Inhibition of mRNA Deadenylation by the Nuclear Cap Binding Complex (CBC)*

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Poly(A)-specific ribonuclease (PARN) is a cap-interacting and poly(A)-specific 3'-exoribonuclease. Here we have investigated how the cap binding complex (CBC) affects human PARN activity. We showed that CBC, via its 80-kDa subunit (CBP80), inhibited PARN, suggesting that CBC can regulate mRNA deadenylation. The CBC-mediated inhibition of PARN was cap-independent, and in keeping with this, the CBP80 subunit alone inhibited PARN. Our data suggested a new function for CBC, identified CBC as a potential regulator of PARN, and emphasized the importance of communication between the two extreme ends of the mRNA as a key strategy to regulate mRNA degradation. Based on our data, we have proposed a model for CBC-mediated regulation of PARN, which relies on an interaction between CBP80 and PARN. Association of CBC with PARN might have importance in the regulated recruitment of PARN to the nonsense-mediated decay pathway during the pioneer round of translation.

The cap structure and the poly(A) tail are the two boundary marks that define the extreme borders of a eukaryotic mRNA (1). Both elements play several important roles in regulating eukaryotic gene expression, and in particular, they influence the fate of mRNA, including its synthesis, maturation, translation, and stability (reviewed in Refs. 1–5). A number of proteins that change dynamically during the mRNA life cycle interact with each of the elements, and it has become evident during recent years that these proteins play critical roles in regulating processes that are influenced by or dependent on the two elements (reviewed in Refs. 1–6).

In the mammalian cell nucleus, the cap is recognized by the nuclear cap binding complex (CBC)4 (7), and the poly(A) tail is associated with nuclear poly(A)-binding protein 1 (PABPN1) (8). CBC is a heterodimeric complex that consists of a small (CBP20) and a large (CBP80) protein subunit and plays direct roles in pre-mRNA splicing (7), 3' end formation (9), and uridylic acid-rich small nuclear RNA export (10). In the cytoplasm, CBC is replaced by the cytoplasmic cap-binding protein, also known as eukaryotic translation initiation factor 4E (eIF4E), which together with factors eIF4A and eIF4G is responsible for initiation of cap-dependent mRNA translation (see Refs. 11 and 12 and references therein). Likewise, PABPN1 is replaced upon transport of the mRNA to the cytoplasm by the cytoplasmic poly(A)-binding protein (PABPC, Pab1p in Saccharomyces cerevisiae). PABPC is one of the key factors that participates in mediating several of the roles the mRNA poly(A) tail has on gene expression, including its stimulatory effect on translation (see Refs. 4 and 8 and references therein).

Both the cap and the poly(A) tail play critical roles during eukaryotic mRNA degradation, and in two of the general pathways poly(A), removal precedes the degradation of the cap (2, 3, 5). The chemical nature of the cap makes the mRNA 5' end inaccessible to 5'-exoribonucleases, and specific decapping activities are required for its degradation (reviewed in Ref. 13). In the deadenylation-dependent decapping pathway, the cap is removed by the Dcp1p/Dcp2p decapping activity after the initial deadenylation step, whereas the scavenger decapping enzyme Dcp5p degrades the cap as one of the final steps in the deadenylation followed by the 3'-5' degradation pathway. Several different poly(A)-degrading activities (reviewed in Refs. 2 and 3) have been identified in eukaryotic cells, although their roles in the different mRNA degradation pathways have not yet been worked out. At least three of these activities are highly poly(A)-specific: the Pan2/3 nuclease in yeast and mammals (14, 15), poly(A)-specific ribonuclease (PARN) in vertebrate cells (16–20) and Arabidopsis (21), and the Ccr4–Pop2–Not complex in yeast (22, 23) and its orthologs in other organisms (see Refs. 2 and 3 and references therein).

