Biochimica et Biophysica Acta 1793 (2009) 878–887

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Selective localization of PCBP2 to cytoplasmic processing bodies

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ARTICLE INFO

Article history:
Received 27 December 2008
Received in revised form 29 January 2009
Accepted 2 February 2009
Available online 20 February 2009

Keywords:
Processing body
PCBP2
FRAP
Post-transcriptional regulation

ABSTRACT

Processing bodies (P-bodies) are cytoplasmic domains that have been implicated in critical steps of the regulation of gene expression, including mRNA decay and post-transcriptional gene silencing. Previously, we reported that PCBP2 (Poly-(rC) Binding Protein 2), a facilitator of IRES-mediated translation, is a novel P-body component. Interestingly, PCBP2 is recruited to only a subset of Dcp1a-positive P-bodies, which may reflect functional diversity among these structures. In this study, we examined the selective P-body localization of PCBP2 in detail. Co-localization studies between Dcp1a and PCBP2 revealed that PCBP2 is present in ~40% of P-bodies. While PCBP2 was more likely to reside in larger P-bodies, P-body size did not seem to be the sole determinant, and puromycin-induced enlargement of P-bodies only modestly increased the percentage of PCBP2-positive P-bodies. Photobleaching experiments demonstrated that the accumulation of PCBP2 to specific P-bodies is a dynamic process, which does not involve the protein's transcription-dependent nucleo-cytoplasmic shuttling activity. Finally, we found that PCBP1, a close relative of PCBP2, localizes to P-bodies in a similar manner to PCBP2. Taken together, these results establish the compositional diversity among P-bodies, and that PCBP2, probably in complex with other mRNP factors, may dynamically recognize such differences and accumulate to specific P-bodies.

1. Introduction

Mammalian processing bodies (P-bodies), or GW bodies, are dynamic cytoplasmic structures that are highly enriched in mRNA decay factors, translational repressors and post-transcriptional gene silencing machinery. Initially, it was proposed that P-bodies represent actual sites of mRNA degradation [1,2]. Subsequently it became apparent that they also serve as storage sites of mRNAs [3,4], and could be involved in short interfering RNA (siRNA)- and micro-RNA (miRNA)-dependent translational repression, [5–8]. The fact that P-bodies are well conserved from yeast to mammals strongly indicates significant roles of these structures in RNA metabolism. While most of the P-body components identified to date seem ubiquitously present in P-bodies, it was recently reported that there could be differences within a population of P-bodies even in a single cell, suggesting the existence of compositional, and possibly, functional diversity among P-bodies [9,10]. Indeed, Pillai et al. showed that translationally repressed mRNA (and let-7 miRNA) was enriched in some, but not all of P-bodies, suggesting that P-bodies may be comprised of two populations, one for storage of mRNA and the other for its degradation [8]. More recently, Mozar et al. and Cougot et al. showed that heterogeneity does exist in P-bodies in mammalian astrocytes/astrocytoma cells and neurons, respectively [11,12]. Previously, we identified the RNA-binding protein PCBP2 (Poly-(rC) Binding Protein 2), or αCP2, as a novel component of P-bodies [13]. PCBP2 has been characterized for its ability to stimulate IRES (Internal Ribosomal Entry Site)-mediated translation [14,15], is reported to associate with polysomes [16], and stabilizes specific transcripts by binding to the 3′ untranslated region [17,18]. PCBP2 is alternately known as hnRNP (heterogeneous nuclear ribonucleoprotein)-E2, a member of shuttling hnRNPs, and may be involved in the export of nascent mRNPs. Interestingly, PCBP2 did not localize to all of the P-bodies as verified by immunostaining of Dcp1a. In this study, we examined such "selective" localization of PCBP2 to P-bodies in detail using immunofluorescence microscopy and photobleaching experiments. Our results indicate that accumulation of PCBP2 to specific P-bodies is a dynamics process that does not seem to involve its nuclear history, and that the presence of this protein likely reflects compositional diversity of repressed mRNP complexes.

2. Materials and methods

2.1. Reagents and antibodies

Actinomycin D, bromo-deoxyuridine (BrdU), puromycin, sodium arsenite and thymidine were purchased from Sigma. Hoechst 33342 was obtained from Molecular Probes. The following primary antibodies were used for immunofluorescence: mouse anti-BrdU antibody (Molecular Probes), rabbit anti-Dcp1a antibody (a kind gift from Dr. Jens Lykke-Andersen), rat anti-HA antibody (Roche), rabbit

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doi:10.1016/j.bbamcr.2009.02.002

anti-hnRNP A3 antibody (a kind gift from Dr. Ross Smith), mouse anti-PCBP2 antibody (Abnova), mouse anti-PTB antibody (Calbiochem) and goat anti-TIA1 antibody (Santa Cruz Biotechnology). Alexa-conjugated secondary antibodies were purchased from Molecular Probes.

2.2. Cell culture and transfections

HeLa cells and Neuro2a cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FCS (Gibco-Invitrogen) as described previously [19]. Differentiation of

