MOLECULAR AND CELLULAR BIOLOGY, Nov. 2007, p. 7791–7801 Vol. 27, No. 22
0270-7306/07/$08.00+0 doi:10.1128/MCB.01254-07

Human TOB, an Antiproliferative Transcription Factor, Is a Poly(A)-Binding Protein-Dependent Positive Regulator of Cytoplasmic mRNA Deadenylation▽†

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Received 12 July 2007/Returned for modification 10 August 2007/Accepted 23 August 2007

In mammalian cells, mRNA decay begins with deadenylation, which involves two consecutive phases mediated by the PAN2-PAN3 and the CCR4-CAF1 complexes, respectively. The regulation of the critical deadenylation step and its relationship with RNA-processing bodies (P-bodies), which are thought to be a site where poly(A)-shortened mRNAs get degraded, are poorly understood. Using the Tet-Off transcriptional pulsing approach to investigate mRNA decay in mouse NIH 3T3 fibroblasts, we found that TOB, an antiproliferative transcription factor, enhances mRNA deadenylation in vivo. Results from glutathione *S*-transferase pull-down and coimmunoprecipitation experiments indicate that TOB can simultaneously interact with the poly(A) nuclease complex CCR4-CAF1 and the cytoplasmic poly(A)-binding protein, PABPC1. Combining these findings with those from mutagenesis studies, we further identified the protein motifs on TOB and PABPC1 that are necessary for their interaction and found that interaction with PABPC1 is necessary for TOB's deadenylation-enhancing effect. Moreover, our immunofluorescence microscopy results revealed that TOB colocalizes with P-bodies, suggesting a role of TOB in linking deadenylation to the P-bodies. Our findings reveal a new mechanism by which the fate of mammalian mRNA is modulated at the deadenylation step by a protein that recruits poly(A) nuclease(s) to the 3′ poly(A) tail-PABP complex.

Deadenylation is the first major step that triggers mRNA decay in eukaryotic cells (reviewed in references 19, 41, and 44). Computational modeling of eukaryotic mRNA turnover indicates that changes in levels of mRNA are highly leveraged to the rate of deadenylation (8). The importance of deadenylation in regulating mammalian mRNA turnover can be observed in several modes of mRNA decay, including decay directed by AU-rich elements in the 3′ untranslated region (4, 10), the rapid decay mediated by destabilizing elements in protein-coding regions (9, 23), the surveillance mechanism that detects and degrades nonsense-containing mRNA (11), and the decay directed by microRNA (59). Shortening of the 3′ poly(A) tail also plays a critical role in rendering mRNAs nontranslatable (26, 46, 58), thus inactivating gene expression. In spite of the importance of deadenylation, relatively little is known about the mechanisms that control it.

Recent progress in identifying key mammalian poly(A) nucleases involved in deadenylation (1, 6, 13, 16, 20, 38, 53, 55) has offered the opportunity to examine the regulation of deadenylation and to characterize the participating regulatory proteins. In mammalian cells, shortening of the poly(A) tail is mediated by the consecutive activities of two different poly(A) nuclease complexes (61). During the first phase, PAN2, presumably complexed with PAN3 (53, 61), shortens the poly(A) tails to ~110 A nucleotides. In the second phase, CCR4, presumably complexed with CAF1 (6, 55, 61), further shortens the poly(A) tail to oligo(A). Decapping mediated by the DCP1-DCP2 complex (36, 54, 56) was found to occur after either the first or the second phase of deadenylation (61).

To identify the potential regulatory factors involved in mammalian deadenylation, we have carried out literature and database searches with a focus on proteins that have the potential to interact with a poly(A) nuclease and/or the cytoplasmic poly(A)-binding protein (PABPC1). A family of antiproliferative genes, termed the *tob*/*btg* family (reviewed in references 24 and 39), emerged from the searches, because they contain a highly conserved N-terminal domain that can interact with CAF1 (7, 42). In humans, this family consists of six members: *tob*, *tob2*, *ana*, *pc3b*, *btg1*, and *btg2*, among which *tob* and *tob2* also encode a C-terminal domain with two putative PABP-interacting motifs (24, 39). Increasing evidence suggests that TOB proteins are involved in negative control of cell growth and can function as tumor suppressors (25, 49, 62). Moreover, TOB is highly expressed in anergic T-cell clones and in unstimulated peripheral blood T lymphocytes (52). The ability of TOB to maintain T-cell quiescence is thought to be due to its modulation of transcription (52). Despite the fact that TOB proteins have been known for a decade to function in antiproliferation and potentially in transcriptional control, the biochemical and molecular mechanisms by which they exert their functions remains unclear.

In this study, our results revealed that TOB proteins mod-

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† Supplemental material for this article may be found at http://mcb.asm.org/.

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▽ Published ahead of print on 4 September 2007.

ulate deadenylation, a key step in mRNA turnover. In addition, TOB protein localizes to RNA processing bodies (P-bodies), cytoplasmic foci that are enriched in 5′-to-3′ decay factors and translationally repressed mRNPs (for a recent review, see reference 17). These findings suggest a novel role for TOB proteins in regulating cytoplasmic deadenylation and identify a new mechanism by which the fate of mammalian mRNA is controlled at the deadenylation step by a protein that interacts with both poly(A) nuclease(s) and PABPC1 and has the potential to direct poly(A)-shortened mRNA intermediates to P-bodies.

MATERIALS AND METHODS

Plasmids. Construction of plasmids pSVα1/GAPDH (12), pTet BBB (60), pTet BBB-PTC (11), HA-CCR4a (9), and pGST-PABP, pGST-PABP-2nd-C, and pGST-PABP-RRM1 (28) has been described previously. The GFP-hDcp1 plasmid was a gift from B. Seraphin (54). When PCR techniques were employed to amplify desired DNA fragments for cloning, PfuUltra DNA polymerase (Stratagene) was used. DNA sequencing was performed to confirm all the desired mutations and in-frame fusions. To construct pTOB-V5, a 1,035-bp BamHI-XhoI fragment from an IMAGE clone (BC031406; ATCC), spanning the TOB coding region, was inserted in frame into the BamHI and XhoI site of pcDNA6/V5-His A (Invitrogen). To construct pTOB2-V5, a 1,032-bp cDNA encoding TOB2 was PCR amplified from a KIAA1663 clone (Kazusa DNA Research Institute, Japan) and inserted between the HindIII and XbaI sites of pcDNA6/V5-His A (Invitrogen). To generate MBP-TOB2, a 1,035-bp cDNA encoding the TOB2 open reading frame was PCR amplified from the KIAA1663 clone and inserted between the SnaBI and XhaI sites of pMal-Y (a gift from S. Ohno). Point mutations PABPC1 (F), PABP-2nd-C (F), TOB (FF), and TOB2 (FF) were created at positions as described below in the text using the QuikChange multisite-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with pGST-PBAP, pGST-PABP-2nd-C, pTOB-V5, and pTOB2-V5 as template.

