Cell cycle-dependent phosphorylation of the translational repressor eIF-4E binding protein-1 (4E-BP1)

Kate J. Heesom, Alexandra Gampel, Harry Mellor and Richard M. Denton

A fundamental control point in the regulation of the initiation of protein synthesis is the formation of the eukaryotic initiation factor 4F (eIF-4F) complex. The formation of this complex depends upon the availability of the mRNA cap binding protein, eIF-4E, which is sequestered away from the translational machinery by the tight association of eIF-4E binding proteins (4E-BPs). Phosphorylation of 4E-BP1 is critical in causing its dissociation from eIF-4E, leaving 4E available to form translationally active eIF-4F complexes, switching on mRNA translation. In this report, we provide the first evidence that the phosphorylation of 4E-BP1 increases during mitosis and identify Ser-65 and Thr-70 as phosphorylated sites. Phosphorylation of Thr-70 has been implicated in the regulation of 4E-BP1 function, but the kinase phosphorylating this site was unknown. We show that the cyclin-dependent kinase, cdc2, phosphorylates 4E-BP1 at Thr-70 and that phosphorylation of this site is permissive for Ser-65 phosphorylation. Crucially, the increased phosphorylation of 4E-BP1 during mitosis results in its complete dissociation from eIF-4E.

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Results and discussion

Cell cycle-dependent phosphorylation of 4E-BP1

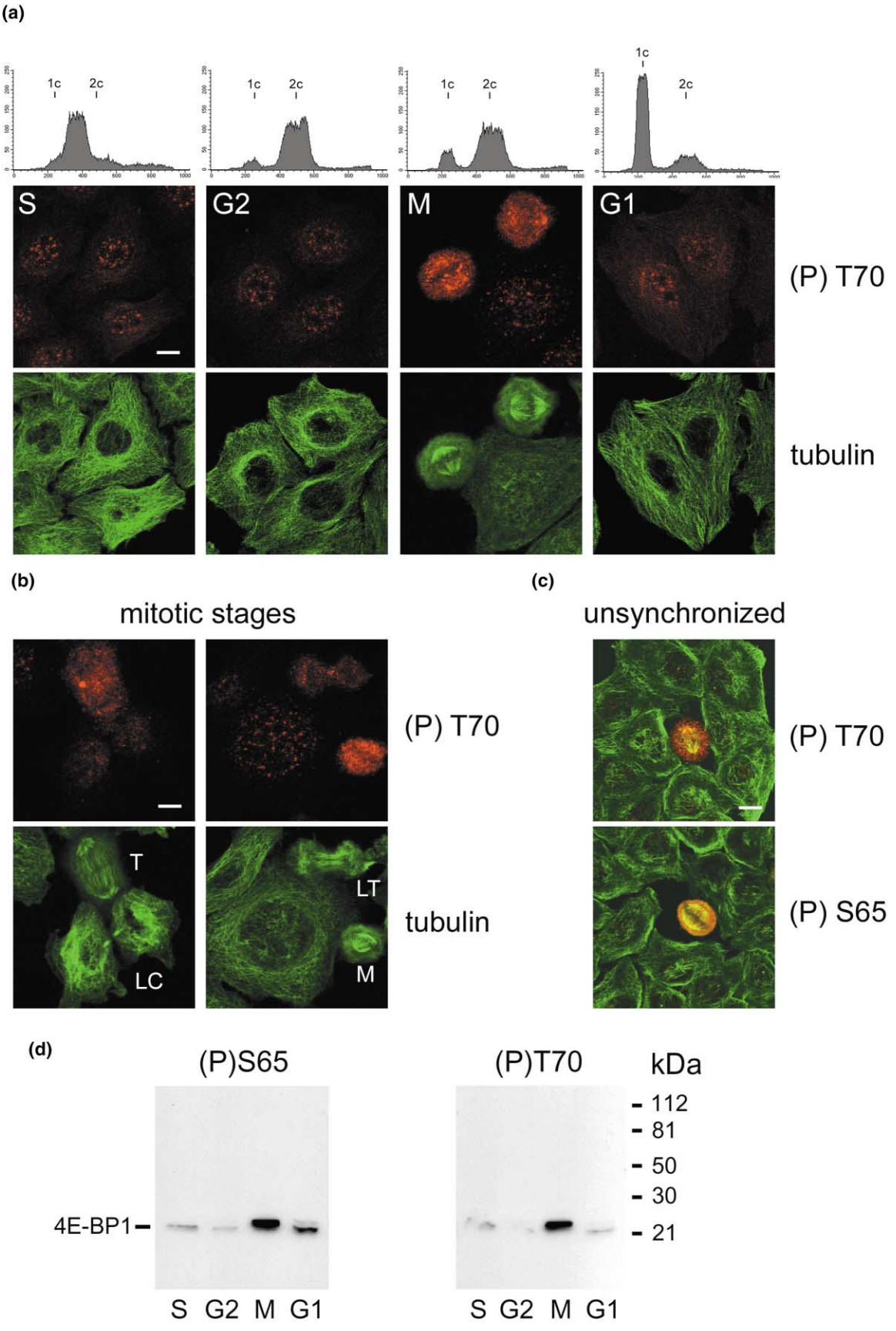
It is well established that the phosphorylation of 4E-BP1 plays an important role in the regulation of cap-dependent translation [1–7]. To investigate the phosphorylation of 4E-BP1 during the cell cycle, HeLa cells were synchronized at the G1/S boundary by a sequential thymidine and aphidicolin block [8]. Cell cycle progression was monitored by flow cytometry of propidium iodide-stained cells and by immunofluorescence staining for tubulin to detect mitotic cells (Figure 1a). Following release from the aphidicolin block, cells entered S-phase after 4 hr and were predominantly in the G2-phase of the cell cycle until 10 hr after release (as determined by flow cytometry). Cells went through mitosis between 10 and 12 hr after release from the aphidicolin block, with the majority of cells being postmitotic 12 hr after release (as determined by immunofluorescence). Cells were then in G1 for an additional ~12 hr.