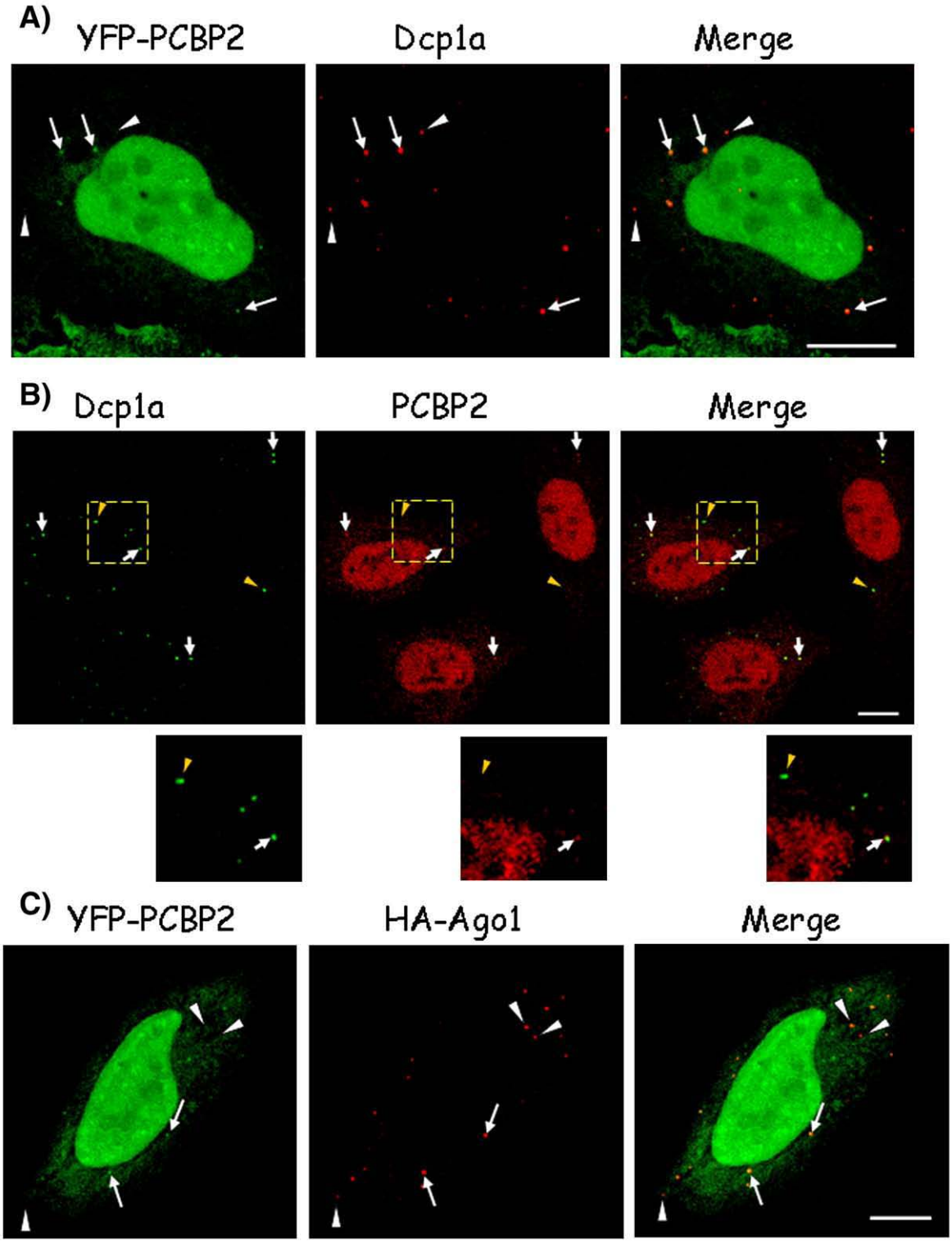

Fig. 1. Selective localization of PCBP2 to P-bodies. (A) HeLa cells expressing YFP-tagged PCBP2 were immunostained for Dcp1a and viewed with a confocal microscope. Z-section images taken in 0.7 μm steps were 3D-reconstructed using Zeiss AIM software. (B) Wild type HeLa cells were doubly immunostained for Dcp1a and PCBP2 and processed as in (A). Arrows indicate co-localization, whereas arrowheads indicate the lack of co-localization. Magnified images of the boxed region (with slight contrast enhancement to emphasize P-body-localization) are shown below. (C) HeLa cells expressing both YFP-tagged PCBP2 and HA-tagged Ago1 were immunostained with anti-HA antibody and processed as in (A). Arrows indicate co-localization, whereas arrowheads indicate the lack of co-localization. Bars = 10 μm.

Neuro2a cells was induced as described previously [19]. Transient transfections of plasmids were performed using Effectene reagent (Qiagen) following the manufacturer's instructions. For cell synchronization, conventional double thymidine block was utilized. Briefly, HeLa cells were cultured for 2 days to reach ~50% confluency, and then incubated in a medium containing 2 mM thymidine for 19 h. Subsequently, cells were washed with PBS, maintained in regular medium for 9 h, followed by secondary incubation in 2 mM thymidine for 16 h. Afterwards, cells were released from thymidine treatment and subjected to immunofluorescence. Pulse-labeling with BrdU was performed to ensure the quality of synchronization.

