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The epidermal growth factor (EGF) receptor subfamily of receptor tyrosine kinases, including Neu/ErbB2, ErbB3 and ErbB4, has been implicated in the development of many breast cancers. Heregulin (HRG) has been identified as the ligand for these receptors, and this ligand-receptor interaction is the first step in a signaling pathway which is presumably overactive in tumors where the above named ErbB receptors are overexpressed. Our goal was to attempt to uncover the downstream effects of HRG in the cell. In particular, we proposed to investigate an 18 kDa nuclear protein, p18, which responded to HRG signals with a dramatically enhanced ability to bind GTP. In this report, we have identified p18 as CBP20, the 20 kDa subunit of the nuclear cap-binding complex (CBC). The CBC binds to capped RNAs, and in so doing, has been shown to facilitate cap-dependent pre-mRNA splicing and export. We have demonstrated that the CBC binds to capped RNAs in response to stimuli, and that cap-dependent RNA splicing is enhanced in cells treated with HRG. Additionally, we now find that multiple signaling pathways may be involved in the activation of the CBC to bind to capped RNAs.

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INTRODUCTION

The primary goal of the original proposal was to identify and characterize molecular components involved in heregulin (HRG)-initiated signal transduction pathways with the belief that identification of these molecules will increase our effectiveness in the intervention and potential cure of breast cancer. The understanding of HRG stimulated signal transduction events is particularly relevant as it applies to breast cancers involving the members of the epidermal growth factor (EGF) receptor subfamily of receptor tyrosine kinases (class I), including Neu/ErbB2, ErbB3, and ErbB4. Heregulin in the physiological ligand for these receptors (1-3), and overexpression of this class of receptors has been correlated with high occurrences of certain breast cancers (4-9). The overexpression of Neu/ErbB2 in human breast cancers (up to 40%) has been correlated to a poor prognosis in patients with breast cancer (5, 7).

The interactions between HRG and this receptor class, as well as consequent interactions amongst receptor family members, has been worked out in some detail. While HRG was shown to lead to the tyrosine phosphorylation of Neu/ErbB2 in some mammary carcinoma cells, it was ineffective in eliciting the same response in ovarian cells overexpressing Neu/ErbB2 (10) suggesting the role of additional cellular factors in receptor activation in response to HRG. ErbB3 and ErbB4 were later found to be the true receptors for HRG (1-3). ErbB3, while capable of binding to HRG, lacks intrinsic tyrosine kinase activity (11). Indeed, it appears that ErbB3, upon heregulin binding, is induced to heterodimerize with Neu/ErbB2. This heterodimerization event is sufficient for the activation of Neu/ErbB2 and subsequent transphosphorylation of ErbB3 on tyrosine residues. The concept of heterodimerization and transphosphorylation among the class I receptors has now become well accepted, supported by observations such as the tyrosine phosphorylation of both Neu/ErbB2 and ErbB3 in response to EGF (these receptors have no apparent affinity for EGF and their phosphorylation is most likely due to a heterodimerization with the EGF receptor (11, 12)), and various ligand-dependent interactions between Neu/ErbB2:ErbB3, Neu/ErbB2:ErbB4, and ErbB3:ErbB4 (13) An interesting outcome of these heterodimer complexes is the different combinations of Src homology 2 (SH2) containing proteins which can be recruited to phosphorylated tyrosines on the cytoplasmic domain of these receptors upon ligand binding (2), potentially capable of generating different downstream responses. The nature of these downstream responses, however, is less clear and prompted us to search for novel HRG-sensitive molecules in an attempt to elucidate the means by which HRG promotes its growth effects on a cellular level.

Our discovery of an 18 kDa, nuclear protein which responded to HRG treatment of cells with a dramatically enhanced GTP-binding activity formed the specific basis for the original proposal. This GTP-binding activity (referred to as p18) was found to be significantly enhanced in HeLa cells in response to HRG, and to a lesser extent, EGF, and in PC-12 cells in response to HRG, EGF, and nerve growth factor (NGF). A similar 18 kDa nuclear GTP-binding activity has been observed in all cell lines which we have examined including several mammary carcinoma cell lines (MDA-MB-453, MDA-MB-468, and SKBR3). Additionally, we found an increased p18 GTP-binding activity specifically associated with the G1/S phase of the cells cycle, suggesting that this protein might have fundamental links to cell growth regulation. We postulated that this protein was involved in mediating HRG effects on mitogenesis, perhaps by its ability to regulate some critical nuclear function. To explore this hypothesis, we devised a series of specific aims, stated in the original proposal. These aims were to identify the protein responsible for this regulated GTP-binding activity, delineate the signal transduction pathways which give rise to the HRG-stimulated GTP-binding, and characterize proteins which directly activated p18 in response to HRG.

Over the last year, we have made considerable advances in addressing these aims. Through a combination of approaches, we have determined that p18 is identical to the RNA

cap-binding protein, CBP20. The CBP20 protein, together with its 80 kDa binding partner, CBP80, forms a functional complex termed CBC (14-17). This nuclear complex binds cotranscriptionally to the monomethylated guanosine cap structure (m^7G) of RNA polymerase II transcribed RNAs (14, 18, 19) and has been reported to play a role in diverse aspects of RNA metabolism: it increases the splicing efficiency of cap proximal introns (14, 20-22), positively affects the efficiency of 3' end processing (23), and is required for the efficient transport of U snRNAs (16). We now show that CBC binding to GTP and m^7G capped RNAs can be regulated by extracellular factors. The finding that HRG induces the CBC to bind capped RNA led to the further discovery that HRG can activate the cellular splicing machinery and therefore influence gene expression at the level of RNA splicing. This result potentially provides one mechanism by which a loss of regulation of the CBC in cells overexpressing ErbB receptors may result in aberrant gene expression and cell growth. More recently, we have undertaken studies to address signaling pathways that culminate in a CBC-RNA binding event. The low molecular weight GTP-binding proteins Ras and Cdc42 are both capable of activating the CBC when transiently expressed in a constitutively active form. This activation can be blocked with the drug rapamycin which implicates the involvement of translational signaling pathways as well. These findings raise the exciting possibility that multiple signal transduction pathways converge in the nucleus at the CBC proteins to input into gene expression by regulating RNA-binding events by the CBC. In the coming year, we plan to expand upon our delineation of this HRG-activated signal transduction pathway to the nucleus, and further explore the effects of HRG on gene expression by modulating RNA processing through the CBC.

BODY

Experimental Procedures

This section will contain some of the more routinely used experimental procedures which are relevant to the results described below.