Phospho-specific anti-4E-BP1 antibodies were used to analyze the phosphorylation of 4E-BP1 at Thr-70 during the cell cycle (Figure 1a–d). In interphase cells, cytoplasmic 4E-BP1 was not significantly phosphorylated at this site, although a small amount of phospho-Thr-70 4E-BP1 was detected in the nucleus of these cells. The nature and role of this nuclear localization is unclear at the present time, although it should be noted that a similar pattern has been observed for nuclear eIF-4E, which is thought to associate with splicing factors in nuclear speckles [9]. As the cells entered mitosis, there was a substantial increase in the phosphorylation of 4E-BP1 at Thr-70 throughout the cell. The increased phosphorylation was detected at the onset of mitosis and decreased in late anaphase and telophase. By the end of mitosis, when the nuclear envelope had reformed, the pattern of phosphorylation had largely returned to interphase levels and staining was mainly detected in the nucleus of the daughter cells (Figure 1b).

The above experiments were also performed using phospho-specific antibodies recognizing 4E-BP1 phosphorylated at Ser-65. The pattern of 4E-BP1 phosphorylation at this site exactly matched that of Thr-70 (data not shown). The increased phosphorylation of 4E-BP1 at Ser-65 and Thr-70 during mitosis was also evident in an asynchronous population of HeLa cells (Figure 1c), and so, it is clearly not an artifact of the cell synchronization process. Specificity of the phospho-specific antibodies for 4E-BP1 was confirmed by analysis of total cell lysates using Western blotting (Figure 1d). The distribution of total 4E-BP1 throughout the cell as determined by immunofluorescence is shown in Figure S1 in the Supplementary material available with this article online.