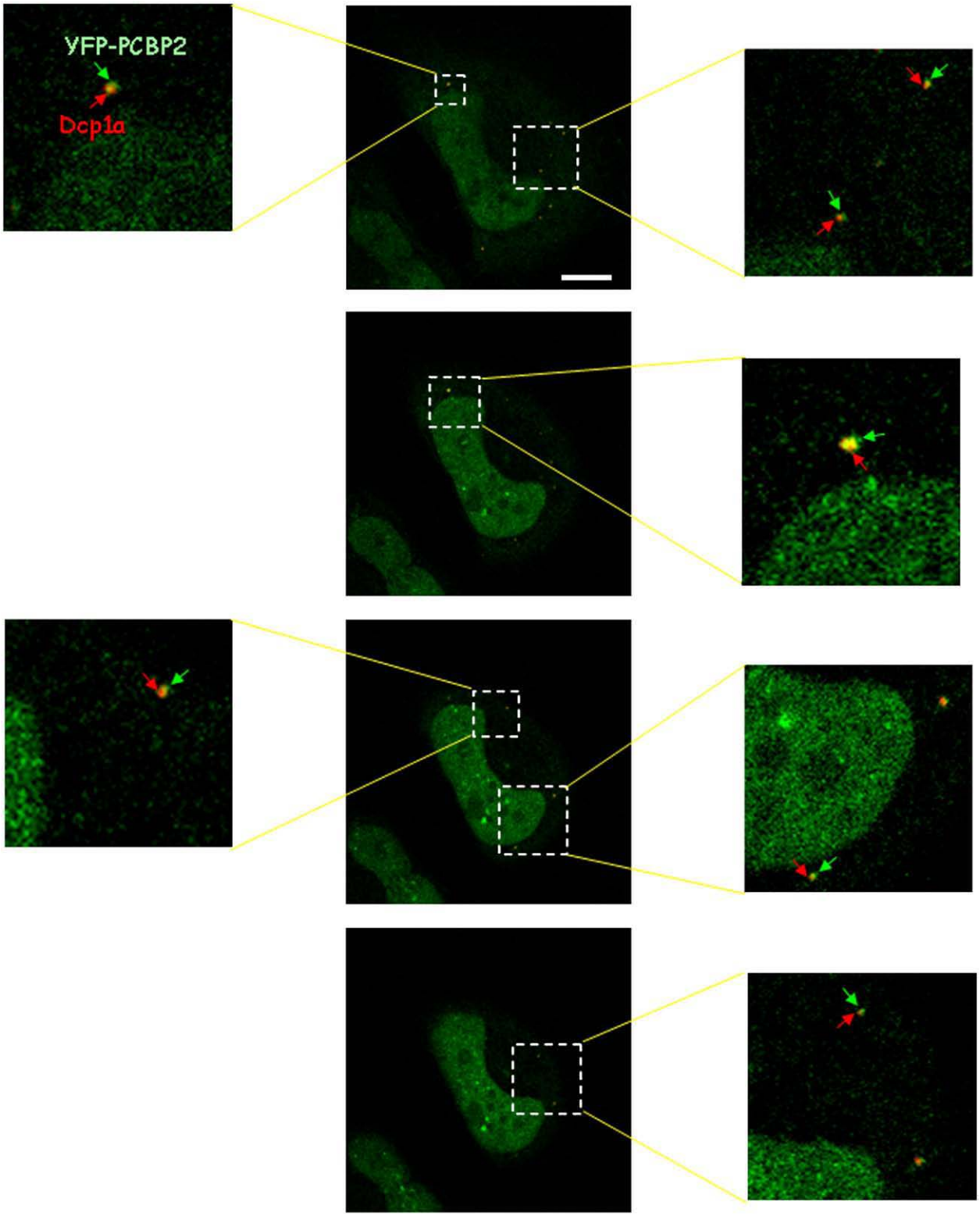

Fig. 2. Incomplete overlapping of fluorescence signals from PCBP2 and Dcp1a. Four consecutive z-sections of a representative HeLa cell expressing YFP-PCBP2 co-stained with Dcp1a using an Alexa-conjugated secondary antibody are presented. Green and red arrows in the magnified images of the boxed region indicate subcompartments within P-bodies enriched in PCBP2 and Dcp1a, respectively. Images were acquired such that no saturation in fluorescence signals would occur. Bar = 10 μm.

2.3. Plasmids

Constructs encoding YFP-tagged and HA-tagged PCBP2 were as described previously [13]. The expression vector for FLAG/HA-tagged Ago1 was purchased from Addgene, which was originally constructed in Dr. Thomas Tuschl's lab [20]. The plasmid encoding CFP-tagged Dcp1a was constructed by inserting the Dcp1a cDNA into ECFP-C1 vector (Clontech). Human PCBP1 cDNA was amplified from I.M.A.G.E. clone (clone ID: 6061411) purchased from Open Biosystems and cloned into EYFP-C1 vector (Clontech).

2.4. Immunofluorescence

Indirect immunofluorescence was performed as described previously [13]. All the primary antibodies were added at a dilution of

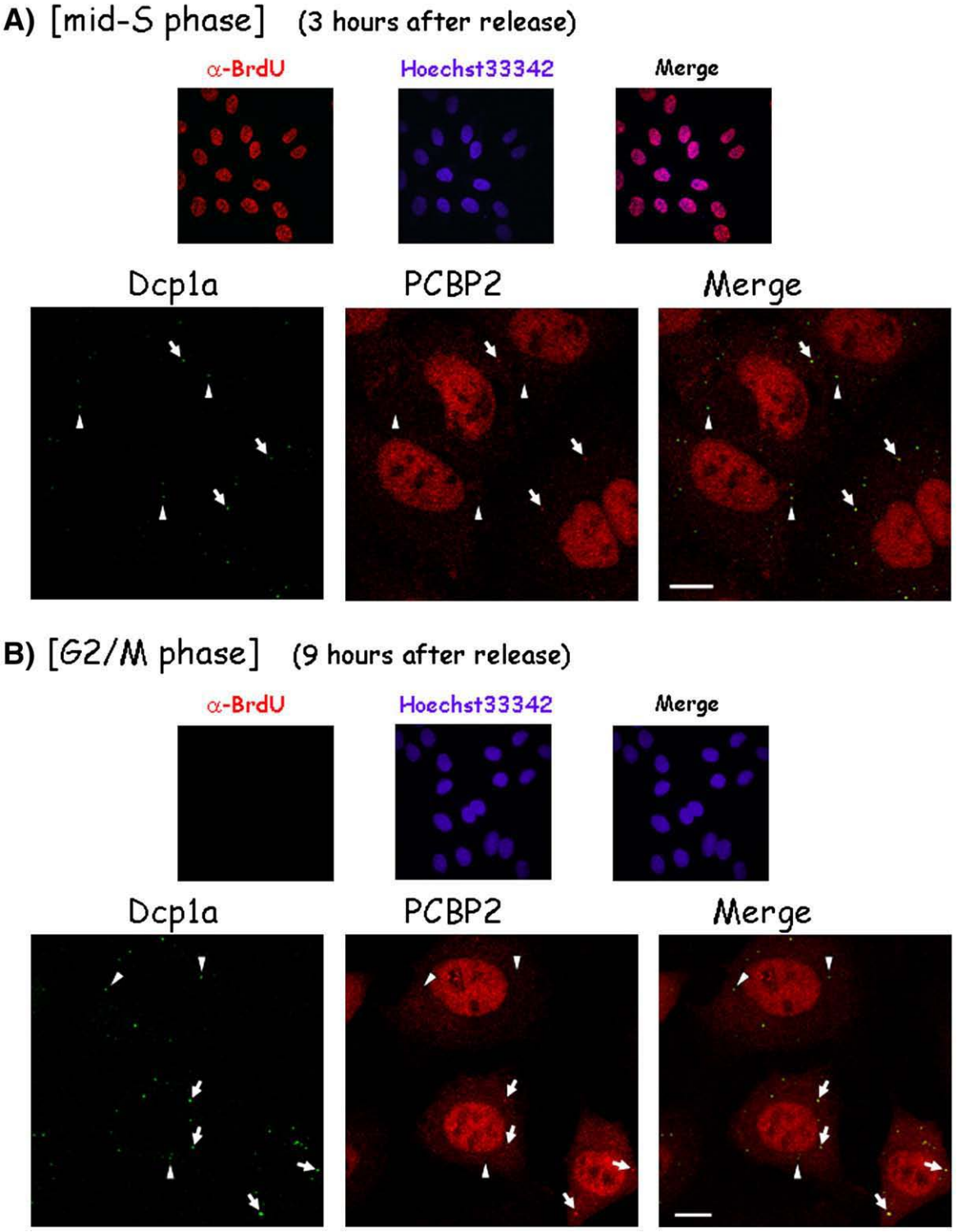

Fig. 3. Cell cycle and P-body-localization of PCBP2. HeLa cells were synchronized by double thymidine blocking and stained for Dcp1a and PCBP2 after 3 h (A) or 9 h (B) release. Upper panels show BrdU staining of the synchronized cells. Note that P-body size increases as cells progress from S-phase into G2/M phase. Bars = 10 μm.