Cell culture and transfection. Mammalian cell culture and DNA and small interfering RNA transfections were carried out as described previously (61). Briefly, NIH 3T3 B2A2 cells were split to a density of 0.6×10^6/6-cm dish 24 h before transfection. A 2.4-μg mixture of DNA (0.07 μg of reporter plasmid, 0.07 μg of internal control plasmid, 0.67 μg of CCR4a plasmid, and 1.59 μg of TOB plasmids) was diluted into 0.4 ml of Dulbecco's modified Eagle's medium containing 10% calf serum, into which 15 μl of PolyFect (QIAGEN) was added and mixed well. The mixture was then added to the culture dish. Time course experiments using the Tet-Off system for transcriptional pulsing were performed as described previously (35, 60).

Preparation of RNA samples and Northern blot analysis. Isolation of total cytoplasmic RNA and Northern blot analysis were conducted as described previously (48). Briefly, total cytoplasmic mRNA was isolated at various time points after the transcription pulse driven by the Tet-Off promoter of the reporter plasmid in transfected cells. Gene-specific DNA probes were prepared by random oligonucleotide priming for Northern blot analysis. A control plasmid encoding a 1.4-kb stable message, termed α-globin/GAPDH, served as an internal standard for normalization of the test messages. The ^{32}P-labeled probes were produced by inclusion of [α-^{32}P]dCTP (>6,000 Ci/mmol; Perkin-Elmer). Data were quantitated using a PhosphorImager (Bio-Rad). RNase H treatment of cytoplasmic mRNA to generate poly(A)$^-$ RNA and analysis of deadenylation and decay curves were carried out as described previously (47). All experiments were performed at least twice with reproducible results.

GST pull-down assay. Glutathione *S*-transferase (GST) pull-down assays were performed using the MagneGST pull-down system (Promega) according to the manufacturer's instructions. Briefly, GST fusion proteins were expressed in *Escherichia coli* BL21 (Amersham) and then induced by 0.5 mM isopropyl β-D-thiogalactoside at 30°C for 3 h. The cells were harvested and lysed in cell lysis reagent containing protease inhibitor cocktail (Roche) and DNase (Promega). The lysates were clarified by centrifugation at 14,000 × *g* for 10 min. The expressed GST fusion protein in the clear lysate was immobilized on MagneGST particles (Promega) and then incubated with 5 μl of the in vitro-translated protein in 250 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail). After incubation with rotating for 1 h at 4°C, the MagneGST particles were washed four times with 400 μl of radioimmunoprecipitation buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate). The bound fraction was eluted in 1× sodium dodecyl sulfate (SDS) loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained by Coomassie blue to show equal loading of the GST fusion protein and then dried for autoradiography to detect pull-down protein labeled with [^{35}S]methionine. For in vitro translation, [^{35}S]methionine-labeled proteins were produced in the rabbit reticulocyte lysate using an in vitro-coupled transcription/translation system (Promega) in the presence of [^{35}S]methionine (Amersham). The in vitro-translated products were treated with DNase I (0.5 U/μl) and RNase A (0.1 mg/ml) for 15 min at 30°C prior to being used in GST pull-down assays.

Gel mobility shift assay. GST-PABP-RRM1 (0.68 pmol) or GST-PABPC1 (0.25 pmol) was incubated with 0.5 pmol ^{32}P-labeled poly(A)$_{25}$ RNA probe (2×10^3 cpm) with 0, 1.7, or 6.8 pmol of MBP-TOB2 for 15 min at room temperature in 15 μl of binding buffer (10 mM HEPES-KOH pH 7.6, 3 mM $MgCl_2$, 100 mM KCl, 5 mM EDTA, 2 mM dithiothreitol, 5% glycerol, 1.5 mg/ml heparin, 0.5% NP-40, 0.2 mg/ml total yeast RNA). The mixtures were separated by electrophoresis on a 6% nondenaturing acrylamide gel (60:1 acrylamide-bisacrylamide) in 0.5× Tris-borate-EDTA buffer. The ^{32}P-labeled poly(A)$_{25}$ RNA probe was prepared as follows. A 10 μM concentration of poly(A) (Dharmacon) was incubated with 150 μCi ATP (6,000 Ci/mmol) and 30 U of T4 polynucleotide kinase (New England BioLabs) at 37°C for 2 h. After phenol and chloroform extraction, the probe was purified by using CHROMA SPIN columns (Clontech).

Coimmunoprecipitation and Western blot analysis. Cytoplasmic lysates were prepared as described previously (45). Briefly, 48 h after transfection, COS7 cells expressing wild-type or mutant TOB-V5 or TOB2-V5 or the control pcDNA6-V5 vector were harvested and lysed at 4°C in 600 μl lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM Na-orthovanadate, 1 mM Na-pyrophosphate, and 1 mM NaF supplemented with a protease inhibitor cocktail [Roche]). Fifty μl of cell lysate was saved as "input" control, and the rest was incubated with anti-V5 epitope antibody-conjugated agarose beads (Sigma) in the presence of 0.1 mg/ml RNase A at 4°C for 4 h. The beads were subject to five washes with the lysis buffer. The coprecipitated proteins were detected by Western blotting with antibodies as indicated in the figures below. For Western blot analysis, the protein samples were resolved on a 10% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). For detecting ectopically expressed V5-tagged or hemagglutinin (HA)-tagged proteins, membranes were incubated with horseradish peroxidase (HRP)-conjugated monoclonal anti-V5 antibody (1/5,000; Invitrogen) or HRP-conjugated monoclonal anti-HA antibody (1/1,000; Roche), respectively. For detecting endogenous proteins, membranes were first incubated with rabbit anti-CAF1-B peptide antibody (1/1,000), rabbit anti-PAN2-B peptide (1/2,000), rabbit anti-PABP-N (1/4,000; R. Lloyd), or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1/10,000; Research Diagnostics). Secondary HRP-conjugated goat anti-rabbit immunoglobulin G antibodies (1/4,000; Bethyl) or goat anti-mouse antibodies (1/5,000; Bio-Rad) were used for detection with chemiluminescence reagent (peroxide/luminol enhancer; Pierce).

Immunofluorescence microscopy. NIH 3T3 cells were transfected and prepared for immunofluorescence microscopy as described previously (61), except that various expression plasmids were delivered into NIH 3T3 cells by using Fugene 6 reagent (Roche). To block translation elongation, cells were cultured in medium containing 7.5 μg/ml cycloheximide for 110 min. Mounting medium containing 4′,6′-diamidino-2-phenylindole (DAPI) was applied to the slides for visualization of cell nuclei. The slides were observed under a Delta Vision deconvolution fluorescence microscope (X. He, Baylor College of Medicine). To detect V5-tagged TOB proteins, the primary and secondary antibodies were mouse antibody to V5 epitope tag (1:1,000 dilution; Invitrogen) and Alexa Fluor 555 goat anti-mouse antibody (1:1,000 dilution; Molecular Probes), respectively. To detect endogenous CENP-F, rabbit primary antiserum against CENP-F (1:50; Santa Cruz) and Alexa Fluor 647 chicken anti-rabbit antibody (1:1,000 dilution; Molecular Probes) were used as primary and secondary antibodies, respectively. For real-time imaging of P-bodies in live cells, 24 h after transfection with GFP-hDcp1 plasmid, the NIH 3T3 cells were reseeded to a glass-bottom dish (MatTek Corporation) in HEPES-buffered medium. After growing for another 22 h, the cells were observed under a Delta Vision deconvolution fluorescence microscope at 37°C. Images of the transfected cells showing P-bodies were taken every 5 min after addition or removal of cycloheximide.