To examine the activity of 4E-BP1 kinases during the cell cycle, extracts prepared from synchronized HeLa cells at various different cell cycle stages were used to phosphorylate recombinant 4E-BP1. Phosphorylated proteins

Figure 1


(a)
1c
2c
S
G2
M
G1
(P) T70
tubulin
(b)
mitotic stages
(P) T70
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LT
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tubulin
(c)
unsynchronized
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(P) S65
(d)
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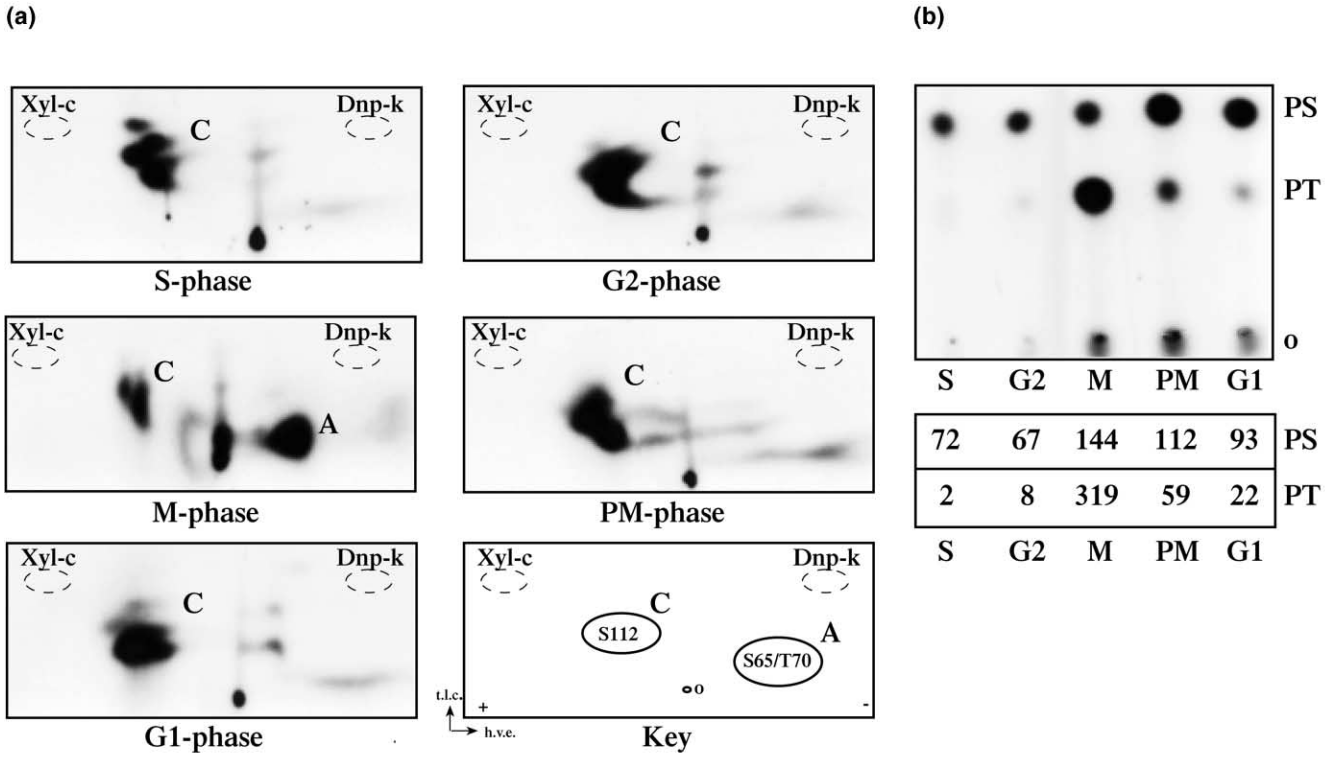
kDa
112
81
50
30
21
4E-BP1
S G2 M G1
S G2 M G1