1. Cell culture conditions and transient transfection.

Rat pheochromocytoma (PC12) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum, 10% horse serum, and antibiotic/antimycotic solution (Sigma Chem. Co.). Mammary carcinoma cell lines, HeLa, and BHK21 cells were maintained in DMEM, with the addition of 10% fetal bovine serum and antibiotic/antimycotic solution. NIH 3T3 cells required 10% calf serum instead of fetal bovine serum. Prior to growth factor treatment, cells were switched to serum-free media for 40 hours. Growth factors [NGF (Gibco-BRL), heregulin b1 (residues 177-244; a generous gift from Dr. Mark Sliwkowski, Genentech), and EGF (Calbiochem), or 25% fetal bovine serum] were then added to the serum-free media in concentrations and for times indicated in the results at 37°C. Following treatment, the growth factor-containing media was removed and the cells were washed (2X) with Tris-buffered saline (TBS: 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, 1.0 mM EDTA), and then lysed (see below). Transient transfections utilize the LipofectAMINE reagent (Gibco BRL) and are performed according to manufacturer's directions.

2. Cell Fractionation and Nuclear Lysis

Tissue culture cells were washed (2X) on the plate with TBS and then lysed in a buffer containing Hank's buffer (20 mM Hepes, pH 7.4, 5 mM KCl, 137 mM NaCl, 4 mM $NaHCO_3$, 5.5 mM D-glucose, 10 mM EDTA), 0.3% (v/v) NP-40, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 µg/ml each of leupeptin and aprotinin. The lysate was then centrifuged for 15 minutes at 800 rpm at 4°C. The resulting supernatant was micro-centrifuged for 10 minutes at 4°C, and this

supernatant saved as the cytoplasmic fraction. The nuclear pellet was then washed (2X) with an equal volume of Hank's buffer with 0.2% (v/v) Triton X-100, and centrifuged for 15 minutes at 800 rpm at 4°C. The resulting pellet was treated as the purified nuclear fraction. The nuclei were then lysed in a buffer containing 50 mM Tris, pH 7.4, 1% Triton X-100 (v/v), 400 mM KCl, 1 mM sodium orthovanadate, 1 mM DTT, and protease inhibitors as described above. The samples were incubated on ice for 30 minutes, microfuged for 10 minutes at 4°C, and the resulting supernatant was used as the whole nuclear fraction.

3. Photoaffinity labeling of proteins using either [α^{32}P]GTP or capped RNA.

Photoaffinity labeling of GTP-binding proteins with [α^{32}P]GTP was performed as previously described (24). In brief, the UV crosslinking reaction was carried out in a buffer that contained 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 minutes at room temperature with an equal volume of crosslinking buffer containing [α^{32}P]GTP (2-3 µCi per sample) (3000 Ci/mmol, New England Nuclear) 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 minutes. After irradiation, samples were mixed with 5X Laemmli buffer and boiled. SDS-PAGE was performed using 15% acrylamide gels. The gels were then typically Silver-stained, dried, and autoradiography was performed (typically overnight) using Kodak X-OMAT XAR-5 film at -80°C. To perform competition experiments, competing nucleotides (m^7GpppG, GpppG [New England Biolabs], m^7GTP, GTP, GMP-PNP, AMP-PNP [Sigma]) were added to the sample prior to the addition of the [α^{32}P]GTP-containing crosslinking buffer. This buffer did not contain AMP-PNP. The samples were then subjected to UV-crosslinking as described above. UV-crosslinking was done essentially as described by Rozen and Sonenberg (25) except that the RNA probe was transcribed from BamHI-cleaved pBSII KS with T3 RNA polymerase (Promega).

4. Purification of p18 from bovine retinal tissue.

Bovine retina were obtained frozen from J.A. & W.L. Lawson Co. (Lincoln, NE). The retina (typically 200/batch) were thawed in a buffer (TKM) containing 50 mM Tris, pH 8.0, 25 mM KCl, and 5 mM $MgCl_2$, together with protease inhibitors as described for cell lysate preparations, and then homogenized with a motor-driven dounce homogenizer. The homogenate was then 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 (26) and the soluble nuclear contents were then extracted. p18 GTP-binding activity was precipitated using 40-75% ammonium sulfate, resuspended in 3-5 ml of Buffer A, and loaded onto a FPLC Superdex-200 Highload 16/60 column as described above. The purification of p18 was monitored both by Silver-staining and UV-crosslinking to [α^{32}P]GTP. The fractions eluted from the Superdex-200 column were assayed for [α^{32}P]GTP-binding to p18, and 6 peak fractions (eluting with molecular mass ~100-150 kDa) were pooled in a final volume of 12 ml and loaded directly onto a FPLC ion exchange, Mono Q 5/5 (Pharmacia) column equilibrated in Buffer B (Buffer A minus KCl). Bound proteins were eluted from the Mono Q 5/5 with a 28 ml linear gradient of 100 mM - 500 mM NaCl. p18 [α^{32}P]GTP-binding activity elutes from the Mono Q 5/5 column with ~ 300 mM NaCl in a volume of 5 ml. Peak p18 [α^{32}P]GTP-binding activity eluted from the Mono Q column was applied directly to a Bio-Gel HPHT hydroxyapatite column (Biorad) equilibrated in Buffer C (10 mM potassium phosphate, pH 6.8, 2.5 mM $MgCl_2$, 0.01 mM $CaCl_2$, 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 mM - 300 mM potassium phosphate. p18 [α^{32}P]GTP-binding activity was found to elute with ~250 mM phosphate.

5. Cloning and Expression of Recombinant CBP20 and CBP80.

CBP20 was cloned by PCR from HeLa cell cDNA (a generous gift from Dr. Wannian Yang, Cornell University). 5' and 3' primers were designed using the published sequence for hsCBP20 (accession# P52298), and the CBP20 gene was then amplified from the HeLa cell cDNA using 40 PCR cycles (1 min at 94°C, 1 min at 55°C, 1 min at 72°C). The 470 base product was inserted into a cloning vector, pCR2.1, using a TA cloning kit (Invitrogen), and then subcloned into the mammalian expression vector, pcDNA3 (Invitrogen), and into the pGEX-KG *E. coli* expression vector.

E. coli transformed with pGEX-KG-CBP20 vector were grown in a one liter culture, and expression of glutathione-S-transferase (GST)-CBP20 protein was induced for three hours using IPTG. Following induction, the cells were pelleted by centrifugation (5000 rpm for 10 minutes in a JA-10 rotor). The harvested cells were resuspended in 15 ml of 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 1 mM DTT, and protease inhibitors (as described above), and then lysed using 15 µg of lysozyme, followed by the addition of 200 mM $MgCl_2$ and 1 µg DNase-1. Following centrifugation (100,00 x *g* for 30 min at 4°C), the supernatant was incubated with glutathione agarose beads for 1 hour at 4°C to bind the GST-CBP20. The 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, 1 mM DTT, 10 µM GTP and protease inhibitors. GST-CBP20 was eluted from the glutathione agarose beads using 10 mM glutathione, pH 8.0, and the GST moiety was cleaved from CBP20 by the addition of 500 U thrombin for 30 minutes at room temperature.