Mechanisms controlling mRNA degradation are still poorly understood at the molecular level, although recent studies have begun to unravel the components involved in modulating RNA degradation (reviewed in Refs. 2, 3, and 5). Among the cis-acting elements, the AU-rich elements (ARE) (24) (reviewed in Refs. 5 and 25), which are frequently located in the 3'-untranslated regions of mRNA, are known to interact with a variety of RNA-binding proteins and participate in regulating mRNA turnover. Besides the AREs, the poly(A) tail is also a primary target at which regulation of mRNA decay occurs, and PABPC/Pab1p has turned out to be both a positive and a negative regulator of poly(A) degradation (2, 3). The Pan2/3 nuclease activity is positively affected by PABPC/Pab1p binding to the poly(A) (15, 26) tail, whereas the activities of PARN (18, 27) and yeast Ccr4–Pop2–Not complex (28, 29) are inhibited by PABPC/Pab1p. In addition to this, it is also known that the poly(A) tail affects decapping (30–32). In the human case, experimental evidence suggests that PABPC protects the cap from Dcp2-mediated decapping by binding simultaneously to both the cap structure and the poly(A) tail (33).

Here we have investigated how the nuclear and cytoplasmic cap-binding proteins CBC and eIF4E affect human PARN-mediated degradation of the poly(A) tail at the 3' end of the mRNA. PARN is unique among the major poly(A) nucleases since it interacts directly with both
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the poly(A) tail at the 3' end of the mRNA and the 5' end-located cap structure during poly(A) hydrolysis (20, 27, 34, 35). We found that CBC inhibited PARN activity. Our data, therefore, suggested a previously unrecognized function for CBC in mRNA deadenylation, identified CBC as a potential regulator of PARN, and once again emphasized the importance of communication between the two extreme ends of the mRNA as a key strategy to regulate mRNA degradation.

MATERIALS AND METHODS

Expression and Purification of Recombinant PARN, CBC, and eIF4E—Recombinant and His-tagged versions of human PARN, PARN(W475A), PARN(E455A, W456A), and PARN(1-470) were expressed and purified to apparent homogeneity as described previously (20). Recombinant human CBP20 and CBP80 were purified, and CBC was reconstituted according to Mazza et al. (36, 37). His-tagged eIF4E was expressed in Escherichia coli strain BL21 (DE3) containing plasmid pET15-eIF4E5 and purified to apparent homogeneity using the same protocol as was used for purification of His-tagged PARN (20). In plasmid pET15-eIF4E, the open reading frame of the human eIF4E mRNA was inserted between the NdeI and BamHI restriction enzyme sites of the vector pET-15b (Novagen Inc.). The reading frame of eIF4E was in-frame with a N-terminally located His tag originating from the cloning vector.

Preparation of RNA Substrates—RNA substrates (16) with or without m7G(5')ppp(5')G at their 5' ends were synthesized by in vitro transcription according to Martinez and Virtanen (unpublished results). For body labeling, the specific radioactivities of the included radioactive mononucleotides were 40 Ci/mmol, whereas for poly(A) tail labeling, the specific radioactivity was 5 Ci/mmol.

Deadenylation Assays—The indicated amounts of capped (m7GpppG) or non-capped 32P-labeled (3A30) RNA were incubated under standard in vitro deadenylation conditions (20 mM HEPES-KOH (pH 7), 1.5 mM MgCl2, 100 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 2.5% polyvinyl alcohol, 10% glycerol, and 0.1–0.2 units of RNAguard (Amersham Biosciences, catalog number 27-0815-01)) as described previously (35). Reactions were mixed at 4 °C and contained the indicated amounts of PARN, eIF4E, CBP20, CBP80, and/or reconstituted CBC. The final concentrations of CBC and eIF4E in the reactions were chosen to be in the range of their dissociation constant values, being ~4 and 100 nM, respectively (38, 39). The reaction volume was 21 μl, and incubations were performed at 30 °C for 30 min. The reaction products were analyzed by TLC or purified and analyzed on 10% polyacrylamide (acylamide/bisacrylamide 19:1, v/v)/7 M urea gels as described elsewhere (16).

