Identification and Characterization of a Novel Cap-binding Protein from Arabidopsis thaliana

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Cap-binding proteins specifically bind to the 7-methyl guanosine (m7G) functional group at the 5' end of eukaryotic mRNAs. A novel Arabidopsis thaliana protein has been identified that has sequence similarity to cap-binding proteins but is clearly a different form of the protein. The most obvious primary sequence difference is the substitution of two of the eight conserved tryptophan residues with other aromatic amino acids in the novel protein. Analogous forms of this novel protein appear to be present in other higher eukaryotes but not in yeast. Analysis of the native and recombinant forms of the novel protein by retention on m7GTP-Sepharose indicate that it is a functional cap-binding protein. Measurements of the dissociation constant for this protein indicate that it binds mGTP 5-20-fold tighter than eukaryotic initiation factor (eIF(iso)4E). The novel protein also supports the initiation of translation of capped mRNA in vitro. Biochemical analysis and yeast two-hybrid data indicate that it interacts with eIF(iso)4G to form a complex. Based on these observations, this protein appears to be able to function as a cap-binding protein and is given the designation of novel cap-binding protein (nCBP).

eIF4E-eIF4G complex depending upon the method of purification (7, 8). Higher plants possess a unique second form of eIF4F designated eIF(iso)4F (9). eIF(iso)4F contains distinct forms of the cap-binding protein (eIF(iso)4E) and the larger subunit (eIF(iso)4G) (10). The two isoenzyme forms of plant eIF4F have the same activities in vitro (9); however, the eIF(iso)4F prefers hypermethylated caps and mRNAs with less secondary structure (11, 12). Recently, a second form of mammalian eIF4G was reported (13). However, this form of eIF4G was not reported to have a distinct form of eIF4E associated with it and does not appear to be the functional equivalent of eIF(iso)4G.

The mammalian eIF4E is known to be phosphorylated at Ser-209, and the phosphorylation state appears to correlate with activity of the protein (reviewed in Refs. 14 and 15). Certain stimuli, including insulin and several growth factors, induce phosphorylation of eIF4E (14). Overexpression of the mammalian eIF4E results in cell transformation, suggesting that eIF4E levels play a critical role in normal growth and/or development (16, 17). The recent discovery of proteins that specifically bind mammalian eIF4E and sequester it from interacting with eIF4G have linked the insulin signaling pathway directly to translation (reviewed in Refs. 14 and 18). These eIF4E binding proteins (4E-BP) are a major target for phosphorylation following insulin or growth factor treatment, and the phosphorylated form of 4E-BP no longer binds eIF4E (18). Thus the availability and phosphorylation state of eIF4E are crucial regulatory mechanisms for translational control in mammals. It is not known if similar types of regulation by phosphorylation or sequestration occur in plants.

In this report, we have identified a novel cap-binding protein (nCBP) from Arabidopsis thaliana that is distinct in both amino acid sequence and m7GTP binding properties from eIF4E or eIF(iso)4E and appears to be present only in higher eukaryotes.

EXPERIMENTAL PROCEDURES

Materials—The cDNA (19) encoding the expressed sequence tag (EST) for the nCBP (7G6T7) was obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University). DNA sequencing of the cDNA (both strands) was performed at the DNA sequencing facility of the Institute for Cellular and Molecular Biology (University of Texas at Austin). Expression vector pET15b and Escherichia coli DE3(HMS174) were obtained from Novagen (Madison, WI). Restriction enzymes and DNA modifying enzymes were obtained from Life Technologies Inc. IPTG and X-gal were from Ambion (Austin, TX) or Research Products International (Mt. Prospect, IL). The A. thaliana suspension culture was generously provided by A. N. S. Reddy (Colorado State University). Preparation of the Arabidopsis extracts and chromatography on m7GTP-Sepharose (Pharmacia Biotech Inc.) will be described elsewhere but were similar to the preparation and fractionation of wheat germ extracts (20). Wheat eIF(iso)4E and eIF(iso)4G were prepared as described previously (21). Protein determinations were by the method of Bradford (22). SDS-PAGE (12.5 or 20% acrylamide) and Western blots were carried out as described previously (23). The silver stain kit was from Novex (San Diego, CA). Antibodies to recombinant...
**Novel Cap-binding Protein**

| Species       | Accession Number |
|---------------|------------------|
| Arabidopsis   | AF028809         |
| Mouse         | U01137           |

Conserved Trp residues are indicated in **italics**. The amino acid substitution for the conserved Trp residues in the nCBP are in **bold**.