Figure 2



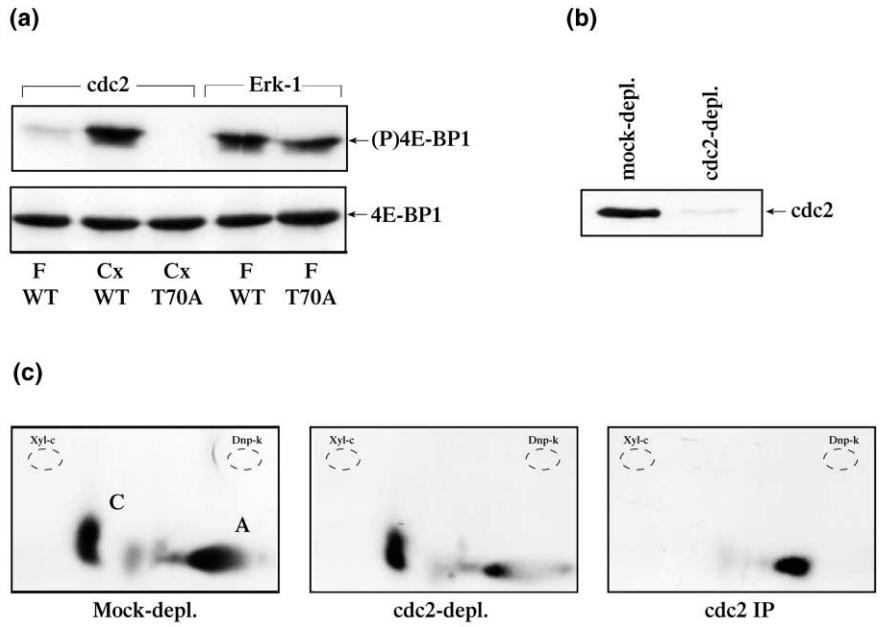
4E-BP1 phosphorylation by cell extracts prepared at different cell cycle stages. **(a)** Cell extracts were prepared from synchronized HeLa cells at the indicated cell cycle stages. Extracts prepared using the mitotic shake-off technique 11 hr after release from the block are labeled M-phase. Extracts prepared at the same time by complete extraction (containing a mixture of mitotic and immediately postmitotic cells) are labeled PM-phase. The cell extracts were used to phosphorylate recombinant eIF-4E/4E-BP1 complex, and the resulting radiolabeled 4E-BP1 was digested with trypsin and subjected to thin layer chromatography. A key shows the migration of the two markers, xylene cyanol (Xyl-c) and dinitrophenol lysine (Dnp-k), and of known tryptic phospho-peptides of 4E-BP1 containing phosphorylated Ser-112 (peptide C) or Ser-65 and Thr-70 (peptide A). **(b)** Radiolabeled 4E-BP1 was subjected to phospho-amino acid analysis. The position of the origin (o) and markers showing the migration of phospho-threonine (PT) and phospho-serine (PS) are shown. To correct for the different levels of protein present in each extract, the table shows the intensity of the phospho-serine/phospho-threonine spot/μg protein in the extract.

were then separated by SDS-PAGE, and the radiolabeled 4E-BP1 was cut from the gel, digested with the protease trypsin, and subjected to two-dimensional thin layer chromatography (Figure 2a) and phospho-amino acid analysis (Figure 2b).

The phosphorylation of 4E-BP1 at Ser-112 (within a tryptic peptide(s) previously described as peptide C [10]) was seen with extracts taken from all points of the cell cycle, suggesting that the kinase(s) responsible for the phosphorylation of this site was active throughout the cycle (Figure 2a and Supplementary material). In contrast, phosphorylation at Thr-70 was only seen with mitotic extracts (as demonstrated by the appearance of tryptic

Phosphorylation of 4E-BP1 at Thr-70 and Ser-65 during the cell cycle. **(a)** HeLa cells were synchronized in G1 with successive thymidine and aphidicolin blocks and then released into fresh media. The progress of the cells through the cell cycle was monitored by flow cytometry of propidium iodide-stained cells at 2-hr intervals. Markers indicate G1 (1c) and G2/M (2c) DNA content. The *x* axis indicates fluorescence, and the *y* axis indicates cell number. At each time point, parallel samples were also fixed and stained with an anti-phospho-specific 4E-BP1 antibody, which recognizes 4E-BP1 phosphorylated at Thr-70 (red). Cells were counterstained for tubulin to detect mitotic cells (green). Time points representative of cycle stages are shown beneath the corresponding flow cytometry trace. **(b)** The panels show additional cells from the 10-hr time point in (a), which is enriched for mitotic cells. Cells caught in various stages of mitosis are shown: M; metaphase, T; telophase, LT; late telophase, LC; late cytokinesis. **(c)** The panel shows unsynchronized cells stained for tubulin (green) and anti-phospho-specific 4E-BP1 antibodies recognizing 4E-BP1 phosphorylated at Thr-70 or Ser-65 (both red). The scale bar represents 10 μm. **(d)** Western blot analysis of total cell lysates using anti-phospho-specific 4E-BP1 antibodies.

