The Nuclear Cap-binding Complex Is a Novel Target of Growth Factor Receptor-coupled Signal Transduction*

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In an attempt to further understand how nuclear events (such as gene expression, nuclear import/export, and cell cycle checkpoint control) might be subject to regulation by extracellular stimuli, we sought to identify nuclear activities under growth factor control. Using a sensitive photoaffinity labeling assay that measured [α-32P]GTP incorporation into nuclear proteins, we identified the 20-kDa subunit of the nuclear cap-binding complex (CBC) as a protein whose binding activity is greatly enhanced by the extracellular stimulation of serum-arrested cells. The CBC represents a 20- and 80-kDa heterodimer (the subunits independently referred to as CBP20 and CBP80, respectively) that binds the 7-methylguanosine cap on RNAs transcribed by RNA polymerase II. This binding facilitates precursor messenger RNA splicing and export. We have demonstrated that the [α-32P]GTP incorporation into CBP20 was correlated with an increased ability of the CBC to bind capped RNA and have used the [α-32P]GTP photoaffinity assay to characterize the activation of the CBC in response to growth factors. We show that the CBC is activated by heregulin in HeLa cells and by nerve growth factor in PC12 cells as well as during the G1/S phase of the cell cycle and when cells are stressed with UV irradiation. Additionally, we show that cap-dependent splicing of precursor mRNA, a functional outcome of CBC activation, can be catalyzed by growth factor addition to serum-arrested cells. Taken together, these data identify the CBC as a nuclear target for growth factor-coupled signal transduction and suggest novel mechanisms by which growth factors can influence gene expression and cell growth.

Growth factor binding to cell-surface receptors can initiate signals that are propagated through the cell by a cascade of protein-protein interactions, ultimately to impact upon specific cellular functions and regulate cell growth. The activities of signaling molecules must be tightly regulated to maintain the integrity of cellular communication, as loss of regulation in these processes can give rise to defects in cell growth and metabolism that may lead to human disease. Given the importance of signaling processes in cell growth, a great deal of effort has gone into the elucidation of proteins participating in signaling pathways that start at the level of receptor activation and culminate in the stimulation of a nuclear activity. Multiple cascades have now been identified that result in the activation of different nuclear mitogen-activated protein kinases, including the extracellular receptor-activated kinases and the stress-responsive c-Jun N-terminal kinase/stress-activated protein kinase and p38 (1, 2). Extracellular receptor-activated kinase activation is the outcome of mitogen-stimulated Ras signaling, whereas c-Jun N-terminal kinase/stress-activated protein kinase and p38 activities are often stimulated by pathways involving the Cdc42 and Rac GTP-binding proteins (2–6). Although these different signaling pathways were originally thought to be independently regulated, later work showed that cross-talk between the individual mitogen-activated protein kinase pathways exists. A common functional outcome of the activation of these signaling pathways is a translocation of the activated mitogen-activated protein kinase to the nucleus and subsequent activation of specific transcription factors and gene expression (2–6).

How other nuclear functions might be influenced in response to extracellular stimulation is less clear. However, it is attractive to envision how critical nuclear activities such as RNA metabolism and export, nuclear protein import, and cell cycle control might be subject to regulation as downstream targets of extracellular stimuli. With this in mind, we set out to identify novel nuclear activities that were growth factor-responsive. Using a photoaffinity labeling approach, we identified the nuclear cap-binding complex (CBC)1 as such an activity based on the enhanced ability of its ~20-kDa subunit (CBP20) to undergo a photocatalyzed incorporation of [α-32P]GTP in response to extracellular stimulation. The CBP20 protein and its 80-kDa binding partner, CBP80, constitute a functional CBC (7–10). This nuclear complex binds cotranscriptionally to the monomethylated guanosine cap structure (m7G) of RNA polymerase II-transcribed RNAs (7, 11, 12) and has been reported to play a role in diverse aspects of RNA metabolism: it increases the splicing efficiency of cap proximal introns (7, 13–15), positively affects the efficiency of 3'-end processing (16), and is required for the efficient transport of U snRNAs (9). We demonstrate that the incorporation of [α-32P]GTP by CBP20 reflects the activation of the CBC and is correlated with its ability to bind capped RNA. A variety of growth factors and other cellular stimuli can activate the CBC under conditions that can give

