells, is shown by open circles. Results are presented as the mean ± SE obtained from three separate experiments. (D) Live cell images of exogenously expressed GFP-Dcp1a (20-min intervals) were obtained after glucose depletion. The number of P-bodies in the two indicated cells is shown in parentheses. Scale bars indicate 20 μm.

effector Rhotekin-RBD (Figure 1B), together with the increase in P-body numbers (Figure 1C). The average number of P-bodies per cell progressively increased by ~3-fold at 6 h after glucose deprivation. There was also a transient increase in GTP-bound RhoA, and the maximum level was observed at earlier time (2 h) than that observed in the maximum number of P-bodies. There was no significant change in the amount of endogenous rck/p54 during the various incubation times (Figure 1B, the lowest panel), indicating that the turnover of rck/p54 in P-bodies was not altered by glucose depletion. We analyzed the effect of glucose depletion on the P-body dynamics using live-cell imaging with exogenously expressed GFP-Dcp1a (Figure 1D). Glucose depletion induced a marked increase in the number of GFP-Dcp1a-positive foci (also see Supplementary Video S1). Live cell imaging suggests that the increase in P-body number appears to be a result of the formation of new P-bodies in the cytoplasm and not from the division of previously formed P-bodies (Supplementary Video S1). Thus, the activation of RhoA appears to correlate with the alteration of P-body dynamics.

GTP-bound forms of RhoA subfamily specifically alter P-body dynamics

In order to determine the role of RhoA activation in the alteration of P-body dynamics, ectopic expression of various RhoA forms was introduced into HeLa cells. When wild-type RhoA (RhoA/WT) was solely over-expressed, there was a marked increase in the number of small rck/p54-positive P-bodies (Figure 2A, panel d, see the transfected cells surrounded by dotted lines). A RhoA/G14V mutant (GTP-bound active form) was also capable of stimulating P-body formation (Figure 2A, panel f), but a RhoA/T19N mutant (GDP-bound inactive form) did not stimulate the creation of P-bodies (Figure 2A, panel h) (36,37). To determine the functional differences among the Rho/Rac/Cdc42 superfamily, other Rho GTPases were also