1:400, incubated for 2 h at room temperature, followed by secondary antibodies added at a 1:400 dilution.

2.5. Fluorescence microscopy

Samples were viewed with a 63× oil-immersion PlanApo lens on an Axiovert135 fluorescence microscope (Carl Zeiss). Images were acquired with a confocal laser scanning microscope (LSM510 Meta, Carl Zeiss), from at least nine fields of view for each experiment, which was performed in triplicate. For imaging of fixed cells, laser power was set at 1% of diode laser (405-nm line), 1% of 25 mW argon ion laser (488-nm line), 20% of 1 mW Helium-neon laser (543-nm line) and 20% of 5 mW Helium-neon laser (633-nm line), to visualize Hoechst33342, YFP or Alexa-488, Alexa-546, and Alexa-647, respectively. For FRAP experiments, laser power was set at 10% of 30 mW argon ion laser (458-nm line) and 1% of 25 mW argon ion laser (514-nm line) to detect CFP and YFP, respectively. Images were acquired 0.7 μm stepwise and 3D reconstructed using Zeiss AIM software (version 3.2). Quantitative co-localization analyses were doubly performed on 3D images by 1) manual counting and 2) using the analysis program provided by Zeiss LSM software. In the former method, line profiles were obtained and fluorescence intensity of P-body-localized PCBP2 and cytoplasmically diffuse PCBP2 was compared when necessary. In the latter method, pixels above background fluorescence were evaluated using the cross-hair function, and the results of the co-localization analysis were superimposed on image channels for quantification of PCBP2-enriched P-bodies. This procedure was repeated for 50 cells in triplicate.

2.6. Fluorescence Recovery after Photobleaching (FRAP)

FRAP experiments were performed essentially as described previously [13]. Briefly, HeLa cells were plated on glass-bottomed dishes and doubly transfected with plasmids encoding CFP-Dcp1a and YFP-PCBP2. After cultured overnight, FRAP experiments were performed using a confocal microscope (LSM510 Meta, Zeiss) using the 458-nm line for detecting CFP, and the 488-nm line to photobleach and detect YFP, with a pinhole adjustment resulting in a 2 μm optical slice. A single P-body containing both CFP-Dcp1a and YFP-PCBP2 was selected, and a 2 μm square area that enclosed the target P-body was defined as ROI (region of interest). The ROI was photobleached, and the fluorescence recovery was measured every 4 s, which was repeated for 15 different cells. For quantitative analyses, background intensity was subtracted and intensities of selected P-bodies were measured over time and normalized using intensities of a region of interest (ROI) in the nucleus of a transfected but non-bleached cell. In the case of actinomycin D-treated cells, normalization was performed using intensities of a ROI in the cytoplasm of a transfected, non-bleached neighboring cell.

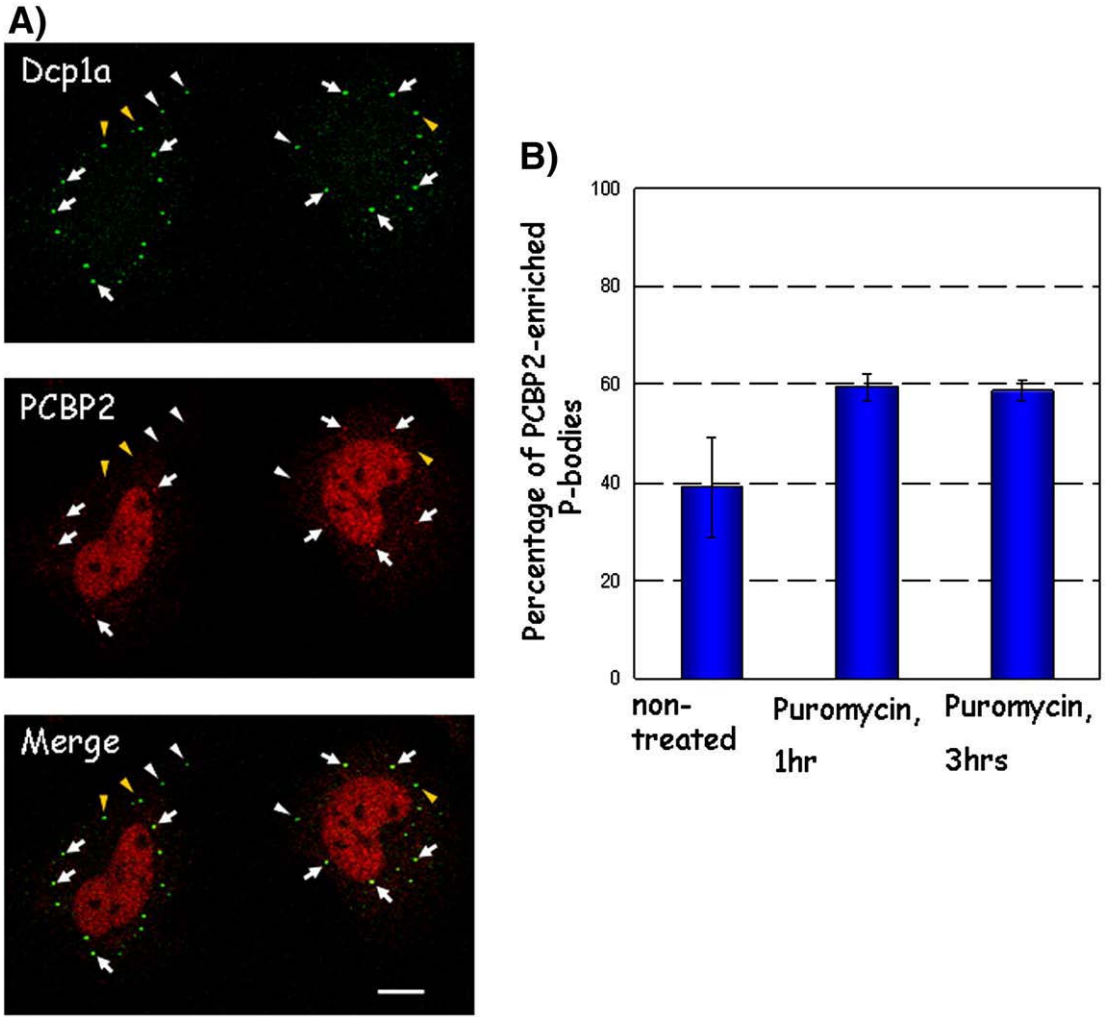

Fig. 4. The effect of puromycin treatment on P-body-localization. (A) HeLa cells were treated with puromycin (25 μg/ml, 1 h) to accumulate non-translating mRNAs into P-bodies, and immunostained for Dcp1a and PCBP2. Arrows indicate co-localization, whereas arrowheads indicate the lack of co-localization. (B) Quantitative analyses of P-body-localization of PCBP2. Percentage of P-bodies enriched in PCBP2 was calculated for cells treated with puromycin for 1 h or 3 h and for control cells. The experiments were performed in triplicate for at least 50 cells. Bars = 10 μm.