1 The abbreviations used are: CBC, cap-binding complex; NGF, nerve growth factor; EGF, epidermal growth factor; TBS, Tris-buffered saline; DTTO, dithiothreitol; AMP-PNP, adenosine 5'-[β,γ-iminophosphosphate]; GMP-PNP, guanosine 5'-[β,γ-iminophosphophosphate]; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; HA, hemagglutinin; eIF, eukaryotic initiation factor; snRNA, small nucleotide RNA.
rise to a stimulation of the splicing of precursor mRNAs in an *in vitro* assay system. The implications of CBP20 functioning as a novel end point in signal transduction highlight the importance of RNA metabolism in regulated cell growth.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions**—Rat pheochromocytoma (PC12) cells were maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 10% horse serum, and antiobiotic/antimycotic solution (Sigma). All other cell types, including HeLa, BHK21, and COS-7 cells, were maintained in Dulbecco's modified Eagle's medium with the addition of 10% fetal bovine serum and antibiotic/antimycotic solution (Sigma). Prior to growth factor treatment, cells were switched to serum-free medium for 40 h. Growth factors (NGF (Life Technologies, Inc.), heregulin β1 (residues 177–244; a generous gift from Dr. Mark Sliwicki, Genentech), and EGF (Calbiochem) or 25% fetal bovine serum) were then added to the serum-free medium in the concentrations and for the times indicated under "Results" at 37 °C. Following treatment, the growth-factor-containing medium was removed, and the cells were washed twice with Tris-buffered saline (TBS; 25 mM Tris, pH 7.4, 140 mM NaCl, and 1.0 mM EDTA) and then lysed (see below). Cell cycle blocks were performed in HeLa cells. A G1 block was achieved by switching to serum-free medium for 22–24 h. For G1/S phase arrest, 2.5 mM 1-butyne was added to the growth medium for 22–24 h. 80 ng/ml nocodazole was added to the growth medium for 22–24 h to achieve arrest in M phase. After treatment, cells were collected, washed twice with TBS, and lysed. To challenge cells with UV irradiation, the medium was removed from serum-starved cells, and the cells were then exposed to UV light for 2 min. Following exposure, cells were replenished with serum-free medium and allowed to recover at 37 °C for the times indicated below.

**Cell Fractionation and Nuclear Lysis**—Tissue culture cells were washed twice on the plate with TBS and then lysed in a buffer containing Hanks' solution (20 mM Hapes, pH 7.4, 5 mM KCl, 137 mM NaCl, 4 mM NaHCO3, 5.5 mM glucose, and 10 mM EDTA), 0.3% (v/v) Nonidet P-40, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and aprostatin. The lysate was then centrifuged at 800 rpm for 15 min at 4 °C. The supernatant was microcentrifuged for 10 min at 4 °C, and then the resulting supernatant was saved as the cytoplasmic fraction. The nuclear pellet was washed twice with an equal volume of Hanks' solution with 0.2% (v/v) Triton X-100 and centrifuged at 800 rpm for 15 min at 4 °C. The resulting pellet was treated as the purified nuclear fraction. The nuclei were then dissolved in a buffer containing 50 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM DTT, 20% (v/v) glycerol, 100 mM NaCl, and 500 μM AMP-PNP. Samples (20 μl) prepared from the cell fractionation procedures, described above, were incubated for 10 min at room temperature with an equal volume of cross-linking buffer containing (α-32P)-GTP (2–3 μCi/sample, 3000 Ci/mmol; NEN Life Science Products) in a 96-well, non-tissue culture-treated plate. The samples were then placed in an ice bath and irradiated with UV light (254 nm for 15 s). After irradiation, the samples were mixed with 5 μl cross-linking buffer and boiled. SDS-PAGE was performed using 15% acrylamide gels. The gels were then typically silver-stained and dried, and autoradiography was performed (typical overnight) using Kodak X-Omat XAR-5 film at ~80 °C. To perform competition experiments, competing nucleotides (mG3ppG and G3ppG (New England Biolabs Inc.) and mGTP and GTP (Sigma)) were added to the sample prior to the addition of the (α-32P)-GTP-containing cross-linking buffer. This buffer did not contain AMP-PNP. The samples were then subjected to UV cross-linking as described above.