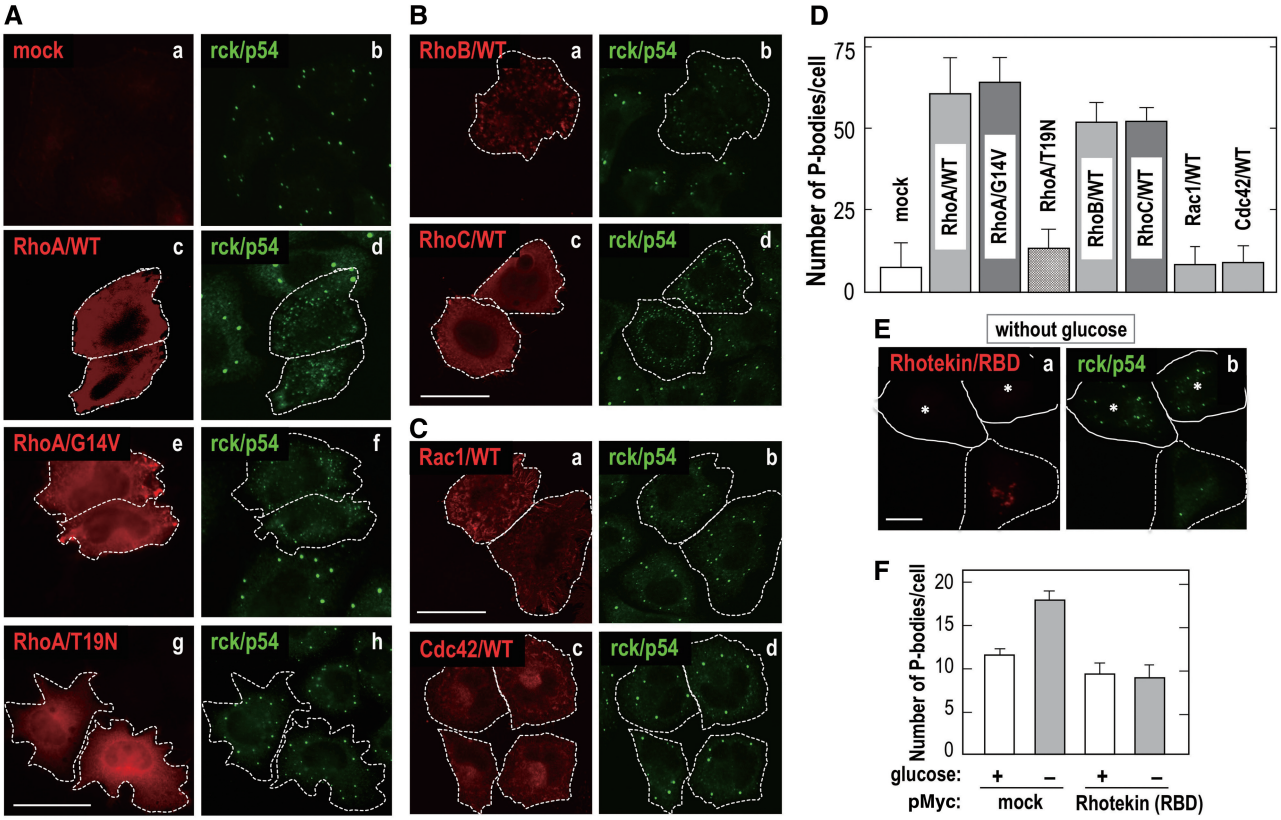

Figure 2. GTP-bound RhoA subfamily specifically alters P-body dynamics. (**A–C**) HeLa cells were transiently transfected with Myc-mock, Myc-RhoA/WT, Myc-RhoA/G14V, Myc-RhoA/T19N (A); Myc-RhoB/WT, Myc-RhoC/WT (B); Myc-Rac1/WT or Myc-Cdc42/WT (C) and cultured for 24 h. The expressed proteins were stained with an anti-Myc antibody (red). The localization of endogenous rck/p54 was determined by immunoblotting with the anti-rck/p54 antibody (green). The transfected cells are surrounded by dotted lines. Scale bars indicate 20 μm. (D) The number of rck/p54-positive P-bodies per cell was counted in approximately 150 cells expressing the various forms of the Rho subfamily. Results are presented as the mean ± SE obtained from three separate experiments. (**E** and **F**) HeLa cells that had been transfected with Myc-Rhotekin/RBD were cultured for 24 h and further incubated with or without glucose for 4 h. The localization of endogenous rck/p54 was determined with the anti-rck/p54 antibody (**E**, green). The transfected and non-transfected cells are surrounded by dotted and solid lines, respectively. The number of rck/p54-positive P-bodies per cell was counted in approximately 150 cells expressing the Rhotekin/RBD (**F**).

introduced into HeLa cells. Both RhoB and RhoC, which have structural and functional similarities to RhoA, were capable of stimulating P-body formation (Figure 2B, panels b and d). However, Rac1 or Cdc42, which belong to another Rho subfamily, does not noticeably alter the dynamics of rck/p54-positive P-bodies (Figure 2C, panels b and d). The effects of these Rho-family GTPases on P-body dynamics are summarized in Figure 2D. These results indicate that the GTP-bound forms of RhoA–C, members of the RhoA-subfamily, are sufficient for inducing small P-body formation, and only the RhoA-subfamily has specific effects on the dynamics of P-bodies. To confirm that activation of the RhoA subfamily is responsible for the glucose-depleted effect on P-bodies, we introduced Rhotekin/RBD into HeLa cells. Rhotekin/RBD is capable of binding to GTP-bound active forms of Rho GTPases thereby specifically inhibiting the Rho-dependent signaling pathway (38). In cells expressing Rhotekin/RBD (RhoA-binding domain), glucose depletion failed to stimulate P-body formation (Figure 2E and F). This finding indicates that activation of the RhoA subfamily is required for the glucose-depleted effect on P-body dynamics.

Since the expression of the RhoA subfamily markedly altered the dynamics of rck/p54-positive foci, we further investigated whether the small rck/p54-positive P-bodies induced by RhoA have similar components to those in non-transfected cells. HeLa cells were transfected with an expression vector encoding GFP-Dcp1a. The P-bodies containing endogenous rck/p54 were observed to be well co-localized with the GFP signals in the mock-transfected control cells (Supplementary Figure S1, panel c). The increased rck/p54-positive P-bodies, characteristic of RhoA-expressing cells, completely merged with GFP-Dcp1a in RhoA-transfected cells (Supplementary Figure S1, panel f). These results indicate that P-bodies identified under both control and RhoA-expressing conditions are similar to one another, at least in terms of their constituent proteins, rck/p54 and Dcp1a.

RhoA activation and glucose depletion impair the nucleation of ARE-mRNAs into P-bodies

AREs are found in the 3′-untranslated region of many mRNAs which undergo translational silencing and rapid decay. The accumulation of ARE-mRNAs in P-bodies is