One-dimensional TLC Assays—Deadenylation reactions with increasing amounts of CBC or eIF4E, as indicated, were performed as described above. Capped (m7GpppG) or non-capped 32P- or poly(A) tail-labeled L3(3A30) RNA was used, as indicated. The released 5'-AMP product was detected by TLC analysis. Essentially, a 1 μl aliquot taken from the 21 μl reaction was spotted onto a polyethyleneimine cellulose F plastic sheet (Merck, catalog number 1.05579), and the chromatogram was developed using 0.75 M KH2PO4, pH 3.5 (H2PO4), as solvent. The plate was dried and scanned by a 4005 PhosphorImager (Amersham Biosciences). For the kinetic analysis, 0.1 mg/ml bovine serum albumin was added in the reactions, and analysis was performed as described elsewhere (35).

Immunoprecipitation—Co-immunoprecipitation reactions were performed according to McKendrick et al. (40). HeLa nuclear extracts were purchased from 4C (Mons, Belgium) and subjected to immunoprecipitation using affinity-purified anti-PARN or anti-human CBP80(116) (7) polyclonal serum. The polyclonal antisemur specific for human PARN was generated by immunizing rabbits using 0.45 mg/rabbit recombinant purified His-tagged PARN polypeptide followed by three boost injections with the same amount of antigen. The extracts were treated before use with 50 μl of protein A-Sepharose CL-4B beads (Amersham Biosciences) in 4 °C for 1 h with rotation followed by centrifugation at 10,000 × g in 4 °C for 10 min. The cleared supernatant was diluted 5-fold in IP150 buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 0.05% Nonidet P-40, and 1% Triton X-100) and incubated with an appropriate volume of the antibody/antisemur in 4 °C for 1 h with rotation. Subsequently, the protein A-Sepharose beads were recovered by centrifugation and incubated at 4 °C with 50 μg of RNase A/ml for 10 min. Finally, the beads were washed three times with 1 ml of ice-cold IP150 buffer and eluted with SDS-PAGE sample buffer, and the recovered proteins were resolved by SDS-PAGE. The resulting gel was transferred to polyvinylidene difluoride membrane, following a standard Western blot procedure (41), and probed with the indicated antibody.

RESULTS

CBC Inhibits PARN Activity—To investigate whether the nuclear (i.e. CBC) or cytoplasmic (i.e. eIF4E) cap-binding proteins had any effect on human PARN activity, we incubated a polyadenylated and 7-methyl guanosine capped RNA substrate, L3(A30), in the presence of PARN and a 1:1 in vitro reconstituted CBC complex or eIF4E. We found that CBC inhibited PARN activity and that the inhibition effect was titratable, whereas eIF4E had no detectable effect on PARN (Fig. 1, A and B, see also below and see Fig. 4). It has previously been observed by Wilusz and co-workers (27) that eIF4E inhibits PARN-mediated deadenylation in an in vitro decay system based on a crude HeLa cell-free S100 extract. Thus, as compared with the study by Wilusz and co-workers (27), we were not able to reconstitute the eIF4E inhibition effect on PARN in our purified system, even if we increased the eIF4E concentration far above the concentrations used in their study. This discrepancy is most likely explained by the use of different in vitro deadenylation systems. We have used a highly purified and defined reconstituted deadenylation system based on recombinant components, whereas Wilusz and co-workers (27) used a crude in vitro decay system. It is therefore likely that the eIF4E inhibition effect they observed requires additional components that are not present in our highly purified in vitro system.

To further examine the CBC inhibition effect, we investigated whether it was cap-dependent and found that CBC inhibited PARN activity even if the RNA substrate lacked a cap structure (Fig. 1C). This suggests that CBC may inhibit PARN through a direct interaction without binding the cap. To investigate whether this could be the case, we incubated PARN with CBC at an RNA substrate concentration that was at least five times higher than the Ks value for recombinant PARN (42), using both capped and non-capped substrates. Fig. 1, B and C, shows that CBC could still inhibit PARN under these conditions, providing further support that CBC inhibits PARN through a direct and cap-independent interaction.

