Cdc42 Stimulates RNA Splicing via the S6 Kinase and a Novel S6 Kinase Target, the Nuclear Cap-binding Complex*

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Cdc42 is a low molecular weight GTP-binding protein that plays a key regulatory role in a variety of cellular activities. The importance of the coordination of different cell functions by Cdc42 is underscored by the fact that a constitutively active Cdc42 mutant induces cellular transformation. In this study, we describe a novel function for Cdc42: its ability to stimulate pre-messenger RNA splicing. This activity is dependent on cysteine 37 in the effector loop of Cdc42 but is not dependent on cell growth. A likely candidate protein for mediating the Cdc42 effects on pre-mRNA splicing is the nuclear RNA cap-binding complex (CBC), which plays a key role in an early step of cap-dependent RNA splicing. Activation of the CBC by Cdc42 can be inhibited by rapamycin. Additionally, phosphatidylinositol 3-kinase (PI3K) and the Cdc42 effector, pp70 S6 kinase, stimulate the RNA cap-binding activity of the CBC. S6 kinase may directly target the CBC in vivo as it can phosphorylate the 80-kDa subunit of the CBC, CBP80, at residues that are subject to a growth-factor-dependent and rapamycin-sensitive phosphorylation in vivo. Together, these data suggest the involvement of a Cdc42-S6 kinase pathway in the regulation of RNA splicing, mediated by an increase in capped RNA binding by the CBC, as well as raise the possibility that the effects of Cdc42 on cell growth may be due in part to its regulation of RNA processing.

Cdc42 was a low molecular weight GTP-binding protein that coordinates multiple cellular events by a regulated binding and hydrolysis of GTP. Transcriptional regulation (1–5), vesicular trafficking (6), actin cytoskeletal arrangement and cell cycle progression (7, 8) all represent activities in which Cdc42 has been implicated. Additionally, it has been shown that Cdc42 plays an important role in cell growth control, as a constitutively active form of Cdc42, Cdc42 F28L, induces malignant transformation (9). We have recently shown that an interaction between Cdc42 and the coatomer subunits of COP-coated vesicles is somehow involved in the transformation signal (6). However, there appears to be a requirement for other Cdc42 targets as well, as deletion of the insert region of Cdc42 blocks transformation (10) independent of any interaction with the coatomer complex. What this suggests is that Cdc42 may be orchestrating distinct cellular functions in order to coordinate regulated cell growth.

In a previous study, we observed that activated forms of Cdc42 were capable of stimulating the nuclear cap-binding complex (CBC) in vivo to bind capped RNAs (11). In binding to RNAs containing a m7G cap structure, the CBC has been implicated in a number of fundamental aspects of RNA processing including pre-mRNA splicing (12–15), U snRNA export (16), and polyadenylation (17). Importantly, the CBC has also been shown to be a nuclear end point for extracellular stimuli such as growth factors and cell stress stimuli (11). In this study we have investigated the possibility that Cdc42 might be involved in the signal regulation of RNA processing via the CBC. Specifically, we find that activated alleles of Cdc42 can stimulate splicing activity in a manner that is dependent on amino acid 37 of the effector loop. As the CBC plays a pivotal role in cap-dependent pre-mRNA splicing, we have investigated the Cdc42 pathway leading to CBC activation and find that this pathway proceeds via the Cdc42 effector, pp70 S6 kinase (S6K). Rapamycin inhibits the Cdc42-induced activation of the CBC, whereas S6K, together with PI3 kinase, will induce the CBC to bind capped RNAs. Additionally, S6K can phosphorylate the CBC in vitro at a site that undergoes a rapamycin-sensitive, growth factor-dependent phosphorylation in vivo. Together, these data suggest a mechanism by which Cdc42 might influence pre-mRNA splicing, through the S6K-mediated activation of the CBC.

MATERIALS AND METHODS

Cell Culture and Mammalian Protein Expression—Mammalian cells were maintained and cell lysates were prepared as previously described (11). For the metabolic labeling of PC12 and HeLa cells, the cells were incubated with phosphate-free DMEM containing 1.5 mCi/ml [32P]orthophosphoric acid (150 mCi/ml) for 3 h followed by treatments with NGF, heregulin, or rapamycin as indicated in the text. Stable NIH 3T3 cell lines expressing either HA-tagged Cdc42 (F28L,C37A) or HA-tagged Cdc42 (F28L,Y40C) from the pJ4H vector were previously described (9). Constructs for transient expression include pcDNA, HA-tagged Cdc42 (Q61L) (5), pcDNA, HA-tagged wild type Cdc42, pcDNA, HA-tagged Cdc42 (F28L) (9), pcCMV myristoylated 110-kDa PI3 kinase catalytic subunit, pJ5H Myc-tagged S6K, and various forms of pcDNA, CT1/CTK, CBP80 (see below).