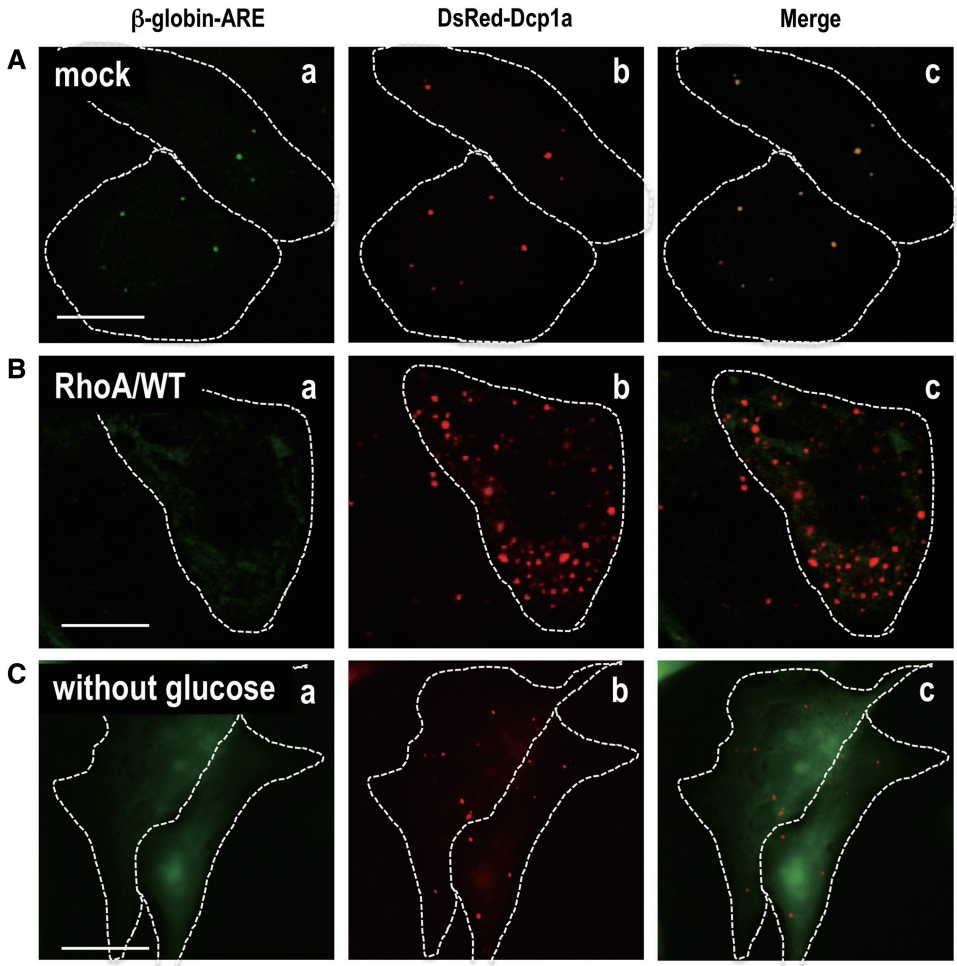

Figure 3. RhoA activation and glucose depletion impair the nucleation of ARE-mRNAs into P-bodies. (**A** and **B**) HeLa cells that had been transfected with β-globin-ARE mRNA, DsRed-monomer-Dcp1a and either Myc-mock (A) or Myc-RhoA/WT (B) were cultured for 24 h. (**C**) HeLa cells that had been transfected with β-globin-ARE mRNA and DsRed-monomer-Dcp1a were cultured for 24 h and further incubated without glucose for 4 h. The β-globin-ARE mRNA was detected by *in situ* hybridization using a FITC-conjugated anti-sense oligonucleotide for the β-globin ORF. β-globin-ARE mRNA (green; panel a), DsRed-monomer-Dcp1a (red; panel b) and merged images (panel c) are shown. Individual cells are surrounded by dotted lines. Scale bars indicate 10 μm.

markedly enhanced when mRNA-decay machinery is inhibited (25). These data suggest that P-bodies may function as reservoirs, sequestering ARE-mRNAs from polysomes to facilitate their decay effectively. We investigated how intracellular localization of ARE-mRNAs was modified by the RhoA-induced rearrangement of P-bodies. In the present assay, the ARE of GM-CSF was chosen, since it is a well-defined target of rapid decay. The β-globin reporter mRNA containing the GM-CSF ARE was expressed in HeLa cells and subjected to *in situ* hybridization. Dcp1a fused to the reporter molecule DsRed-monomer was used as a marker for the localization of P-bodies. The *in situ* hybridization assay clearly indicates that β-globin-ARE mRNA was co-localized with foci containing Dcp1a (Figure 3A), indicating that the ARE-mRNA is nucleated into the intact P-bodies to serve as substrates of decay enzymes under glucose-loaded normal conditions. However, the ARE-mRNA was also localized in the cytoplasm, in addition to the rearranged Dcp1a-positive P-bodies, when the HeLa cells had been transfected with RhoA/WT (Figure 3B). Furthermore, the localization of ARE-mRNAs was evident in the cytoplasm, along with Dcp1a-positive P-bodies, under glucose-depleted conditions (Figure 3C).

Glucose depletion and RhoA activation diminish TTP protein and impair the rapid degradation of ARE-mRNAs

Previous study has shown that TTP targets ARE-mRNAs to P-bodies for their translational silencing (25). We therefore investigated whether glucose depletion and RhoA activation exerted their influence on the amount of TTP protein. Endogenous TTP protein in HeLa cells could not been detected by using several antibodies commercially available (data not shown). Therefore, CMV-drived FLAG-tagged TTP was introduced into HeLa cells, and the cells were further incubated with or without glucose. As shown in Figure 4, there was progressive decrease in the protein level of TTP upon glucose depletion (Figure 4A, lanes 1–3 versus 4 and B). Furthermore,

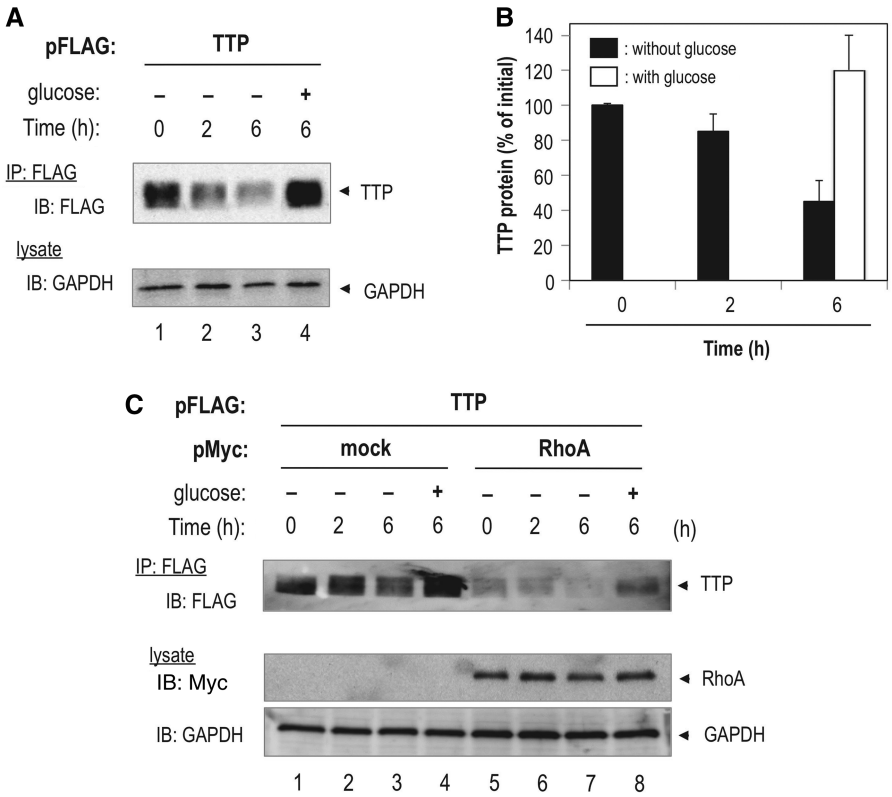

Figure 4. RhoA activation and glucose depletion diminish the protein amount of TTP. HeLa cells that had been transfected with FLAG-TTP (**A**) and further with either Myc-mock (**C**, lanes 1–4) or Myc-RhoA/WT (C, lanes 5–8) were cultured for 24 h. The cells were further incubated with or without glucose for the indicated times. Lysates and proteins immunoprecipitated with anti-FLAG beads were subjected to immunoblot analysis to detect the indicated proteins. The intensity of proteins in (A) was densitometrically analyzed, and the relative ratio of TTP/GAPDH obtained from three separate experiments is shown as the mean ± SE as percentages of the 0-time value (**B**).