RESULTS

TOB can interact simultaneously with PABPC1 and CCR4-CAF1. Previously, we hypothesized that PABPC1 plays a critical role in deadenylation by modulating the activities of PAN2-PAN3 and CCR4-CAF1 poly(A) nucleases in vivo (61).

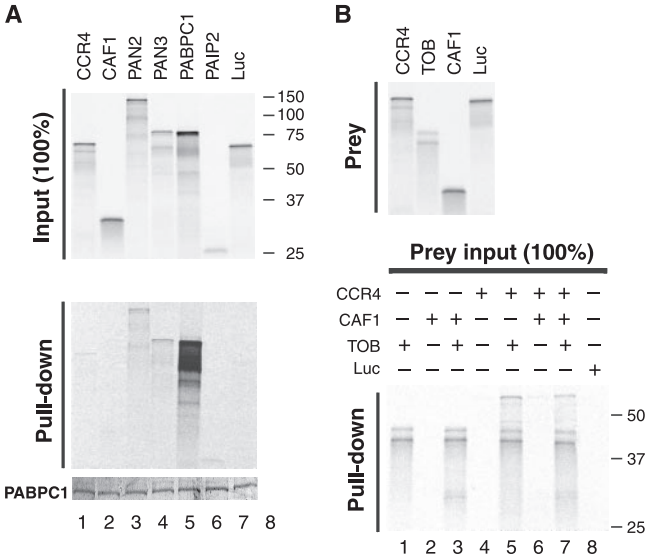

FIG. 1. Interactions between PABPC1, poly(A) nucleases, and TOB. (A) GST pull-down assays examining the interactions between PABPC1 and poly(A) nucleases. In vitro-translated and [^{35}S]methionine-labeled human CCR4, CAF1, PAN2, and PAN3 (top panel) were incubated with MagneGST particle-immobilized GST-PABPC1, and the resulting complexes were resolved by SDS-PAGE followed by Coomassie blue staining (bottom panel) for equal bait loading and then autoradiography (middle panel). PABPC1 and Paip2 were used as positive controls, whereas luciferase (Luc) was used as a negative control. (B) CCR4, CAF1, and TOB (top panel) were in vitro translated, [^{35}S]methionine labeled, and incubated in combinations with MagneGST particle-immobilized GST-PABPC1. GST pull-down (bottom panel) was performed as described for panel A. Luciferase was used as a negative control.

In the present study, we first performed GST pull-down experiments to address whether PABPC1 directly interacts with PAN2-PAN3 and CCR4-CAF1 poly(A) nucleases. Our results (Fig. 1A) showed that PAN2, PAN3, and two positive controls (PABPC1 itself and PABP-interacting protein 2 [Paip2]) can be pulled down by GST-PABPC1. In contrast, a very modest amount of CCR4 and no appreciable amount of CAF1 or luciferase could be pulled down by GST-PABPC1.

As the CCR4-CAF1 complex is a major player in eukaryotic deadenylation (5, 50, 51, 61), we then asked whether there might be a PABP-interacting protein(s) that mediates the association between this complex and the 3′ poly(A) tail-PABP complex, thus serving as a regulatory factor(s) in deadenylation. Database and literature searches drew our attention to TOB, due to its ability to interact with CAF1, its potential to bind PABPC1, and its critical role in regulation of gene expression (see the introduction). A direct interaction between TOB protein and PABPC1 was confirmed by a GST pull-down assay, and the interactions among TOB, CCR4, and CAF1 were also addressed (Fig. 1B). The presence of TOB led to an appreciable pull-down of CAF1 and/or CCR4 by GST-PABPC1 (Fig. 1B, lanes 3, 5, and 7), whereas no such pull-down was detected in the absence of TOB (Fig. 1B, lanes 2, 4, and 6). These data indicated that CCR4-CAF1 and PABPC1 interact at different sites on the TOB protein, allowing TOB to associate with both of them simultaneously. These in vitro findings suggest that TOB may regulate mRNA turnover by modulating the deadenylation rate through a direct interaction with poly(A) nucleases or by mediating the association between the CCR4-CAF1 complex and the 3′ poly(A)/PABP complex.

TOB can enhance deadenylation in vivo. To test whether TOB can modulate deadenylation, we carried out transfection experiments in NIH 3T3 cells and examined the effect of TOB overexpression on deadenylation by monitoring decay of β-globin mRNA (BBB) transcribed from the cotransfected plasmid pTet-BBB under control of the Tet-Off-driven promoter (35, 60). As shown in Fig. 2A, overexpression of TOB (Fig. 2A, middle panel) resulted in accelerated deadenylation of BBB message accompanied by the appearance before the 1-h time point of an intermediate bearing an oligo(A) tail, which did not appear until the 8-h time point in control cells (Fig. 2A, left panel). These findings indicated an earlier commencement of the second phase of deadenylation in cells overexpressing TOB. The poly(A)-shortening profile of the BBB mRNA for each Northern blot assay was also graphed to illustrate how the deadenylation kinetics were affected by TOB (see Fig. S1A, left, in the supplemental material). The data showed that overexpression of TOB caused a shift of the deadenylation curve towards the position of the cognate poly(A)$^-$ RNA and, again, that a significant amount of the oligo(A)-containing intermediate appeared before the 1-h time point when TOB was overexpressed.

Previously, we have shown that CCR4 plays a key role in the second phase of deadenylation, when mRNAs with poly(A) tails of ~110 nucleotides are further shortened to oligo(A) (61). The observations that TOB can interact with CCR4 and CAF1 (Fig. 1B) and that overexpression of TOB enhances the second phase of deadenylation (Fig. 2A; see also Fig. S1 in the supplemental material) prompted us to ask whether coexpression of TOB and CCR4 would further speed up deadenylation. Figure 2B shows that overexpression of CCR4 enhanced the second phase of deadenylation of BBB mRNA (left panel). Deadenylation was even more rapid when TOB and CCR4 were coexpressed (Fig. 2B, middle panel), compared with expression of CCR4 alone. Note that to better evaluate the fast deadenylation kinetics, a 0.5-h time point was included and the 8-h time point was omitted for this set of experiments. The nonsynchronous pattern and the presence of an oligo(A)-mRNA intermediate were evident before the 0.5-h time point (Fig. 2B, middle panel). Deadenylation profiles (see Fig. S1B, left, in the supplemental material) for the Northern blot assays also illustrate that the deadenylation of BBB mRNA was further accelerated in the presence of TOB. Given that the level of CCR4 expression was much lower when coexpressed with TOB than when expressed alone (Fig. 2E), the stimulatory effect of TOB on the deadenylation of BBB mRNA is likely to be synergistic.