**Purification of an 18-kDa Protein from Bovine Retinal Tissue That Incorporates (α-32P)-GTP—Bovine retinas were obtained frozen from J. A. & W. L. Lawson Co. (Lincoln, NE). The retinas (typically 200–500 mg) were homogenized in a buffer containing 50 mM KCl, 5 mM MgCl2, and protease inhibitors as described for cell lysates preparations and then homogenized with a motor-driven Dounce homogenizer. The homogenate was centrifuged at 2500 rpm in a swinging bucket rotor to yield a crude nuclear pellet. The nuclei were purified from this crude preparation using the method described by Blobel and Potter (19), and the soluble nuclear contents were then extracted as described previously. The 18-kDa activity was eluted using 50–75% ammonium sulfate, resuspended in 3–5 ml of Buffer A (50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20 mM KCl), and loaded onto a fast protein liquid chromatography Superdex-200 Highload 16/60 column as described above. The purification of this activity was monitored by both silver staining and UV cross-linking to (α-32P)-GTP. The fractions eluted from the Superdex-200 column were then analyzed by SDS-PAGE, and specific radioactivity of (α-32P)-GTP was determined. Six peak fractions (eluting with molecular masses of ~100–150 kDa) were pooled in a final volume of 12 ml and loaded directly onto a fast protein liquid chromatography ion-exchange Mono Q 5/5 column (Amersham Pharmacia Biotech) equilibrated in Buffer A minus KCl. Bound proteins were eluted from the Mono Q 5/5 column with a 28-ml linear gradient of 100–500 mM NaCl. (α-32P)-GTP—incorporating activity eluted from the Mono Q column with a 500 mM KCl linear gradient. Peak activity as assayed by (α-32P)-GTP incorporation was eluted from the Mono Q column and applied directly to a Bio-Gel HPHT hydroxylapatite column (Bio-Rad) equilibrated in 10 mM potassium phosphate, pH 6.8, 2.5 mM MgCl2, 0.01 mM CaCl2, and 1 mM DTT. Bound proteins were then eluted, first by stepping the potassium phosphate to 100 mM and then by a 20-ml linear gradient of 100–300 mM potassium phosphate. Peak activity as assayed by the light-catalyzed incorporation of (α-32P)-GTP was eluted from this column with ~250 mM phosphate.

**Cloning and Expression of Recombinant CBP20—**CBP20 was cloned by polymerase chain reaction from HeLa cell cDNA (a generous gift from Dr. Wannian Yang, Cornell University). Five- and 3'-primers were designed using the published sequence for *Homo sapiens* CBP20 (GenBank™ accession P52298), and the CBP20 gene was then amplified from the HeLa cell cDNA using 40 polymerase chain reaction cycles (1 min at 94 °C for denaturation, 1 min at 55 °C for primer annealing, and 1 min at 72 °C). The 470-base product was inserted into a cloning vector (pCR2.1) using a TA cloning kit (InviGen) and then subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and into the Escherichia coli expression vector pGEX-2TK.

**E. coli** cells transformed with the pGEX-2TK-CBP20 vector were grown in a 1-liter culture, and expression of glutathione S-transferase (GST-CBP20) protein was induced by the addition of 250 mM phosphate. The protein was then purified from *E. coli* cells transformed with the pGEX-2TK-CBP20 vector using glutathione-agarose beads for 1 h at 4 °C to bind the GST-CBP20 protein. Glutathione-agarose-bound CBP20 was washed with 50 mM Tris-HCl, pH 8.0, 0.5% (v/v) Triton X-100, 200 mM KCl and 1 mM DTT and then stored in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 μM GTP, and protease inhibitors. GST-CBP20 was eluted from the glutathione-agarose beads using 10 μM glutathione, pH 8.0, and the GST moiety was removed from CBP20 by the addition of 500 units of thrombin for 30 min at room temperature.

Using the LipofectAMINE protocol (Life Technologies, Inc.), a hemagglutinin-tagged form of CBP20 (HA-CBP20) was transiently transfectected into BHK21 cells according to the manufacturer's directions. Following a 5-h incubation with serum-free medium containing the lipid-DNA complex, the medium was removed and replaced with medium containing 10% fetal bovine serum. Cells were then incubated in the presence of serum for ~20 h and then were switched to serum-free medium for 40 h prior to stimulation with serum.

**Immunoprecipitation and Western Immunoblotting—**A polyclonal antibody generated against recombinant CBP20 (cCBP20) was prepared as described previously (7). Cytosolic and nuclear lysates were prepared as described above. Prior to immunoprecipitation, the cytosolic lysate was adjusted to 100 mM NaCl, and the nuclear lysate was 5.127.8.2018 from http://www.jbc.org/ by guest on July 27,
antibody or final volume of 20 mL.