CBP80 Constructs—CBP80 was cloned from a human testis cDNA library using 5’ and 3’ primers designed from the published sequence for hsCBP80 (GenBank™ accession number 1705654). A recombinant CBP80 baculovirus was generated according to the manufacturer’s directions (PharMingen), and a NLS mutant (K17A,R18A) CBP80 virus was a generous gift from Dr. Iain Mattaj (EMBL, Heidelberg). Recombinant CBP80 proteins were generated by infecting 6 × 10^6 SF21 cells per 75-cm² flask with 50 µl of virus, and the recombinant CBP80 was then purified over a GST-CBP20 (11) affinity column. CBP80 NLS

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1 The abbreviations used are: CBC, cap-binding complex; PI3 kinase, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; NGF, nerve growth factor; DTT, dithiothreitol; PCR, polymerase chain reaction; NLS, nuclear localization signal; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; FRAP, FK506-binding protein-12/rapamycin-associated protein kinase; eIF, eukaryotic initiation factor; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Activated Cdc42 induces cap-dependent RNA splicing in NIH 3T3 cells in a manner dependent on position 37 in the effector loop. A, NIH 3T3 cells stably transfected with Cdc42 F28L or vector alone were analyzed for their ability to support the splicing of either a specific m'GpppG-capped or nonspecific ApppG-capped probe. The lower diffuse band represents the unspaced precursor RNA probe, and the upper band is the lariat formation splice product. B, splicing experiments were performed using NIH 3T3 cells stably expressing Cdc42 F28L (designated L28), Cdc42 F28L, C37A (L28/37A), and Cdc42 F28L, Y40C (L28/C40). The left-hand panel shows the results from the splicing experiment, and the right-hand panel depicts a Western blot of the Cdc42 expression levels using an antibody against the HA-tag of the expressed Cdc42 protein.

Fig. 1. Activated Cdc42 induces cap-dependent RNA splicing in NIH 3T3 cells in a manner dependent on position 37 in the effector loop. A, NIH 3T3 cells stably transfected with Cdc42 F28L or vector alone were analyzed for their ability to support the splicing of either a specific m'GpppG-capped or nonspecific ApppG-capped probe. The lower diffuse band represents the unspaced precursor RNA probe, and the upper band is the lariat formation splice product. B, splicing experiments were performed using NIH 3T3 cells stably expressing Cdc42 F28L (designated L28), Cdc42 F28L, C37A (L28/37A), and Cdc42 F28L, Y40C (L28/C40). The left-hand panel shows the results from the splicing experiment, and the right-hand panel depicts a Western blot of the Cdc42 expression levels using an antibody against the HA-tag of the expressed Cdc42 protein.

RESULTS AND DISCUSSION

Previously, we have demonstrated that activated forms of Cdc42 can promote the nuclear CBC to bind capped RNA substrates (11). One of the important functional outcomes of this binding event is an enhancement of capped RNA splicing (12). This leads to the intriguing suggestion that Cdc42-stimulated signaling pathways can promote cap-dependent RNA splicing. To test this possibility, splicing lysates were prepared from NIH 3T3 cells that were stably transfected with a constitutively active form of Cdc42, Cdc42 F28L (9), or vector alone, after a 24-h growth period in low serum. The activity of these splicing lysates was then assessed in an in vitro splicing assay using either a specific m'GpppG-capped probe or a nonspecific ApppG-capped probe. As shown in Fig. 1A, Cdc42 F28L facilitates the generation of RNA splicing products as compared with vector alone, and this effect is greatly enhanced when the RNA probe contains a specific m'GpppG cap.