RhoA/WT overexpression in the cells markedly diminished TTP protein without the cell exposure to glucose-depleted medium (Figure 4C, lanes 1–3 versus 5–7). Importantly, the reduction of the protein amount of TTP protein by glucose depletion was clearly abolished with the addition of glucose (Figure 4A, lane 4 and C, lanes 4 and 8), and these results are consistent with low expression of TTP under SV40 promoter and terminator (data not shown).

RhoA activation by glucose depletion impair the rapid degradation of ARE-mRNAs

We next investigated how the P-body alteration induced by RhoA and glucose depletion affected the degradation rate of β-globin-ARE mRNA. Actinomycin D was used to shut down transcription, and subsequent mRNA degradation was monitored by northern blot analysis. The reporter ARE-mRNA was rapidly degraded in the control NIH3T3 cells that had been transfected with a mock plasmid (Figure 5A, upper lanes 1–3). Over-expression of RhoA/WT markedly reduced the degradation rate of the reporter mRNA (Figure 5A, upper lanes 4–6). The rapid degradation of β-globin-ARE mRNA was also impaired under glucose-depleted conditions (Figure 5B, upper lanes 4–6). As expected, β-globin-MS2 mRNA lacking the ARE (Figure 5C, upper panels) remained stable throughout Actinomycin D treatment, and RhoA over-expression or glucose depletion did not alter their degradation rate.

RhoA protein itself is essentially required for P-body formation

We also performed knockdown of endogenous RhoA with siRNA to confirm the requirement of RhoA for the glucose depletion-induced alteration of P-bodies. Immunoblot analysis indicated that treatment with RhoA-siRNA was very effective in reducing the endogenous protein and that glucose depletion did not alter the diminished RhoA level (Figure 6A). Surprisingly, there was no P-body formation in the RhoA-knockdown cells even under glucose-loaded control conditions, and glucose depletion failed to stimulate P-body formation (Figure 6B and C). The re-introduction of silent-mutated RhoA at the siRNA target site recovered the formation of P-bodies (Supplementary Figure S2A and B), indicating that the loss of P-bodies with RhoA-siRNA was certainly due to reducing the level of RhoA. Introduction of RhoA with silent mutations conferred resistance to RNA interference as expected (Supplementary Figure S2C). Thus, RhoA protein itself appears to be essentially required for P-body formation.

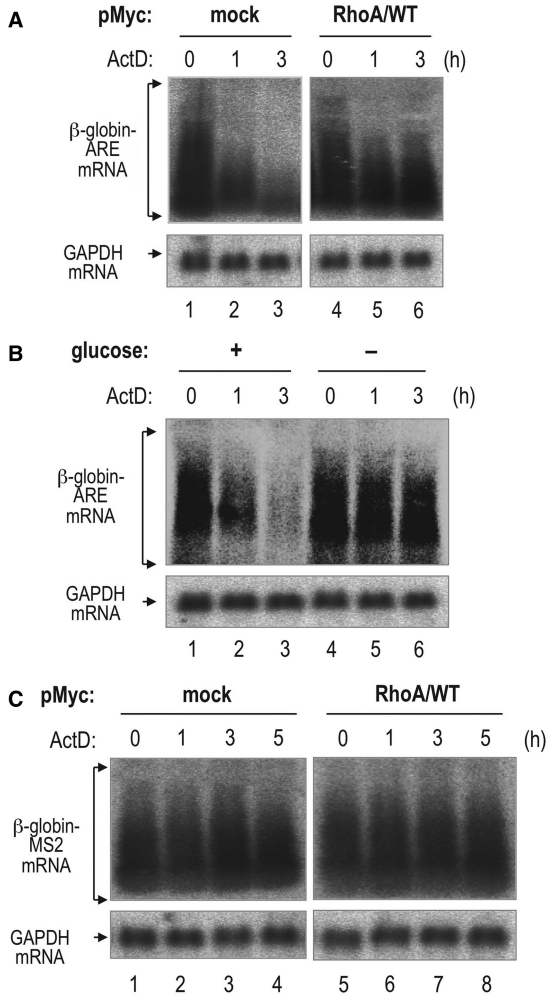

Figure 5. RhoA activation and glucose depletion impair the rapid degradation of ARE-mRNAs. NIH3T3 cells that had been transfected with β-globin-ARE mRNA (**A** and **B**) or β-globin-MS2 mRNA (**C**) and Myc-mock (A and C, left panels) or Myc-RhoA/WT (A and C, right panels) were cultured for 24 h. The transfected cells were also further incubated with (B, lanes 1–3) or without (B, lanes 4–6) glucose for 4 h. These cells were treated with Actinomycin D (ActD) to inhibit transcription and further incubated for the indicated times. Total RNA was extracted from the cells and subjected to northern blot analysis to estimate the amounts of β-globin-ARE mRNA (A and B) and β-globin-MS2 mRNA (C). GAPDH mRNA was used as a loading control.

The RhoA knockdown exhibiting total P-body disassembly might be rather an unexpected phenotype, since overexpression of the RhoA subfamily stimulated small P-body formation, and since P-bodies with normal size were still observed when RhoA activation had been inhibited by the expression of Rhotekin/RBD or the dominant-negative RhoA/T19N mutant (Figure 2). Therefore, we further investigated how P-body disassembly induced by the RhoA knockdown exerted its influence on the amount of TTP protein and the degradation of ARE-mRNAs. As shown in Figure 6D, TTP protein level was markedly decreased in RhoA-depleted cells (Figure 6D, lanes 1 versus 2). Moreover, the rapid degradation of ARE-mRNAs was markedly impaired by RhoA knockdown (Figure 6E, upper lanes 1–3 versus 4–6). These results indicate that RhoA protein itself is required not only for the formation of P-bodies but also for rapid degradation of ARE-mRNAs.