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**RESULTS AND DISCUSSION**

Analysis of the EST data base for *A. thaliana* cap-binding proteins, eIF4E and eIF(iso)4E, revealed a sequence that appeared to be a novel form of eIF4E. This novel cap-binding protein, termed nCBP, differed significantly in sequence from other known eIF4E or eIF(iso)4E sequences. One of the obvious differences was the substitution of two of the conserved tryptophan residues (Trp-1 and -3) with other aromatic residues.

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**Novel Cap-binding Protein**

- **ncBP** and eIF(iso)4E were added to the reaction mixture containing 20 pmol of eIF(iso)4E. The amount of [14C]leucine incorporated was determined as described previously (20). Capped β-globin mRNA was prepared as described (24).

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**Fluorescence Measurements**—Fluorescence measurements were carried out at 23 °C in 20 mM HEPES, pH 7.6, 1 mM MgCl₂. The concentration of protein was 0.25 μM for both nCBP and eIF(iso)4E. The data were collected and analyzed as described previously (25). Excitation was at 280 nm and emission monitored at 330 nm. Background fluorescence was subtracted.

**Affinity Chromatography of nCBP and eIF(iso)4E on m7GTP-Sepharose**—A mixture containing 130 μg of recombinant nCBP and 130 μg of recombinant wheat eIF(iso)4E was applied to a 0.5 ml column of m7GTP-Sepharose equilibrated in buffer B containing 0.1 mM KCl, and the flow-through fractions (400 μl) were collected. The matrix was washed with an additional 5 ml of buffer. The retained protein was eluted with buffer B containing 0.3 mM KCl and 400 μM m7GTP, and fractions (400 μl) were collected. The flow-through and eluate fractions were analyzed by SDS-PAGE.

**Yeast Two-hybrid Analysis**—The coding regions of nCBP and Arabidopsis eIF(iso)4E were placed into the binding domain plasmid, pGBT9. The constructs were confirmed by DNA sequencing. The wheat eIF(iso)4E in pGBT9/N and wheat eIF(iso)4G in pGAD424 were pre-

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**nCBP were raised in rabbits at the University of Texas M. D. Anderson Cancer Center Veterinary Services (Bastrop, TX). The Matchmaker Two-hybrid kit was obtained from CLONTECH.**

**Purification of Novel Cap-binding Protein**—The coding region for *Arabidopsis* nCBP was amplified with the appropriate primers containing Xhol and BamHI sites. The amplified DNA was cloned into pET15b restricted with Xhol and BamHI and in frame with the 6-histidine tag. The plasmid construct was confirmed by DNA sequencing. The plasmid containing the nCBP coding region was transformed into *E. coli* DE3(HMS174). Expression of the recombinant protein was carried out at 23 °C in 20 mM HEPES, pH 7.6, 1 mM MgCl₂. The concentration of protein was 0.25 μM for both nCBP and eIF(iso)4E. The data were collected and analyzed as described previously (25). Excitation was at 280 nm and emission monitored at 330 nm. Background fluorescence was subtracted.

**Affinity Chromatography of nCBP and eIF(iso)4E on m7GTP-Sepharose**—A mixture containing 130 μg of recombinant nCBP and 130 μg of recombinant wheat eIF(iso)4E was applied to a 0.5 ml column of m7GTP-Sepharose equilibrated in buffer B containing 0.1 mM KCl, and the flow-through fractions (400 μl) were collected. The matrix was washed with an additional 5 ml of buffer. The retained protein was eluted with buffer B containing 0.3 mM KCl and 400 μM m7GTP, and fractions (400 μl) were collected. The flow-through and eluate fractions were analyzed by SDS-PAGE.

**Yeast Two-hybrid Analysis**—The coding regions of nCBP and Arabidopsis eIF(iso)4E were placed into the binding domain plasmid, pGBT9 (26), after amplification of the coding region with the appropriate primers. The constructs were confirmed by DNA sequencing. The wheat eIF(iso)4E in pGBT9/N and wheat eIF(iso)4G in pGAD424 were prepared as described previously (26). The β-galactosidase color assay with X-gal was performed according to the protocol of the manufacturer.

