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# In vivo interaction between CDKA and eIF4A: a possible mechanism linking translation and cell proliferation

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**Abstract In a proteomics-based screen for proteins interacting with cyclin-dependent protein kinase (CDK), we have identified a novel CDK complex containing the eukaryotic translation initiation factor, eIF4A. Reciprocal immunoprecipitations using antibodies against eIF4A indicate that the interaction is specific. The CDKA–eIF4A complex is abundant in actively proliferating and growing cells but is absent from cells that have ceased dividing. The CDKA–eIF4A complex contains kinase activity that is sensitive to the CDK-specific inhibitor roscovitine. This interaction points to a possible molecular mechanism linking cell proliferation with translational control.**



*Key words:* Cyclin-dependent kinase; CDK; eIF4A; Cell cycle control; Translation; *Arabidopsis*

## 1. Introduction

Cyclin-dependent kinases (CDKs) are a family of serine/threonine protein kinases that are involved in controlling cell cycle progression. Their activity is modulated at a number of levels including phosphorylation and binding to associated regulatory proteins such as cyclins and low molecular weight inhibitory proteins. Cyclins act as rate-limiting activating subunits that are produced and destroyed as necessary. Thus different cyclins act at different points during the cell cycle: broadly, D-type cyclins in G1/S phase and B-type cyclins in G2/mitosis [1]. Cyclins are believed to target CDKs to specific substrates and thus differentially control processes associated with progression through the cell cycle.

A large number of cellular processes are differentially regulated during the cell cycle and it is not always clear that a given process is directly regulated by CDK activity. One such process is protein translation. The translation of transcripts depends on the access of ribosomes to the mRNAs and the eukaryotic translation initiation factor (eIF4) proteins facilitate and control such access; mRNA is recruited to the ribosome by the eIF4 complex that binds to the m7GpppX cap present on the 5′ end of most transcripts. eIF4-mediated translation is dramatically reduced during mitosis in mammalian cells and most protein translation ceases [2]. eIF4E is the cap-binding component of eIF4, its function can be inhibited by an eIF4E-binding partner (4E-BP). During mitosis 4E-BP can be phosphorylated in vitro by human CDK1 [3], possibly regulating the rate of translation. However, the 4E-BPs have not yet been found in any plant genome, suggesting an alternative regulatory mechanism or that the function of 4E-BPs is undertaken by a structurally divergent protein.

These associations between growth and translation suggest a direct link between the cell cycle and the regulation of translation. Here we report a novel protein–protein interaction between CDKA and eIF4A. We show that eIF4A can be immunoprecipitated with CDKA and vice versa, that roscovitine-sensitive histone H1 kinase activity precipitates with eIF4A, and that the CDKA–eIF4A complex is most abundant in actively proliferating cells.

## 2. Materials and methods

*Arabidopsis* cultures were maintained as described in [4]. Cells were subcultured weekly into fresh Murashige and Skoog's medium with 3% (w/v) sucrose, 0.5 mg/l NAA and 0.05 mg/l kinetin.

Extracts were prepared using a modified protocol from [5]. Protein extracts were prepared by grinding 4-day-old *Arabidopsis* cells to a fine powder in liquid nitrogen. The powder was resuspended in 'extraction buffer 1' (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 0.1% (v/v) Triton X-100) or in 'extraction buffer 2' for kinase assays (25 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM NaF, 0.5 mM $Na_3VO_4$, 15 mM β-glycerophosphate, 15 mM 4-nitrophenylphosphate, 0.1% (v/v) Tween-20, 0.5 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin). The mixture was centrifuged (18 000×*g*, 20 min) and then used for immunoprecipitation.

For immunoprecipitations, 7m-GTP Sepharose and $p13^{suc1}$ affinity purification, protein extracts were added onto 40 μl of protein G beads (Sigma) with 5–20 μl of antibody or 7m-GTP Sepharose (Amersham) or $p13^{suc1}$ beads made as described [6]. This mixture was incubated at 4°C overnight. The beads were washed four times with bead buffer (50 mM Tris–HCl p H8.0, 150 mM NaCl, 5 mM EGTA, 5 mM NaF, 0.01% (v/v) NP-40). The proteins were eluted from the beads with Laemmli sample buffer and boiled for 5 min.