CBP80 was cloned by PCR from a human testis cDNA library (a generous gift from Dr. Nena Winand, Cornell University). 5' and 3' primers were designed using the published sequence for hsCBP80 (accession# Q09161), and the CBP80 gene was then amplified from the human testis cDNA library using 40 PCR cycles (30 sec. at 94°C, 30 sec. at 50°C, and 1 min at 72°C). The 2600 base product was inserted into a TA cloning vector as described above and subcloned into the mammalian expression vector pcDNA3 and the baculovirus expression system vector pVL1393 (Pharmingen). For expression of CBP80 in *Spodoptera frugiperda* cells, a recombinant baculovirus containing the CBP80 gene was generated according to manufacturer's directions. To express CBP80 protein in *Spodoptera frugiperda* cells, cells are infected with the recombinant virus for three days. The cells are then harvested, and lysed in a buffer containing 50 mM Tris pH 8.0, 300 mM NaCl, 0.4% CHAPS, and protease inhibitors as described above. The lysate is cleared by centrifugation at 40,000 *x g* in a Beckman ultracentrifuge. To generate rCBC protein complex, *E. coli* expressed GST-CBP20 protein attached to glutathione agarose beads is incubated with CBP80-expressing insect cell lysates. These beads are washed extensively to remove nonspecific CBP20-binding proteins, and the rCBC complex is then eluted from the glutathione beads using 10 mM glutathione. This complex can then be further purified using gel filtration and ion exchange chromatography as described in the purification of p18 above.

6. Pre-mRNA Splicing Reactions.

Splicing extracts were prepared from HeLa cells (serum-starved for 40 hours prior to stimulation with 100 nM heregulin for 24 hours) as described by Lee and Green (27). pBSAd1 precursor linearized by SauIIIA was transcribed using T3 RNA polymerase in the presence of m^7GpppG cap analog. Splicing reactions were then carried out as described by Izaurralde et. al. (14). In brief, 60 µg of splicing extract was pre-incubated for 15 minutes at 30 °C with 1 mM $MgCl_2$, 5 mM creatine phosphate, 1.5 mM ATP, 2.5 x 10^4 cpm m^7GpppG capped, labeled precursor mRNA, and an additional 1 mM $MgCl_2$ were then added in a final volume of 20 µl and the reactions were incubated for 2 hours at 30°C. Splice products were visualized by separation on a 10% denaturing polyacrylamide gel, followed by autoradiography.

7. S6 kinase reactions.
For *in vitro* kinase reactions, recombinant CBC was incubated with partially purified S6 kinase (Upstate Biotechnology Inc.) in a kinase reaction buffer containing 10 mM Hepes pH 8.0, 10 % glycerol, 5 mM $MgCl_2$, 20 μM ATP, and 10 μCi/reaction [γ^{32}P]ATP for 15 minutes at room temperature. The proteins were then separated on a 12 % SDS-polyacrylamide gel. The gel was stained with Coomassie Blue, and then dried and exposed for autoradiography.

Results and Discussion

Identification of the HRG-stimulated, 18 kDa nuclear GTP-binding activity as the 20 kDa subunit of the nuclear cap binding complex (CBC). One of the major efforts of the original proposal was to determine the molecular identity of the 18 kDa GTP-binding activity, p18. Our strategy for making an identification was to use classical chromatography techniques to purify sufficient quantities of this activity so that the identity could be revealed using microsequencing analysis. Bovine retina was chosen as a starting source for two primary reasons: the ease of working with this particular tissue and a high observable p18 GTP-binding activity. From this tissue source, nuclei were prepared by cell fractionation and lysed to release the soluble p18 activity. This activity was then separated from the majority of the contaminating low molecular weight proteins by a series of chromatographic procedures: ammonium sulfate precipitation, gel filtration chromatography, anion exchange, and hydroxyapatite chromatography. These steps resulted in a significant enrichment of the p18 GTP-binding activity. Additionally, we began to resolve an 80 kDa protein band (as assessed by Silver staining) which appeared to copurify with the GTP-binding activity through the four steps outline above. However, we had great difficulty purifying sufficient amounts of the protein corresponding to the 18 kDa GTP-binding activity, suggesting that p18 was a low abundancy protein. This was true even when extremely large starting sources (2000 bovine retina) were used.

Simultaneously, we employed a computer based approach to search the data base for putative low molecular weight GTP-binding proteins. We believed this type of approach might be fruitful since we had determined that this activity was present in the yeast *S. cerivisaie*, and the genome for this organism has been sequenced in its entirety. This search failed to yield a GTP-binding protein with characteristics similar to p18. However, it did reveal an 18 kDa nuclear cap-binding protein, CBP20, which had many biochemical features in common with the GTP-binding activity. Besides a similar molecular weight and cellular location, CBP20 formed a stable heterodimer with an 80 kDa protein, CBP80, reminiscent of the copurification of p18 with an 80 kDa protein. Additionally, complex formation between the two subunits is required for the RNA-cap binding activity. Our preliminary studies had suggested that p18 was active to bind GTP within a 100 kDa complex (as assessed by gel filtration analysis), but could not support nucleotide binding as a monomer. Finally, CBP20 RNA-binding could be assayed using a UV-crosslinking approach similar to the one employed in our study. This is significant as this crosslinking approach is not effective for all proteins. The substrate of the CBC is the m^7GpppN-cap structure on RNAs transcribed by RNA polymerase II. Effectively, this structure is identical to GTP with the addition of a methyl group attached to the 7' position of the guanosine ring. Therefore, it was possible that GTP could be used to read out an RNA cap-binding event.

To exam the possibility that p18 was the CBP20 protein, we contacted the lab of Iain Mattaj at the EMBL in Heidelberg, Germany. This lab had been responsible for identifying the CBP20 and CBP80 proteins as nuclear receptors for RNA-cap structures, and further demonstrating the role of these proteins in cap-dependent RNA splicing and in

the export of U snRNAs (14, 16). In collaboration with the Mattaj lab, we examined the possibility that p18 was CBP20 by Western blotting analysis. This analysis demonstrated the copurification and enrichment of CBP20 and CBP80 with the 18 kDa GTP-binding activity. While this result was very suggestive, we could not dismiss the possibility that CBP20 and CBP80 were copurifying nonspecifically with p18, as this activity had not been purified to homogeneity. In order to conclude that p18 was CBP20, three criteria had to be fulfilled: (1) p18 must associate with CBP80, (2) p18 must demonstrate a higher affinity toward RNA cap analogs than for GTP, and (3) recombinant CBC proteins must be able to bind and be crosslinked to [α^{32}P]GTP, and show a growth factor sensitivity in this binding.

Using p18 from both bovine retina preparations and PC12 cells (one cell line where the regulation of this activity had been previously studied), we determined that p18 had a much higher affinity for cap analogs than for GTP. This was determined using a competition approach where we assayed the ability of cap analogs (m^7GpppG, GpppG or m^7GTP) or GTP to compete with [α^{32}P]GTP for binding to p18 in a UV-crosslinking assay (Figure 1A). The highest affinity cap analog, m^7GpppG competed with [α^{32}P]GTP-binding to p18 approximately one thousand times more effectively than GTP (approximate kD for m7GpppG binding to p18 was 5 nM, while only 5 μM for GTP). This experiment demonstrated p18 was a cap-binding protein. Additionally, we were able to coimmunoprecipitate p18 GTP-binding activity as well as CBP20 protein when we performed an immuoprecipitation of CBP80 using a specific CBP80 antiserum (Figure 1B).

