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13. ABSTRACT ***(Maximum 200 Words)***
This project involves structural studies of an interesting new nuclear target for the EGF receptor, and the related Neu/ErbB2 tyrosine kinase, named the CBC for RNA-capped binding protein complex. The CBC consists of two subunits, CBP20 (Mr ~20 kDa) and CBP80 (Mr ~80 kDa), and undegoes a growth factor (EGF, heregulin)-dependent binding of RNAs transcribed by RNA polymerase II at a 5′ cap structure that consists of a guanosine residue methylated at the N7 position. This represents a first key step in the cap-dependent splicing of precursor messenger RNA (mRNA) and in the nucleocytoplasmic transport of U snRNAs which are necessary for the formation of spliceosome complexes. While CBC activity is stimulated by EGF, it is most strongly stimulated by heregulin, an activator of the Neu/ErbB2 tyrosine kinase, and appears to be constitutive in breast cancer cells where Neu/ErbB2 expression is high. Thus, we believe that the CBC represents an exciting nuclear target for receptor tyrosine kinases, linking growth factor-dependent gene expression to RNA processing. We have obtained (2.35 Å) X-ray diffraction data for the CBC. Further experiments using heavy atom derivatives are being conducted at this time and will provide us with enough information to be able to solve the structure of the CBC.

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INTRODUCTION

The original intention of my predoctoral studies was to perform a structural analysis of the interactions of the c-Cbl protein with the EGF receptor. However, before I was able to initiate this work, a structural study of the c-Cbl protein was reported. Thus, I set out to re-design my research project so that it would maintain a structure-function emphasis on a signaling system that is operating in mammary cells and potentially relevant to breast cancer. After a good deal of consideration, I decided to join Dr. Richard Cerione's laboratory at Cornell because they were about to undertake a comprehensive structure-function analysis of an mRNA binding protein that is activated in response to growth factors (in particular, heregulin) and which plays an essential role in RNA processing at the level of splicing and certain aspects of nucleocytoplasmic transport. This mRNA binding protein is the RNA capped-binding protein complex (CBC) which consists of a 20 kDa subunit (CBP20) and an 80 kDa subunit (CBP80) and undergoes a growth factor-dependent binding of RNAs transcribed by RNA polymerase II at a 5' cap structure that contains a guanosine residue methylated at the N7 position. It has been proposed that the regulation of CBC activity by growth factors like heregulin may serve as a key checkpoint for cell growth by preventing the processing of inappropriate transcripts.

A letter that was sent to the US Army Medical Research and Material Command from Dr. Cerione, together with a proposed change in the statement of work, is included in the Appendix. Below, I will present background on the CBC and its potential involvement in growth factor-coupled signaling and cell growth regulation, as well as the progress that I have made during the past several months on the structural determination of the CBC by X ray crystallography.

Background: Growth factor signaling to the CBC and its relevance to different aspects of RNA processing-

Normal and malignant human mammary epithelial cells are able to synthesize and to respond to various different, locally acting growth factors through specific receptors. Among these are the type 1 family of growth factor receptors, which consist of the epidermal growth factor receptor, ErbB-2/Neu, ErbB-3, and ErbB-4 (1-6). They are required for normal mammary development and lactation and are aberrantly expressed in approximately 40% of breast carcinomas. Indeed, in human breast cancer cases the prognosis of a patient is inversely correlated with the overexpression and/or amplification of this receptor family. The physiological ligand for these receptors has been shown to be heregulin (7-9). Interaction of heregulin with ErbB3 induces a heterodimerization between ErbB2 and ErbB3, which results in the transphosphorylation and activation of the ErbB2 tyrosine kinase. Phosphorylation of this receptor initiates signaling cascades which in turn can impact upon cell function, growth and division.

Wilson *et al.* (16) have identified a novel nuclear target for heregulin signaling which responds to the growth factor treatment of cells with an increased ability to incorporate labeled GTP. They identified this target as the 20 kDa subunit of the nuclear cap binding complex (CBC) and demonstrated that the CBC is stimulated to bind to capped RNAs in response to heregulin. Based on these observations, Wilson *et al* suggested that heregulin can impact upon cell growth by modulating gene expression at the level of RNA processing via the CBC. They further suggested that in a situation where the heregulin signal is constitutive, the active CBC could affect gene expression by amplifying the rate of RNA processing, and thus contribute to unregulated cell growth and division.