E. coli

Following UV cross-linking, proteins were separated by 15% SDS-PAGE and visualized by Coomassie Blue staining (A).

UV cross-linking buffer and were incubated with [α-32P]GTP and UV cross-linked as described above.

For Western blot analysis, proteins were transferred to polyvinylidene difluoride membranes following SDS-PAGE. The polyvinylidene difluoride membranes were blocked with 2.5% (w/v) bovine serum albumin in TBS plus 0.1% Tween 20 for 1 h at room temperature. Following the blocking, the membranes were incubated with either 12CA5 or CBP80 monoclonal antibody or aCBP80 polyclonal antibody. Following the first incubation, 40 μl of protein A-Sepharose beads were added to each sample, and the samples were incubated for another hour at 4 °C. The samples were then centrifuged, and the immunoprecipitated pellets were washed four times with 50 mM Tris-HCl, pH 8.0, 133 mM KCl, 0.33% Triton X-100, 1 mM DTT, and 1 mM sodium orthovanadate. The resulting immunoprecipitated pellets were resuspended in 20 μl of UV cross-linking buffer and were incubated with [α-32P]GTP and UV cross-linked as described above.

For Western blot analysis, proteins were transferred to polyvinylidene difluoride membranes following SDS-PAGE. The polyvinylidene difluoride membranes were blocked with 2.5% (w/v) bovine serum albumin in TBS plus 0.1% Tween 20 for 1 h at room temperature. Following the blocking, the membranes were incubated with either 12CA5 or aCBP80 antibody for 1 h at room temperature, washed with several changes of TBS and 0.1% Tween 20, and incubated for 30 min at room temperature with sheep anti-rabbit or sheep anti-mouse horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech) as appropriate. Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

RNA Binding Assays—UV cross-linking was done essentially as described by Rozen and Sonenberg (20), except that the RNA probe was transcribed from HeLa cells (serum-starved for 40 h prior to stimulation with 100 nM EGF) as described by Izaurralde et al. (7). In brief, 60 μg of splicing extract were preincubated for 15 min at 30 °C with 1 mM MgCl₂, 5 mM creatine phosphate, 1.5 mM ATP, 2.5 × 10⁴ cpm of labeled precursor mRNA, and an additional 1 mM MgCl₂ were then added in a final volume of 20 μl, and the reactions were incubated for 2 h at 30 °C. Splice products were visualized by separation on a 10% denaturing polyacrylamide gel, followed by autoradiography.

RESULTS

The overall goal of these studies was to identify nuclear activities that could represent novel downstream targets in receptor-coupled signaling pathways. One of the assays we used to identify such activities was the photocatalyzed incorporation of [α-32P]GTP into nuclear proteins. The rationale for this approach was that it would provide a sensitive assay for identifying guanine nucleotide-binding activities in the nucleus, in a manner analogous to the use of phosphorylation assays to identify growth factor-sensitive phosphoproteins.

Using this assay, we identified an 18-kDa protein that strongly incorporated [α-32P]GTP in serum-treated but not serum-starved cells (see below). We found this activity to be exclusively nuclear and present in every cell line we examined, including HeLa, PC12, COS-7, and BHK21 cells, as well as in various mammary epithelial cells. A similar activity was also observed in the yeast Saccharomyces cerevisiae.

A purification scheme was developed using bovine retinal nuclei, which were a particularly rich source of this 18-kDa nuclear activity. A series of three chromatography steps resolved the activity, as assayed by [α-32P]GTP incorporation, from the majority of contaminating low molecular mass proteins (see “Experimental Procedures”). These steps also resolved an 80-kDa protein (designated p80), detected by silver staining, which co-purified with the 18-kDa activity. This putative protein complex was reminiscent of the nuclear CBC, as the CBC comprises an 18-kDa nuclear protein, CBP20 (for cap-binding protein 20), stably complexed with an 80-kDa protein, designated CBP80. The formation of the CBP20-CBP80 heterodimer enables the CBC to bind a guanine derivative, the 7-methylguanosine cap structure (m7GpppN), on RNAs transcribed by RNA polymerase II (7–10). The similarities between the 18-kDa nuclear activity and CBP20 (both in complex formation and substrate binding) led us to investigate whether the CBC was a nuclear target for extracellular signals.