Figure 3



Phosphorylation of 4E-BP1 by cdc2. **(a)** Recombinant cdc2-cyclin B complex (lanes 1–3) or Erk-1 (lanes 4 and 5) were assayed for their ability to phosphorylate either wild-type 4E-BP1 (WT) or 4E-BP1 in which threonine at position 70 had been mutated to alanine (T70A). The 4E-BP1 used was either free (F) or in a complex with eIF-4E (Cx). Proteins were separated by SDS-PAGE and visualized by auto-radiography for phospho-4E-BP1 (upper panel) or by Western blotting for total 4E-BP1 (lower panel). **(b)** Extracts prepared using the mitotic shake-off technique were depleted of cdc2, as described in the Supplementary materials and methods. The presence of cdc2 in depleted extracts was assessed by Western blotting. **(c)** Depleted extracts and immunoprecipiated material were used to phosphorylate recombinant 4E-BP1, and the resulting radiolabeled 4E-BP1 was digested with trypsin and subjected to thin layer chromatography.

peptide A [10] [Figure 2a] and a corresponding increase in phospho-threonine residues [Figure 2b]). Phospho-amino acid analysis of tryptic peptide A produced from 4E-BP1 phosphorylated by mitotic extracts identified both phospho-serine and phospho-threonine residues, indicating that Ser-65-directed kinase activity also increased in mitotic extracts (see Figure S2a in the Supplementary material). Phosphorylation of Thr-70 and Ser-65 by mitotic extracts was confirmed by Western blotting using anti-phospho-specific antibodies (Figure S2b). Kinase activity able to phosphorylate 4E-BP1 at Ser-65 and Thr-70 decreased in PM extracts (containing a mixture of mitotic and immediately postmitotic cells) and was further reduced in G1 extracts (Figure 2a).

Phosphorylation of 4E-BP1 by cdc2

The timing of the increased phosphorylation of 4E-BP1 at Thr-70 and Ser-65 suggested a potential role for the cyclin-dependent kinase cdc2-cyclin B complex, which is activated at the onset of mitosis. In addition, the consensus sequence for cdc2 (K/R-T-P-P/R-K/R/H) [11, 12] exactly matches that surrounding Thr-70 in the 4E-BP1 protein.

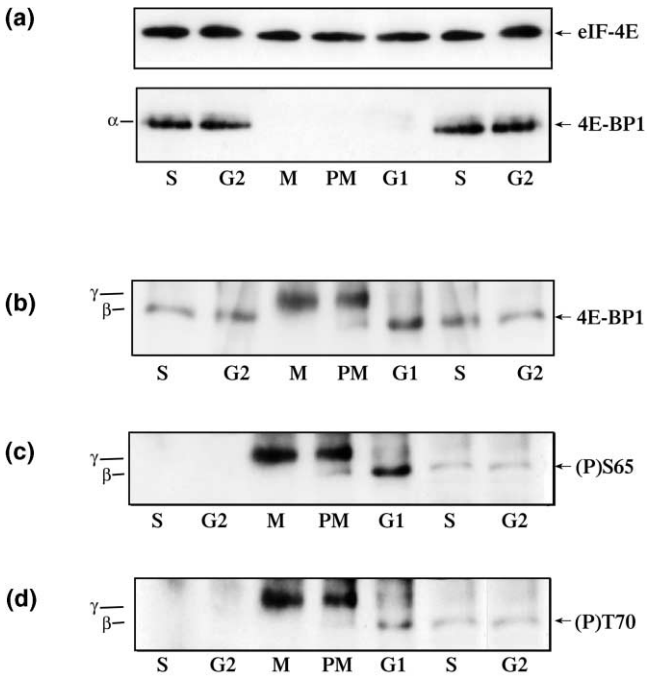
We therefore investigated the ability of a recombinant cdc2-cyclin B complex to phosphorylate 4E-BP1 (Figure 3a). Under the conditions used, cdc2 phosphorylated eIF-4E-bound BP1 more efficiently than free 4E-BP1 (mean fold increase $\pm$ SEM = 6.5 $\pm$ 1.7; n = 4). Two-dimensional thin layer chromatography and phospho-amino acid analysis showed that the 4E-BP1 was phosphorylated predominantly at threonine residues within tryptic peptide A (see Figure S3a,b in the Supplementary material), identifying Thr-70 as the site that is phosphorylated. The ability of cdc2 to phosphorylate 4E-BP1 on Thr-70 was confirmed using a recombinant eIF-4E/4E-BP1 complex in which Thr-70 of 4E-BP1 had been mutated to alanine. The T70A mutant complex was not a substrate for cdc2-cyclin B, although the free form of this mutant was a substrate for Erk-1, which is known to phosphorylate 4E-BP1 at Ser-65 (Figure 3a). In addition, 4E-BP1 phosphorylated by cdc2 was detected using phospho-specific antibodies to Thr-70 (Figure S3c).