In order to assess the ability of recombinant CBC proteins to bind [α^{32}P]GTP in a UV-crosslinking assay, it was first necessary to clone these proteins and join them with appropriate expression vectors. CBP20 and CBP80 were cloned by PCR, from HeLa cDNA and a human testis cDNA library respectively, using primers designed to match the proteins sequence in the data base. These PCR products were then subcloned into vectors for protein expression in *E. coli* (pGEX-KG) and mammalian cells (pcDNA3). Using recombinant proteins expressed in and purified from *E. coli*, we were able to demonstrate that CBP20 was able to bind [α^{32}P]GTP in the UV-crosslinking assay, and this binding was significantly enhanced by the addition of CBP80. This result verified that like p18, CBC proteins could bind and be UV-crosslinked to [α^{32}P]GTP. Finally, in order to demonstrate the growth factor sensitivity of recombinant CBC proteins, CBP20 (as a hemaglutinin (HA)-tagged construct) was transiently transfected into BHK21 cells. These cells were serum starved and then stimulated with serum to achieve a growth signal. Subsequently, the HA-CBP20 was immunoprecipitated from both cytosolic and nuclear extracts and assayed for [α^{32}P]GTP-binding (Figure 2). The nuclear HA-CBP20 specifically demonstrated an ability to bind to [α^{32}P]GTP, exclusively under stimulated conditions.

Taken together, these experiments have addressed the identity of the HRG-sensitive 18 kDa nuclear GTP-binding activity, p18. We have found that p18 is the 20 kDa subunit of the nuclear cap-binding complex. The implications of this finding are that CBC proteins will bind to capped RNAs in response to growth factors, including HRG. We have verified that both endogenous p18 and recombinant CBP20 (in a transfection system) will bind to capped RNAs in response to stimulus. To our knowledge this is the first demonstration that one outcome of a HRG signal is an RNA-binding event. Given the importance of CBC recognition of cap structures in RNA processing events such as splicing and export, we hypothesized that these events will be susceptible to HRG regulation. We have examine the ability of HRG to promote RNA splicing. Using HeLa cells (a system well adapted to studying splicing), we have demonstrated that the addition of HRG to serum arrested cells greatly enhances the ability of nuclear extracts to promote splicing of a radiolabelled, precursor RNA probe (Figure 3). This result is very significant as it provides a potential mechanism for HRG effects on cell growth regulation. HRG may mediate cell growth by regulating gene expression at the level of RNA processing through

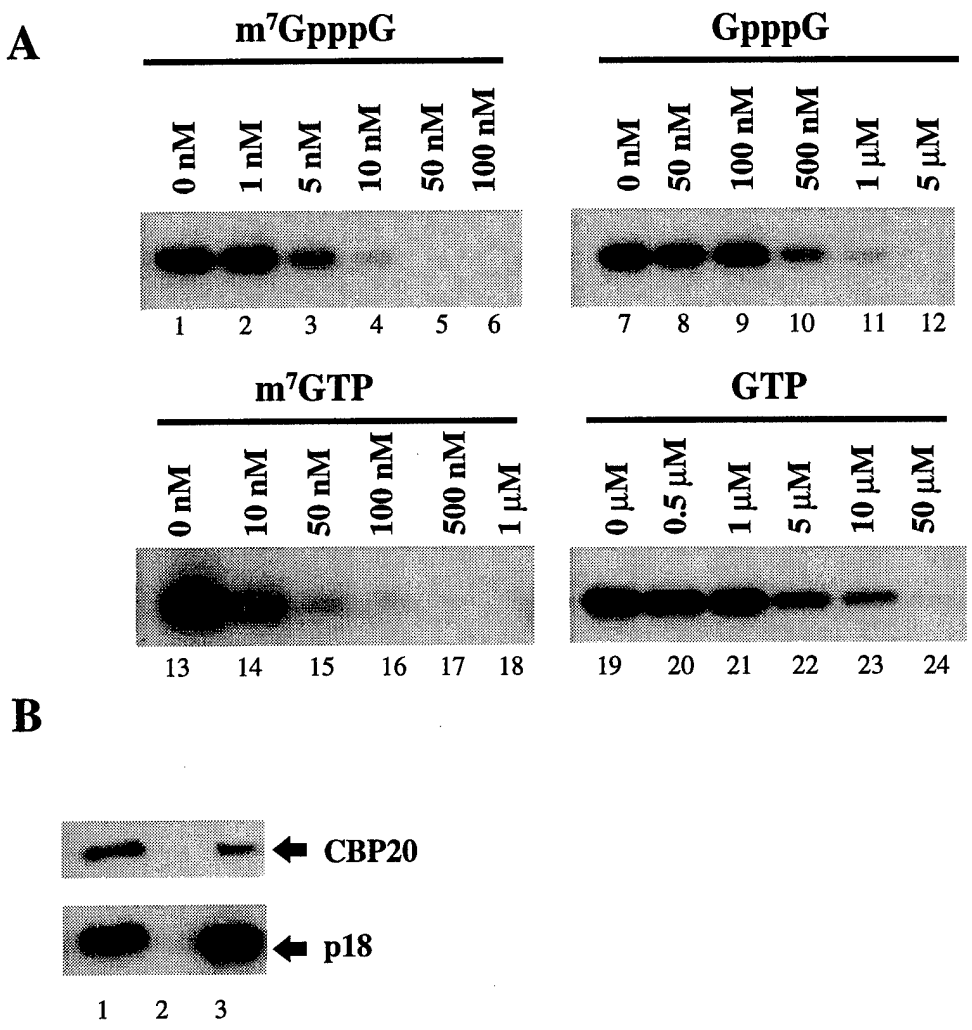

Figure 1- p18 GTP-binding activity corresponds to the nuclear cap-binding protein, CBP20. (A) [α32P]GTP-binding to p18 from PC12 cells is blocked by the addition of RNA cap analogs. CBP80 was immunoprecipitated from asynchronously growing PC12 cells as described in (B). The immunoprecipitates were then assayed for [α32P]GTP-binding to p18 in the presence of: m^7GpppG (0 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, lanes 1-6), GpppG (0 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM, lanes 7-12), m^7GTP (0 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, lanes 13-18), and GTP (0 mM, 0.5 µM, 1 µM, 5 µM, 10 µM, 50 µM, lanes 19-24). Following crosslinking, proteins were separated by 15% SDS-PAGE and the gel was dried and autoradiography was performed. (B) p18 [α32P]GTP-binding activity co-immunoprecipitates with CBP80 from PC-12 cells. Nuclear extracts were prepared from PC-12 cells growing asynchronously in culture. Two hundred micrograms of lysate were then immunoprecipitated with either 5 µl of preimmune serum (lane 2) or with 5 µl of aCBP80 antiserum (lane 3). The immunoprecipitates (resuspended in 30 µl of UV-crosslinking buffer), or 100 µg of protein from the nuclear extract (lane 1) were then assayed for [α32P]GTP-binding by UV crosslinking (lower panel). Endogenous CBP20 protein was detected by Western blotting using a specific CBP20 antiserum (upper panel).