3. Results

3.1. Selective P-body-localization of PCBP2

To confirm the limited localization of PCBP2 to P-bodies, we examined the subcellular distribution of PCBP2 by 1) expression of YFP-tagged PCBP2, and 2) immunostaining endogenous PCBP2 using an antibody specific to PCBP2, accompanied by immunostaining of Dcp1a to visualize P-bodies. Images were 3D reconstructed to make sure that all of the P-bodies and fluorescence signals were captured in the analysis. As shown in Fig. 1A and B, in both cases, PCBP2 was enriched only in a subset of P-bodies. Quantitative

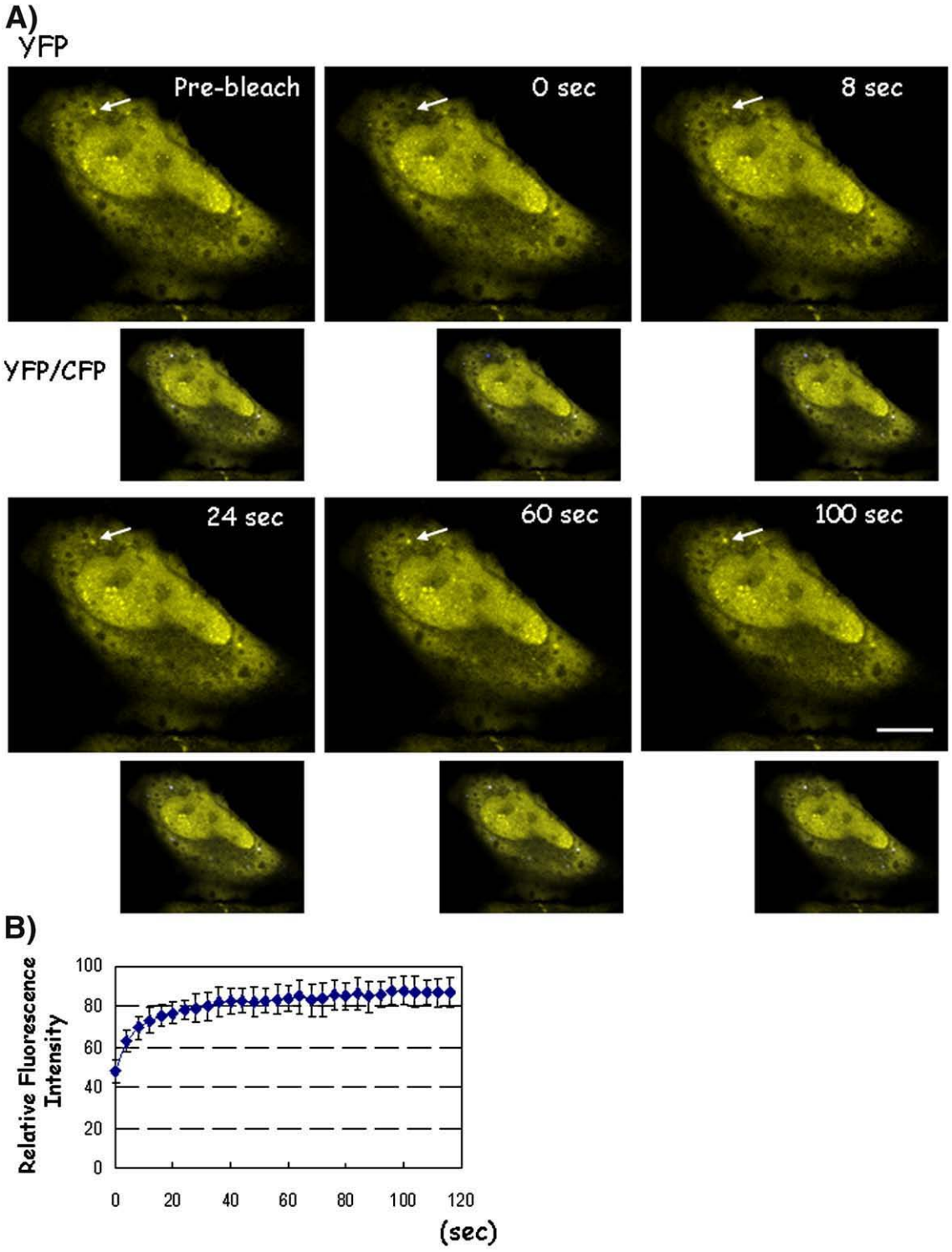

Fig. 5. Dynamic accumulation of PCBP2 to P-bodies. (A) FRAP experiments. HeLa cells doubly transfected with plasmids encoding YFP-PCBP2 and CFP-Dcp1a were subjected to FRAP analysis as described in Materials and methods. The recovery kinetics of YFP-PCBP2 is presented in (B). (C) HeLa cells were treated with actinomycin D (5 μg/ml, 3 h) and immunostained for Dcp1a and PCBP2. Nuclei were counterstained with Hoechst 33342. A single optical section is presented. Arrows indicate co-localization between Dcp1a and PCBP2. (D) FRAP experiments were performed for doubly transfected HeLa cells treated with actinomycin D. The upper panel shows the recovery kinetics, whereas the lower panel shows time-lapse micrographs of a photobleached cell. Bars = 10 μm.