As a test of the generality of TOB's enhancing effect on deadenylation, the deadenylation kinetics of β-globin mRNA bearing a nonsense codon (BBB+PTC) in the second exon known to induce nonsense-mediated mRNA decay (3, 14) were monitored in cells overexpressing TOB protein or coexpressing TOB and CCR4 (Fig. 2C and D). The results showed that TOB also enhances deadenylation of BBB+PTC mRNA (Fig. 2C, middle panel; see also Fig. S1C, left, in the supplemental material). Coexpression of TOB and CCR4 caused

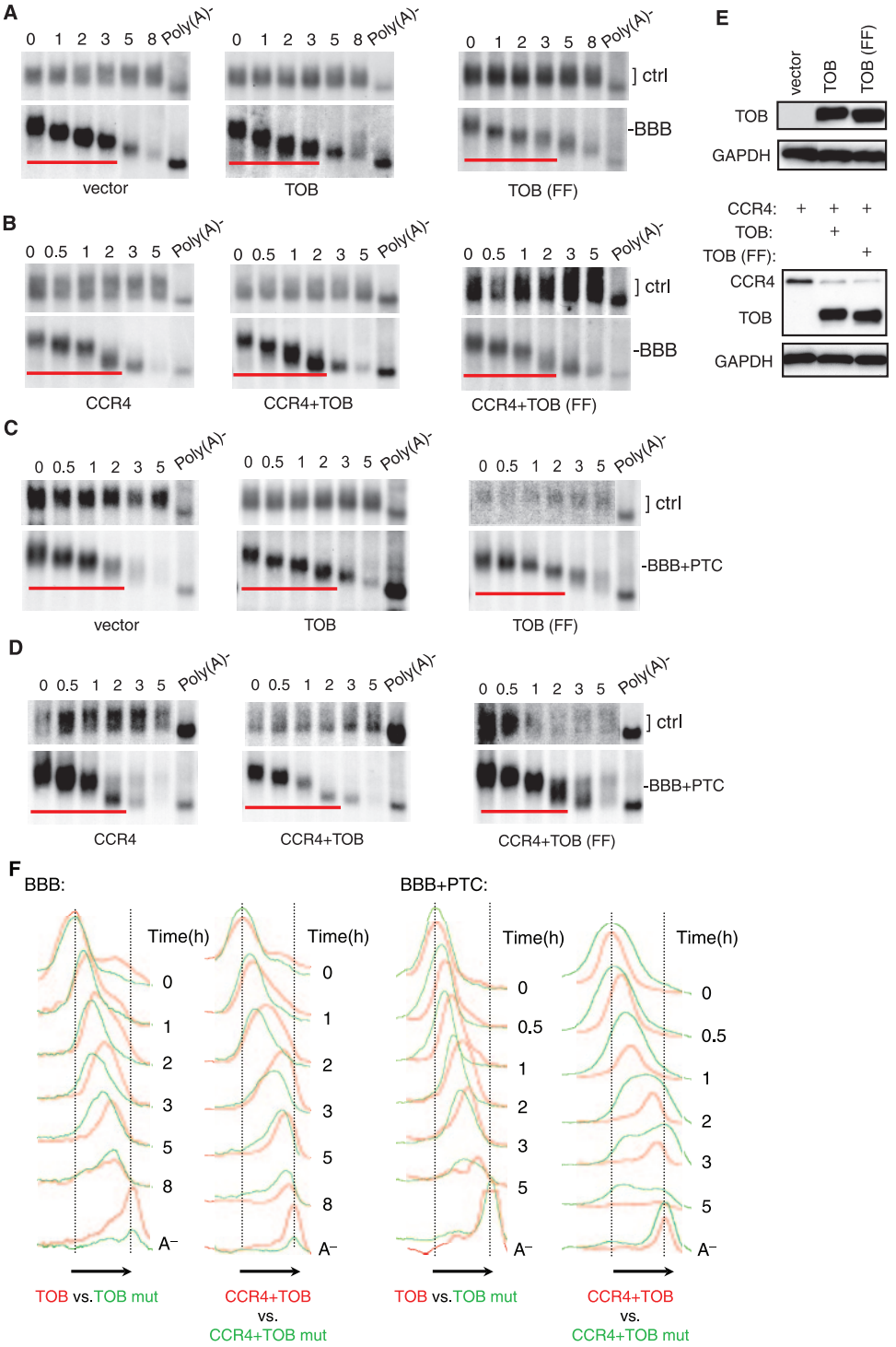

FIG. 2. PABP-dependent enhancement of deadenylation by TOB. (A to D) Northern blots showing deadenylation and decay of the β-globin (BBB) (A and B) or BBB+PTC (D and D) mRNA in the absence (vector) or presence of ectopically expressed wild-type TOB, mutant TOB(FF), CCR4 and wild-type TOB, or CCR4 and mutant TOB(FF). NIH 3T3 B2A2 cells were transiently cotransfected with the reporter plasmid pTetBBB (A and B) or pTetBBB-PTC (C and D), together with a control plasmid (pSVα-globin/GAPDH) and plasmid(s) encoding wild-type TOB, TOB(FF) mutant, CCR4, their combination, or an empty vector, as indicated underneath each panel. The α-globin/GAPDH mRNA was expressed constitutively and served as an internal standard for transfection efficiency and sample handling. The times given at the top correspond to hours after tetracycline addition. Poly(A)$^-$ RNA was prepared in vitro by treating the RNA sample from an early time point with oligo(dT) and RNase H. (E) Western blot analysis of total lysates, showing that ectopically expressed TOB-V5 and HA-CCR4 can be readily detected by anti-V5 or anti-HA antibody and that wild-type and mutant TOB proteins are expressed to a similar level. (F) Deadenylation profiles were drawn on the basis of raw data collected from the corresponding Northern blots in panels A to D. For each panel (A to D), the profile for wild-type TOB (red) was superimposed on that for mutant TOB (green) to illustrate the difference in deadenylation kinetics.

even more rapid deadenylation (Fig. 2D, middle panel; see also Fig. S1D, left, in the supplemental material). Moreover, TOB2, a paralog of TOB, also exhibited a similar enhancing effect on the deadenylation of both BBB and BBB+PTC mRNAs (see Fig. S2 in the supplemental material). This enhancing effect was further illustrated by subjecting the β-globin mRNA samples to oligonucleotide-directed RNase H cleavage prior to Northern blotting (see Fig. S3 in the supplemental material), which displayed a better resolution as the 3′ fragment of the message was shortened by 280 nucleotides.

To further substantiate the above conclusion, we also examined the effect of TOB overexpression on the poly(A) size distribution of BBB mRNA when the message was constitutively transcribed in NIH 3T3 cells under stationary or starvation conditions (see Fig. S4 in the supplemental material). Consistent with TOB proteins enhancing deadenylation, the distribution of the BBB mRNA shifted toward a smaller size when TOB proteins were overexpressed. Collectively, we conclude that TOB proteins can function as positive regulators of deadenylation in vivo.

TOB can form a complex with poly(A)-PABP via interaction with PABPC1. Interactions with cytoplasmic PABP have been shown to be critical for PABP-interacting proteins to exert their functions in translation and mRNA turnover (9, 27, 37). To test whether the binding of TOB to PABPC1 is important for its function in enhancing deadenylation, we first investigated the interaction between TOB and poly(A)-PABP with gel mobility shift assays. GST fused to PABPC1 (GST-PABPC1) or to the first RNA recognition motif of PABPC1 (GST-PABP-RRM1) was expressed in *E. coli*. These fusion proteins were then purified and incubated with a labeled 25-mer poly(A) sequence to permit complex formation (Fig. 3, lanes 2 and 6). Addition of purified TOB fused to maltose-binding protein (MBP-TOB) caused a dramatic supershift of the poly(A)-PABP complex, which resulted in an increase of the poly(A)-PABP-TOB complex near the wells and a concomitant reduction of the poly(A)-PABP complex (Fig. 3, lanes 7 and 8). By contrast, there was only a very modest supershift of poly(A)-PABP-RRM1 by TOB (Fig. 3, compare lanes 2 and 3 and lanes 3 and 8). Moreover, TOB itself did not form a complex with the poly(A) sequence (Fig. 3, lanes 4 and 5). Together, these results indicate that TOB can form a complex with poly(A)-PABP via TOB's interaction with PABPC1, suggesting that binding to PABPC1 is important to the function of TOB in enhancing deadenylation. These findings rule out the possibility that TOB enhances deadenylation by destabilizing the poly(A)-PABP complex.