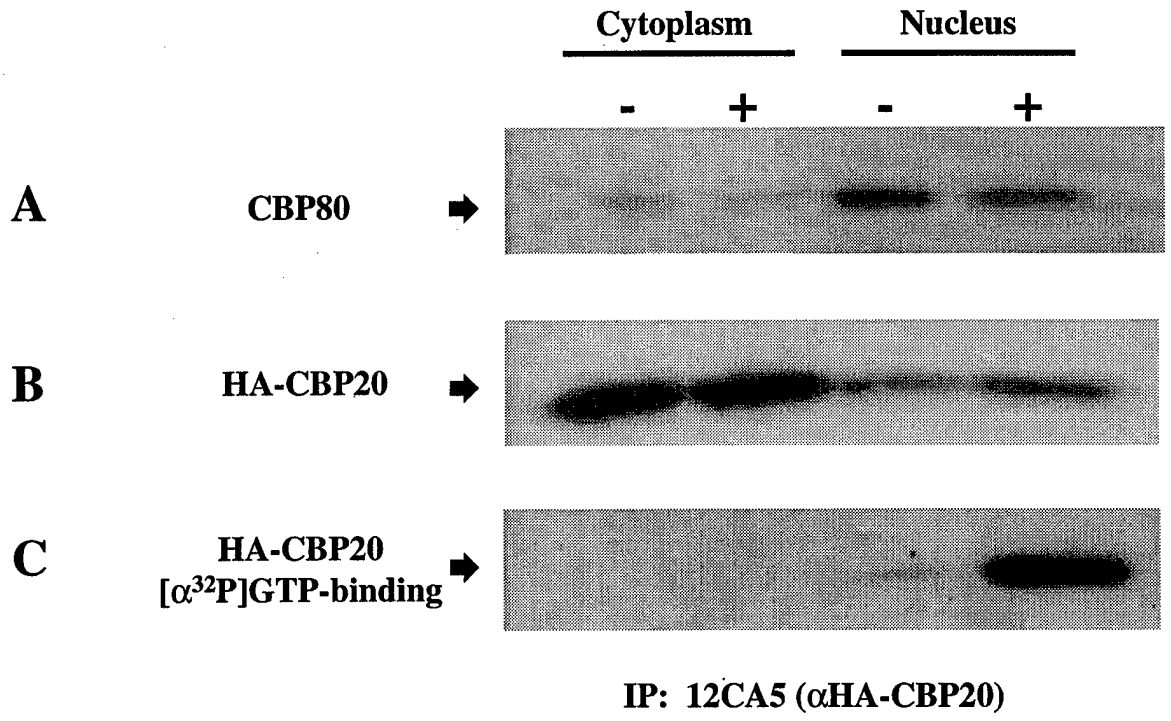

Figure 2. Serum-dependent binding of [a32P]GTP to recombinant CBP20 expressed in BHK21 cells. Human CBP20 was cloned by PCR from HeLa cell cDNA and then subcloned into the mammalian expression vector, $pcDNA_3$, to express a hemaglutinin (HA)-tagged form of the protein. BHK21 cells were transiently transfected with HA-CBP20 (using 8 μg DNA per 100 mm plate of BHK21 cells). The transfected cells were serum-starved for 40 hours (-) and then stimulated with 25% fetal bovine serum (+) for 1.5 hours. HA-CBP20 was immunoprecipitated from cytosolic or nuclear lysates using the 12CA5 monoclonal antibody. Immunoprecipitates were then assayed for [α32P]GTP binding by UV-crosslinking. Proteins were separated by 15% SDS-PAGE and transferred to immobilon for Western blot analysis and auto-radiography. Panel A shows the CBP80 protein co-immunoprecipitating with HA-CBP20 primarily from the nuclear lysates as detected by Western blotting using a CBP80 anti-serum. Panel B is a Western blot using the 12CA5 antibody to detect the immunoprecipitated HA-CBP20 from cytosolic and nuclear lysates. The [α32P]GTP-binding activity corresponding to the immunoprecipitated HA-CBP20 is shown in Panel C.

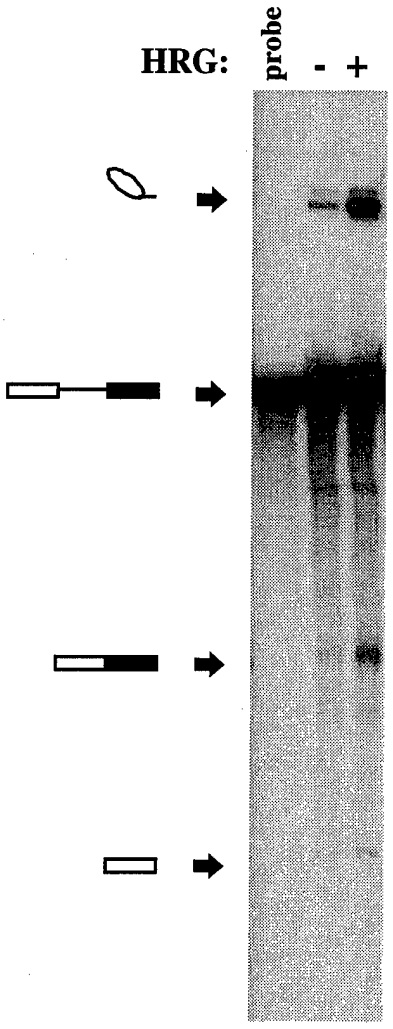

Figure 3. RNA splicing is initiated by HRG-stimulated signal transduction pathways to the nucleus. Splicing extracts were prepared from HeLa cells which were either serum-starved, or starved and then stimulated with HRG (100 nM) for 24 hours. These lysates were then assayed for their ability to support splicingof an m^7GpppG-capped RNA probe. The mature splice products and intermediates of the splicing reaction are indicated diagramatically on the left.

an activation of the CBC to bind capped RNA substrates. In breast cancers where ErbB family members are overexpressed, constitutive activation of HRG signaling pathways and the CBC proteins may result in deregulated and aberrant processing of RNA transcripts, thus impact significantly upon proper gene expression.