The CBC is comprised of a stable heterodimer between a 20 kDa subunit and an 80 kDa subunit (designated as CBP20 and CBP80, respectively). The CBC by recognizing the 7-methylguanosine-cap structure on mRNA and UsnRNA has been shown to be an important factor involved in multiple levels of RNA metabolism including RNA splicing, export, 3' processing and mRNA translation initiation *in vitro* (10-15,17).

The export of UsnRNA has been coupled to nuclear protein import by an association between CBC and importin-α (18). Importin-α is one subunit of the importin heterodimer (comprised of importin-α and importin-β) which is involved in the recognition and import of

classical nuclear localization sequence (NLS)-containing proteins. The α-subunit recognizes and binds the NLS while the β-subunit (which is primarily cytosolic) is responsible for docking at the nuclear pore. CBC binds to importin-α through a NLS present within the amino terminal region of the CBP-80 subunit. This complex can support capped mRNA binding *in vitro* and UsnRNA *in vivo* and is localized mainly in the nucleus. Interestingly, binding of importin-β to the CBC releases the bound mRNA.

Ribosome binding to the 5' end of cellular mRNAs involves interactions mediated by a cytosolic mRNA cap-binding protein complex designated the eukaryotic initiation factor 4F (eIF4F). eIF4F consists of the eukaryotic initiation factors eIF4E, eIF4G and eIF4A (19). The largest eIF4F component (170 kDa), eIF4G, has binding sites for:

- eIF4E (aa 382-486) which tethers the complex to the mRNA cap (20).
- eIF3 which is believed to tether the 40S ribosomal subunit to eIF4F (21).
- eIF4A (aa 542-883) which catalyzes ATP-dependent RNA helicase activity that may promote ribosomal scanning along structured mRNA.
- the poly (A) binding protein PAB (aa 188-299) which may promote interactions between the 3' and 5' ends of the mRNA (22) and,
- CBC (aa 486-592); interestingly CBC binding is reduced by eIF4E even though eIF4E binds at a different site on eIF4G (17).

The eIF4E-eIF4G interaction is of central importance for cap-dependent mRNA translational initiation, and can be blocked by small regulatory proteins that bind to eIF4E (the 4E binding proteins 4E-BPs) (23). Inhibition by 4E-BPs is inversely dependent on the phosphorylation state of these proteins (24), whereby the hypophosphorylated forms bind most tightly to eIF4E (25). Cell transformation caused by overexpression of eIF4E is reversed by concomitant overexpression of the 4E-BP gene. Biochemical studies have determined that the binding sites for eIF4G and the 4E-BPs overlap within a surface of eIF4E (27). Moreover, 4E-BPs are targets of MAP-kinase (Erk, JNK and p38) pathways and FRAP, linking mRNA translational regulation to growth factor regulated signaling pathways.

During the course of our experiments we noticed that binding of importin-α to the CBC significantly increased its affinity for the RNA cap analog (see below). In a recent report, Ptushkina *et al* (28) showed that binding of 4E-BP to eIF4E enhances the binding of capped mRNA, thus trapping eIF4E in inactive complexes. Based on these observations, we can propose a model where the CBC first binds capped mRNA (or UsnRNA), followed by an interaction between importin-α and the CBC, which locks the mRNA in place. The mRNA is then spliced and transported out of the nucleus. In the cytosol, the CBC-importin-α-cap mRNA complex binds to eIF4G with two possible outcomes:

- Translation is initiated by the CBC-eIF4F complex.
- eIF4E binds to eIF4G and importin-β binds to importin-α leading respectively to the displacement of the CBC from eIF4G and the release of cap mRNA.

The apparent symmetry of the interactions between 1) the capped mRNA-bound CBC and importin-α, versus capped mRNA-bound eIF4E and 4E-BP, 2) the CBC and eIF4G versus eIF4E and eIF4G, and 3) the growth factor induced phosphorylation of 4E-BP and CBC, could potentially lead to new insights into the mechanisms underlying the regulation of mRNA translation. The high resolution structure of CBC, alone and in complex with importin-α and eIF4G would provide valuable insights into the mechanistic basis of RNA splicing and translation. This information could be used for the pharmacological manipulation of these growth factor regulated activities and potential treatment of malignancies, particularly breast cancer.