It has previously been shown that PARN interacts with the 5' end cap structure (20, 27, 34, 35) and that this interaction stimulates PARN activity by increasing the rate of hydrolysis, which in turn amplifies PARN processivity. It was therefore of interest to investigate whether the cap binding property of PARN influenced CBC inhibition of PARN activity. For this purpose, we used three PARN mutants, the point mutant PARN(W475A), the double point mutant PARN(E455A, W456A), and the C-terminally deletion mutant PARN(1-470), all being active in deadenylation (Fig. 2) but severely defective in their cap binding properties (supplemental Table 1). Fig. 2 shows that mutants PARN(W475A) and PARN(E455A, W456A)
were efficiently inhibited by CBC, whereas the deletion mutant PARN (1–470) was unaffected. From these data, we conclude that the C-terminal region of PARN is essential, whereas the cap binding property of PARN is not a prerequisite for CBC-mediated inhibition. Importantly, the PARN(1–470) data show that PARN-mediated deadenylation can occur in the presence of CBC. Thus, the CBC inhibitory effect was not caused by CBC interfering with the RNA substrate, for example, by making the RNA substrate inaccessible to PARN hydrolysis (see also below and see Fig. 4).

The 80-kDa Subunit of CBC Inhibits PARN Activity—CBC is composed of two subunits, CBP20 and CBP80 (7). The primary function of CBP20 (36, 43) is to physically interact with the cap, whereas CBP80 has at least two separate functions (37): (i) to interact with and induce a conformational change of CBP20 and (ii) to provide a large surface for binding to proteins involved in different aspects of mRNA and cap-dependent functions, such as interaction with translation factor eIF4G (12, 40, 44) or phosphorylated adaptor for RNA export (45). In light of this, we investigated whether the entire CBC complex was required for PARN inhibition or whether either of the two subunits alone could inhibit PARN. Fig. 3A shows that CBP80 inhibited PARN in a cap-independent manner, whereas CBP20 had no effect on PARN activity. As expected, we also found that both cap binding site mutants, PARN(E455A,W456A) and PARN(W475A), were inhibited by CBP80, whereas the C-terminal deletion mutant PARN(1–470) was unaffected by the addition of CBP80 (Fig. 3B). These data, which are in keeping with the observation that CBC inhibition of PARN is cap-independent, show that CBP80 alone inhibits PARN activity and strongly suggest that the inhibition is caused by a direct interaction between PARN and the CBP80 subunit of CBC.

The Mechanism of Inhibition—To investigate the mechanism of inhibition in detail and further characterize the role of the cap, we performed several sets of kinetic analyses using either PARN or...
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PARN(W475A) RNA as the source of enzyme. Capped and non-capped L3(A30) RNA was titrated at various concentrations of CBC or CBP80, and the results were plotted using Lineweaver-Burk formalism (Fig. 4). In the case of capped L3(A30) RNA being hydrolyzed by PARN, the CBC inhibition pattern followed the kinetics of non-competitive inhibition, whereas the kinetic pattern corresponded to non-competitive inhibition when non-capped substrate was used (Fig. 4, A and B). The cap binding-deficient PARN(W475A) mutant was inhibited non-competitively by CBC, irrespective of whether the RNA substrate was capped or not (Fig. 4, C and D), and the same mode of inhibition was also observed when CBP80 inhibited PARN (Fig. 4, E and F). Thus, a simple competitive mechanism of inhibition was not observed in any of the cases, suggesting that: (i) PARN has a binding site for CBC that is separate from its active site and (ii) CBC does not inhibit PARN by binding and interfering with the RNA substrate. Furthermore, the kinetic determination inhibition constants \( K_e \) (Table 1) were all similar, ranging from 15 to 25 nm, suggesting that the inhibition effect in all cases were caused by a similar molecular interaction between PARN and CBC or CBP80.