We have shown that the Cdc42 F28L mutant causes the transformation of NIH 3T3 cells (9), raising the possibility that Cdc42 F28L-mediated effects on splicing are not a direct outcome of a signaling pathway initiated by Cdc42 but rather reflect the fact that cells expressing Cdc42 F28L are able to grow in low serum. Therefore, we sought a Cdc42 mutant that would uncouple RNA splicing and cell growth. Splicing experiments were performed using lysates from NIH 3T3 cells that stably expressed either Cdc42 F28L or two effector loop mutants of Cdc42 in the activated background, Cdc42 F28L/C37A and Cdc42 F28L, Y40C (Fig. 1B). In contrast to Cdc42 F28L, neither of the effector loop mutants will support the growth of cells in low serum (data not shown). The Cdc42 F28L, Y40C double mutant activates the splicing machinery, whereas Cdc42 F28L, C37A does not (Fig. 1B). Thus, the activation of splicing by Cdc42 is not dependent on cell growth. Furthermore, this mutational analysis suggests that the Cdc42 targets known to utilize position 40 in the effector loop, such as the CRIB (Cdc42/Rac-interacting binding) domain-containing proteins PAK, ACK, and WASP, are not involved in signaling to...
FIG. 3. S6K phosphorylates CBP80 in vitro at a position within the N-terminal NLS. A, S6K was immunoprecipitated from COS-7 cells which coexpressed S6K and the myristoylated 110-kDa subunit of PI3 kinase. CBC was added to the S6K in a phosphorylation reaction and incubated for increasing periods of time. CBP80 phosphorylation was detected by autoradiography (top panel), and S6K levels were detected by Western blotting against the Myc-tag on the recombinant S6K. B, immunoprecipitated Myc-S6K was incubated with increasing concentrations of CBC for 30 min in a phosphorylation reaction. The top panel represents CBP80 phosphorylation, and the bottom panel shows S6K levels in the assay. C, partially purified S6K was incubated with recombinant wild type CBC (wt CBP80) or an NLS mutant (K17A,R18A CBP80) in a phosphorylation reaction for 30 min. CBP80 protein levels were detected by Coomassie Blue stain (top panel), and phosphorylation of CBP80 was visualized by autoradiography (bottom panel).

Fig. 4. CBP80 undergoes a growth factor-responsive and rapamycin-sensitive phosphorylation within the N-terminal NLS in vivo. A, PC12 cells were metabolically labeled with [32P]orthophosphoric acid for 3 h and then stimulated with NGF (100 ng/ml) for 15 min. CBP80 was then isolated by immunoprecipitation, and its phosphorylation state was assessed by autoradiography. B, PC12 cells were metabolically labeled for 3 h with [32P]orthophosphoric acid. Thirty minutes prior to NGF treatment, 50 ng/ml rapamycin (Rap) was added as indicated. The cells were harvested and fractionated, and the cytosolic and nuclear lysates were immunoprecipitated using a specific CBP80 antiserum. Immunoprecipitated proteins were detected by autoradiography. C, N-terminal CBP80 sequence. The bipartite NLS is underlined and labeled as NLS1 or NLS2, and alanine mutations were made as shown. D, wild type and mutant CBP80 constructs were transfected into HeLa cells, and the proteins were expressed for 24 h. The cells were metabolically labeled with [32P]orthophosphoric acid for 3 h and treated with heregulin for 15 min. Lysates were prepared, and the transfected CBP80 proteins were isolated using an α-KT3 antibody. The immunoprecipitated proteins were separated by SDS-PAGE, and CBP80 was detected by Western blotting using a specific CBP80 antibody (top panel) and by autoradiography (bottom panel).

The identification of Cdc42 effectors for which position 37 is necessary has not been described to date, but it would appear that an effector of this class plays a pivotal role in the Cdc42-RNA processing pathway.

Given that the CBC is a downstream target for Cdc42, it seems likely that the regulation of CBC activity underlies the effects of Cdc42 on pre-mRNA splicing. To further explore how signals are conveyed from Cdc42 to the CBC, we tested the abilities of two small molecule inhibitors, rapamycin and wortmannin, to inhibit the activation of the CBC by Cdc42. These drugs inhibit FRAP and PI3 kinase, respectively. HeLa cells were transiently transfected with Cdc42 F28L, and 30 min prior to harvesting, either rapamycin or wortmannin was added. The cells were fractionated and the nuclear fractions were assayed for CBC activity by measuring the photolyzed incorporation of [α-32P]GTP into CBP20 as has been described previously (11). Rapamycin reduced CBC activity in the presence of Cdc42 F28L, whereas wortmannin did not (Fig. 2A), suggesting that FRAP, but not PI3 kinase, is downstream of Cdc42 in the CBC pathway.