EXPERIMENTAL PROCEDURES

1. Protein Purification

a. CBC

Insect cells expressing CBC are lysed using 0.4% CHAPS in a solution containing 150 mM NaCl, 30 mM Tris pH 8.0, 2 mM DTT and 1 mM sodium azide (Buffer A). Lysates are centrifuged at 40,000 rpm for 50 minutes. The supernatant is loaded on to a Q-sepharose resin and eluted with Buffer A using a NaCl gradient from 150–450 mM. The fraction containing CBC is loaded on to a 7-methyl GTP sepharose (Pharmacia) column and eluted with 100 μM 7-methyl-GTP-G (cap structure analog, New England Biolabs) in Buffer A. The protein is then loaded on to a Q-resource column (Pharmacia) and purified with a 200-500 mM NaCl gradient in Buffer A using an AKTA system (Pharmacia). As a final step, the protein is concentrated to 3 ml and loaded on to a Superdex 200 (26/60 Pharmacia) gel filtration column in Buffer A with no NaCl. SDS-PAGE shows that the complex is >95% pure.

b. CBC-Importin α complex

CBC expressing insect cells are lysed, centrifuged and loaded on to a Q-sepharose column (see above). Protein is eluted with a NaCl gradient and the fractions containing CBC are saved. *E. coli* cells expressing hexahistidine (His)-tagged importin-α are lysed in buffer A minus DTT using a French press system. Lysates are centrifuged at 40,000 rpm and the supernatant is loaded on to a Nickel resin and eluted with 200 mM imidazole. CBC and importin-α fractions are pooled together and are loaded on to a Resource-Q column and are then subjected to gel filtration chromatography (see above).

2. Assays of the exchange of [α^{32}P]GTP for capped RNA

When CBC and importin-α fractions were pooled together and loaded on to the 7-methyl GTP sepharose column, the importin-CBC complex could not be eluted from the column with 100 μM 7-methyl-GTP-G. This made us suspect that importin-α could block the binding of capped mRNA to the CBC. We set out to prove this hypothesis by observing the exchange of [α^{32}P]GTP for capped RNA on the CBC in the presence and absence of importin-α (see below).

RESULTS AND DISCUSSION

1. Structural studies of the CBC:

During the past several months, we have made exciting progress on the three dimensional structural analysis of the CBC. I have succeeded in generating crystals that diffract to better than 2.5 Å. I am currently establishing conditions with heavy atom derivatives to solve the phase problem and I expect to be able to complete the structure of the complex within the period of my fellowship. Additional details are provided below.

a. Crystallization experiments-

CBC crystals have been obtained at 4°C by sitting drop vapor diffusion. As a first step, 2 μl of protein solution (12 mg/ml) were mixed with the same volume of a solution containing 22% PEG400, 100 mM Tris-HCl pH 7.25, 125 mM $MgCl_2$, 10% glycerol and 2 mM DTT, yielding crystals that grew in 2-3 days but diffracted only to 8.0 Å. Refinement of the original crystallization conditions was performed using 10 mM glycine and 3% ethylene glycol. This yielded crystals that diffracted to 2.8 Å. Finally, dehydration experiments using 27% PEG400 was performed for two days followed by treatment with 32% PEG400 for 1 week. This led to crystals diffracting up to 2.3 Å (figure 1).

b. Diffraction experiments and data processing-

All diffraction experiments have been collected using synchrotron radiation at the Cornell High Energy Synchrotron Source (CHESS, Ithaca NY) and at the Advanced Photon Source (APS, Chicago, IL) with both facilities being equipped with a Quantum-4 CCD detector. An oscillation step of 1° has been used throughout. The crystal to detector distance has been varied from 225 to 250 mm. Raw reflection intensities have been reduced with Mosfilm. Crystal unit cell parameters determined from the reflections by these programs were as follows: a=111.03, b=111.03, c=176.42, $\alpha=\beta=90$, $\gamma=120$. The space group was determined to be P6222 using reciprocal lattice characteristics. The crystals contain 2 molecules in the asymmetric unit. Table 1 shows the crystallographic data for CBC. Figure 2 shows the diffraction pattern for CBC.