It is not obvious how the differences in the modes of inhibition, i.e. non-competitive versus uncompetitive, should be interpreted. Traditionally, a non-competitive inhibitor can interact with the enzyme, regardless of whether a substrate is bound or not, whereas an uncompetitive inhibitor can only bind to the enzyme-substrate complex, suggesting in both cases that the active site is separate from the inhibitor binding site and implying in the uncompetitive case that substrate binding induces a conformational change of the enzyme that reveals the binding site for the inhibitor. However, in the case of CBC-mediated inhibition of PARN, which is cap-independent, the uncompetitive mode of inhibition was only observed when both PARN and CBC could

### Table 1

| Reaction | Inhibition constants |
|----------|----------------------|
| \( m^G\text{GpppG L}_3(A_{30}) \) | \( p^\text{pG L}_3(A_{30}) \) |
| PARN/CBC | 15 ± 3 | 25 ± 5 |
| PARN(W475A)/CBC | 20 ± 2 | 22 ± 5 |
| PARN/CBP80 | 16 ± 4 | 20 ± 6 |

* PARN or PARN(W475A) was incubated under deadenylation conditions with CBC or CBP80, as indicated.

* The listed inhibition constants, \( K_e \), were determined kinetically as described under "Materials and Methods." The structure of the X-axis of the RNA substrate was as indicated. The given values are the average ± experimental error of two independent experiments where each experiment was based on several independent data-points.
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FIGURE 5. A CBP80-PARN complex. A, HeLa nuclear extracts were subjected to immunoprecipitation (IP), in the presence or absence of RNase A (as indicated, see "Materials and Methods"), using an affinity-purified anti-PARN serum. The precipitate was fractionated by SDS-PAGE and subsequently analyzed by Western blotting using anti-CBP80 (lanes 2 and 3) or preimmune sera (pre, lane 4) as the probe (as indicated). In lane 1, Hela cell nuclear extract was fractionated, without prior immunoprecipitation, and subsequently probed by using affinity-purified anti-PARN. The positions of PARN and CBP80 are indicated with 74,000 (74) and 80,000 (80), respectively. B, HeLa nuclear extracts were subjected to immunoprecipitation, in the presence of RNase A, using anti-CBP80 serum. The precipitate was fractionated by SDS-PAGE and subsequently analyzed by Western blotting using preimmune sera (lane 2) or anti-PARN (lane 3) (as indicated). In lane 1, Hela cell nuclear extract was fractionated, without prior immunoprecipitation, and subsequently probed by using anti-CBP80. The positions of PARN and CBP80 are indicated according to their molecular massies 74,000 (74) and 80,000 (80), respectively.

FIGURE 6. A model for CBC-mediated inhibition of PARN (see "Discussion" for a detailed discussion of the model). A schematic drawing of CBC-mediated inhibition of PARN in which CBC, consisting of CBP20 (small gray circle) and CBP80 (large open oval), interacts with the mRNA cap structure (open diamond) and one subunit of PARN (large gray circle) is shown. PARN, which interacts with the mRNA poly(A) tail, becomes inactivated since it physically interacts with the CBP80 subunit of CBC. The interaction between CBC and the cap structure is not a prerequisite for CBC-mediated inhibition of PARN. PARN is shown as a dimeric structure consisting of two identical subunits in which each of the subunits contains an active site (small open oval). The oligomeric composition of PARN was initially proposed based on biochemical studies (20), and recently, a dimeric composition was observed in a crystal structure of PARN (55).

FIGURE 6A. Schematic representation of the mechanism of inhibition of PARN mediated by CBC. CBC binds to PARN in a subunit-specific manner. The inhibition by CBC is not through a direct interaction with the cap structure but through a direct interaction with PARN. The inhibition is thought to be mediated through a complex that contains both CBC and PARN.

interaction between the two components, although direct experimental evidence for a physical interaction remains to be obtained. Our proposal relies primarily on several observations in which CBC inhibited the activity of PARN (Figs. 1–4 and Table 1). The importance of the CBP80 subunit is most evident by the fact that the CBP80 subunit alone could inhibit PARN (Figs. 3 and 4 and Table 1). An interaction between the CBP80 subunit and the C-terminal region of PARN is supported by the observations that the PARN C-terminal region was essential for inhibition (Fig. 3) and that the inhibition could not be released by increasing the RNA substrate concentration far above saturation (Fig. 1) as well as by the results of the kinetic analyses of mechanism of inhibition (Fig. 4). In particular, the kinetic analyses provided strong support for a direct physical interaction between PARN and CBP80 as a key component behind the mechanism of inhibition. Most importantly, the inhibition could not be released competitively by titrating the RNA substrate (Fig. 4). Finally, we note that CBP80 alone could inhibit PARN activity without any need for the cap structure (Figs. 3 and 4). This observation is also in keeping with a model of inhibition based on an interaction between PARN and CBP80 since CBP80 requires CBP20 to interact with the cap structure (7, 37). It is worthwhile to mention that our observation that elf4E alone could not inhibit PARN is consistent with our suggestion that the cap binding property of CBC is not the primary reason behind its inhibition effect on PARN.