First, we assayed directly the ability of recombinant E. coli-expressed CBP20 to incorporate [α-32P]GTP. Fig. 1A shows GST-CBP20, thrombin-cleaved CBP20, and the complexed CBP proteins (His-tagged CBP20 (9) and CBP80 (7)) as visualized by staining with Coomassie Blue. Fig. 1B shows that the recombinant CBP20 proteins were all capable of incorporating [α-32P]GTP in a photoaffinity labeling assay. This activity was greatly enhanced by the presence of CBP80 (see lane 5), consistent with previous studies that have demonstrated that complex formation between CBP20 and CBP80 is necessary for
capped RNA binding. The GST control did not show any cross-linking to [α-32P]GTP.

We next examined whether the ability of CBP20 to incorporate [α-32P]GTP could be regulated in response to serum. BHK21 cells were transiently transfected with a HA-tagged CBP20 construct. Following 40 h of serum starvation, the cells were stimulated with 25% fetal bovine serum for 1.5 h, and HA-CBP20 was immunoprecipitated (IP) from cytosolic or nuclear lysates using 12CA5 monoclonal antibody. Immunoprecipitates were then assayed for [α-32P]GTP incorporation. Proteins were separated by 15% SDS-PAGE and transferred to Immobilon for Western blot analysis and autoradiography. A shows the CBP80 protein co-immunoprecipitating with HA-CBP20 from the nuclear lysates as detected by Western blotting using CBP80 antiserum. B is a Western blot using 12CA5 antibody to detect immunoprecipitated HA-CBP20 from cytosolic and nuclear lysates. The [α-32P]GTP incorporation corresponding to immunoprecipitated HA-CBP20 is shown in C.

Given that the m7GpppN RNA cap structure is a known substrate for the CBC, the stimulated incorporation of [α-32P]GTP into CBP20 may reflect an enhanced ability of the CBC to bind the cap structure on RNA. To address this issue, we first examined the relative binding affinities of the CBC for different cap analogs by testing their ability to inhibit the incorporation of [α-32P]GTP into CBP20. PC12 cell nuclear lysates were immunoprecipitated with antibodies generated against CBP80 (i.e. the binding partner of CBP20) (7), and the immunoprecipitates were then assayed for photocatalyzed incorporation of [α-32P]GTP into CBP20 in the absence and presence of RNA cap analogs or GTP. CBP20 proteins that co-immunoprecipitated with CBP80 could be efficiently labeled with [α-32P]GTP. This activity was strongly inhibited by the addition of low concentrations of cap analogs to the [α-32P]GTP cross-linking assay and yielded the following binding specificity: m7GpppG > m7GTP > GpppG > GTP (Fig. 3A). Indeed, the m7GpppG analog competed with [α-32P]GTP for binding to CBP20 ~1000 times more effectively than GTP, suggesting that the CBC most likely binds RNA, rather than GTP, in cells.

We further examined whether the CBC shows a regulated binding to capped RNAs using a PC12 cell line that stably expresses HA-tagged CBP20. Following starvation, these cells were stimulated with NGF. HA-CBP20 was then immunoprecipitated from the cytoplasmic and nuclear lysates and assayed in the presence of either [α-32P]GTP (lanes 1–4) or m7GpppN-capped RNA (lanes 5–8).
FIG. 4. Characterization of the growth factor-induced capped RNA-binding activity of the CBC (assayed by CBP20 [α-32P]GTP incorporation). A, HeLa cells (lanes 1–6) were serum-starved (control [CON]; lanes 1 and 4) and then treated with 100 ng/ml EGF (lanes 2 and 5) or 30 nm heregulin (HRG; lanes 3 and 6) for 15 min at 37 °C. PC12 cells (lanes 7–14) were serum-starved (control; lanes 7 and 11) and then treated with 30 nm HRG (lanes 8 and 12), 100 ng/ml EGF (lanes 9 and 13), or 100 ng/ml NGF (lanes 10 and 14). The cells were lysed, separated into cytoplasmic (lanes 1–3 and 7–10) and whole nuclear (lanes 4–6 and 11–14) fractions, and assayed for [α-32P]GTP incorporation into CBP20 using 45 μg of protein from cell lysates. B, a dose-response experiment was performed with the addition of either heregulin (lanes 2–4) or NGF (lanes 5–7) to serum-starved PC12 cells (control; lane 1) for 30 min at 37 °C. For each dose of heregulin or NGF, 50 μg of total nuclear lysate protein were assayed for the incorporation of [α-32P]GTP into CBP20, and then 15% SDS-PAGE was performed. The resulting gel was dried and exposed to x-ray film for 5–15 h. C, a time course of 100 ng/ml NGF treatment was performed in serum-starved PC12 cells (control; lane 1) with NGF addition for 7.5 min (lane 2), 15 min (lane 3), 30 min (lane 4), 60 min (lane 5), or 24 h (lane 6). 50 μg of protein from the nuclear lysates were assayed for incorporation of [α-32P]GTP into CBP20, followed by 15% SDS-PAGE and autoradiography overnight.

