Translation elongation in eukaryotes is mediated by the concerted actions of elongation factor 1A (eEF1A) that delivers aminoacylated-tRNA to the ribosome, elongation factor 1B (eEF1B) complex that catalyzes the exchange of GDP to GTP on eEF1A, and eEF2 that facilitates ribosomal translocation. Here we present evidence in support of a novel mode of translation regulation by hindered tRNA delivery during mitosis. A conserved consensus phosphorylation site for the mitotic kinase CDK1 on the catalytic delta subunit of eEF1B (termed eEF1D) is required for its post-translational modification during mitosis, resulting in lower affinity to its substrate eEF1A. This modification is correlated with reduced availability of eEF1A-tRNA complexes, as well as reduced delivery of tRNA to and association of eEF1A with elongating ribosomes. This mode of regulation by hindered tRNA delivery, although first discovered in mitosis, may represent a more globally-applicable mechanism employed under other physiological conditions that involve down-regulation of protein synthesis at the elongation level.

Global translation, a tightly-regulated step in eukaryotic gene expression, is known to be down-regulated during mitosis (1-2). Early studies have shown that the down-regulation in mammalian cells is attributed to phosphorylation events that interfere with the formation of eIF4F complex (3) and eIF2-GTP-tRNA_{\text{Met}} ternary complex (4), both required for the initiation step. However, according to a more recent report, while mitotic polysomes become significantly less active in protein synthesis, they do not exhibit ribosomal runoff or disassembly, which is the expected outcome of inhibition at the initiation step (5). This work suggested that polysomes remain intact during mitosis due to simultaneous down-regulation of translation at both the initiation and post-initiation steps (5). Evidence in support of mitosis-specific translational modulation at the elongation step include (i) absence of stress granules, which typically form due to ribosomal runoff in response to inhibitors of translation initiation; (ii) reduced ribosome disassembly in response to puromycin, which only affects actively elongating ribosomes; (iii) decreased β-actin protein synthesis despite the retention of β-actin mRNA in heavy polysomes; and (iv) increased ribosome transit time, indicative of reduced elongation rate (5). The present study was designed to investigate mechanisms controlling translation elongation during mitosis.
and two catalytic subunits (eEF1Bα and eEF1Bδ, termed eEF1B2 and eEF1D, respectively, according to NCBI Entrez Gene nomenclature), as well as valine-tRNA synthetase (Val-RS) (9). eEF1B2 is highly conserved throughout eukaryotes (9) and, being the only catalytic subunit, essential for normal growth in yeast (10). However, yeast deficient in their catalytic subunit can be rescued from lethality by eEF1A overexpression (11). eEF1D, which originated from gene duplication of an eEF1B2 ancestor and fusion with a leucine zipper domain (12), is unique to higher eukaryotes, while a divergent catalytic subunit, termed eEF1Bβ, has developed in plants (13). Although eEF1B2 and eEF1D share a common function and highly-homologous catalytic domains, they have been suggested to bind their substrate eEF1A through different sites, based on the observation that binding of eEF1A to eEF1B2 results in masking of a CK2 phosphorylation site, whereas a similarly located site on eEF1D is not masked by binding to eEF1A (14). The large leucine zipper motif within eEF1D, which is absent from eEF1B2 and from plant eEF1Bβ, suggests interactions of this subunit with other proteins (15), but there is no direct interaction between eEF1B2 and eEF1D (14). The structural subunit, eEF1G, functions as a scaffold protein, holding together the two catalytic subunits. eEF1G does not interact directly with eEF1A or Val-RS; it does, however, interact directly with the two catalytic subunits, eEF1B2 and eEF1D (14) (16). It can bind both subunits simultaneously, but never two subunits of the same type, suggesting the presence of two non-interchangeable binding sites. Depletion of the structural subunit in yeast is not lethal and does not result in repression of translation, but instead leads to increased resistance to oxidative stress and several antibiotics (17).

In sea urchin embryos (18) (18), as well as mammalian HeLa cells (5), the translation elongation rate decreases in synchrony with CDK1 activation during M-phase. Xenopus oocytes eEF1D was reported to undergo phosphorylation by CDK1 during metaphase at the initial stages of early development. More specifically, two CDK1 phosphorylation sites were found, Thr131 and an unidentified serine residue, which was responsible for mobility retardation on SDS-PAGE (19-20). While the above threonine-containing motif is not present in human eEF1D, two other consensus CDK1 target sites were identified, namely Ser133 and Thr147. Indeed, human eEF1D was shown to be phosphorylated by CDK1 on Ser133 in-vitro (21). In-vivo phosphorylation of this site was confirmed by a multitude of phosphoproteome (mass-spectrometry) analyses (www.phosphosite.org). Still, there is lack of information regarding the implication of mitosis-specific eEF1D phosphorylation to the regulation of translational elongation. In this study, we demonstrate that Ser133 is essential for reduced interaction of eEF1D with its substrate eEF1A during mitosis. We show that less eEF1A-tRNA complexes are available for delivering charged aa-tRNA to elongating ribosomes in mitotic cells, leading to slow-down of translation elongation.