c. Attacking the phase problem-

To solve the phase problem, CBC crystals have been soaked in crystallization solution containing 0.5 M NaI for 30, 60 or 120 seconds or in 1 M NaBr for 30 or 60 seconds. Seven data sets were collected last weekend at CHESS. Diffraction ranged from 2.5 and 2.9 Å for NaBr to 2.8-3.9 Å for NaI. Further experiments using other heavy metal derivatives will be conducted this coming weekend at CHESS.

2. Exchange of [α^{32}P] GTP for capped RNA:

Based on observations made during our attempts to co-purify and co-crystalize the complex between the CBC and α-importin, we set out to examine whether α-importin might increase the affinity of the CBC for capped RNA. Our working hypothesis is that when α-importin is bound to the CBC it traps the complex in a high affinity state for capped RNA, thus preventing the dissociation of processed (spliced) mRNA and not allowing the binding of a new precursor mRNA molecule. In essence, we would argue that α-importin blocks the exchange of precursor mRNA for spliced mRNA on the CBC. Presumably, a growth factor-dependent signal will convert the CBC into an exchangeable species, perhaps through the binding of β-importin to the CBC-α-importin complex. We have begun to test the first part of this model by examining how α-importin affects the exchange of [α^{32}P]GTP for pre-bound cap analog on CBC. As shown in Figure 3, we have found that α-importin causes a dramatic reduction in exchange activity as read-out by an inhibition of the incorporation of [α^{32}P]GTP. We now plan to examine whether β-importin catalyzes exchange activity and if this in fact is an important aspect of the growth factor-dependent activation of the CBC.

Recommendations in relation to the Statement of Work (see the new Statement of Work and accompanying letter from Dr. Cerione)

Task 1: Development of recombinant expression systems for crystallization trials and biochemical studies of the EGF/heregulin-responsive RNA cap-binding complex. (1-12 months).

Task 1a has been fulfilled; we have developed an expression system using a Baculovirus-insect cell system that expresses CBC (protein expression varies from 10-40 mg per liter of culture).

Task 1b- these experiments were performed in collaboration with Kristin Wilson, a Postdoctoral Research Associate working in the Cerione laboratory. Using [α^{32}P] GTP we were able to demonstrate that importin-α binding to the CBC increases the affinity for capped RNA.

Task 1c has also been accomplished. I have obtained crystals of the CBC in complex with methyl –7 GTP-G. I have refined the crystallization conditions during a period of 8 months. I now have crystals diffracting to 2.3 Å. Data obtained in the diffraction studies of native CBC along with data obtained from heavy metal derivatives should allow me to solve the structure of the CBC in complex with methyl –7 GTP-G.

Task 2: Collect and analyze X-ray diffraction data for the three dimensional structure of the CBC (months 6-24)

Task 2a is finished. I have collected diffraction data at 2.3 Å for native CBC.
Task 2b is in progress. I have collected (last weekend) the first data sets using heavy atom derivatives. I will continue to collect data at CHESS at least 3 times before the end of August and we have scheduled a trip to APS in Chicago at the end of September. I hope to obtain useful data in the following months that will allow me to complete task 3.
I feel very comfortable with my results, as so far my experiments are on schedule.

KEY RESEARCH ACCOMPLISHMENTS

- Binding of importin-α to the CBC increases its affinity for capped RNA.
- Crystallization of the CBC in complex with 7-methyl-GTP-G.
- X-ray diffraction studies of the CBC yielding data to 2.35 Å resolution.

REPORTABLE OUTCOMES

Nothing as yet

CONCLUSIONS

The overexpression of the ErbB2 and ErbB3 receptor tyrosine kinases is an important factor in many breast cancers. Indeed, up to 40% of all breast cancers show an increase in ErbB2 levels (1-6). The physiological ligand for these receptors has been shown to be heregulin (7-9). An interaction between heregulin and ErbB3 induces a heterodimerization between ErbB2 and ErbB3 which results in the activation of ErbB2 and transphosphorylation of ErbB3. The phosphorylation of ErbB3 serves to initiate signaling cascades which in turn can impact upon normal cell function, growth and division. It is presumed that signals from ErbB2 in the absence of ligand contributes to the development of breast cancer.