Kinase assays were performed using a protocol modified from [5]. Immunoprecipitations were performed as above, except the protein G beads were washed with kinase wash buffer (20 mM HEPES pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT). Once washed the beads were incubated for 30 min in kinase wash buffer supplemented with 74 kBq $^{32}P$[ATP], 10 mM ATP, 1 mg/ml histone H1 (GibcoBRL). The reaction was stopped with the addition of Laemmli sample buffer. Final extracts were run on 12.5% SDS–PAGE gels and radioactive incorporation was detected using X-OMAT film (Kodak).

Proteins were identified from gels by mass spectrometry (MS) as described previously [7].

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*Abbreviations:* CDK, cyclin-dependent kinase; eIF4, eukaryotic translation initiation factor; 4E-BP, eIF4E-binding partner

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## 3. Results

### 3.1. eIF4A interacts in vivo with CDKA

CDKA was immunoprecipitated (Fig. 1) using an antibody directed to the PSTAIRE region of CDKA (anti-PSTAIRE, Sigma), anti-CDKA and anti-cyclin D3 [8] (both gifts from J.A.H. Murray). Mouse IgG and a blank immunoprecipitation without antibody were used as controls. Anti-PSTAIRE and anti-CDKA specifically precipitate CDKA, as determined by Western blotting (Fig. 1B) and anti-cyclin D3 co-precipitates CDKA as previously described [8]. A band corresponding to the predicted molecular weight of CDKA (Fig. 1A, closed arrow) was excised from the gel, cleaved using trypsin and the fragments analysed by MS as previously described [7]. The MS fingerprint contained eleven tryptic fragments that matched to predicted tryptic fragments of *Arabidopsis* CDKA (tolerance set to 50 ppm), confirming the identity of CDKA. Another major band at about 45 kDa, marked with an open arrow, was also analysed by MS identification of peptide fragments. The resulting mass fingerprints corresponded to *Arabidopsis* AteIF4A1 or AteIF4A2 as potential proteins present in that location of the gel. AteIF4A1 and AteIF4A2 are extremely similar, sharing 96% similarity at the amino acid level [9] and thus they could not be differentiated by the MS fingerprint.

To confirm the specificity of this interaction we used eIF4A antibodies to carry out a reciprocal immunoprecipitation and tested for the presence of CDKA. The eIF4A antibody (gift of C. Kuhlemeier) was raised against a tobacco protein so we first tested the specificity of the anti-eIF4A antibody on *Arabidopsis* extracts. Fig. 2A shows a Western blot of *Arabidopsis* and tobacco whole cell extracts probed with the eIF4A antibody. This antibody blots a doublet in tobacco, in agreement with [10], and cross-reacts with a single band in *Arabidopsis* whole cell extracts. The band recognised by the antibody in

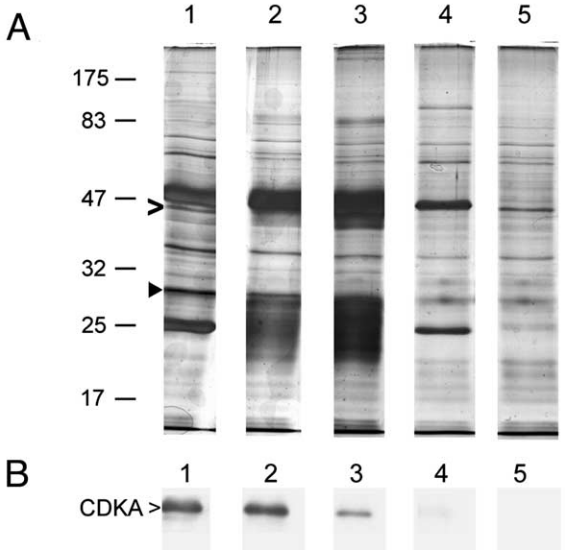


Fig. 1. CDKA complexes contain eIF4A. A: Silver stained 10% gel of immunoprecipitations from extracts of 4-day-old *Arabidopsis* cells using the following antibodies: (1) anti-PSTAIRE, (2) anti-CDKA, (3) anti-cyclin D3, (4) mouse IgG and (5) protein G beads only (no antibody). Closed arrow – CDKA location. Open arrow – protein band identified as eIF4A by MS. B: Western blot of the gel shown in panel A, probed with anti-PSTAIRE antibody.