DISCUSSION

In the present study, we find that glucose depletion not only alters P-body dynamics but also activates RhoA in HeLa cells (Figure 1). Among Rho/Rac/Cdc42 super family, the RhoA subfamily specifically alters the P-body dynamics, and inhibition of the RhoA-subfamily activation abolishes the glucose-depleted effect on P-body dynamics (Figure 2). Importantly, both glucose depletion and RhoA activation diminish TTP protein (Figure 4) and inhibit the nucleation of ARE-mRNAs into the rearranged P-bodies and their degradation (Figures 3 and 5). Moreover, the loss of TTP protein and the impaired degradation of ARE-mRNAs were also observed in P-body-disassembled cells induced by RhoA knockdown (Figure 6). Although it is well known that the RhoA-subfamily small GTPase regulates a wide range of cellular responses, this is the first report demonstrating that activation of the RhoA subfamily is involved in the stress-induced rearrangement of P-bodies and the release of nucleated ARE-mRNAs.

Significance of the rearranged P-bodies created by RhoA activation

The size and number of P-bodies in glucose-depleted or RhoA-expressing cells appear to be microscopically smaller than those in the steady-state HeLa cells. The small P-bodies created by RhoA activation contain two P-body marker proteins, rck/p54 and Dcp1a, and the microscopic images indicate that their co-localization is undistinguishable from that in control cells (Supplementary Figure S1). Core components of P-body formation and also some mRNA decay machinery are still present in the rearranged small P-bodies. According to an estimate of the rck/p54- or Dcp1a-immunostained density of P-bodies, their total volumes per single cell may be almost constant, in spite of the alteration of P-body dynamics (data not shown). Moreover, we show a marked decrease of P-bodies in cells where RhoA has been knocked down (Figure 6B and C). Re-introduction of a wild-type RhoA-siRNA resistant mutant could re-organize the P-body (Supplementary Figure S2A and B). These results indicate that RhoA is essentially required for P-body formation. Assuming that the total mass of the P-body component remains the same, the components of P-bodies in RhoA-knockdown cells are nearly diffused and invisible in cytoplasm. This indicates that phenotypes of overexpression and knockdown might go partly in the same direction. Supportively, both RhoA overexpression and knockdown on the decrease of TTP protein level under glucose depletion and the stabilization of ARE-mRNAs are rather similar (Figures 3–6). It thus seems likely that the balance between activated RhoA and inactivated RhoA is precisely regulated and that RhoA activation and RhoA knockdown might result in