It has previously been proposed that PARN is regulated by at least four different strategies: (i) stimulation of PARN activity through a direct interaction between PARN and the mRNA cap structure (20, 27, 34, 35); (ii) inhibition of PARN activity by PABPC (18, 27), probably caused by PABPC binding and interfering with the poly(A) substrate.; (iii) promotion of poly(A) degradation by ARE and associated proteins (46–49); and (iv) competition between PARN and elf4E for cap binding (27, 50). The outcome of the competition between PARN and elf4E appears to be modulated by the phosphorylated state of PARN and elf4E (50). The mechanism behind ARE-mediated regulation of PARN is at present poorly understood, and models based on both direct (e.g. K homology splicing regulatory protein (47) and RNA helicase associated with AU-rich element (49)) and indirect (e.g. tristetraprolin (48)) interaction between PARN and the ARE-associated proteins have been suggested. As compared with these strategies, our study therefore defines a novel strategy by which PARN-mediated deadenylation is regulated and emphasizes the importance of communication between the two extreme ends of the mRNA as a key strategy to regulate mRNA function (reviewed in Refs. 1–5).

Regulation of PARN activity by CBC will most likely be prevalent in the nucleus in accordance with the primary localization of CBC (51). Along this line, we note that nuclear extracts of *Xenopus* oocytes have
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been reported to be inactive in deadenylation unless they are further purified (17, 52). Interestingly, it has been reported that PARN isolated from *Xenopus* oocytes co-purifies with two polypeptides being 74 and 62 kDa in sizes, the latter most likely being a proteolytic product that lacks the C terminus of the former (19, 52). The 74-kDa form is localized in the nucleus and is catalytically active in vitro if purified, even if nuclear extracts prepared from *Xenopus* oocytes are inactive in deadenylation (52). To explain this, Copeland and Wormington (17) suggested that nuclear proteins precluded PARN to access its RNA substrate and that these nuclear proteins must dissociate from the RNA substrate, presumably during the remodeling process of the ribonucleoprotein complexes that is associated with the nuclear-cytoplasmic transport. CBC alone harbors all the properties to be involved as a key component responsible for the nuclear inactivation of deadenylation in *Xenopus* oocytes. Further studies are required to investigate whether this indeed is the case. Besides the possibility to inhibit PARN activity in the nucleus, we envisage that the interaction between CBC and PARN could represent a mechanism by which PARN is recruited to the nascent pre-mRNA before the mature mRNA has been generated. In this scenario, the CBC-mediated inhibition would ensure that PARN does not degrade the mRNA poly(A) tail unless PARN is activated, which could occur by, for example, a remodeling process, as discussed above.

PARN has been implicated in nonsense-mediated decay (NMD) (reviewed in Refs. 53) in mammalian cells since it co-purifies with essential NMD factors and its down-regulation abrogates NMD (54). Furthermore, Maquat and co-workers (11) have suggested that NMD occurs after a pioneer round of mRNA translation, which occurs while CBC still is bound to the mRNA cap structure (12). Thus, CBC-mediated regulation of PARN could play a function in regulating NMD. For example, it is conceivable that CBC could play two independent roles: (i) to inhibit PARN activity until the pioneer round of translation has been completed and (ii) to recruit PARN to its mRNA substrate without the requirement for PARN to interact directly with the mRNA. Tentatively, if a premature termination codon is encountered, PARN could be released from CBC to participate in NMD or, alternatively, if no premature termination codon is recognized during the pioneer round of translation, CBC together with PARN may be replaced by translation initiation factor eIF4E.

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