**RESULTS AND DISCUSSION**

Analysis of the EST data base for *A. thaliana* cap-binding proteins, eIF4E and eIF(iso)4E, revealed a sequence that appeared to be a novel form of eIF4E. This novel cap-binding protein, termed nCBP, differed significantly in sequence from other known eIF4E or eIF(iso)4E sequences. One of the obvious differences was the substitution of two of the conserved tryptophan residues (Trp-1 and -3) with other aromatic residues (Fig. 1A). Further inspection of the EST data base revealed...
many mouse and human ESTs that were more similar to the Arabidopsis nCBP than to mammalian eIF4E. The number of independent mouse and human nCBP ESTs that appear in the EST data base suggest that the mammalian nCBP mRNAs are highly expressed. This prediction was confirmed by Northern blot analysis of RNA from various mouse tissues that showed high levels of nCBP mRNA expression in all tissues.2 Temeles et al. (27) reported several mouse cDNA sequences for transcripts that were highly expressed in preimplantation embryonic mouse tissue, but the protein products were not all identified. One of these unidentified cDNAs (GenBank DataBank accession number U01137) appears to encode the mouse nCBP protein (Fig. 1B). Sequence comparison of the nCBP from Arabidopsis and mouse shows that these two proteins are more similar to each other (note Trp-1 and -3 are altered also in mouse nCBP) than to their cognate normal cap-binding proteins (compare Fig. 1, panels A and B). Two of the non-conservative amino acid changes in the Arabidopsis nCBP are also present in the mouse nCBP, e.g., L136→E and T171→N (numbering for Arabidopsis nCBP). These two amino acids are absolutely conserved among eIF4E from plants, yeast, Drosophila, Xenopus, and mammals, suggesting that the mouse and Arabidopsis nCBP are more related to each other than to their cognate eIF4E.

Three of the conserved tryptophans (Trp-3, -5, and -8 in Fig. 1A) were recently shown to be involved in the binding of m7GDP by solving the structures of mouse eIF4E and yeast eIF4E with bound m7GDP (4, 5). Mutagenesis studies with yeast (28) and human (29) eIF4E indicated that mutation of the conserved Trp residues (with the exception of Trp 4 in yeast) either eliminated or reduced the ability of eIF4E to bind m7G functional columns. It was, therefore, of great interest to determine if the nCBP, which is lacking Trp-1 and -3, is able to bind m7G functional groups. The m7GTP-Sepharose column was applied to a m7GTP-Sepharose column to test its ability to interact with m7G functional groups. The m7GTP-Sepharose column (2.0 ml) has an estimated capacity of 1.5 mg. We find that fewer nonspecific proteins are retained on the column when the capacity of the column is slightly exceeded. Consequently, only 70% of the soluble recombinant nCBP remaining after ultracentrifugation was retained on the column as judged by densitometry (Fig. 2, lanes 1 and 2). The nCBP was eluted with m7GTP (Fig. 2, lanes 3–8) and approximately 1.2 mg of protein was recovered. It was observed that the nCBP appeared to elute less well from the m7GTP-Sepharose column than either eIF(iso)4E or eIF4E. Increasing the concentration of m7GTP from 100 μM to 400 μM appeared to enhance the elution and the recovery. This observation suggested that the nCBP may have a more avid interaction with m7G functional groups.

Initial measurements using fluorescence spectroscopy indicate at least a 5–20-fold difference in the binding affinity of nCBP and eIF(iso)4E for m7GTP (Fig. 3). The high affinity of m7GTP for nCBP precludes accurate determination of the binding constant by fluorescence due to limiting signal at low protein concentrations. Nonetheless, the evident difference in binding affinities provides an explanation for the difficulty in elution of nCBP from m7GTP-Sepharose. Further analysis with other cap analogs and capped oligonucleotides will be essential to elucidate the basis for the differences in observed binding constants.

To obtain an estimate of the relative amount of nCBP present in Arabidopsis, an extract was prepared from Arabidopsis suspension cultures and applied to a m7GTP-Sepharose column. Fig. 3A shows a silver-stained gel of the m7GTP eluate (lane 1) and purified recombinant nCBP (lanes 2 and 3). The Arabidopsis eIF4E and eIF(iso)4E in the eluate were identified by Western blot analysis using mouse antiserum raised to recombinant wheat eIF(iso)4E (Fig. 4B, lane 1) and by mass

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2 J. Schaunessy, University of Arkansas Medical Center, personal communication.