Wilson et al. (16) in our laboratory have described a novel nuclear target for heregulin signaling, the cap binding complex (CBC) which consist of an 80 kDa subunit (CBP80) and a 20 kDa subunit (CBP20). The CBC by recognizing the 7-methylguanosine-cap structure on mRNA and UsnRNA has been shown to be an important factor involved in multiple levels or RNA metabolism including RNA splicing, export, 3' processing and mRNA translation initiation *in vitro* (10-15,17). These findings suggest that heregulin can impact upon cell growth by modulating gene expression at the level of RNA processing via the CBC and indeed, they show that heregulin can stimulate cap splicing.

During the course of our experiments we noticed that the binding of importin-α to the CBC increases the affinity of CBC for the cap analog. We set out to prove this hypothesis by assaying the exchange of [α^{32}P]GTP for capped RNA. We observed that importin-α, indeed, increases the affinity of CBC for the cap analog. This increased affinity could be important for ensuring that the spliced mRNA remains associated with the CBC during nuclear export. We expect that the binding of capped mRNA to the CBC will be subject to multiple regulatory events (e.g. phosphorylation). Interestingly, Ptushkina *et al* (28) showed that binding of phosphorylated 4E-BP to eIF4E enhances cap binding thus trapping eIF4E in inactive complexes with high affinity for capped mRNA.

We have obtained high-resolution (2.35 Å) X-ray diffraction data for the native CBC. Further experiments using heavy atom derivatives are being conducted at this time and will

provide us with enough information to be able to solve the structure of the CBC. The high-resolution structure of CBC and possibly CBC in complex with importin-α (we have crystals of this complex that need to be refined) would give invaluable information towards understanding the molecular mechanisms at the atomic level underlying CBC function.

The crystal structure of two other cap binding proteins eIF4E (29) and VP39 (30), together with protein mutagenesis experiments and physicochemical analysis of model small molecule compounds, have lead us to a closer understanding of the mechanism of mRNA cap recognition and binding. Enhanced stacking interactions between the electron deficient nucleobase and two electron rich aromatic residues seem to play a dominant role in cap recognition (31). Solving the CBC structure would possibly uncover a novel atomic mechanism for cap binding since its two subunits are necessary for cap RNA binding, whereas eIF4E and VP39 each consist of only a single subunit. This information could be used for the pharmacological manipulation of the growth factor-regulated pathways that impact on RNA processing and thereby provide new strategies for the treatment of malignancies, particularly of breast cancer.

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APPENDIX 1

Table 1. Statistics for data collection of CBC

	CBC native	CBC NaBr	CBC NaI
Beamline	A1 CHESS	A1 CHESS	A1 CHESS
Wavelength (A)	0.9243	0.9243	0.9243
Data Processing	Mosfilm/Scala	Mosfilm/Scala	Mosfilm/Scala
Resolution	2.35 (2.35-2.7)	2.5 (2.5-2.9)	2.9 (2.9-3.8)
Rsym	6.7%	7.5%	7.1%
Completeness	99%	97%	94%
$< I > / < \sigma(I) >$	2.3	2.1	2.7