Characterization of signal transduction pathways resulting in the activation of the CBC. More recently, with the identity of p18 as CBP20 established, we have been in position to begin the characterization of heregulin signaling pathways to the nucleus that activate this novel endpoint. These studies were initiated by first looking at CBP20 activity levels in various mammary carcinomas (Figure 4). Interestingly, cell lines overexpressing ErbB2, ErbB3 and ErbB4 receptors, such as SKBR3, showed very high CBP20 activity, even under conditions of serum starvation. The levels could not be increased with HRG treatment, suggesting the overexpression of these receptors was sufficient for full activation of CBP20. Another cell line, 231, which does not have these receptors but highly overexpresses the EGF receptor, has much lower CBP20 basal activity. Deregulated CBC activity may therefore be more relevant in cancers utilizing the ErbB2, ErbB3, and ErbB4 receptors than in cancers typified by overexpression of the EGF receptor.

In the original proposal, we wished to examine the possibility that low molecular weight GTP-binding proteins might be functioning downstream of receptor activation in pathways leading to the CBC. The Ras protein is one GTP-binding protein classically described in the literature for its role in receptor tyrosine kinase signaling pathways and thus, was one likely candidate protein to be involved in signaling to the CBC. Alternatively, we were interested in the idea that members of the Rho subfamily of GTP-binding proteins, Cdc42 and Rac, could be involved since the CBC appeared to be activated in response to conditions of cellular stress (e.g. UV irradiation) as well as by growth factors. Cdc42 and Rac are known to be positioned upstream from the stress activated MAP kinases, c-Jun kinase (JNK1) and p38 (28-31). To examine the ability of Cdc42 or Ras to activate the CBC, constitutively active forms of these proteins (either L61 Cdc42, L28 Cdc42, or V12 Ras) or wild type Cdc42 were transiently transfected into either the breast cancer cell line SKBR3 or NIH 3T3 cells. These cells were consequently serum starved, and nuclear lysates prepared from these cells were assayed for CBC activation using the [α^{32}P]GTP UV-crosslinking assay (Figure 5). Neither lysates from vector controls, nor from wild type Cdc42 transfection demonstrated significant CBC activity. However, both activated forms of Cdc42 and Ras could enhance the CBC activation, circumventing the requirement of growth factors. Such experiments suggest that multiple GTP-binding protein pathways may lead to the nucleus and result in a CBC response.

Additionally, we examined the ability of the drug, rapamycin, to block activation of the CBC in the presence of activated Cdc42 or Ras. Rapamycin is a small molecule inhibitor which causes cell cycle arrest in G1/S by targeting a protein fundamental in translational signaling pathways, FRAP (FKBP-rapamycin associated protein) (32, 33). The ability of rapamycin to block activation of the CBC would implicate the input of yet another signaling pathway, the translational signaling pathway mentioned above. NIH 3T3 cells were transiently transfected with either L28 Cdc42 or V12 Ras, serum starved, and thirty minutes before harvest, rapamycin was added to the cells. When nuclear lysates from these cells were assayed for CBC activation, we found that rapamycin blocked activation of the CBC by either activated forms of Cdc42 or Ras. Thus, translational signaling pathways feed into the CBC somewhere downstream of either the Cdc42 or Ras GTP-binding proteins. This is particularly interesting both because it points to a high level of regulation of the CBC (as demonstrated by the participation of multiple signaling pathways in its regulation) and because it suggests that their might exist coordinate regulation between CBC-dependent RNA processing in the nucleus, and translation initiation in the cytoplasm.

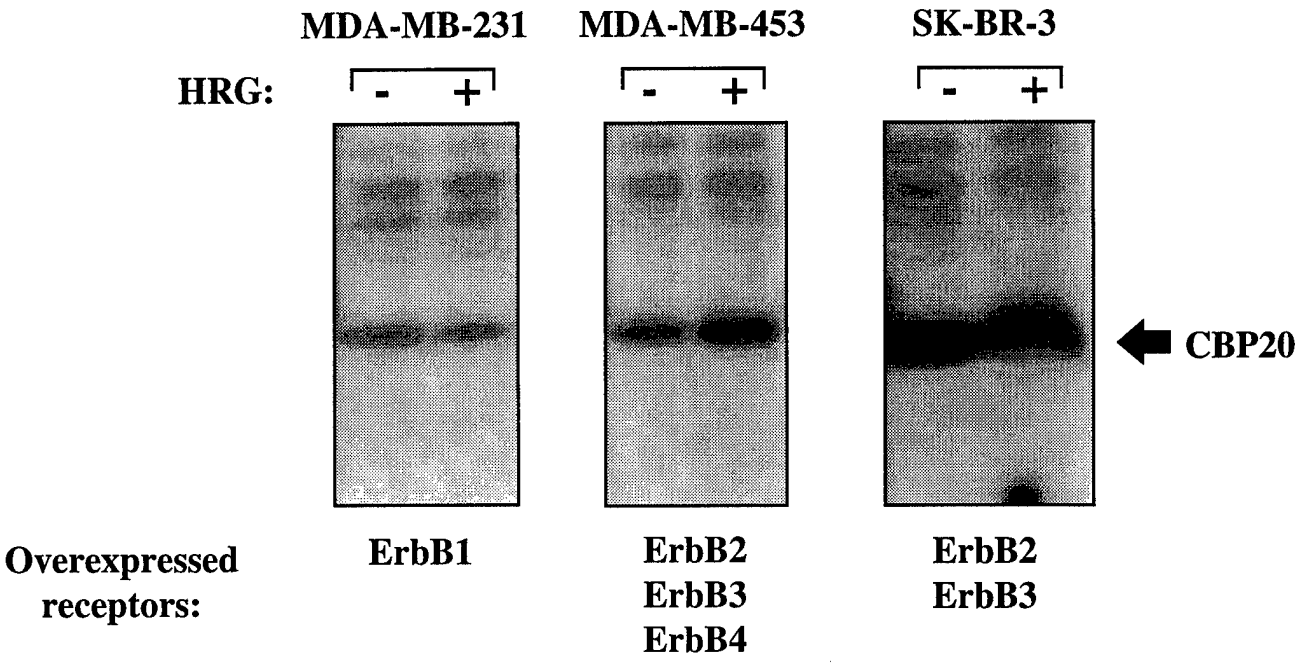

Figure 4. CBP20 activity levels in different mammary carcinoma cell lines. Different mammary carcinoma cell lines (MDA-MB-231, MDA-MB-453 and SK-BR-3) were serum starved and then stimulated with 100 nM HRG for 1 hour. Nuclear lysates were then prepared from these cells and assayed for CBP20 activity using a photoaffinity labeling assay with [α^{32}P]GTP. Proteins were then separated by SDS-PAGE, and the resulting gel was dried and autoradiogrammed.3