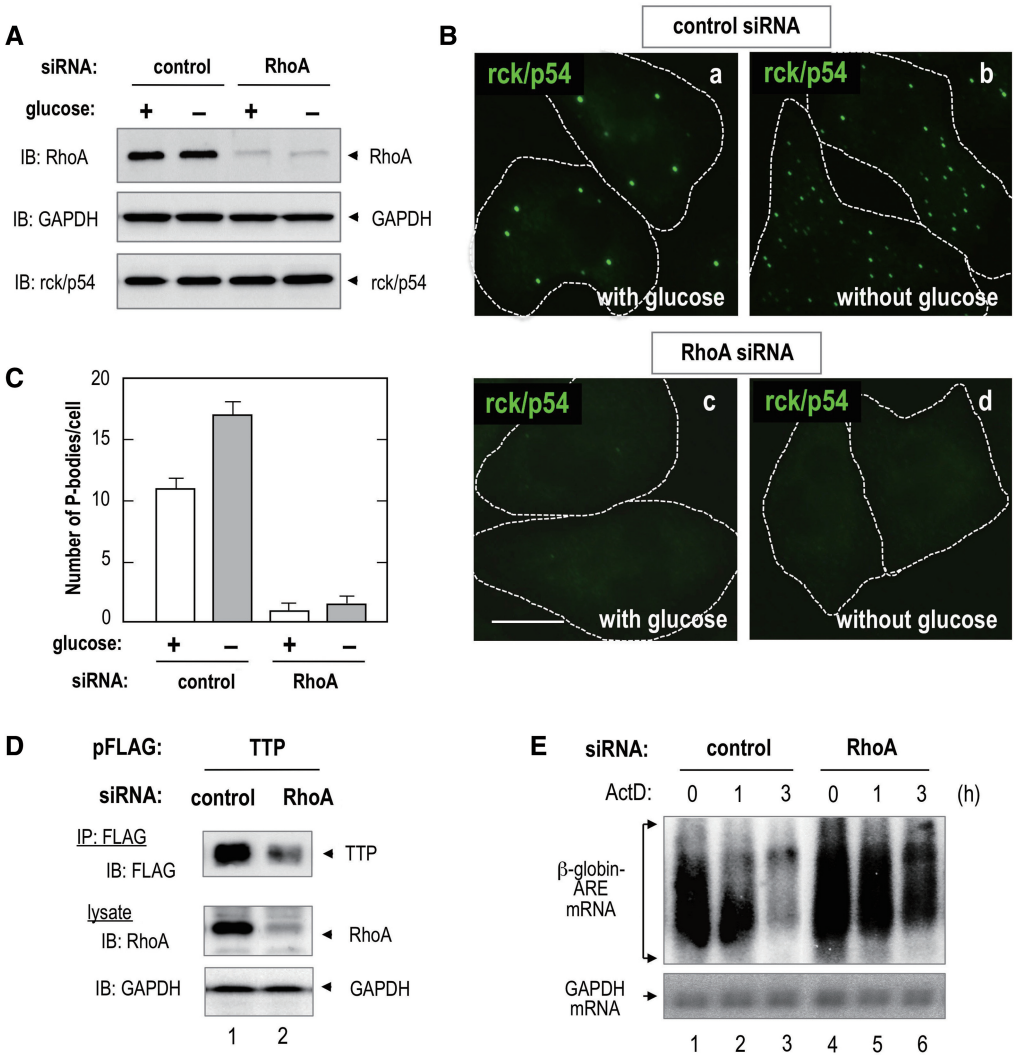

Figure 6. The effects of RhoA knockdown on P-body formation and the degradation of ARE-mRNAs. (**A–C**) HeLa cells that had been transfected with RhoA-specific siRNA or control siRNA were cultured for 48 h and further incubated with or without glucose for 4 h. The cells were subjected to immunoblot analysis with the indicated antibodies (A). Localization of endogenous rck/p54 in the treated cells was determined with the anti-rck/p54 antibody (B, green). The cells are surrounded by dotted lines. Scale bars indicate 10 μm. The number of rck/p54-positive P-bodies per cell was counted in approximately 150 cells (C). Results are presented as the mean ± SE obtained from three separated experiments. (**D**) HeLa cells that had been transfected with RhoA-specific siRNA or control siRNA were further transfected with FLAG-TTP and cultured for 24 h. Lysates and proteins immunoprecipitated with anti-FLAG beads were subjected to immunoblot analysis to detect the indicated proteins. (**E**) NIH3T3 cells that had been transfected with RhoA-specific siRNA or control siRNA were cultured for 24 h were further transfected with β-globin-ARE mRNA. The cells were treated with Actinomycin D (ActD) to inhibit transcription and further incubated for the indicated times. The amount of β-globin-ARE mRNA was measured as described in Figure 5.

the similar rearrangement for P-bodies and the stabilization of ARE-mRNAs. It has been established that Rho-family GTPases regulate primarily cytoskeletal reorganization in response to extracellular signals in mammalian cells. Indeed, microtubule disruption leads to a decrease of P-body mobility and an increase in P-body abundance (15). Concomitantly, we have examined effects of microtubule disruption and observed that nocodazole treatment has the similar effect on RhoA activation and P-bodies dynamics as glucose depletion (Supplementary Figure S3A and B). The nocodazole-induced increase of P-bodies is inhibited by overexpression of Rhotekin/RBD (Supplementary Figure S3C and D). These findings suggest that the glucose-depleted effects on P-body dynamics and ARE-mRNA metabolism are linked to RhoA activation-dependent cytoskeletal regulation. The live-cell imaging during glucose depletion indicated that the increase in small P-bodies is not due to the division of pre-existing large P-bodies (Supplementary Movie S1). These findings suggest that a key component(s) required for P-body formation is diffusely distributed as cytoplasmic pool and that rearrangement of P-bodies is properly induced depending on cellular conditions. Based on this assumption, it can be speculated that aggregation into large P-bodies provides a mechanism to effectively increase the local concentrations of mRNA substrates. Indeed, catalytic enzymes of mRNA decay, hDcp2 and hXrn1, are

concentrated in P-bodies (4,5) and their knockdown results in accumulation of ARE-mRNAs in P-bodies lacking the decay activity (25).

Proposed mechanisms for RhoA activation and P-body rearrangement

In this study, inhibition of the Rho-dependent signaling by means of Rhotekin/RBD overexpression inhibits P-body formation under glucose depletion (Figure 2E). However, a dominant-negative form of RhoA (RhoA/T19N) or Rhotekin/RBD fails to alter the number of P-bodies under non-stressed conditions (Figure 2A and E). We show that RhoA exists as a slightly activated (GTP-bound) state and that further activation occurs under conditions of cellular stress (Figure 1B). The expression of RhoA/T19N and Rhotekin/RBD was not able to fully suppress the Rho-dependent pathway, suggesting that slightly activated RhoA and its effector may be sufficient at least for organizing P-bodies (Figure 2A and E). During glucose depletion, Rhotekin/RBD inhibits further RhoA activation, and therefore the number of P-bodies could not increase. Alternatively, we propose the notion that another compensated pathway for the P-body formation is activated during RhoA suppression under steady-state conditions. The rearrangement pathway for P-bodies may be stimulated by Rho and/or signaling proteins, which are activated by the internal cell cycle or the external environment.

The large families of guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs) play roles in regulating Rho activity, in response to cellular stress, but upstream and specific targets for the rearrangement of P-bodies remain unclear in the present study. We have examined overexpression of ROCK, mDia and Smurf as the possible downstream effectors of Rho GTPase on the organization of P-bodies, but their overexpression did not affect the P-body dynamics. An additional study to identify the Rho effectors is required to define the molecular mechanisms for the stress-induced P-body rearrangement.

Relationship between the RhoA-induced rearrangement of P-bodies and ARE-mRNA decay and translation

Recent studies show that P-body formation is not causative of neither rate-limiting for general mRNA decay (6,39–41) nor miRNA-mediated translational control (42,43) in P-body component-knockdown cells. In yeast cells, P-bodies may not be the predominating sites for general mRNA decapping (44). We hypothesize that ARE-mRNA decay is a special case. ARE-mRNAs are specifically delivered to P-bodies and subjected to translational repression by proteins including TTP (25). This suggests that inhibition and/or degradation of TTP induce mislocalization of ARE-mRNAs. In the present study, we observe that both glucose depletion and RhoA activation diminish exogenous TTP protein and inhibit the nucleation of ARE-mRNAs into the rearranged P-bodies (Figures 3 and 4). These results confirm the notion that TTP protein regulates localization of ARE-mRNAs. The reduction of TTP was also observed with two different promoters for the exogenous protein, suggesting that the TTP reduction is attributed to post-transcription, not to promoter or terminator. We tested whether TTP might be proteolysed under glucose-depleted conditions, however, addition of a proteasome inhibitor (MG132) or a reversible inhibitor of serine and cysteine proteases (leupeptin) did not inhibit the TTP reduction (data not shown). Further investigations are necessary to elucidate the molecular mechanism of TTP down-regulation. The degradation of ARE-mRNAs is impaired in cells with both overexpression and knockdown of RhoA. Although P-body formation appears to link to ARE-mRNA metabolism (7,25), the requirement of their localization in P-bodies for rapid degradation has not been fully understood. Our present findings that mislocalized ARE-mRNAs in cytoplasm are escaped from efficient degradation shed light on this question (Figures 3 and 5).