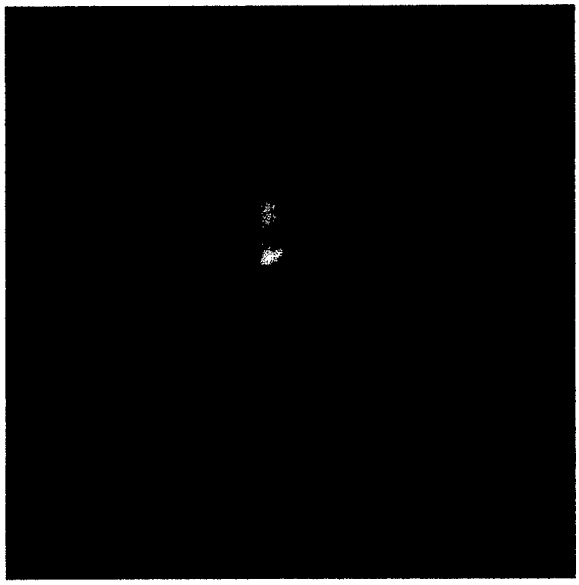

Figure 1. CBC crystal

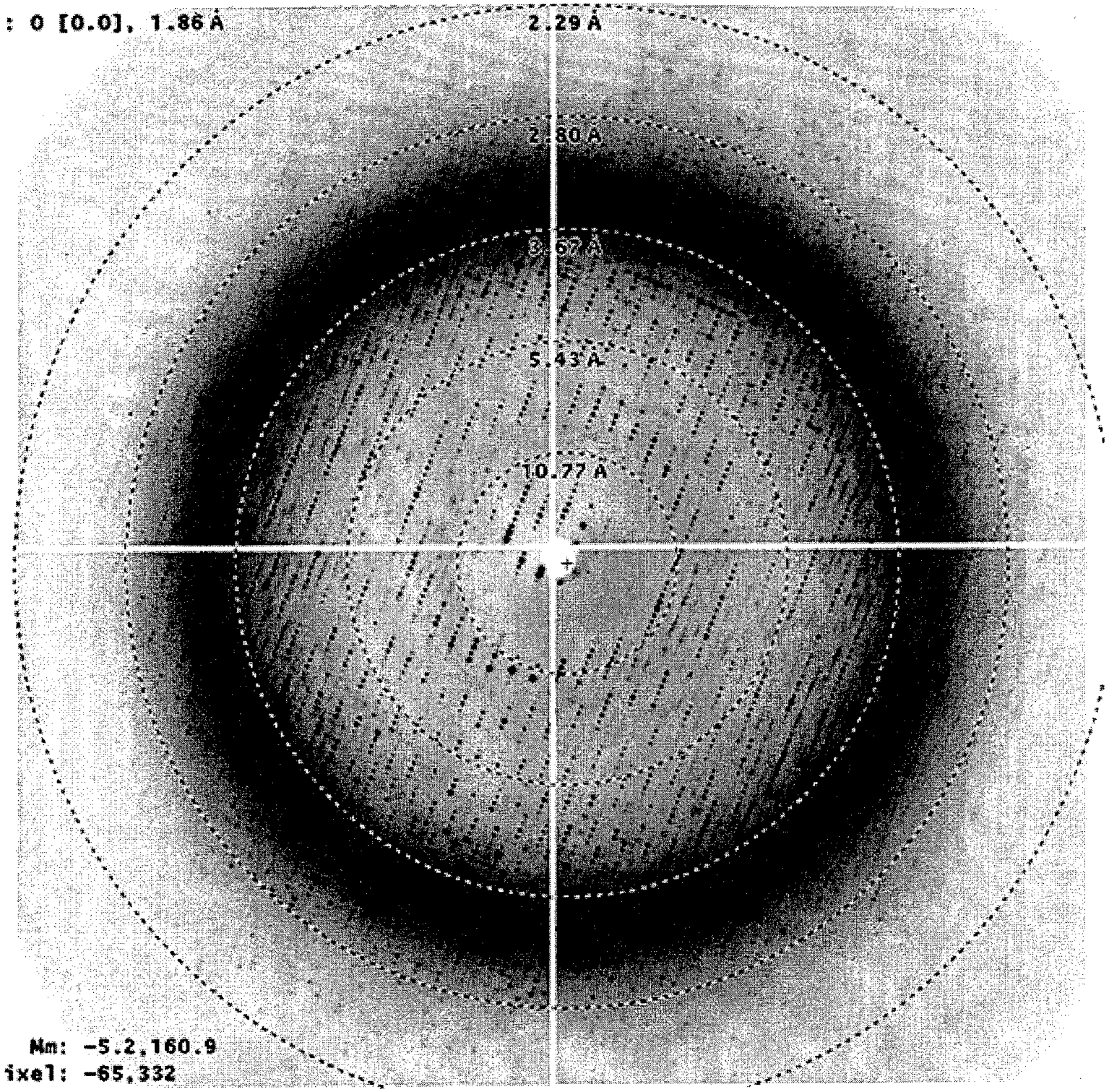

Figure 2. Diffraction pattern of native CBC collected at A1 station at CHESS.