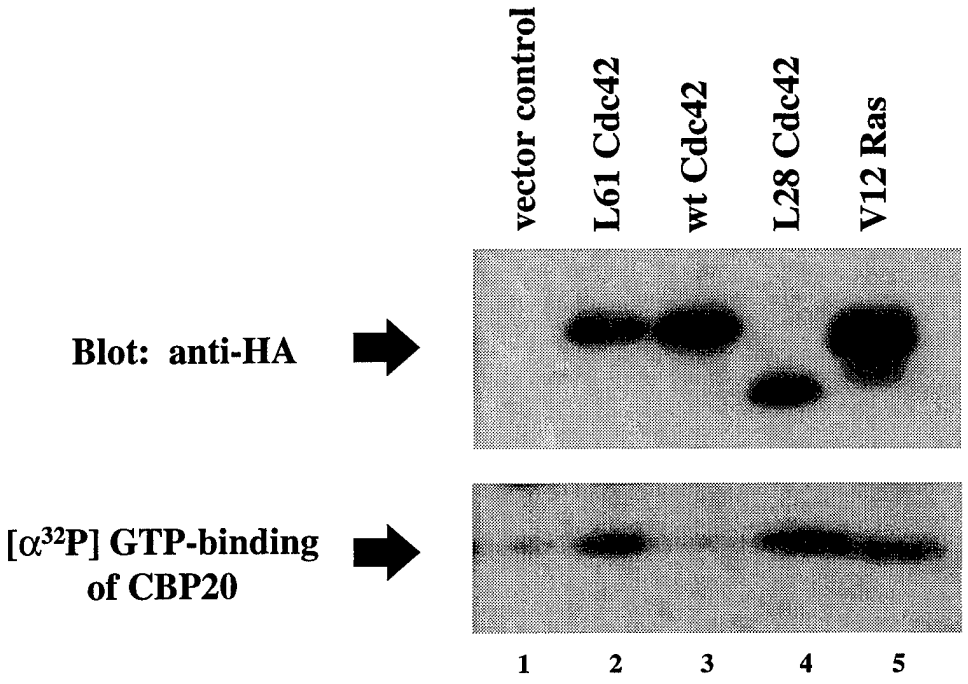

Figure 5. CBP20 is stimulated by activated forms of Cdc42 and Ras. NIH 3T3 cells were transiently transfected with constitutively active forms of Cdc42 (L61, lane 2 or L28, lane 4), wild type Cdc42 (lane 3) and constitutively active Ras (V12, lane 5). 24 hours before harvesting, the cells were switched to serum free medium. The cells were harvested and cytoplasmic and nuclear lysates were prepare. 30 µgs of cytoplasmic lysate was used to check the protein expression level of the transfected protein by Western blotting using an antibody directed against the HA-tag on the proteins (upper panel). 30 µgs of nuclear lysate was used to assay the activation of CBP20 in a photoaffinity labeling assay using [α^{32}P]GTP (lower panel).

Finally, we are now pursuing the possibility that one Cdc42 target in particular, the p70 S6 kinase may be responsible for exerting both the Cdc42 and rapamycin effects on the CBC. The p70 S6 kinase has long been recognized as a mitogen-sensitive kinase whose activation leads to the increased phosphorylation of the ribosomal S6 protein, which in turn has a positive effect on protein translation rates (34). The activity of this kinase is extremely sensitive to the drug rapamycin (35-37), and additionally it has been shown to be activated by Cdc42. It seems plausible, therefore that this protein may be involved in transduction events to the CBC downtstream of Cdc42 and FRAP, and might in fact phosphorylate the CBC directly. Thus far, we have examined the ability of a partially purified S6 kinase enzyme to phosphorylate the CBC *in vitro*. We find that S6 kinase is highly active in phosphorylating both the CBP80 and CBP20 subunits of the CBC, but cannot phosphorylate CBP20 alone. We have further mapped the site of this phosphorylation to the extreme N-terminal 13 amino acids which contain a polybasic nuclear localization sequence. CBP80 mutants lacking these amino acids do not get phosphorylated by S6 kinase. Interestingly, CBP20 when complexed to this mutant CBP80 cannot be phosphorylated either. This data leads us to the very attractive possibility that S6 kinase may phosphorylate the CBC *in vivo*, and this phosphorylation, in turn, may result in a CBC RNA-binding event. Future studies will address the *in vivo* phosphorylation of CBP80, and the continued involvement of the S6 kinase.

Recommendations in relation to the Statement of Work

We believe we have made substantial progress in performing the Tasks outlined in the Statement of Work in our original proposal. Task 2, the purification and molecular cloning is complete in its entirety. We have identified p18 as the CBP20 subunit of the CBC, and have shown that the CBC is activated to bind capped RNAs in response to growth factors such as HRG. Both CBP20 and CBP80 have been cloned by PCR, and we have developed systems for the expressions of these proteins in *E. coli*, *Spodoptera frugiperda*, and mammalian cells. Additionally, we have made significant in roads into accomplishing Task 1. This task was to develop cell systems for examining the involvement of Cdc42 and related signaling molecules in the activation of p18. As described above, we have found that both Cdc42 and Ras GTP-binding proteins are able to activate the CBC in NIH 3T3 and SKBR3 cells. The original task involved first using an NIH 3T3 cell line in which ErbB3 had been stabley transfected, and then verifying our findings in breast cancer cells. Our preliminary results in SKBR3 cells suggest that there is no need to work in this intermediate model 3T3 cell system, and at this point our work can be performed directly in breast cancer cells such as SKBR3. We plan to expand upon these findings by determining which Cdc42 effector molecules mediate signals to the CBC. In particular we will pursue the possibility that this signal is propagated through the p70 S6 kinase. In the event that p70 S6 kinase is not involved, we will explore the roles of other Cdc42 effectors such as the PAK kinase, or a newly identified effector protein, COOL, in CBC activation.

The third Task is more problematic as proposed in its original form. This task was to perform biochemical studies of the regulation of p18, with two specific proposals: examine a direct interaction between the RCC1 protein and p18, and purification of a guanine nucleotide exchange factor (GEF) for p18. Our original studies in a temperature sensitive cell line, tsBN2, revealed that functional loss of the RCC1 protein resulted in a substantial increase in p18 activity. Additionally we had observed p18 GTP-binding activity coimmunoprecipitating with the RCC1 protein. This led us to propose that RCC1 might bind to p18 and inhibit its activation. Upon learning the identity of p18, we examined the ability of CBP20 and CBP80 to coimmunoprecipitate with RCC1. Using Western blotting techniques, we have not been able to confirm this association, and no longer feel that this is a viable avenue to pursue.