We took further advantage of the high sensitivity of the [α-32P]GTP incorporation assay to examine the abilities of different growth factors to activate the endogenous CBC. Fig. 4A (left panel) shows the results obtained when HeLa cells were first serum-starved and then treated with EGF and heregulin (the ligand for the Neu-ErbB2/ErbB3 and Neu-ErbB2/ErbB4 heterodimers (22, 23)). Endogenous CBP20 present in nuclear lysates from HeLa cells was strongly stimulated to incorporate [α-32P]GTP by heregulin as well as, to a lesser extent, by EGF. Similarly, in PC12 cells, endogenous CBP20 present in nuclear lysates was activated by growth factors (Fig. 4A, right panel). In this case, the incorporation of [α-32P]GTP into CBP20 was most strongly stimulated by NGF (as observed in Fig. 3B), followed by heregulin and then EGF. Fig. 4B shows that in all cases, the growth factor-stimulated activation of CBP20 was dose-dependent.

In our initial experiments, the incorporation of [α-32P]GTP into CBP20 was assayed after relatively short periods of growth factor treatment (~15 min). Although this was sufficient to detect incorporation of the radiolabeled GTP, more complete time course experiments indicated that near maximal incorporation occurred following treatment with growth factors for 1 h. An example for PC12 cells is shown in Fig. 4C. In this experiment, serum-starved PC12 cells were challenged with 100 ng/ml NGF for increasing time periods, up to 24 h. The results show that near maximal incorporation of [α-32P]GTP into CBP20 was observed after ~1 h of growth factor addition and that this level of incorporation was maintained through 24 h. A similar time course was obtained when PC12 cells were treated with heregulin (data not shown).

Nuclear lysates from asynchronously growing cells also contain activated CBP20, suggesting that the growth factor regulation of the CBC activity may be associated with a particular phase of the cell cycle. This is illustrated in Fig. 5A. HeLa cells were arrested in G1 phase by serum starvation, in G1/S phase by thymidine addition, and in M phase by nocodazole treatment. Cytoplasmic and nuclear fractions were then prepared (or a mitotic pellet was prepared in the case of M phase-arrested cells), and the resulting lysates were assayed for the ability of CBP20 to incorporate radiolabeled GTP. We found that CBP20 did not incorporate [α-32P]GTP in cells arrested in either G0 or M phase of the cell cycle. However, CBP20 strongly incorporated [α-32P]GTP in HeLa cells arrested in G1/S phase. Thus, the activation of the CBC appears to be sensitive to cell cycle-dependent as well as growth factor-dependent regulation.

To determine whether the CBC might respond to a broader range of stimuli, we assayed the ability of CBP20 to incorporate radiolabeled GTP under conditions of cellular stress. PC12 cells were first serum-starved and then exposed to UV radiation for 2 min. Following this exposure, the cells were allowed to recover for 30 min or 1 h, and then endogenous CBP20 was assayed for its ability to incorporate [α-32P]GTP. Fig. 5B shows that CBP20 was strongly stimulated to incorporate radiolabeled GTP in cells that had been UV-irradiated. We found a similar stress activation of endogenous CBP20 in COS-7 and HEK-293 cells (data not shown).