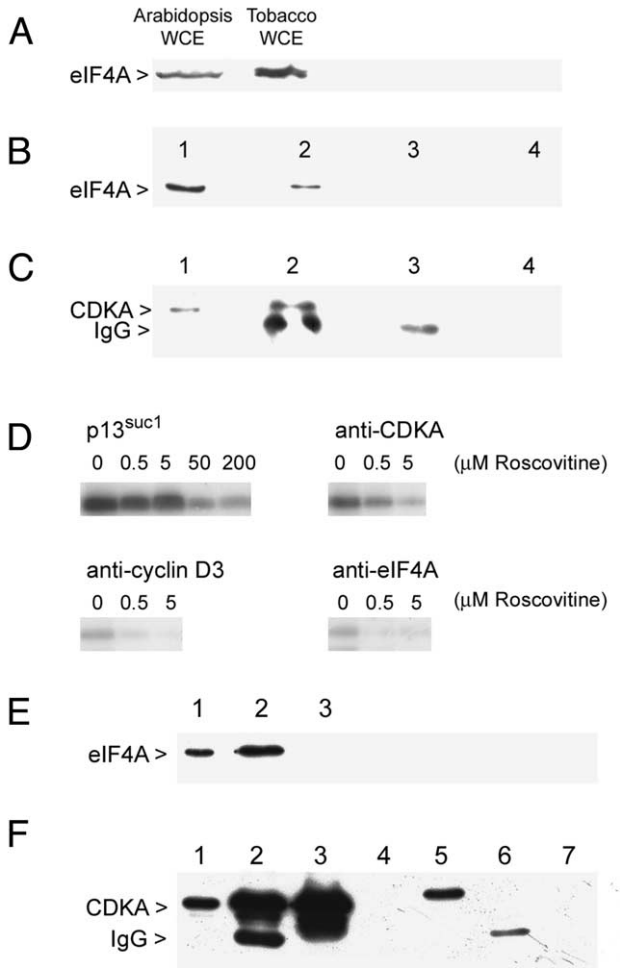


Fig. 2. eIF4A complexes contain an active CDKA. A: *Arabidopsis* and tobacco whole cell extracts were probed with anti-eIF4A. B: Western blot probed with anti-eIF4A. Lanes: (1) whole cell extract before immunoprecipitation, (2) immunoprecipitation with anti-PSTAIRE, (3) immunoprecipitation with mouse IgG and (4) protein G beads only (no antibody). C: A reciprocal immunoprecipitation; eIF4A was immunoprecipitated and probed for co-precipitating CDKA. The lower bands in lanes 2 and 3 correspond to the light chain of IgG. D: Histone H1 kinase assays were performed on protein extracts from 4-day-old *Arabidopsis* cells. Kinase activity was purified using $p13^{suc1}$, anti-CDKA, anti-cyclin D3 and anti-eIF4A. Increasing concentrations of the CDKA inhibitor roscovitine were added to the kinase reaction as indicated. E: 7m-GTP Sepharose can co-precipitate eIF4A. Lanes: (1) whole cell extract before immunoprecipitation, (2) precipitation with 7m-GTP Sepharose beads and (3) with Sepharose 4B beads. The eluted proteins were probed for the presence of eIF4A. F: CDKA is not a member of the mRNA cap-binding complex. Lanes: (1) whole cell extract before immunoprecipitation, (2) immunoprecipitation with anti-PSTAIRE, (3) with anti-CDKA, (4) with 7m-GTP-Sepharose, (5) $p13^{suc1}$, (6) mouse IgG and (7) no antibody. The eluted proteins were probed for the presence of CDKA with anti-PSTAIRE antibody. The lower band corresponds to the light chain of IgG.