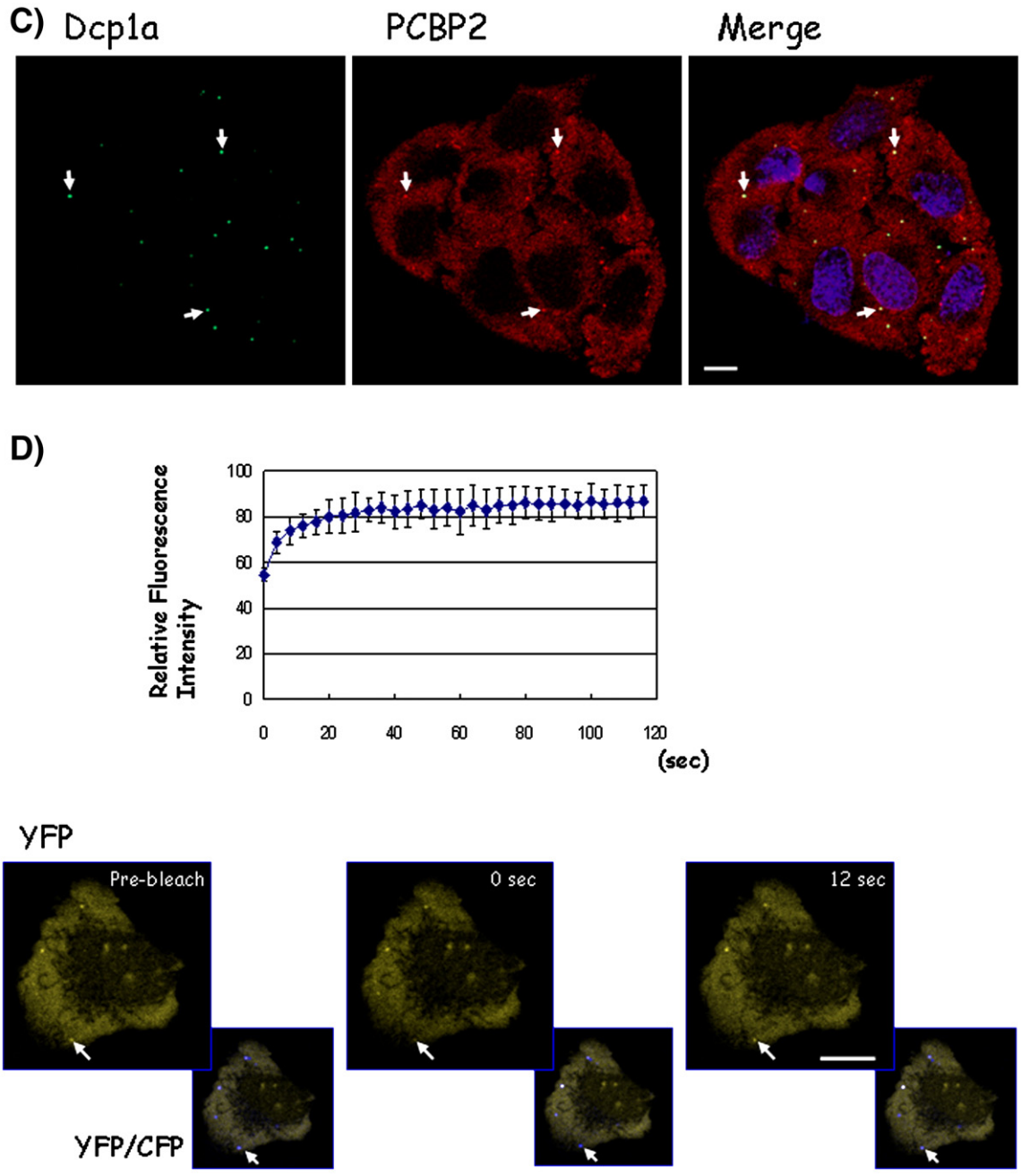

Fig. 5 (*continued*).

analysis revealed that the ratio of PCBP2-positive P-bodies was approximately 40% (shown in Fig. 4B). We tested this selectivity using another P-body marker, Ago1 [6]. When HA-tagged Ago1 was expressed in HeLa cells and co-stained with PCBP2, PCBP2 was again localized to a subpopulation of Ago1-positive P-bodies, while fluorescence signals from Ago1 predominantly overlapped with those of Dcp1a (Fig. S1A). These results reaffirmed the limited P-body localization of PCBP2. Generally, PCBP2 was more likely enriched in larger P-bodies, even though this was not always the case, since some above-average sized P-bodies lacked PCBP2 (Fig. 1B, yellow arrowheads). Furthermore, an up-close look at individual P-bodies revealed that the fluorescence signals of Dcp1a and PCBP2 were not always completely overlapped, when PCBP2 was detected by the expression of YFP-tagged fusion protein (Fig. 2) or by immunostaining of endogenous PCBP2 (Fig. S1B): especially at the periphery of each P-body, the two signals were distinguishable, suggesting that PCBP2 assumes distinct distribution within individual P-bodies from Dcp1a.

3.2. Correlation with the cell cycle

Previously, it was reported that P-body size and number dramatically changes during cell cycle [21]. To study the correlation of cell cycle with the selectivity of P-body-localization, routine thymidine block and release method was employed. After synchronized at G1/S phase, HeLa cells were released from thymidine treatment and subjected to immunofluorescence using antibodies against Dcp1a and PCBP2. In parallel experiments, synchronized HeLa cells were pulse-labeled with bromo-deoxyuridine (BrdU) and immunostained using anti-BrdU antibody to monitor the efficiency of synchronization. At 3 h, bright BrdU staining was observed for almost 100% of cells, suggesting that the synchronized cells were

uniformly in S phase (Fig. 3A, upper row). At 9 h, no BrdU staining was observed, and subsequently the cells underwent mitosis, indicating that the synchronized cells progressed into G2 phase at this period (Fig. 3B, upper row). During S phase, P-body size was generally smaller (Fig. 3A, lower row), whereas it became larger in G2 phase (Fig. 3B, lower row), in agreement with the report by Yang et al. [21]. In accordance with the above observation that PCBP2 is likely present in larger P-bodies, PCBP2 seemed more prevalently localized to P-bodies in G2 phase than in S phase, indicating that its P-body localization may be cell cycle-dependent. However, PCBP2 was present in P-bodies of above-average size during S phase (Fig. 3A, arrows), and a portion of P-bodies, especially smaller ones, were PCBP2-negative even during G2 phase (Fig. 3B, arrowheads). These observations led us to speculate that P-body-localization of PCBP2 appeared dependent on cell cycle only as a consequence of reduced (S phase) or increased (G2 phase) prevalence of larger P-bodies, and that P-body size may be a more dominant factor.