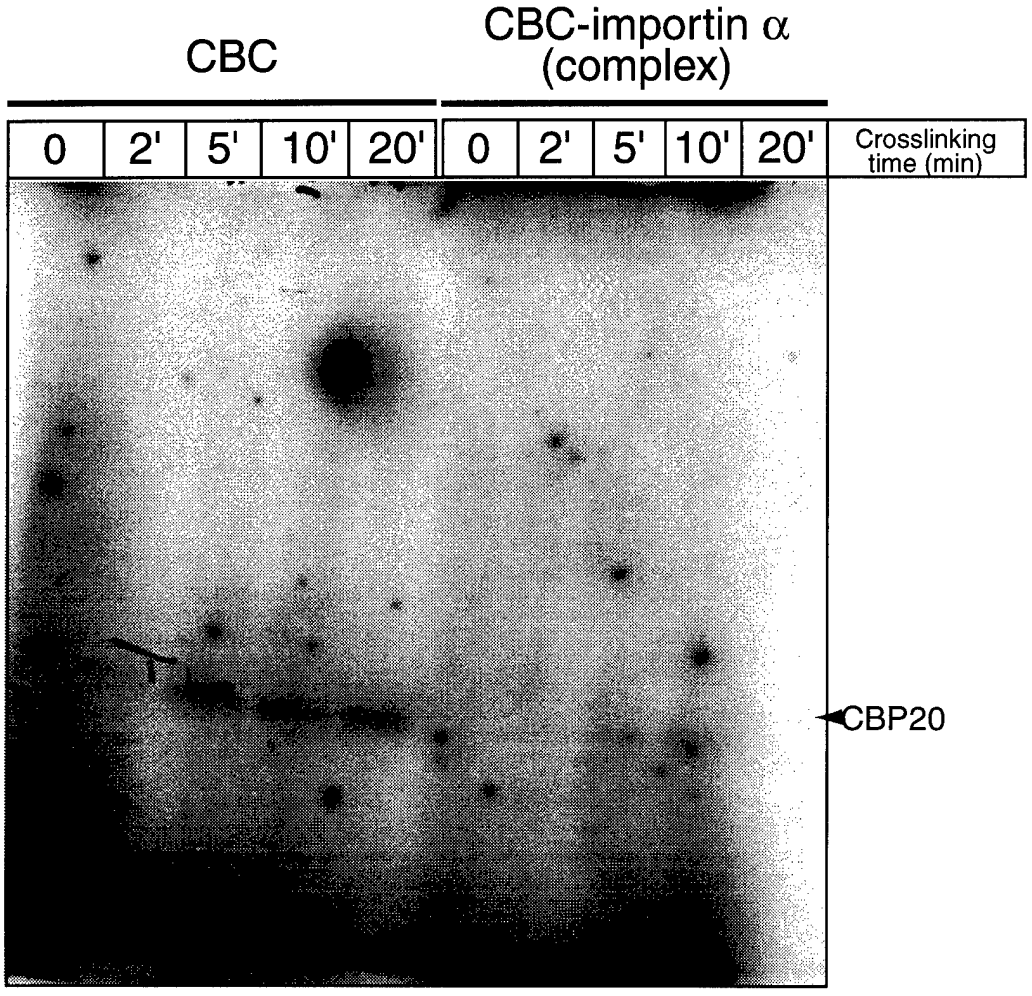

Figure 3 Exchange of [α^{32}P]GTP for capped RNA
Incorporation of [α^{32}P]GTP into the CBC in the absence and presence of importin α. Photoaffinity crosslinking of radiolabeled GTP to the CBP20 subunit was performed as previously described (1b).
CBC = 250 ng; CBC-α-importin complex = 375 ng total protein

Statement of Work

Structural studies of a new nuclear target for EGF receptor tyrosine kinases

Task 1. Development of recombinant expression systems for crystallization trials and biochemical studies of the EGF/heregulin-responsive RNA cap-binding protein complex (CBC).