*Arabidopsis* extracts has the predicted molecular weight for eIF4A proteins (AteIF4A1 and AteIF4A2 which are both 47 kDa). This antibody was then used to probe the CDKA immunoprecipitations originally used to identify eIF4A by MS. In this way, we confirmed the presence of eIF4A in the anti-PSTAIRE immunoprecipitation and showed that it is absent in both controls, immunoprecipitation with mouse

IgG and without antibody (Fig. 2B, lanes 3 and 4). Finally, we performed a reciprocal immunoprecipitation using the anti-eIF4A antibody to immunoprecipitate eIF4A and then used Western blotting to determine if CDKA co-precipitates. Fig. 2C shows that CDKA is present in the anti-eIF4A immunoprecipitations, confirming the interaction.

Next, we measured the histone H1 kinase activity associated with eIF4A and compared this to the total kinase activity as isolated by other methods. In Fig. 2D, $p13^{suc1}$ pellets the maximal histone H1 kinase activity, probably representing multiple CDK-related complexes. The anti-CDKA pellet has less kinase activity than $p13^{suc1}$ precipitates, and is considerably more sensitive to the CDKA-specific inhibitor roscovitine. The $p13^{suc1}$ precipitate requires in excess of 5 μM roscovitine for inhibition, suggesting the presence of diverse kinases, whereas the anti-CDKA-associated kinase activity is sensitive to only 0.5 μM roscovitine, suggesting a smaller pool of more specific kinase. In all of the experiments the selective CDKA inhibitor roscovitine was used to determine the activity of CDKA as opposed to other histone H1 kinases. The anti-cyclin D3 antibody precipitates only CDKA activity bound to cyclin D3 [8], and reveals a significantly lower kinase activity reflecting the fact that the antibody targets a single cyclin and therefore precipitates a small subpool of CDKA kinase activity. Anti-eIF4A also precipitates roscovitine-sensitive histone H1 kinase activity. Anti-eIF4A isolates a smaller amount of kinase activity compared to that precipitated by $p13^{suc1}$ or anti-CDKA, but is comparable to that isolated by anti-cyclin D3.

To test if CDKA is a component of the eIF4F mRNA cap-binding complex, we isolated the cap complex from cell extracts using 7m-GTP Sepharose beads. Sepharose 7m-GTP is commonly used to purify the eIF4F complex [9,11–13]. We found that eIF4A could be intermittently detected by Western blot (Fig. 2E). However, CDKA was never detected bound to 7m-GTP beads, whether eIF4A was present or not (Fig. 2F).

### 3.2. CDKA and eIF4A interact in proliferating cells

Both CDKA and eIF4A are constitutively expressed in both proliferating and non-proliferating cells [9,14] (Fig. 3A), but their function is regulated by phosphorylation, protein localisation and protein–protein interactions. Therefore, we examined if and how the interaction between CDKA and eIF4A changed during the growth cycle. Over the course of eight days the *Arabidopsis* culture goes through a characteristic growth cycle [8,15]: a lag phase before day 1, followed by an exponential growth phase from days 2 to 4, then a cessation of cell division and an increase in biomass from days 5 to 7. Finally, after day 7, the cell enters stationary phase.

For each day we prepared protein extracts and performed an anti-PSTAIRE immunoprecipitation on those extracts. Fig. 3A shows Western blots on the whole cell extracts prior to immunoprecipitation. The amount of CDKA (anti-PSTAIRE) remains constant for the first 4 days and then declines slightly, cyclin D2 is constant from days 2 to 4, before becoming undetectable as the mitotic index of the culture declines. Despite this decline in mitosis the eIF4A protein remains at a constant level from days 2 through 7, suggesting that the regulation of eIF4A function is post-translational. When CDKA was immunoprecipitated (Fig. 3B) a constant amount of CDKA was precipitated from days 2 through 7. However, the amount of eIF4A co-precipitating with CDKA