3.3. The effect of puromycin on the selectivity

Several studies reported P-body size and number increase by puromycin, a translational inhibitor that triggers premature termination of polypeptide chain and polysome disassembly [22–24]. Puromycin treatment leads to the accumulation of non-translatable mRNPs, thereby rendering the mRNA degradation machinery limiting [25], resulting in significant enlargement of P-bodies. To test the possibility that puromycin-induced enlargement of P-bodies causes more ubiquitous P-body localization of PCBP2, PCBP2 and Dcp1a were immunostained in cells treated with this drug. As shown in the left panel of Fig. 4A, puromycin treatment (25 μg/ml, 1 h) resulted in significant enlargement of Dcp1a-positive P-bodies. Quantitative analysis revealed that the percentage of PCBP2-positive P-bodies increased from approximately 40% to around 60%, underscoring the importance of P-body size in recruiting PCBP2. However, considerable portion (~40%) of the P-bodies, including large ones (yellow arrowheads), still lacked PCBP2 (Fig. 4A, arrowheads; Fig. 4B) even after longer hours of treatment (Fig. 4B, 3 h). Collectively, these data indicate that while puromycin-induced loading of repressed mRNPs, and the resulting enlargement of P-bodies, led to more prevalent P-body localization, they are not sufficient for ubiquitous recruitment of PCBP2.

3.4. Dynamic accumulation of PCBP2 to P-bodies

Next, to address whether PCBP2 remains anchored to specific P-bodies or it could exchange between P-bodies and the cytoplasm, we performed Fluorescence Recovery after Photobleaching (FRAP) experiments. HeLa cells were doubly transfected with plasmids encoding CFP-Dcp1a and YFP-PCBP2, and FRAP experiments were performed on YFP-PCBP2 using a confocal microscope. A single P-body enriched in both CFP-Dcp1a and YFP-PCBP2 was chosen, the YFP signal was photobleached and the fluorescence recovery was monitored. As shown in Fig. 5A and B, YFP-PCBP2 localized in P-bodies exhibited rapid fluorescence recovery, suggesting that PCBP2 is not tethered to particular P-bodies but dynamically moves in and out of them. This also indicates that PCBP2 diffusely distributed in the cytoplasm is capable of recognizing only specific P-bodies. Since PCBP2 is also known as a shuttling hnRNP, it may have the ability to escort nascent mRNPs from the nucleus to storage particles in the cytoplasm, reminiscent of maternal germ granules [4]. Therefore, we examined the involvement of the nucleo-cytoplasmic shuttling activity of PCBP2 in P-body-localization. To this end, HeLa cells were treated with the transcription inhibitor, actinomycin D (actD). A short period (3 h) of actD treatment did not significantly affect P-body integrity in our experiments, unlike in long-term treatments [1], and neither PCBP2 localization nor P-body integrity was perturbed when incubated with DMSO vehicle (data not shown). PCBP2 became almost completely relocalized to the cytoplasm upon actD treatment (Fig. S1C), indicating that this protein belongs to the class of hnRNPs whose nuclear import is coupled to transcription [26], and that its shuttling activity was stalled by the drug treatment. However, its localization to P-bodies was not perturbed (Fig. 5C). Furthermore, dynamics of P-body-localized PCBP2 was barely affected by actD, as demonstrated by FRAP experiments using actD-treated HeLa cells (Fig. 5D). Taken together, these results indicate that recognition of particular P-bodies by PCBP2 is a dynamic process which does not require its nucleo-cytoplasmic shuttling activity and probably, its nuclear history.

3.5. P-body-localization of PCBP1, a homolog of PCBP2

Lastly, to test if there is any other factor that exhibits similar P-body localization, we focused on PCBP1 (Poly-(rC) Binding Protein 1, alternately hnRNP-E1), a protein that shows greater than 80% homology in its amino acid sequence. PCBP1 is also functionally related to PCBP2, particularly in its ability to stimulate IRES-mediated translation and to stabilize specific transcripts [13,27]. We transfected a plasmid encoding human PCBP1 fused to YFP into HeLa cells and studied PCBP1 localization. Consistent with a previous report [28], we found that PCBP1 was predominantly nuclear, with particular enrichment in nuclear speckles (data not shown). However, a small portion of the protein was cytoplasmic, and as shown in Fig. 6A, similar to PCBP2, PCBP1 was enriched only in a subset of Dcp1a-stained P-bodies. Interestingly, PCBP1 appeared to occur in P-bodies less frequently than PCBP2. Upon exposure to arsenite, PCBP1 similarly became localized to both P-bodies and SGs (Fig. S2). To study if P-body-localization of PCBP1 and PCBP2 is correlated, we co-expressed YFP-tagged PCBP1 and HA-tagged PCBP2 and examined their localization alongside with Dcp1a (We decided to detect PCBP2 using HA-tagged fusion protein to avoid the possibility that PCBP2 antibody might label exogenously expressed PCBP1 because of the sequence similarity). Co-staining of PCBP1 and PCBP2 revealed that PCBP1 was indeed more selective in P-body localization, since a portion of the P-bodies that are enriched in PCBP2 lacked PCBP1 (Fig. 6B, yellow arrowheads). Importantly, P-bodies containing PCBP1 were almost always positive for PCBP2 (Fig. 6B, arrows), and P-bodies lacking PCBP2 appeared invariably negative for PCBP1. Therefore, PCBP1 seemed present only in PCBP2-positive P-bodies, suggesting a correlation between the P-body-localization of the two proteins. Since PCBP1 has been known to associate with PCBP2 [29,30], PCBP1 may be recruited to specific P-bodies in complex with PCBP2, and possibly other mRNP factors.

4. Discussion

Our results indicate that PCBP2, possibly in association with PCBP1 and other mRNP complexes, dynamically recognizes and accumulates in specific P-bodies, likely regardless of its nuclear history. Given that PCBP2 localizes only to larger P-bodies and neither its overexpression nor depletion affects the size and number of P-bodies [13], PCBP2 is likely recruited to P-bodies only at later stages of P-body formation. It has become increasingly clear that amassment of mRNPs committed to silencing or degradation forms the first step of P-body assembly, which progresses into microscopically visible P-bodies utilizing several scaffolding proteins like GW182 and Ge-1 and by further accumulating non-translating mRNPs [22]. Since such “building blocks” are ubiquitously present in P-bodies, it does not seem probable that interaction with these proteins alone will result in the recruitment of PCBP2. Furthermore, puromycin-induced loading of repressed mRNPs could not bring about ubiquitous P-body-localization of PCBP2 (Fig. 4), suggesting that the presence of PCBP2 likely reflects intrinsic difference among P-bodies rather than stochastic accumulation, probably originating from the initial step of their