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**Fig. 2.** Affinity chromatography of recombinant nCBP on m7GTP-Sepharose. nCBP was purified from E. coli by affinity chromatography on m7GTP-Sepharose as described under “Experimental Procedures.” The fractions were separated on a 12.5% acrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, input to column (15 μl); lane 2, effluent from column (15 μl); lanes 3–8, m7GTP eluate (15 μl).
spectrum of polypeptide synthesis activity of the m7GTP eluate shown in lane 1 of panel A resolved on a 20% SDS-gel and blotted to nitrocellulose; lane 1, mouse antiserum to recombinant wheat eIF(iso)4E (1/500); lane 2, rabbit antiserum to recombinant Arabidopsis nCBP (1/500). The primary antibodies were incubated for 3 h at 25 °C. Goat anti-mouse (lane 1) or goat anti-rabbit (lane 2) horseradish peroxidase second antibody was incubated for 2 h at 25 °C. Mouse antiserum to recombinant wheat eIF(iso)4E cross-reacts with both Arabidopsis eIF(iso)4E and eIF4E. However, note that the rabbit antiserum to nCBP does not cross-react with other cap-binding proteins (eIF4E and eIF(iso)4E) present in the Arabidopsis extract, nor does the mouse antiserum to wheat eIF(iso)4E cross-react with nCBP.

Fig. 5. Ability of nCBP to support polypeptide synthesis in a wheat germ fractionated system and inhibition by m7GTP. The reaction mixtures (100 μl) described under “Experimental Procedures” contained 5 pmol of in vitro transcribed rabbit β-globin mRNA and were incubated at 25 °C for 30 min. The amount of [3H]leucine incorporated was determined as described (20). A, comparison of polypeptide synthesis activity of nCBP (●) and eIF(iso)4E (■). B, comparison of m7GTP inhibition of polypeptide synthesis containing 20 pmol of nCBP (●) or 20 pmol of wheat eIF(iso)4E (■), in the presence of 20 pmol of eIF(iso)4G, and the indicated concentration of m7GTP.

spectrometry identification of peptides (data not shown). The amount of nCBP present in the m7GTP eluate (Fig. 4A, lane 1) appears to be at least 10-fold lower than that of either eIF4E or eIF(iso)4E, suggesting that this protein is present in very low amounts in Arabidopsis. nCBP in the Arabidopsis m7GTP eluate was identified with antiserum to recombinant nCBP (Fig. 4B, lane 2). Antibody raised to recombinant wheat eIF(iso)4E cross-reacts with both Arabidopsis eIF4E and eIF(iso)4E (Fig. 4B, lane 1), indicating that these proteins are closely related. However, note that there is no cross-reaction between the nCBP and other Arabidopsis cap-binding proteins with either the antiserum to nCBP or the antiserum to wheat eIF(iso)4F (Fig. 4B, lanes 1 and 2). The lack of cross-reaction between nCBP and eIF4E or eIF(iso)4E, and the amino acid sequence data suggest that nCBP is a more distantly related form of a cap-binding protein.

To further test the interaction of the nCBP with m7GTP functional groups, the ability of nCBP to translate a capped mRNA was determined. As shown in Fig. 5A, the nCBP is able to substitute for wheat eIF(iso)4E, in the presence of wheat eIF(iso)4G. The nCBP is able to support initiation of translation at about 30% of the level of eIF(iso)4E. This result implies that nCBP interacts with a capped mRNA but also forms a functional complex with eIF(iso)4G. We have also obtained data that show nCBP is also able to interact with eIF4G (data not shown). It is not clear at this time whether the lower protein synthesis activity is an innate characteristic of nCBP itself or is due to some other unknown reason (e.g. not having the correct binding partner).

The inhibition of translation initiation by m7GTP in the presence of eIF(iso)4E or nCBP was also determined. As shown in Fig. 5B, the nCBP appears to be much less sensitive to the presence of m7GTP in the translation assay than eIF(iso)4E. At least 5–6-fold more m7GTP was required to obtain 50% inhibition. The order of addition of the m7GTP did not appear to have any affect on the level of inhibition. This result implies that the interaction of capped β-globin mRNA is much stronger with the nCBP than with eIF(iso)4E and therefore requires higher amounts of m7GTP to dissociate the complex. Additional fluorescence binding experiments with capped RNAs will be necessary to determine the dissociation constants relative to that of m7GTP.