Previous studies have used immuno-depletion to show the role of cdc2 in vivo [13]; thus, we have investigated the ability of mitotic cell extracts that had been immunodepleted of cdc2 to phosphorylate 4E-BP1. The efficiency of depletion was shown to be greater than 90% by immunoblotting (Figure 3b). Incorporation of ^{32}P into 4E-BP1 by cdc2-depleted extracts was 2- to 3-fold lower than for mock-depleted extracts. Furthermore, depletion of cdc2 resulted in a substantial decrease in the amount of tryptic peptide A (Figure 3c) and a corresponding decrease in phospho-threonine residues (Figure S4). In addition, the immunoprecipitated cdc2 phosphorylated 4E-BP1 at Thr-70, confirming cdc2 as the Thr-70-directed kinase in mitotic cell extracts.

Cell cycle-dependent dissociation of 4E-BP1 from eIF-4E

To examine the effects of cell cycle-dependent phosphorylation of 4E-BP1 on the association of this protein with eIF-4E, extracts were prepared from synchronized cells at various points throughout the cell cycle and then incu-

Figure 4



The dissociation of 4E-BP1 from eIF-4E during the cell cycle. **(a)** HeLa cell extracts were tumbled with m^{7}GTP-Sepharose to bind eIF-4E and associated 4E-BP1. Material that bound to the m^{7}GTP-Sepharose was separated by SDS-PAGE and analyzed by Western blotting for eIF-4E and total 4E-BP1. **(b)** 4E-BP1 that was not brought down with the m^{7}GTP-Sepharose pellet above was immunoprecipitated, separated by SDS-PAGE, and analyzed by Western blotting for total 4E-BP1 or for **(c)** 4E-BP1 phosphorylated on Ser-65 or **(d)** 4E-BP1 phosphorylated on Thr-70.

bated with the mRNA 5′-cap analog, m^{7}GTP-Sepharose. The presence of eIF-4E (and any associated 4E-BP1) within the m^{7}GTP-Sepharose pellet was determined by Western blotting (Figure 4a). 4E-BP1 was found to be associated with eIF-4E during S- and G2-phases of the cell cycle, presumably reducing mRNA translation as cells prepare for mitosis. This eIF-4E-bound form of 4E-BP1 was not recognized by the phospho-specific antibodies, indicating that it was not phosphorylated on Ser-65 or Thr-70 (data not shown). In mitotic cells, however, the 4E-BP1 had completely dissociated from eIF-4E and did not reassociate until S-phase of the next cycle (Figure 4a).