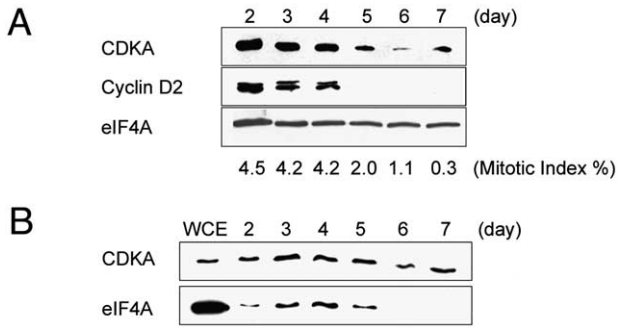


Fig. 3. The CDKA–eIF4A interaction varies during the growth cycle. A: Western blots of whole cell extracts prepared daily from *Arabidopsis* cells were probed with anti-PSTAIRE, anti-cyclin D2 and anti-eIF4A to detect the levels of CDKA, cyclin D2 and eIF4A respectively. The percentage of cells in mitosis (mitotic index) for that particular day is shown beneath each lane. B: The protein extracts from panel A were subjected to anti-PSTAIRE immunoprecipitations and the precipitated proteins were probed for the presence of CDKA (using an anti-PSTAIRE antibody) and eIF4A. Whole cell extract was used to indicate the position of CDKA and eIF4A on the Western blots.

between days 2 and 5 varied with the age of the cell culture; as the cell began to exit the cell cycle around day 5 the amount of CDKA–eIF4A declined and was undetectable at day 6.

## 4. Discussion

Here we show that CDKA physically interacts with eIF4A in vivo. This is based on reciprocal immunoprecipitations using antibodies against eIF4A and CDKA. Furthermore, immunoprecipitation of eIF4A co-precipitates histone H1 kinase activity that is sensitive to the specific CDKA inhibitor roscovitine, indicating that the CDKA–eIF4A complex contains an active kinase. In addition, the interaction is associated with cell proliferation since it can no longer be detected late in the growth cycle when cells cease growing.

There is a considerable body of evidence that growth and cell proliferation are tightly linked with translation but the molecular basis of this linkage is uncertain. Translation and growth may be coupled through the eIF4E subunit, primarily through the action of mTOR and $p70^{s6}$ kinases [16]. CDKA has been implicated in the regulation of cap-dependent translation, phosphorylating and activating the inhibitory protein 4E-BP [3]. However, the 4E-BPs have not been found in plant genomes [9], so it is unclear if this form of translational regulation exists in plants.

Anti-eIF4A precipitates histone H1 kinase activity that can be inhibited by the CDKA inhibitor roscovitine. The level of kinase activity and its roscovitine sensitivity was very similar to that of the cyclin D-associated kinase, suggesting that CDKA bound to eIF4A represents a small subpool of the total CDKA activity. That histone H1 kinase activity co-precipitates with eIF4A indicates that eIF4A bound CDKA is active and may phosphorylate eIF4A; eIF4A does contain a potential CDKA phosphorylation site and eIF4A is known to be phosphorylated in plants in response to various stimulations [17,18]. However, the identity of the kinase responsible and the phosphorylated sites remain unknown.

Anti-PSTAIRE does not co-precipitate an active CDKA

complex (data not shown), as the anti-PSTAIRE antibody is thought to interfere with cyclin binding [19]. However, precipitation of active histone H1 kinase activity using an anti-eIF4A antibody suggests that the CDKA is bound to an as yet unknown cyclin. The first outcome of these observations is that CDKA can bind to eIF4A independently of a cyclin, possibly binding through unknown intermediate proteins. The nature and identity of the cyclin remains unknown. Determining the identity of the cyclin would provide valuable clues as to the cell cycle phase-specific activity of the CDKA–eIF4A complex.

This study demonstrates that the eIF4A–CDKA complex is present in cells that are rapidly dividing and growing. The complex becomes less abundant as the cells switch from a phase characterised by small cycling cells, to one marked by a decrease of the mitotic index and a rise in cell expansion at about day 5. Despite this drop in mitotic index the biomass of the cells in the expansion phase continues to increase dramatically. This indicates that the interaction occurs only in proliferating cells and is not required for cell expansion. Our work suggests that eIF4A may be part of a complex that targets CDKA to the translation machinery.

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