**EXPERIMENTAL PROCEDURES**

**Cells, synchronization, and cell cycle analysis**

HeLa S3 cells were grown in Dulbecco modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (Biological Industries). For double-thymidine block (DTB), cells were treated with 2 mM thymidine (Sigma) for 16 hr, released from G1/S block in fresh DMEM for 9 hr, treated again with 2 mM thymidine for 16 hr, released in fresh DMEM and harvested at 9 hr for mitosis. For synchronization to mitosis using nocodazole, cells were treated with 1 mM nocodazole (Sigma) for 16 hr, released from G1/S block in fresh DMEM for 9 hr, treated again with 2 mM thymidine for 16 hr, released in fresh DMEM and harvested at 9 hr for mitosis. For synchronization to mitosis using nocodazole, cells were treated with 1 mM nocodazole (Sigma) for 16 hr. For synchronization to mitosis using DTB-2me2, cells were treated twice with thymidine as described above and 660 ng/mL 2me2 (Sigma) was added 4 hr before harvesting. For cell cycle analysis, cells were analyzed using flow cytometry on a Becton Dickinson FACSort instrument using the Cell Quest software, as previously described (22).

**Generation of DNA expression vectors and stable cell lines**

Retroviral-based pQCXIP vector (Clontech) was used as the backbone for all vectors described, except as indicated, followed by generation of lentiviral particles. To generate pQCXIP-Flag, FLAG1 and FLAG2 oligonucleotides were annealed and ligated into NotI and BamHI sites of pQCXIP. These oligonucleotides also introduce an XhoI site, which is not otherwise present in pQCXIP. eEF1A and eEF1B2 were cloned from HeLa cells by PCR using eEF1A Fwd and eEF1A
Rev or eEF1B2 Fwd and eEF1B2 Rev oligonucleotides, respectively. eEF1D was cloned from pCMV-eEF1D (23) by PCR using eEF1D Fwd and eEF1D Rev oligonucleotides. Amplified fragments were ligated into XhoI and EcoRI sites of pQCXIP-Flag. Murine eEF1G was cloned from pGFP-eEF1G (24) by digestion with HindIII (filled-in) and XhoI followed by ligation into filled-in BamHI and XhoI of pQCXIP-Flag. pQCXIP-Flag-eEF1D(T147A), pQCXIP-Flag-eEF1D(S133A), pQCXIP-Flag-eEF1D(T147A;S133SA) and pQCXIP-Flag-eEF1D(S133E) were generated using primer extension according to a protocol provided by Promega Corporation, using eEF1D T/A Rev, eEF1D S/A Fwd, eEF1D S/E Rev, eEF1D S/E Fwd oligonucleotides. Transfection of all pQCXIP-based and pLK0.1-based recombinant plasmids was performed into HEK293T cells using the calcium phosphate procedure. pVSVG and pGPT (Clontech) were used to generate retroviral particles for infection in order to generate stable cell lines. Retroviral infection of HeLa S3 cells was performed according to the protocol provided by Clontech Labs Inc.

DNA primers for cloning of PCR fragments
FLAG1: 5’ggccgcatggactacaaagacgatgacgacgacaagctcgagg3’; FLAG2: 5’gatccctcgagcttgtcgtcatcgtctttgtagtccatgc3’; eEF1A Fwd: 5’ggggctcgagatgggaaaggaaaagactc3’; eEF1A Rev: 5’gggggaattcaaacagttctgagaccgttc3’; eEF1D FWD: 5’ggggctcgagatggctacaaacttcctagc3’; eEF1D-Rev: 5’gggggaattcgtctttaatcgtggcagggc3’; eEF1D T/A Rev: 5’cctctgcaggtgcggctggctt3’; eEF1D S/A Fwd: 5’gcacgtagctcccatgcgcaa3’; eEF1D S/E Rev: 5’cagacccagcacgtagaacccatgcgcc3’; eEF1D S/E Fwd: 5’cacttggcgcatgggttctacgtgctgg3’.

Antibodies
Rabbit polyclonal antibodies against eEF1A, eEF1B2, phospho-histone H3 were from Abcam, against β-actin from Cell Signaling, against eIF2α P-Ser51 from MBL and against PABP from Santa Cruz. Mouse monoclonal antibodies against eEF1G and eEF1D were from Abcam and against Flag from Sigma. Mouse monoclonal antibody against eIF2α were from (25). HRP-conjugated goat anti-mouse, anti-rabbit secondary antibodies for Western blotting were from Jackson Immuno Research. Secondary antibodies for immunofluorescence were all multilabeling grade from Jackson Immuno Research Laboratories, Inc.