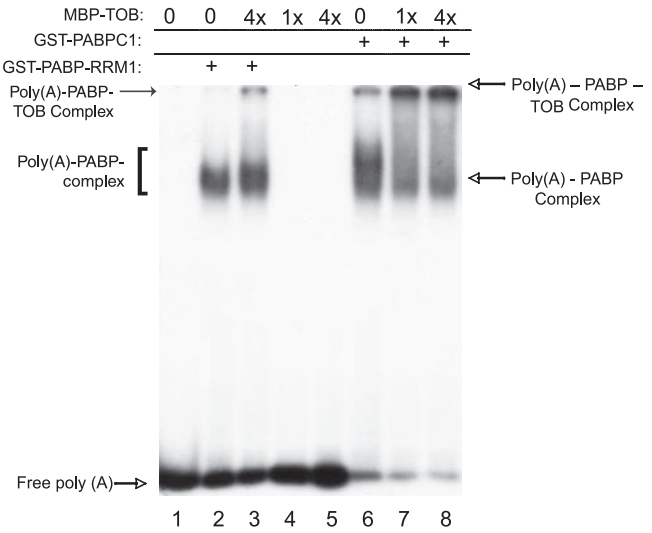

FIG. 3. TOB can form a complex with PABP-poly(A). A gel mobility shift assay was carried out to determine TOB's effect on PABPC1-poly(A) complex formation. ^{32}P-labeled poly(A)$_{25}$ RNA was incubated at room temperature alone (lane 1), with 0.68 pmol of purified GST-PABP-RRM1 (lanes 2 and 3), or with 0.25 pmol of purified GST-PABPC1 (lanes 6 and 7), followed by the addition of a molar excess of MBP-TOB2 (1.7 pmol for lanes 3 and 6 and 6.8 pmol for lane 7) as indicated. As a control, the probe was also incubated with MBP-TOB2 alone (1.7 pmol for lane 4 and 6.8 pmol for lane 5). The two arrowheads denote signals for complexes containing poly(A)-PABP and poly(A)-PABP-TOB.

F567 in the PABC domain of PABPC1 is critical for TOB binding. To examine whether the physical interaction between TOB and PABPC1 is important for enhancement of deadenylation, we set out to identify the binding sites on each protein and test the effects of mutations that eliminate the interaction on deadenylation. We first characterized the TOB-interacting site(s) on PABPC1. Previously, we had shown that a C-terminal sequence motif (PABC) (31) in the second half of the PABPC1 C-terminal domain, previously known as PABP-C2 (9) and now referred to as PABP-2nd-C to avoid confusion with the nomenclature for the mammalian PABPC1, contains the TOB-binding domain (34). The phenylalanine at position 567 (F567) (Fig. 4A) of human PABPC1 was identified as a critical residue for the interaction between the PABC motif and two other PABP-interacting proteins, Paip-1 and Paip-2 (31). Accordingly, we introduced the single point mutation F567A into GST-PABPC1 and GST-PABP-2nd-C and examined the effect on interaction with TOB. The F567A mutation in the PABC motif effectively decreased the ability of PABPC1 and PABP-2nd-C to interact with TOB proteins (Fig. 4B). In contrast, PABPC1 itself remained able to interact with the mutant PABPC1 but not with wild-type PABP-2nd-C, since the PABPC1-PABPC1-interacting domain was on the first half of the C-terminal domain (40). Intriguingly, this mutation appeared to enhance the PABPC1-PABPC1 interaction. An additional control was further provided by showing that both GST-PABPC1 and its F567A mutant, but not the PABP-2nd-C nor its mutant, could pull down Paip2, consistent with a previous report (28) that PABPC1 has a strong Paip2-binding site in the N-terminal RRM-containing domain. Taken together, our data show that PABPC1 associates with the TOB proteins via the PABC motif and that F567 in that motif is critical to the interaction.

Highly conserved phenylalanines in the PAM2 motifs of TOB are critical for its interaction with PABPC1. A consensus PABC recognition site, termed PABP-interacting motif 2 (PAM2) (2), has been used to identify PABP-binding partners, and one highly conserved phenylalanine residue in PAM2 has been found to be critical for interaction with PABC (30). TOB and TOB2 each contain two potential PAM2 motifs in their C-terminal domains (Fig. 5A). We mutated the two conserved phenylalanine residues in the potential PAM2 motifs of both TOB proteins to alanine, namely, F139A and F274A in TOB