It is interesting to consider Rho GTPase activation with respect to specific aspects of ARE-mRNA metabolism and translation. Pro-inflammatory proteins such as TNF-α, GM-CSF and cyclooxygenase-2 are encoded by short-lived mRNAs that are inefficiently translated (17,32,45–47). The protein products of these mRNAs are barely detectable under steady-state conditions, although cell stimulation leads to expression of these proteins. Several reports support this idea (48–51) as the stimuli used in these experiments have been reported to activate the Rho-family GTPases (52,53). We expected elevated ARE-mRNAs to be translated efficiently, however, increase of exogenously transfected ARE-mRNAs was not apparently detected in both control and RhoA-activated cells (data not shown). These results suggested that the reporter ARE-mRNAs released from P-bodies might be translationally silent under the present assay conditions. RhoA activation may be insufficient for the translational stimulation of ARE-mRNAs, and trans-acting factors, such as TIA-1 and TIAR, might be involved in the translational regulation of ARE-mRNAs. These important issues are currently under investigation in our laboratory.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Paul Anderson (Harvard Medical School, MA, USA) for his generous gifts of plasmids.

FUNDING

Funding for open access charge: The Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; Japan Society for the Promotion of Science (JSPS).

Conflict of interest statement. None declared.

REFERENCES

1. van Dijk,E., Cougot,N., Meyer,S., Babajko,S., Wahle,E. and Seraphin,B. (2002) Human Dcp2: a catalytically active mRNA decapping enzyme located in specific cytoplasmic structures. *EMBO J.*, **21**, 6915–6924.
2. Lykke-Andersen,J. (2002) Identification of a human decapping complex associated with hUpf proteins in nonsense-mediated decay. *Mol. Cell. Biol.*, **22**, 8114–8121.
3. Ingelfinger,D., Arndt-Jovin,D.J., Luhrmann,R. and Achsel,T. (2002) The human LSm1-7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrnl in distinct cytoplasmic foci. *RNA*, **8**, 1489–1501.
4. Sheth,U. and Parker,R. (2003) Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science*, **300**, 805–808.
5. Cougot,N., Babajko,S. and Seraphin,B. (2004) Cytoplasmic foci are sites of mRNA decay in human cells. *J. Cell Biol.*, **165**, 31–40.
6. Kshirsagar,M. and Parker,R. (2004) Identification of Edc3p as an enhancer of mRNA decapping in Saccharomyces cerevisiae. *Genetics*, **166**, 729–739.
7. Ferraiuolo,M.A., Basak,S., Dostie,J., Murray,E.L., Schoenberg,D.R. and Sonenberg,N. (2005) A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay. *J. Cell Biol.*, **170**, 913–924.
8. Kedersha,N., Stoecklin,G., Ayodele,M., Yacono,P., Lykke-Andersen,J., Fritzler,M.J., Scheuner,D., Kaufman,R.J., Golan,D.E. and Anderson,P. (2005) Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J. Cell Biol.*, **169**, 871–884.
9. Teixeira,D., Sheth,U., Valencia-Sanchez,M.A., Brengues,M. and Parker,R. (2005) Processing bodies require RNA for assembly and contain nontranslating mRNAs. *RNA*, **11**, 371–382.
10. Andrei,M.A., Ingelfinger,D., Heintzmann,R., Achsel,T., Rivera-Pomar,R. and Luhrmann,R. (2005) A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. *RNA*, **11**, 717–727.
11. Brengues,M., Teixeira,D. and Parker,R. (2005) Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science*, **310**, 486–489.
12. Coller,J. and Parker,R. (2005) General translational repression by activators of mRNA decapping. *Cell*, **122**, 875–886.
13. Serman,A., Le Roy,F., Aigueperse,C., Kress,M., Dautry,F. and Weil,D. (2007) GW body disassembly triggered by siRNAs independently of their silencing activity. *Nucleic Acids Res.*, **35**, 4715–4727.
14. Yang,W.H., Yu,J.H., Gulick,T., Bloch,K.D. and Bloch,D.B. (2006) RNA-associated protein 55 (RAP55) localizes to mRNA processing bodies and stress granules. *RNA*, **12**, 547–554.
15. Aizer,A., Brody,Y., Ler,L.W., Sonenberg,N., Singer,R.H. and Shav-Tal,Y. (2008) The dynamics of mammalian P body transport, assembly, and disassembly in vivo. *Mol. Biol. Cell.*, **19**, 4154–4166.
16. Sweet,T.J., Boyer,B., Hu,W., Baker,K.E. and Coller,J. (2007) Microtubule disruption stimulates P-body formation. *RNA*, **13**, 493–502.
17. Chen,C.Y. and Shyu,A.B. (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci.*, **20**, 465–470.
18. Gueydan,C., Droogmans,L., Chalon,P., Huez,G., Caput,D. and Kruys,V. (1999) Identification of TIAR as a protein binding to the translational regulatory AU-rich element of tumor necrosis factor alpha mRNA. *J. Biol. Chem.*, **274**, 2322–2326.
19. Wilusz,C.J., Wormington,M. and Peltz,S.W. (2001) The cap-to-tail guide to mRNA turnover. *Nat. Rev. Mol. Cell Biol.*, **2**, 237–246.
20. Shim,J. and Karin,M. (2002) The control of mRNA stability in response to extracellular stimuli. *Mol. Cells*, **14**, 323–331.
21. Zhang,T., Kruys,V., Huez,G. and Gueydan,C. (2002) AU-rich element-mediated translational control: complexity and multiple activities of trans-activating factors. *Biochem. Soc. Trans.*, **30**, 952–958.
22. Mazan-Mamczarz,K., Lal,A., Martindale,J.L., Kawai,T. and Gorospe,M. (2006) Translational repression by RNA-binding protein TIAR. *Mol. Cell. Biol.*, **26**, 2716–2727.
23. Piecyk,M., Wax,S., Beck,A.R., Kedersha,N., Gupta,M., Maritim,B., Chen,S., Gueydan,C., Kruys,V., Streuli,M. *et al.* (2000) TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. *EMBO J.*, **19**, 4154–4163.
24. Lopez de Silanes,I., Galban,S., Martindale,J.L., Yang,X., Mazan-Mamczarz,K., Indig,F.E., Falco,G., Zhan,M. and Gorospe,M. (2005) Identification and functional outcome of mRNAs associated with RNA-binding protein TIA-1. *Mol. Cell. Biol.*, **25**, 9520–9531.
25. Franks,T.M. and Lykke-Andersen,J. (2007) TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements. *Genes Dev.*, **21**, 719–735.
26. Hall,A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279**, 509–514.
27. Sahai,E. and Marshall,C.J. (2002) RHO-GTPases and cancer. *Nat. Rev. Cancer*, **2**, 133–142.
28. Ridley,A.J. (2006) Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol.*, **16**, 522–529.
29. Heasman,S.J. and Ridley,A.J. (2008) Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat. Rev. Mol. Cell Biol.*, **9**, 690–701.
30. Kontani,K., Tada,M., Ogawa,T., Okai,T., Saito,K., Araki,Y. and Katada,T. (2002) Di-Ras, a distinct subgroup of ras family GTPases with unique biochemical properties. *J. Biol. Chem.*, **277**, 41070–41078.
31. Takahashi,S., Araki,Y., Sakuno,T. and Katada,T. (2003) Interaction between Ski7p and Upf1p is required for nonsense-mediated 3′-to-5′ mRNA decay in yeast. *EMBO J.*, **22**, 3951–3959.
32. Chen,C.Y., Xu,N. and Shyu,A.B. (1995) mRNA decay mediated by two distinct AU-rich elements from c-fos and granulocyte-macrophage colony-stimulating factor transcripts: different deadenylation kinetics and uncoupling from translation. *Mol. Cell. Biol.*, **15**, 5777–5788.
33. Ura,S., Nishina,H., Gotoh,Y. and Katada,T. (2007) Activation of the c-Jun N-terminal kinase pathway by MST1 is essential and sufficient for the induction of chromatin condensation during apoptosis. *Mol. Cell. Biol.*, **27**, 5514–5522.
34. Ren,X.D., Kiosses,W.B. and Schwartz,M.A. (1999) Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.*, **18**, 578–585.
35. Jacinto,E., Loewith,R., Schmidt,A., Lin,S., Ruegg,M.A., Hall,A. and Hall,M.N. (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat. Cell Biol.*, **6**, 1122–1128.
36. Feig,L.A. (1999) Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. *Nat. Cell Biol.*, **1**, E25–27.
37. Ridley,A.J. (2001) Rho GTPases and cell migration. *J. Cell Sci.*, **114**, 2713–2722.
38. Krendel,M., Zenke,F.T. and Bokoch,G.M. (2002) Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. *Nat. Cell Biol.*, **4**, 294–301.
39. Decker,C.J., Teixeira,D. and Parker,R. (2007) Edc3p and a glutamine/asparagine-rich domain of Lsm4p function in processing body assembly in Saccharomyces cerevisiae. *J. Cell Biol.*, **179**, 437–449.
40. Reijns,M.A., Alexander,R.D., Spiller,M.P. and Beggs,J.D. (2008) A role for Q/N-rich aggregation-prone regions in P-body localization. *J. Cell Sci.*, **121**, 2463–2472.
41. Stalder,L. and Muhlemann,O. (2009) Processing bodies are not required for mammalian nonsense-mediated mRNA decay. *RNA*, **15**, 1265–1273.
42. Chu,C.Y. and Rana,T.M. (2006) Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *PLoS Biol.*, **4**, e210.
43. Eulalio,A., Behm-Ansmant,I., Schweizer,D. and Izaurralde,E. (2007) P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol. Cell. Biol.*, **27**, 3970–3981.