Stress response pathways have been shown to be mediated by the low molecular mass GTP-binding proteins Cdc42 and Rac and to culminate in transcriptional activation through the stimulation of the nuclear mitogen-activated protein kinases JNK1 and p38/HOG1 (3–5, 24). Thus, we examined whether the transient expression of activated Cdc42 would result in a growth factor-independent activation of the CBC. The results in Fig. 5C indicate that this is the case. We found that the transient expression of either a GTPase-defective Cdc42 mutant (Cdc42 Q61L) or a transforming Cdc42 mutant that is capable of undergoing the spontaneous exchange of GTP for GDP (Cdc42F28SL) strongly activated CBP20, whereas expression of wild-type Cdc42 showed no activation. We also have found that expression of V12-Ras stimulates the incorporation of [α-32P]GTP into CBP20 as well as activated Rac and RhoA (data not shown), although thus far, Cdc42 appears to be the most effective activator.
FIG. 5. The CBC shows a cell cycle- and cell stress-dependent activation and can be activated by the low molecular mass GTP-binding proteins Cdc42 and Ras. A, HeLa cells were arrested in G$_0$ phase by serum starvation (lanes 1 and 4), in G$_1$/S phase by 2.5 mM thymidine addition (lanes 2 and 5), and in M phase with 80 ng/ml nocodazole (lanes 3 and 6). The cells were then separated into cytoplasmic (lanes 1–3) and whole nuclear (or a mitotic pellet was prepared for M phase arrest) (lanes 4–6) fractions, and then for each fraction, 50 μg of protein were assayed for [$\alpha$-$^{32}$P]GTP incorporation into CBP20, followed by 15% SDS-PAGE and autoradiography. B, PC12 cells were serum-starved and then exposed to UV light for 2 min. Following exposure, cells were replenished with serum-free medium and allowed to recover for 30 min or 1 h. Cells were then harvested; nuclear lysates were prepared; and 50 μg of nuclear lysate protein were assayed for [$\alpha$-$^{32}$P]GTP incorporation into CBP20 by 15% SDS-PAGE and autoradiography. C, HeLa cells were transiently transfected with GTPase-defective Cdc42 Q61L (L61 Cdc42), constitutively active Cdc42 F28L (L28 Cdc42), or GTPase-defective Ras G12V (V12 Ras) for 24 h, followed by serum starvation for 40 h. The cytoplasmic lysates were analyzed for expression of the transfected proteins by Western blotting using an anti-HA antibody directed against the HA tag on the recombinant GTP-binding proteins (upper panel). The nuclear lysates were assayed for [$\alpha$-$^{32}$P]GTP incorporation into CBP20 (lower panel).

Taken together, these data suggest that the ability of the CBC to bind RNA cap structures is a tightly regulated process. Previous work by others has defined a role for CBC binding to capped RNAs in important RNA metabolic processes, including pre-mRNA splicing (7, 13–15), U snRNA export (9), and 3′-end processing (16). The ability of growth factors to stimulate the capped RNA-binding activity of the CBC suggests that those metabolic processes that benefit from the recognition of the RNA cap by the CBC (such as pre-mRNA splicing) will also be subject to extracellular regulation. To test this prediction, splicing extracts were prepared from quiescent cells that were either serum-starved or starved and then stimulated with heregulin for 24 h (i.e. conditions that lead to maximal stimulation of CBP20 activity in nuclear lysates (see Fig. 4B)). Creatine phosphate, ATP, and m$^7$GpppG-capped precursor adenovirus mRNA were added to initiate splicing (see “Experimental Procedures”). Extracts prepared from quiescent cells were not competent to splice the m$^7$GpppG-capped precursor RNA (Fig. 6). However, splicing of the m$^7$GpppG-capped RNA was markedly stimulated in extracts prepared from hreegulin-treated cells and was ~5-fold higher than the splicing of a nonspecific AappG-capped RNA probe by the same extract (data not shown). These results indicate that under conditions where growth factor signaling activates CBC, there is a corresponding stimulation in capped precursor mRNA splicing. Because we also observed some increase in AappG-capped RNA splicing, the possibility exists that other targets, perhaps acting in conjunction with the CBC, may be important in mediating the observed growth factor effect in cap-dependent RNA splicing. Thus, cap-dependent RNA splicing, in addition to CBC-capped RNA binding, is a functional end point for growth factor-coupled signaling pathways leading to the nucleus.

**DISCUSSION**

The original goal of these studies was to identify novel nuclear activities that were susceptible to growth factor regulation to further our understanding of how growth factors exert their effects in the nucleus. Using a photolabeling approach to detect nuclear proteins that specifically incorporate [$\alpha$-$^{32}$P]GTP, we detected an 18-kDa nuclear activity that was highly sensitive to the addition of growth factors to G$_0$ phase-arrested cells. The fundamental role of this activity in cell growth regulation is underscored by its response to growth factors, its specific association with the G$_1$/S phase of the cell cycle, its activation under conditions of cell stress, and the fact that we have found this activity in every cell and tissue type examined thus far. It was therefore interesting to find that the 18-kDa activity corresponds to the RNA cap-binding protein CBP20, suggesting a necessity for a regulated nuclear cap binding event in cell growth control.