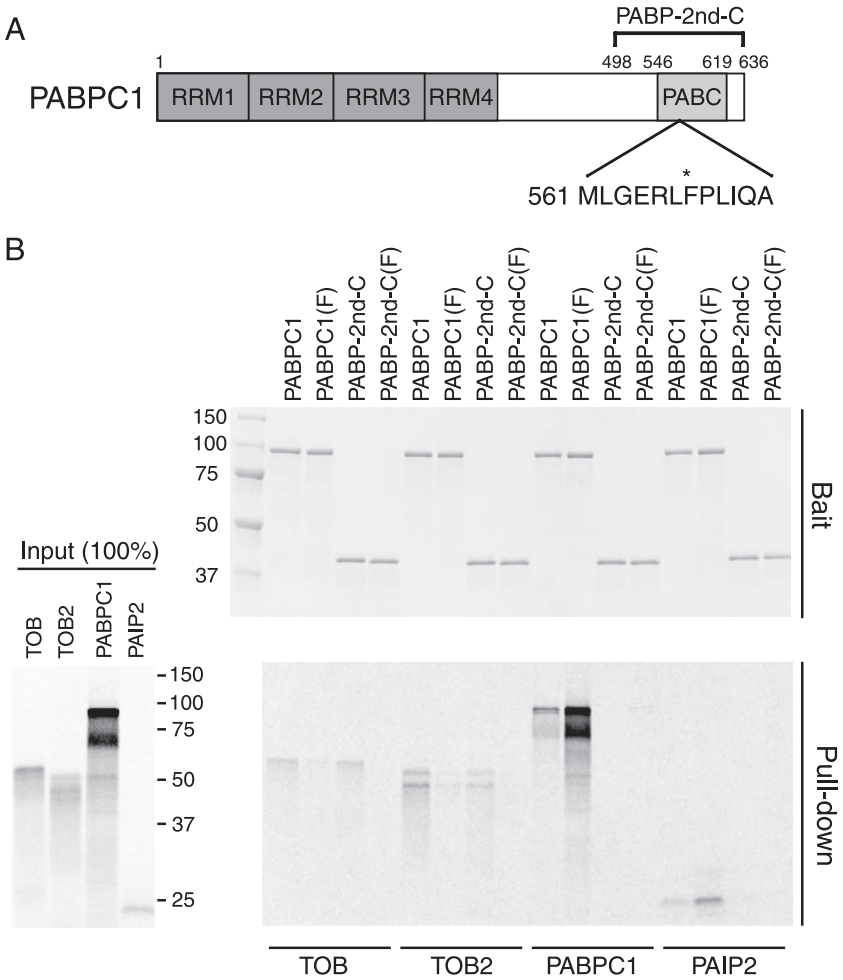

FIG. 4. F567 in the PABC domain of PABPC1 is important for interaction with TOB. (A) Schematic representation of human PABPC1. RRM, RNA recognition motif; PABP-2nd-C, the second half of the PABPC1 C-terminal domain, previously known as PABP-C2; PABC, poly(A)-binding protein C-terminal motif. Phenylalanine (F) at position 567 is indicated with an asterisk and was mutated to alanine to create the PABPC1(F) or PABP-2nd-C(F) mutant. (B) GST pull-down assay examining the TOB-binding region in PABPC1. In vitro-translated and [^{35}S]methionine-labeled TOB, TOB2, PABPC1, and Paip2 (left panel) were incubated with MagneGST particle-immobilized GST-PABPC1, GST-PABPC1(F), GST-PABP-2nd-C, and GST-PABP-2nd-C(F) (top panel). GST pull-down was performed as described for Fig. 1.

and F140A and F260A in TOB2, to create TOB(FF) and TOB2(FF). GST pull-down experiments (Fig. 5B) showed that these double mutations effectively diminish the ability of both TOB proteins to interact with PABPC1, via its PABC domain. These results demonstrate that the highly conserved phenylalanine residues in the PAM2 motifs of TOB are critical for PABP binding.

In vivo interaction of TOB with PABPC1 and CCR4-CAF1. To further substantiate the above GST pull-down data, coimmunoprecipitation and Western blotting experiments were performed to address the in vivo association of TOB proteins with both PABPC1 and CCR4-CAF1. In these experiments, TOB proteins tagged with the V5 epitope were expressed in transfected COS7 cells and immunoprecipitated by anti-V5 antibody (Fig. 6A). Consistent with the GST pull-down results, both wild-type TOB and TOB2 proteins could pull down endogenous PABPC1, whereas TOB proteins with mutated PAM2 motifs were largely unable to pull down endogenous PABPC1. Endogenous CAF1 was coimmunoprecipitated readily with both TOB proteins, with or without the PAM2 mutations, whereas PAN2 poly(A) nuclease was not pulled down by TOB proteins (Fig. 6A). Due to the lack of a strong antibody against endogenous CCR4, coimmunoprecipitation of CCR4 with TOB proteins was carried out using HA-tagged CCR4 (Fig. 6B). The results show that TOB proteins, wild type or mutant, pulled down only a modest amount of HA-CCR4 (Fig. 6B). No CCR4 pull-down could be seen in the control (Fig. 6B, right panel). Also, no GAPDH could be detected in the precipitates (Fig. 6A and B, GAPDH control). These results indicate that either a weak or an indirect interaction occurs between TOB proteins and CCR4, perhaps mediated by CAF1. Taken together, these data confirm our in vitro experiments that identified the TOB-PABPC1 interaction sites on both TOB proteins (Fig. 5) and PABPC1 (Fig. 4). They further demonstrate that CCR4-CAF1 and PABPC1 interact with the TOB proteins at different sites, allowing the TOB proteins to associate simultaneously with both of them (Fig. 1B). These findings demonstrate that an mRNP complex can be formed between TOB, poly(A) nucleases, and poly(A)-PABP in mammalian cells.

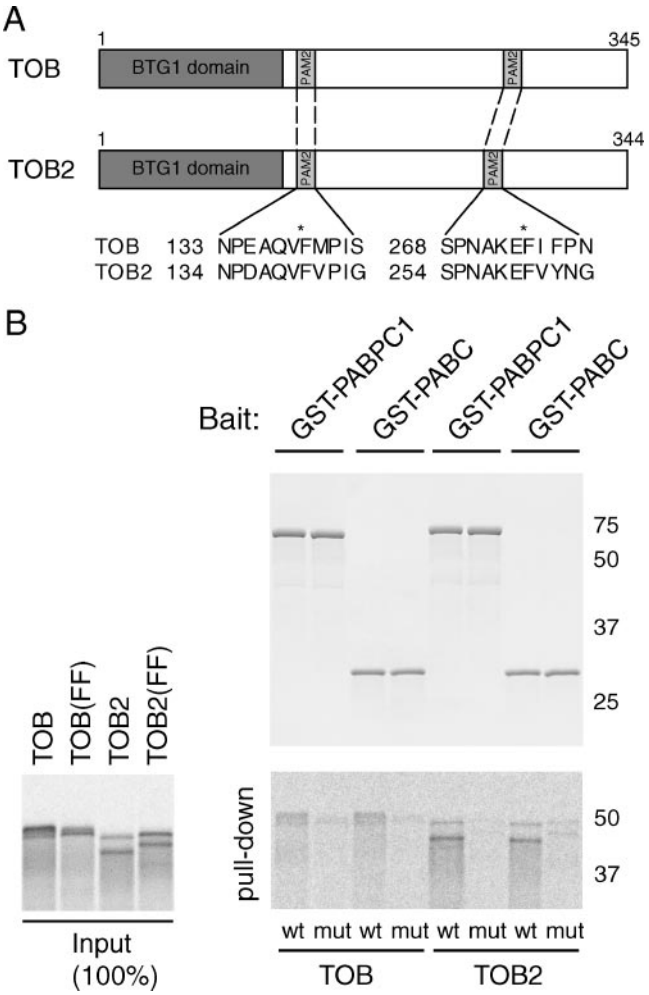

FIG. 5. PABP binds PAM2 motifs in TOB. (A) Schematic representations of human TOB and TOB2, showing PAM2. Phenylalanines (F) at positions 139 and 274 in TOB as well as positions 140 and 260 in TOB2 are indicated with asterisks and were mutated to alanines to create the TOB(FF) or TOB2(FF) mutants (mut). (B) GST pull-down assay examining PABPC1-binding sites in TOB proteins. In vitro-translated and [^{35}S]methionine-labeled TOB, TOB(FF), TOB2, and TOB2(FF) (left panel) were incubated with MagneGST particle-immobilized GST-PABP and GST-PABC (top panel). GST pull-down was performed as described for Fig. 1.