The ability of nCBP to support polypeptide synthesis in the

3 K. A. Ruud, S. R. Lax, and K. S. Browning, unpublished data.
presence of eIF(iso)4G implies the ability to form a complex. If
the two proteins form a stable complex, the complex will be
retained on m7GTP-Sepharose. We have shown previously (21)
that eIF(iso)4G is not retained on m7GTP-Sepharose except in
the presence of eIF(iso)4E. Recombinant wheat eIF(iso)4G and
recombinant nCBP were incubated together and applied to a
m7GTP-Sepharose column. Elution of the column with m7GTP
shows that both the nCBP and eIF(iso)4G were bound (Fig. 6).
These results confirm that the recombinant nCBP interacts
with eIF(iso)4G to form a functional complex. Further evidence
of the interaction of the nCBP with eIF(iso)4G was obtained in
the yeast two-hybrid system. We have previously shown that
wheat eIF(iso)4G and eIF(iso)4E interact strongly in the yeast
two-hybrid system (26). The nCBP and Arabidopsis eIF(iso)4E
were placed into a binding domain vector (pGBT9/N) and co-
transformed into yeast with wheat eIF(iso)4G in an activation
domain vector (pGAD424). Specific interaction with the nCBP
and wheat eIF(iso)4G was observed (Table I) as well as inter-
action of the control wheat or Arabidopsis eIF(iso)4E with
wheat eIF(iso)4G. No interaction of nCBP with eIF4A or eIF4B
was observed in this system (data not shown). These results
further confirm the potential for nCBP to specifically interact
with eIF(iso)4G or a similar protein.

We have identified a new and novel protein that functions as
a cap-binding protein and can interact with eIF(iso)4G to form
a complex that supports the protein synthesis initiation of a
capped mRNA. This protein appears to be ubiquitous in higher
eukaryotes. ESTs for the nCBP are present for maize, Brassica
napus, mouse, and human; also, a Caenorhabditis elegans pre-
dicted gene product appears to be very similar to the nCBP.
Interestingly, there does not appear to be a homolog of this
protein in yeast. Careful inspection of the yeast genome did not
reveal a hidden gene that is similar to the nCBP. Therefore
nCBP may be necessary for a function specific to multicellular
organisms (i.e. differentiation). The mouse nCBP cDNA was
among a group of cDNAs found in a screen for mRNAs that
were highly expressed in preimplantation embryos (27), a tis-
sue undergoing rapid differentiation.

The precise biological role of the nCBP is not obvious. The
higher binding affinity of nCBP for m7GTP would imply a
discriminatory role for the nCBP protein during initiation of
translation. However, the lower activity of nCBP in translation
suggests that a possible function may be to sequester mRNAs
and either slow down or prevent their translation. We have
been unable to demonstrate any competition in vitro of the
nCBP with eIF4E or eIF(iso)4E (data not shown). However, we
have preliminary evidence that the translation of some mRNAs
is not supported by the nCBP (data not shown), suggesting that
discrimination and/or sequestering may be the functional role
of nCBP. We are currently determining the expression levels of
nCBP mRNA and protein in various

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**TABLE I**

| Interaction assay | Activation domain, pGAD | Binding domain, pGBT | Interaction
|------------------|------------------------|---------------------|--------------|
| Wheat eIF(iso)4G | pGBT (no insert)        | nCBP                | +++          |
| Wheat eIF(iso)4G | pGBT (no insert)        | Arabidopsis nCBP    | +++          |
| Wheat eIF(iso)4G | pGBT (no insert)        | Arabidopsis eIF(iso)4E | +++          |
| Wheat eIF(iso)4G | pGBT (no insert)        | nCBP                |             |
| pGAD (no insert) | pGAD (no insert)        | Arabidopsis nCBP    |             |
| pGAD (no insert) | pGAD (no insert)        | Arabidopsis eIF(iso)4E |             |
| pGAD (no insert) | Wheat eIF(iso)4E        |                     |             |

*Scored by the intensity of blue color formation.*

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