44. Hu,W., Sweet,T.J., Chamnongpol,S., Baker,K.E. and Coller,J. (2009) Co-translational mRNA decay in Saccharomyces cerevisiae. *Nature*, **461**, 225–229.
45. Shaw,G. and Kamen,R. (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*, **46**, 659–667.
46. Kontoyiannis,D., Pasparakis,M., Pizarro,T.T., Cominelli,F. and Kollias,G. (1999) Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity*, **10**, 387–398.
47. Dixon,D.A., Balch,G.C., Kedersha,N., Anderson,P., Zimmerman,G.A., Beauchamp,R.D. and Prescott,S.M. (2003) Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1. *J. Exp. Med.*, **198**, 475–481.
48. Andersson,K. and Sundler,R. (2000) Signalling to translational activation of tumour necrosis factor-alpha expression in human THP-1 cells. *Cytokine*, **12**, 1784–1787.
49. Brooks,S.A., Connolly,J.E. and Rigby,W.F. (2004) The role of mRNA turnover in the regulation of tristetraprolin expression: evidence for an extracellular signal-regulated kinase-specific, AU-rich element-dependent, autoregulatory pathway. *J. Immunol.*, **172**, 7263–7271.
50. Vasudevan,S. and Steitz,J.A. (2007) AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. *Cell*, **128**, 1105–1118.
51. Deleault,K.M., Skinner,S.J. and Brooks,S.A. (2008) Tristetraprolin regulates TNF TNF-alpha mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways. *Mol. Immunol.*, **45**, 13–24.
52. Mehta,D., Rahman,A. and Malik,A.B. (2001) Protein kinase C-alpha signals rho-guanine nucleotide dissociation inhibitor phosphorylation and rho activation and regulates the endothelial cell barrier function. *J. Biol. Chem.*, **276**, 22614–22620.
53. Shimizu,S., Tahara,M., Ogata,S., Hashimoto,K., Morishige,K., Tasaka,K. and Murata,Y. (2007) Involvement of nuclear factor-kB activation through RhoA/Rho-kinase pathway in LPS-induced IL-8 production in human cervical stromal cells. *Mol. Hum. Reprod.*, **13**, 181–187.