The migration of 4E-BP1 separated by SDS-PAGE largely depends upon the phosphorylation state of the protein, with more highly phosphorylated forms migrating more slowly than nonphosphorylated 4E-BP1. 4E-BP1 that had dissociated from eIF-4E during mitosis was completely shifted to the slowest migrating γ form (Figure 4b). This is indicative of complete phosphorylation of 4E-BP1, which results in the dissociation of this protein from eIF-4E. In addition, an increase in signal obtained in Western blots using the phospho-specific antibodies was also detected in 4E-BP1 that had dissociated from eIF-4E in mitotic extracts, confirming that the phosphorylation of 4E-BP1 at Ser-65 and Thr-70 increased during mitosis (Figure 4c,d). As cells entered the G1-phase of the cell cycle, the 4E-BP1 returned to its more rapidly migrating β form but did not reassociate with eIF-4E until S-phase of the next cycle, presumably due to the association of the eIF-4E with eIF-4G and other components of the translational machinery.

General conclusions

Our studies have shown that, as cells enter mitosis, 4E-BP1 becomes phosphorylated on Ser-65 and Thr-70, and a concomitant increase in kinase activity able to phosphorylate 4E-BP1 at these sites is seen in mitotic cell extracts. Immuno-depletion studies indicate that the Thr-70-directed kinase in these extracts is the cyclin-dependent kinase, cdc2, a conclusion supported by the finding that purified cdc2-cyclin B is able to phosphorylate 4E-BP1 at Thr-70. This represents the first identification of a kinase that is able to phosphorylate eIF-4E-bound 4E-BP1 selectively at this site. Although we cannot entirely exclude the possibility that another kinase phosphorylates 4E-BP1 at Thr-70 in vivo, our combined data strongly suggest that cdc2 is the kinase responsible for the phosphorylation of this site. In light of this, it is interesting to note that cdc2 has been shown to phosphorylate a number of other proteins involved in the regulation of translation, including p70S6 kinase [14] and protein elongation factor-1 [15], although the functional consequences of these phosphorylations are not clear at present.

The increased phosphorylation of 4E-BP1 at Ser-65 and Thr-70 that is seen at mitosis is associated with the complete dissociation of 4E-BP1 from eIF-4E. The 4E-BP1 remains dissociated during G1-phase and does not reassociate with eIF-4E until S-phase of the next cell cycle. This provides the first direct evidence to link changes in the phosphorylation of 4E-BP1 to its dissociation from eIF-4E during the cell cycle. The phosphorylation of 4E-BP1 at Thr-70 alone by recombinant cdc2 does not result in the dissociation of 4E-BP1 from eIF-4E (data not shown). However, we have found that phosphorylation of wild-type 4E-BP1 by mitotic extracts results in the dissociation of up to 50% of the 4E-BP1 from eIF-4E and that 4E-BP1 that has dissociated is phosphorylated on Ser-65. In contrast, the T70A mutant 4E-BP1 is not phosphorylated on Ser-65 and does not dissociate from eIF-4E (Figure S5). It would appear, therefore, that the phosphorylation of 4E-BP1 at Thr-70 is critical in facilitating the phosphorylation of the protein at Ser-65, which in turn results in its dissociation from eIF-4E (in general agreement with other studies concerned with the regulation of translation by insulin [16, 17]).

It has been reported previously that protein synthesis is reduced during mitosis [18, 19, 20]. However, studies in synchronized HeLa cells have shown that this inhibition is relieved by late telophase [21] and that overall protein synthesis increases rapidly as cells enter G1-phase [18]. Allowing a short time delay for the completion of a round of translation, this is entirely consistent with the release of eIF-4E triggered by the mitotic hyperphosphorylation of 4E-BP1 reported here. We propose that the mitotic phosphorylation of 4E-BP1 on Thr-70 by cdc2 acts to restimulate cap-dependent translation as cells enter G1.

Supplementary material

Additional details, including Materials and methods; discussion of the nature of multiple spots representing tryptic peptide C; and figures showing phospho-amino acid analyses, phospho-specific Western blots, cellular distribution of 4E-BP1, and the effect of phosphorylation by mitotic extracts on the dissociation of wild-type or T70A mutant 4E-BP1 from eIF-4E are available at http://images.cellpress.com/supmat/supmatin.htm.

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