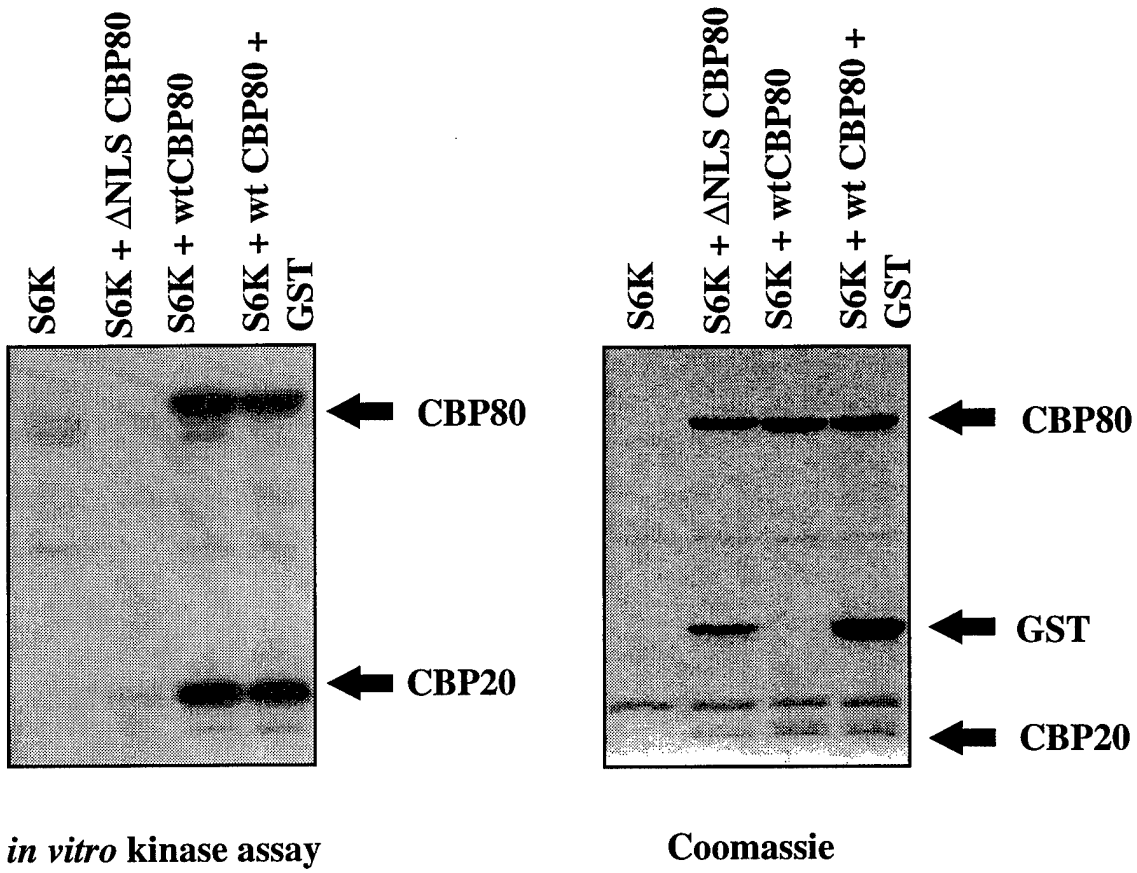

Figure 6. S6 kinase can phosphorylate the CBC *in vitro*, but not CBC containing a ΔNLS mutant of CBP80. In vitro kinase experiements were performed with partially purified S6 kinase and either wtCBC or CBC containing ΔNLS CBP80. The right hand panel is a coomassie stain showing the protein levels, and the left hand panel is the corresponding autoradiogram.

Our rational for exploring the possibility of a putative GEF for the CBC was based on chromatography studies which demonstrated that p18 GTP-binding activity eluted from a gel filtration column as part of a high molecular weight complex (100 kDa), and complex formation was required for binding (i.e. monomeric p18 would not bind GTP). We reasoned that some component of this complex, namely a GEF, must be conferring activity on to p18. We now know that this entity is CBP80, and that complex formation between CBP20 and CBP80 is necessary for substrate binding. In this regard, we have identified the putative GEF as CBP80. Further studies will address the mechanism of activation of the CBC in response to growth factor. One attractive possibility is that the CBC (on one or both subunits) is subject to a HRG-dependent phosphorylation which culminates in an activation of the CBC to bind capped-RNA. This hypothesis can be tested by performing metabolic labeling of cells to detect an *in vivo* phosphorylation of the CBC in response to growth factor treatment. Additionally, we will continue to look for growth factor-induced binding partners of the CBC, in the event they might influence CBC binding to RNA. Again, we will use a metabolic labeling approach, where cells are labeled with ^{35}S methionine. After labeling, the CBC will be immunoprecipitated, and we will look for ^{35}S radiolabeled bands which associate with the CBC in a growth factor dependent manner. If we find specific proteins, we will attempt to purify them using a CBC affinity column in an attempt to obtain amino acid sequence information on these binding proteins. We anticipate that these sorts of approaches will begin to address the regulation of the CBC by growth factors.

CONCLUSIONS

As members of the ErbB family of receptor tyrosine kinases have been found to be overexpressed in a number of human mammary carcinomas, and HRG is the ligand for ErbB3 and ErbB4 in conjunction with Neu/ErbB2, we believe that trying to understand the signal response elicited from the interaction of this ligand with its receptors could shed light on the molecular basis for how the overexpression of these receptors leads to oncogenesis. In particular we were interested in looking for nuclear endpoints for HRG which might suggest functional consequences of the HRG signal in the nucleus. The original proposal described our finding of an 18 kDa nuclear protein, p18, which could be crosslinked to GTP when cells were exposed to growth factors, including HRG. We believed that p18 could represent a new member of the family of low molecular weight GTP-binding proteins which includes Ras. It was our goal, therefore, to identify this HRG-sensitive nuclear GTP-binding protein, and then investigate the series of events which culminate in its activation.

As demonstrated in this report, we have identified p18 as the 20 kDa subunit of the nuclear cap binding complex, CBP20. Although we found p18 to be truly a capped RNA-binding protein rather that a GTP-binding protein, we are extremely excited about the implications of this finding. Previous work by others (14, 16, 22) has demonstrated that the recognition and binding of the CBC to the cap structure on certain RNAs is critical for efficient, cap-dependent pre-mRNA splicing and export of U snRNAs. We have now shown that this recognition of substrate RNA by the CBC is induced by growth factor-generated signals. By extension, our findings suggest that cellular functions which involve the CBC, such as splicing and export, will be subject to extracellular regulation. Indeed we have demonstrated that the HRG stimulation of serum-arrested cells results in nuclear extracts which are greatly enhanced in their ability to support slicing *in vitro*. It is plausible that the CBC is one HRG target whose activation is critical in order for splicing to commence. These data also suggest mechanisms by which HRG influences cell growth-by regulating gene expression at the level of RNA processing.

In addition to identifying CBP20 as an important new, nuclear target for HRG, we have begun to elucidate some of the signaling components which relay the signal from the

cell surface receptors to the nucleus. Interestingly, we have found that multiple signaling pathways are likely to converge at the CBC to result in its activation. The low molecular weight GTP-binding proteins Cdc42 and Ras can activate the CBC, circumventing the need for growth factors. The activation by Cdc42 and Ras can be blocked by the drug rapamycin, which implicates the additional involvement of the FRAP translational signaling pathway. Clearly the regulation of the CBC is going to be very complicated. However, having a nuclear readout for HRG, namely the CBC, is going to continue to be very useful in dissecting the molecular components of HRG signaling pathways. The more information which is garnered in this regard, the greater the likelihood of identifying points in the signaling pathway where therapeutic intervention may be feasible. Our continued efforts will be directed toward further elucidation of these signaling pathways as well as toward the study of the molecular mechanisms of CBC activation in response to HRG.

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