The m$^7$G(5′)ppp(5′)N cap structure on RNAs transcribed by RNA polymerase II has been known for some time to be important for the stability of these RNAs (25, 26) and to facilitate different aspects of RNA metabolism, including translation initiation, pre-mRNA splicing, and nuclear transport. In recent years, CBP20 and its 80-kDa binding partner, CBP80 (collectively termed CBC), have been identified as the protein complex that binds to the cap structure in the nucleus and mediates the cap-dependent enhancement of pre-mRNA splicing and export of U snRNAs (7, 9). To our knowledge, this is the
Regulation of the Nuclear Cap-binding Complex

First report describing a regulated binding activity by the CBC and thus implies that RNA metabolic processes ascribed to the CBC will be regulated as well. This is supported by our finding that growth factors regulate the in vitro splicing of precursor mRNA in nuclear lysates from HeLa cells.

An understanding of the signaling processes that lead to CBC activation could shed light on how mitogens influence gene expression by modulating RNA metabolism. All indications are that the CBC may receive inputs from multiple pathways. The Ras-Raf-MEK-extracellular receptor-activated kinase signaling cascade is one pathway that is central to mediating growth factor effects in the nucleus, and we have observed that expression of oncogenic Ras G12V in cells results in an activation of the CBC. Stress-activated signaling pathways also induce CBC activation. There are a number of lines of evidence that indicate that signaling pathways stimulated by Rho-like GTP-binding proteins (e.g. Cdc42 and Rac) both participate in cellular stress responses (4–7, 27) and are under growth factor control (25–27). In fact, we have found that activated forms of Cdc42 give rise to an effective activation of the CBC. Given that Cdc42 has been suggested to input into rapamycin-sensitive pathways involving FRAP (FKB12/rapamycin-associated protein) by activating the p70 S6 kinase (27), it is interesting to consider whether the regulation of the CBC is linked to translational control. The cytosolic mRNA-capping protein eIF-4E, which plays a critical role in a number of mRNA translational events (29), is also susceptible to growth factor regulation. The phosphorylation of eIF-4E occurs in response to multiple growth factors (including NGF in PC12 cells) and cell cycle arrest (28, 30) and appears to occur downstream of multiple signaling pathways, including the extracellular receptor-activated kinase, c-Jun N-terminal kinase/ stress-activated protein kinase, and p38 kinase pathways (29).

In addition to its direct phosphorylation, the activity of eIF-4E is also regulated by two other growth factor-responsive factors, the eIF-4E-binding proteins 4E-BP1 and 4E-BP2 (30), and recently, 4E-BP1 has shown to be phosphorylated by the phosphatidylinositol 3-kinase-related kinase FRAP (31). Thus, it will be interesting to see if the cytosolic cap-binding protein eIF-4E and the CBC are similarly or even coordinately regulated through growth factor-initiated signals.

A growth factor-dependent phosphorylation of CBP20 could have a direct effect on its RNA cap-binding activity (similar to eIF-4E), although thus far, we have not been able to detect a growth factor-stimulated phosphorylation of CBP20 in vivo. The cellular levels of CBP20, its ability to bind CBP80, and its nuclear localization are not affected by growth factor stimulation (see Fig. 2). We are currently examining whether growth factors influence the interactions between the CBC and specific regulatory proteins to stimulate the binding of the CBC to capped RNA in a manner analogous to the growth factor-regulated interaction between eIF-4E and the 4E-BP proteins.

Our demonstration that the CBC is susceptible to extracellular regulation, in conjunction with the previously defined role for the CBC in RNA processing, makes the CBC an attractive candidate for translating growth factor signals into altered gene expression by affecting the metabolism of specific subsets of RNAs. However, given that the CBC affects both the processing and transport of RNAs transcribed by RNA polymerase II, the growth factor-dependent binding of the CBC to capped RNA may result in a general regulation of gene expression. The reduced ability of the CBC to bind capped RNAs in the absence of a growth factor signal could serve as a checkpoint for cell growth by guarding against the further processing of inappropriate or "leaky" transcripts. This suggests that altered levels and/or mutations of the CBC might be capable of deregulating cell growth. Future studies will be directed toward determining how growth factors influence different aspects of RNA processing (including precursor mRNA splicing and RNA export) through the CBC and how overexpression and/or mutation of the CBC impacts upon normal cell growth.

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