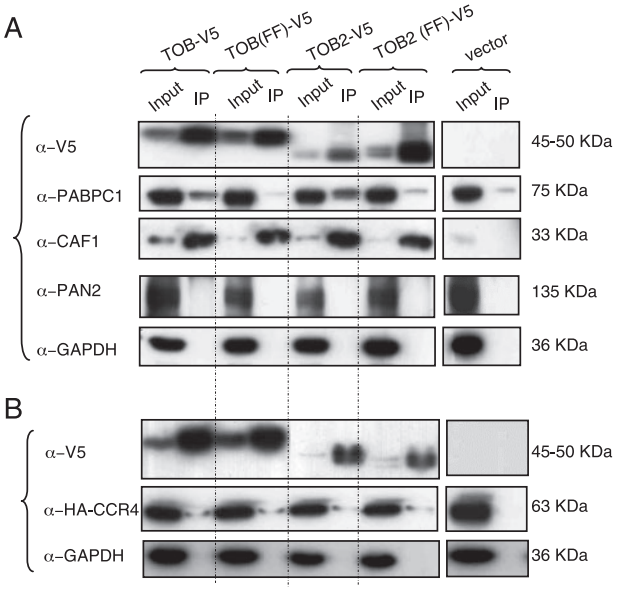

FIG. 6. Interactions between TOB proteins, PABPC1, and poly(A) nucleases. COS7 cells were transfected with plasmids expressing V5-tagged TOB, TOB(FF) mutant, TOB2, TOB2(FF) mutant, or pcDNA6-V5 (as control) (A) or along with plasmid expressing HA-tagged CCR4 (B). RNase A-treated cell extracts were first subjected to immunoprecipitation using anti-V5 antibody conjugated to agarose beads. Immunoprecipitates were then separated by SDS-PAGE and analyzed by Western blotting using various antibodies as indicated to the left. The molecular masses of each protein detected are indicated to the right. In both panels, 5% of total lysate used for immunoprecipitation was included as an input, for comparison.

Interaction of TOB proteins with PABPC1 is critical for enhancement of deadenylation. To assess whether PABP binding is important to TOB's enhancement of deadenylation, we examined the effect of the TOB(FF) double mutant, which cannot bind PABPC1 effectively (Fig. 5B and 6A), on the deadenylation of BBB or BBB+PTC mRNA. The results (Fig. 2A, C, and F; see also Fig. S1A and C in the supplemental material) showed that in contrast to the wild type, the TOB(FF) mutant does not accelerate the deadenylation kinetics of either BBB mRNA or BBB+PTC mRNA. These results indicate that the deadenylation-stimulatory function of TOB requires its interaction with PABPC1.

The above results were further corroborated by examining the deadenylation of BBB and BBB+PTC mRNA when the TOB(FF) mutant was ectopically coexpressed with CCR4, which is involved in the second phase of deadenylation of mRNA (61). Overexpression of wild-type TOB with CCR4 further enhanced the second phase of deadenylation (Fig. 2B and D, middle panel; see also Fig. S1B and D, left sides, in the supplemental material). In contrast, overexpression of the TOB(FF) double mutant slowed CCR4-mediated deadenylation (Fig. 2B and D [compare the later time points in the right and left panels] and F, deadenylation profiles; see also Fig. S1B and D in the supplemental material). Taken together, we conclude that TOB proteins enhance deadenylation in a PABP-dependent manner.

TOB proteins colocalize with P-bodies in NIH 3T3 cells. Recently, we have shown that decapping of mRNA does not occur until the second phase of deadenylation, a step that involves CCR4 (61), is under way. This step is stimulated by TOB proteins, as shown from the results described above. It is well established that shortening of the 3′ poly(A) tail is closely related to the movement of mRNPs from the translated pool to the nontranslated pool, which may facilitate the entry of mRNPs into P-bodies or to nucleate P-body formation. Accordingly, we examined the subcellular distribution of TOB in relation to P-bodies. Green fluorescent protein (GFP) fusion of DCP1a, a P-body component necessary for mRNA decapping (5, 18, 32, 36, 54), has been widely used as a marker to visualize P-body foci (15). In the cytoplasm of proliferating NIH 3T3 cells, GFP-DCP1a was distributed in a focal pattern (see Fig. S5A, upper panels, in the supplemental material). As expected for P-bodies (17), the cytoplasmic foci marked by

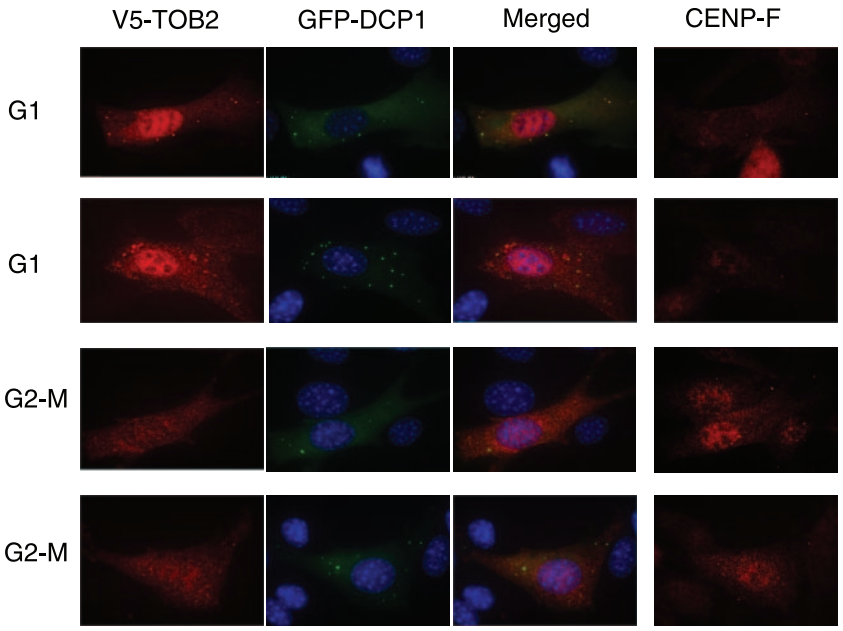

FIG. 7. TOB2 colocalizes with P-bodies in a cell-cycle-related manner. Immunofluorescence microscopy experiment results show the subcellular distributions of V5-TOB2 and P-bodies marked by GFP-DCP1a in proliferating mouse NIH 3T3 cells. V5-TOB2 was visualized using primary mouse anti-V5 antibody followed by secondary goat anti-mouse Alexa 555 antibody. To distinguish cells in G_2 and M phases from cells in G_1 and S phases, cells were stained for a centromere marker protein, CENP-F, by the rabbit primary antiserum against CENP-F and donkey anti-rabbit Alexa 647 antibody. Cell nuclei were detected by DAPI staining.

GFP-DCP1a disappeared after cycloheximide treatment (see Fig. S5A, lower panels, and S5B, upper panels, in the supplemental material). Moreover, real-time imaging of live cells showed that upon removal of cycloheximide, GFP-DCP1a foci reappeared in the cytoplasm (see Fig. S5B, lower panels, in the supplemental material), further validating GFP-DCP1a as a P-body marker in NIH 3T3 cells.

Immunoblotting has detected endogenous TOB in NIH 3T3 cells in the G_0/G_1 phase (25, 49), but anti-TOB antibodies suitable for immunofluorescence microscopy are currently unavailable. Therefore, we used V5 epitope-tagged TOB2 protein (TOB2-V5) expressed in transfected NIH 3T3 cells to examine the subcellular distribution of TOB protein. CENP-F, a centromere protein present at peak levels in the nuclear matrix in the G_2 and M phases (33), was used to distinguish cells in G_2 and M phases from those in G_1 and S phases. Figure 7 shows that TOB2-V5 colocalizes with P-bodies in cells with little or no nuclear staining of CENP-F, whereas no obvious TOB-V5 foci could be detected in cells with strong nuclear staining of CENP-F, although P-bodies were still visible in those cells. Taken together, these results indicate that TOB colocalizes with P-bodies in NIH 3T3 cells in a manner correlating with its cell-cycle-regulated expression.