Months 1-12:

a. Continue to express and purify sufficient amounts of the E. coli-expressed CBP20 subunit and insect cell-expressed CBP80 subunit of the CBC for structure-function studies.
b. Develop assays using radiolabeled GTP and capped RNA analogs to characterize the binding of 7 methyl guanosine caps and related analogs to the CBC. Test the idea that the growth factor-stimulated binding of capped RNA to the CBC is mediated by the nucleocytoplasmic transport proteins, the importins.
c. Continue crystallization trials for the CBC in the presence and absence of RNA cap analogs

Task 2. Collect and analyze X-ray diffraction data for the three dimensional structure of the free CBC

Months 6-24:

a. Obtain diffraction data for the CBC.
b. Develop methods for solving the phase problem using heavy atom derivatives and seleno-methionine based multiple anomalous diffraction (MAD phasing).

Task 3. Comparison of the structures for the CBC in the presence and absence of RNA cap-analogs

Months 12-24:

a. Generate electron density maps for the free CBC and solve its three dimesnional structure
b. Obtain diffraction data and generate an electron density map for the CBC bound to a capped RNA analog, using molecular replacement based on the structure for the free CBC

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July 12, 2000

Commander
US Army Medical Research
and Material Command
1077 Patchel Street
Fort Detrick, MD 21702-5024

Dear Commander:

I am writing with regard to a graduate student Guillermo Calero (M.D.) who is currently being supported by a predoctoral fellowship from the Department of Defense Breast Cancer program, entitled "Structural studies of c-Cbl interaction with the EGF receptor." Because of some circumstances that have developed over the past few months, I am requesting that Dr. Calero be allowed to transfer to my research laboratory to pursue a new, although related, project. This project involves structural studies of an interesting new nuclear target for the EGF receptor, and the related Neu/ErbB2 tyrosine kinase, named the CBC for RNA-capped binding protein complex. The CBC consists of two subunits, CBP20 (Mr ~20 kDa) and CBP80 (Mr ~80 kDa), and undegoes a growth factor (EGF, heregulin)-dependent binding of RNAs transcribed by RNA polymerase II at a 5' cap structure that consists of a guanosine residue methylated at the N7 position. This represents a first key step in the cap-dependent splicing of precursor messenger RNA (mRNA) and in the nucleocytoplasmic transport of U snRNAs which are necessary for the formation of spliceosome complexes. While CBC activity is stimulated by EGF, it is most strongly stimulated by heregulin, an activator of the Neu/ErbB2 tyrosine kinase, and appears to be constitutive in breast cancer cells where Neu/ErbB2 expression is high. Thus, we believe that the CBC represents an exciting nuclear target for receptor tyrosine kinases, linking growth factor-dependent gene expression to RNA processing. We have proposed that the reduced ability of the CBC to bind capped RNAs in the absence of a growth factor (e.g. heregulin) signal may serve as a checkpoint for cell growth by preventing the processing of inappropriate or 'leaky' transcripts.

During the course of Dr. Calero's initial work on the structural studies of c-Cbl, reports of its structure were published, thus compromising the original goal of the predoctoral proposal. Given that Dr. Calero has shown outstanding promise, we have worked to develop another project that we believe is novel and likely to yield fundamental insights into how growth factors like heregulin influence gene expression in the nucleus. This project involves X-ray crystallographic studies of the CBC complex, and in just a short period of time, Dr. Calero has made outstanding progress in developing this study. The reason that Dr. Calero is requesting a change of mentorship is that my laboratory has been engaged in X-ray crystallographic studies of signaling proteins and thus can provide the environment and infrastructure for this undertaking. We believe that we are in a good competitive position to successfully pursue this line of study and I will do all that is necessary to provide Dr. Calero with the required resources and environment for the successful completion of the work. I have enclosed a new "Statement of Work" for the next two years (i.e. the time remaining on Dr. Calero's original fellowship) as well as my own biographical sketch.

It is my sincere hope that you will be able to grant this request, as Dr. Calero is a talented young student whom we all feel is exactly the type of young investigator who should be supported by the breast cancer program. Please do not hesitate to contact me if you feel that you need to discuss this matter further or require any type of additional information.

Sincerely,

Richard A. Cerione, Ph.D.
Professor of Pharmacology and
Chemistry and Chemical Biology

RAC:cw