FRAP is thought to play a pivotal role in signaling pathways that regulate mRNA translation and is well documented as an upstream activator of S6K (18–21). Thus, rapamycin-sensitive phosphorylation within the NLS region, an NLS mutant of CBP80 was examined. This mutant, CBP80 K17A,R18A, has been shown previously to inhibit the nuclear localization of CBP80 (23) and would no longer contain the S6K consensus site at NLS2. Indeed, this mutant fails to serve as a substrate for S6K phosphorylation (Fig. 3C).

Thus we are presented with an interesting possibility for how signaling from Cdc42 to the CBC proceeds, via a direct S6K-catalyzed phosphorylation of CBP80. Previously, we had
shown that the CBC is strongly activated by NGF in PC12 cells and by heregulin in HeLa cells (11). Therefore, PC12 cells were metabolically labeled with $^{32}$Porthophosphoric acid in the presence or absence of NGF. The labeled cells were fractionated, and the CBC was immunoprecipitated from cytosolic and nuclear lysates using a CBP80 antiserum or preimmune serum. Fig. 4A shows that nuclear CBP80 is phosphorylated in a NGF-dependent manner in cells. Phosphorylation of CBP20 was not observed. Additionally, the NGF-dependent phosphorylation of CBP80 was blocked by a 30-min pretreatment of cells with rapamycin (Fig. 4B).

N-terminal alanine mutant constructs of CBP80 were generated to confirm that the in vivo phosphorylation occurs within the S6K phosphorylation consensus sites (Fig. 4C). These CBP80 constructs were transiently transfected into HeLa cells that were then metabolically labeled and treated with heregulin to promote CBC activation. The mutant constructs were isolated from cell lysates and their phosphorylation states were assessed. The in vivo phosphorylation of CBP80 was reduced in the S7A and the T21A,S22A mutants and completely blocked in the CBP80 S7A,T21A,S22A mutant (Fig. 4D). All mutant constructs showed wild type nuclear localization as assessed by immunofluorescence (data not shown). Thus, the in vivo phosphorylation of CBP80 is consistent with a direct phosphorylation by S6K.

Finding that CBP80 is phosphorylated at two positions in cells was unexpected based on our in vitro phosphorylation experiments with S6K and the CBC. Phosphorylation of CBP80 by S6K may be restricted to the NLS2 site, and another, yet to be identified kinase could be responsible for the phosphorylation at NLS1. The CBC is nuclear in the steady state, however, it has been suggested that the CBC accompanies the RNA particle through the nuclear pore (24), and releases its cargo in the cytosol to the cytosolic cap-binding protein eIF-4E before rapidly shuttling back to the nucleus. Although mainly cytosolic, the S6K has also been reported in the nucleus, both as a longer isoform containing a NLS sequence (25–27) and as a kinase capable of the nuclear phosphorylation of the transcription factor, CREM (28). Given that the phosphorylation is occurring within the NLS of CBP80, it might be anticipated that a cytosolic phosphorylation would block importin binding to the CBC and thus nuclear localization. Such has been found to be true for diacylglycerol kinase ζ (29). However, we have not found phosphorylation of CBP80 by S6K to block an interaction with the importins in vitro nor have we observed that aspartic acid mutants, designed to mimic a phosphorylated CBP80, lose their nuclear localization. Thus, at present both cytosolic and nuclear phosphorylations of CBP80 by S6K remain a viable mechanism.

S6K has been previously identified as an effector for Cdc42 (30) although this is the first report to suggest a functional consequence of this pathway, RNA splicing. FRAP, in addition to activating S6K, participates in the growth factor-regulated activation of the translation initiation complex, eIF-4F via the phosphorylation of 4E-BP1 (31–33). It is interesting that we have identified a potentially new role for these translational signaling players in CBC activation and RNA processing. This observation suggests a necessary coordination among aspects of gene expression that require the m7G cap structure: cap-dependent RNA splicing, export, and translation.

The work reported here also raises some new possibilities regarding how Cdc42 controls cell growth. It has recently been suggested that the involvement of Cdc42 in vesicular trafficking through customer interactions is necessary but not sufficient for Cdc42-induced transformation (6). The fact that the Cdc42 F28L,C37A double mutant is both transformation-defective and unable to stimulate RNA splicing suggests an interesting connection between the ability of Cdc42 to mediate RNA processing and induce malignant transformation. Thus, we are currently examining the possibility that CBC mutants, such as phosphorylation-defective CBC, will block Cdc42-induced transformation. Such data would underscore both the importance of Cdc42 in coordinating diverse cellular functions including RNA splicing and the role of CBC-mediated RNA processing in regulated cell growth.

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