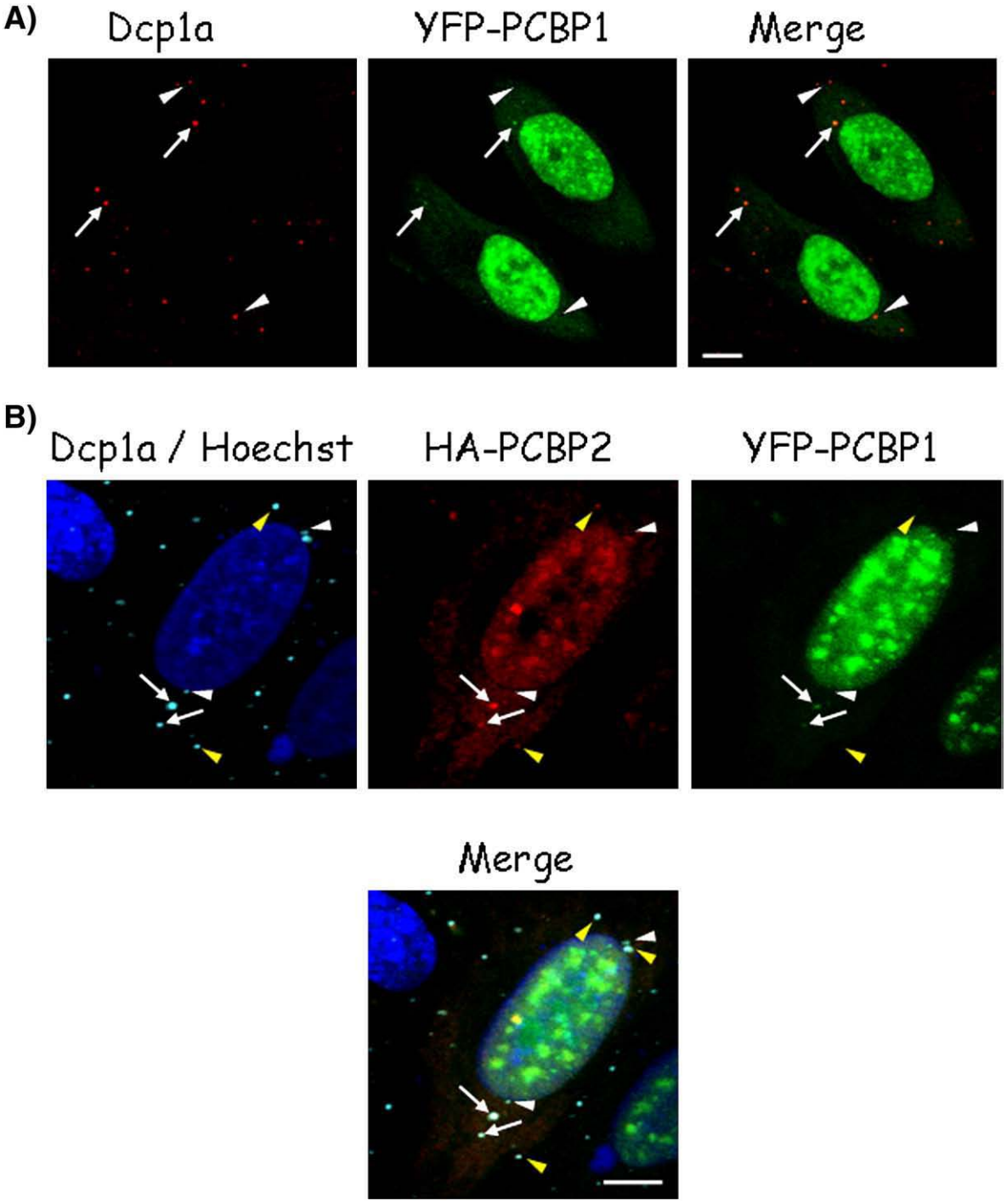

Fig. 6. PCBP1 localizes to a subset of PCBP2-positive P-bodies. (A) HeLa cells transfected with a plasmid encoding YFP-tagged PCBP1 were immunostained for Dcp1a. Arrows indicate the presence of PCBP1 in P-bodies, whereas arrowheads indicate the absence of PCBP1 in P-bodies. (B) HeLa cells doubly expressing HA-tagged PCBP2 and YFP-tagged PCBP1 were immunostained using anti-Dcp1a (light blue) and anti-HA antibodies. PCBP1 and PCBP2 were occasionally found to localize concurrently to specific P-bodies (indicated by arrows). White arrowheads indicate P-bodies lacking both proteins, whereas yellow arrowheads indicate P-bodies containing PCBP2 but not PCBP1. Nuclei were counterstained with Hoechst 33342. Bars = 10 μm.

assembly, i.e. the accumulation of mRNPs in a specific repressed state. Since PCBP2 (and PCBP1) is well known to enhance translation of certain mRNAs and to associate with polysomes, their presence to particular P-bodies may confer specialized functions, such as the ability to reverse repression of specific transcripts in response to changes in the surrounding environment [3]. Further, recent reports have shown that miRNAs are required for P-body formation [23,31]. There is a possibility that PCBP2 might be recruited to P-bodies largely because of its association with mRNAs or proteins involved in miRNA functions. Indeed, some mRNAs that are reported to associate with PCBP2, such as transcripts for c-myc [15] and A-Raf-1 kinase [32], are demonstrated to be targets of specific miRNAs [33,34]. Moreover, PCBP2 was identified to associate with Ago1 (and possibly Dicer) in a large ribonucleoprotein complex in a recent proteomic study [35], further lending support to the above hypothesis. It may be that the observed heterogeneity among P-bodies reflects localization of

miRNAs and their intended targets bound to PCBP2 to specific P-bodies. While the involvement of PCBP2 in miRNA function has not been established, it will be of great interest to investigate whether its unique distribution to P-bodies is related to miRNA-mediated regulation. Together with our observations that PCBP2 localizes to various types of RNA granules, including stress granules (SGs), neuronal RNA trafficking granules and perinucleolar compartment (PNC) (Supplementary Fig. S3), our data further argue for the possibility that PCBP2, in association with a defined set of mRNP complexes, modulates post-transcriptional regulation of gene expression in a distinct manner.

Acknowledgments

We would like to express our gratitude to Dr. Jens Lykke-Andersen and Dr. Ross Smith for generously providing antibodies. We would also like to thank the members of the lab for continuous encouragement. K. Fujimura is supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists. This work was supported, in part, by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

This manuscript is dedicated to the memory of Ms. Maiko Ebina, a dear friend and colleague.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2009.02.002.

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