DISCUSSION

The poly(A) nucleases involved in deadenylation of mRNA in various organisms have started to be identified and characterized in the last decade. The CCR4-CAF1 complex accounts for the major deadenylation activity in *Saccharomyces cerevisiae* and *Drosophila melanogaster* (50, 51). In mammals, a second poly(A) nuclease complex, PAN2-PAN3, has recently been shown to initiate deadenylation prior to CCR4-CAF1 action (61). Given that deadenylation of mRNA has the important consequence of shutting off translation of the transcript, a void in our understanding of how deadenylation is controlled, particularly in mammalian somatic cells during mRNA turnover, has prompted us to examine the underlying mechanisms.

Previous studies (29, 41, 53, 61) suggested that communication between poly(A) nuclease complexes PAN2-PAN3 and CCR4-CAF1 and the 3′ PABP-poly(A) tail complex of the mRNA in mammalian cells can greatly influence the poly(A) nuclease activity and, thus, the rate and extent of deadenylation. In this study, we set out to identify protein factors that mediate or modulate the interaction between the CCR4-CAF1 complex and the PABP-poly(A) complex. Our results led to three main findings. First, TOB, previously considered a transcription factor, can function as a positive, PABP-dependent regulatory factor in the deadenylation of both wild-type stable and nonsense-containing unstable β-globin mRNAs. Second, TOB can associate simultaneously with cytoplasmic PABP via TOB's C-terminal PAM2 motifs and with CAF1 through TOB's N-terminal domain. Third, TOB colocalizes with P-bodies in the G_1 phase but not in the G_2 or M phase.

Although it was previously reported that TOB can separately interact with CAF1 (25) and bind PABP (25, 43), those studies did not determine whether TOB can simultaneously interact with both PABP and CAF1. Moreover, the functional significance of these interactions for regulating deadenylation remains unclear. The present results (Fig. 2 and 6) not only demonstrate a direct interaction between TOB and PABPC1 but also reveal that the PAM2 motifs in TOB and the PABC

motif in PABPC1 are necessary for the interaction, thus establishing TOB as a genuine PABP-interacting protein.

Our observation of an enhancing effect of TOB on deadenylation raises an important question as to whether TOB directly destabilizes the poly(A)-PABP complex. In a gel mobility shift assay, we showed that while TOB does supershift the PABP-poly(A) complex, it has little effect on the complex formed between poly(A) and a truncated PABP that contains only the first RNA-binding domain (Fig. 3). These results suggest that instead of destabilizing the poly(A)-PABP complex, TOB promotes deadenylation through its interaction with the poly(A)-PABP complex, possibly by recruiting poly(A) nucleases, such as CCR4-CAF1, to the 3′ poly(A) tail and/or by modulating their poly(A) nuclease activity. It is of particular interest that TOB's deadenylation-enhancing effect is dependent on its interaction with PABP (Fig. 2; see also Fig. S1 in the supplemental material). When a double point mutation was introduced into the two PAM2 motifs in TOB proteins, their ability to interact with PABPC1 was greatly diminished. This finding is consistent with the above notions.

The ability of TOB to modulate deadenylation via the CCR4-CAF1 complex is reminiscent of the actions of yeast PUF proteins, a family of RNA-binding proteins that interact with the 3′ untranslated regions of specific mRNAs and stimulate their deadenylation (57). One of the yeast PUF proteins, Mpt5p, has been shown to physically bind Pop2p, a homolog of mammalian CAF1 (21, 22). By binding Pop2p, Mpt5p recruits the cytoplasmic Ccr4p-Pop2p deadenylase complex to the target transcript, thus promoting removal of its poly(A) tails and consequent repression of its translation. Thus, it appears that both mammalian TOB and yeast Mpt5p proteins regulate mRNA decay through recruiting the CCR4-CAF1 complex to stimulate deadenylation. Yet, there are distinct differences between the yeast and mammalian systems. Whereas the PUF proteins act in an RNA sequence-specific manner, TOB proteins interact with the PABPC1 protein of the 3′ poly(A)-PABP complex and thus are likely to function in an RNA sequence-independent way. Another difference is that there is no known TOB ortholog in yeast, but the interaction between PUF and POP2 is conserved in yeast, *Drosophila*, and humans (24, 39). Moreover, TOB belongs to a family of antiproliferative proteins whose levels of expression change during the cell cycle, while expression of PUF proteins is not known to be related to the cell cycle. Thus, two distinct families of unrelated proteins, TOB and PUF, that share no homology and play different roles in embryogenesis, cell growth, and cell differentiation appear to have evolved independently to accomplish their functions by converging on deadenylation.

What may be the biological significance or consequence of enhanced deadenylation by TOB? Our experiments show that TOB enhances deadenylation without significantly changing the overall decay rate of the deadenylated RNA body. Several observations in this and other studies suggest a role for the TOB proteins in facilitating the exit of mRNPs from the translation pool to the nontranslated pool residing in P-bodies. First, the enhancement of deadenylation observed with TOB could help to stop translation initiation by abolishing the interaction between the 5′ cap and the 3′ poly(A) tail. Second, TOB interacts with the C-terminal PABC domain of PABP, a region that is also used during translation for interactions with Paip1, Paip2, eukaryotic initiation factor 4B, eRF3, and the 60S ribosomal subunit (2). One might imagine that TOB could compete with these factors and thus decrease translation efficiency. The above notions are consistent with the role for TOB in translation repression implicated in a recent report that overexpression of TOB in an NIH 3T3 cell line, stably expressing exogenous inducible PABP, results in reduction of interleukin-2 protein expression from a simultaneously transfected interleukin-2 plasmid (43). Third, TOB colocalizes in the G_1 phase with P-bodies, structures linked to silencing translation and/or for storing translationally repressed mRNPs (for a recent review, see reference 17). One possibility is that after promoting deadenylation, TOB may facilitate remodeling of mRNPs and their movement into P-bodies, thus repressing translation.

In summary, our data suggest that TOB provides functional links among deadenylation, P-bodies, and cell growth arrest. Consistent with this notion is a study that showed that overexpression of CAF1 leads to cell growth arrest (7). It will be interesting to learn how knockdown of TOB expression may change the mRNA expression profile in cells arrested in the G_0/G_1 phase. The information gathered has the potential to provide important insight into TOB's function as an antiproliferative factor. Given that phosphorylation of TOB at specific serines inactivates its antiproliferative function when cells exit the G_0/G_1 phase (49), it will also be important to address whether and how those posttranslational modifications may regulate TOB's function in modulating deadenylation.

ACKNOWLEDGMENTS

We thank R. Kulmacz and J. Lever for critical reading of the manuscript and their valuable comments, X. He for assistance in microscopy study, B. Seraphin for plasmid GFP-hDcp1, S. Ohno for the expression vector *pMal-Y*, and Kazusa DNA Research Institute for the human TOB2 cDNA clone.

This work was supported by the National Institutes of Health (GM 46454) and in part by the Houston Endowment, Inc., to A.-B.S. A.-B.S. is a recipient of a Senior Investigator Award from the Sandler Program for Asthma Research.

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