Immunoprecipitation and immunoblot analysis
Cells were lysed in Lysis buffer (10 mM HEPES, pH 7.5, 0.5% NP-40, 100 mM NaCl, 10 mM MgCl₂, 1 mM sodium orthovanadate, 10 mM NaF, 20 mM β-glycerolphosphate, 1.4 µg/ml pepstatin, 2 µg/ml leupeptin, EDTA-free protease inhibitor cocktail (Complete; Roche), and 0.1 µM microcystin). For immunoprecipitation, 2-5 mg of total protein extracts was incubated with anti-Flag antibody, followed by addition of Protein G agarose beads (Santa Cruz). Proteins were eluted from the beads by boiling in Laemmli sample buffer. Western blot analyses were performed following 10% SDS-PAGE according to standard procedures. Detection was by enhanced chemiluminescence (GE Healthcare). Densitometry of protein bands was performed using ImageJ software.

Immunofluorescence
Cells were grown on coverslips were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton-X100 in PBS, blocked with 5% goat serum (Biological industries) and incubated primary antibodies overnight at 4°C. Cells were then incubated with secondary antibodies (multilabeling grade; Jackson Immuno Research Laboratories) in 2% BSA in PBS supplemented with 50 ng/ml Hoechst dye (Sigma) for 1 hr at room temperature. Images were acquired using Yokogawa CSU-22 Confocal Head; Axiocvert 200M, (Zeiss) using SlideBook™ (Intelligent Imaging Innovations). Quantification of co-localization was performed by ImageJ software.

In-vitro phosphorylation of eEF1D
eEF1D was immunoprecipitated from non-synchronized HeLa cells expressing Flag-eEF1D as described under Immunoprecipitation, except that the bead-bound eEF1D was washed 3 times with assay buffer (20mM MOPS pH=7.5, 25 mM βGP, 5 mM EGTA, 1 mM DTT, 7.5 mM MgCl₂ and, 1 mM sodium vanadate) and incubated at 37°C for 2 hr with 100 mM ATP and 500 µg of total protein from either non-synchronized or mitotic HeLa cells homogenized in assay buffer,
either with or without 150 µM roscovitine (Sigma), a specific CDK1 inhibitor. Beads were then washed 3 times with assay buffer, boiled in sample buffer, resolved on a 10% SDS-PAGE and subjected to immunoblot analysis with anti-Flag antibody.

**Polysomal profile analysis and extraction of proteins from sucrose gradients**

Polysomal profiles were performed as described earlier (5). For protein extraction, each 0.5 ml fraction was diluted 1:1 with 20 mM Tris pH 7.5, followed by incubation with 7 µl of StrataClean resin (Stratagene) overnight at 4°C. Bound proteins were eluted by boiling in Laemmli sample buffer.

**RNA extraction and analysis**

For rRNA extraction from sucrose gradients, 0.4 ml of 8 M guanidine hydrochloride (Sigma) and 1 ml of ice cold ethanol was added to each 0.5 ml fraction, which was then incubated for 48 hr at -20°C and centrifuged for 30 min at 20,000 x g. Next, RNA was extracted from each fraction by TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions, except that the final precipitation was performed with 1 ml ethanol for 24 hr at -20°C.

For tRNA extraction from Flag-eEF1A immunoprecipitates, TRIZOL reagent was used according to instructions, with the abovementioned exception concerning final precipitation. For Northern blot analysis, RNA was boiled for 2 min in loading buffer containing 49% formamide and 5 mM EDTA, separated on a 12% 8 M UREA PAGE and blotted onto Hybond-N membrane (Amersham). [32P]-labeled DNA probe was prepared as described (26) (26). The following DNA probes were used: [rRNA-lysine: 5'cgccgcagaagggaccttgccctcgacctgatctctacggctgctatcc3'; tRNA-Arginine: 5'gggctccgcaatggataacgct-3'; tRNA-Glutamine: 5'ttccgctgagaatggccggcgcgcggcgcggcgcgctatcagatgctc3'. Analysis of tRNA aminoacylation status was performed essentially as described (27) (27). In brief, total RNA was extracted under acidic conditions using TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions, except that RNA was resuspended in 10 mM sodium acetate pH=4.0. One µg of total RNA was then treated with 0.2 M Tris HCl pH=9.5 for 30 min at 37°C and each sample of treated or untreated RNA was mixed with sample buffer (0.1 M sodium acetate pH=5.0 and 8 M urea) and loaded on a 14% PAGE with 8 M urea and 0.3 M sodium acetate pH=5.0 as gel buffer and 0.3 M sodium acetate as running buffer. The gel was then stained with ethidium bromide.

**RESULTS**

**Less tRNA is associated with polysomes during mitosis**

As an initial step, we reconfirmed that polysomes remain intact in HeLa cells synchronized to mitosis by release from a double-thymidine block (DTB), in contrast to cells synchronized using nocodazole, a cytoskeleton-stabilizing agent often used for mitotic arrest. Indeed, polysomal profiles analyzed on sucrose gradients demonstrated that mitotic cells synchronized by DTB, but not nocodazole, contain intact heavy polysomes (Fig. 1A). We also reconfirmed that mitotic polysomes are less sensitive to inhibitors of translation initiation (Supplemental Figure 1).

To better characterize the mechanism responsible for elongation slow-down during mitosis at the biochemical level, we first optimized the synchronization protocol by using double-thymidine block followed by incubation with 2-methoxyestradiol (2me2), a microtubule-stabilizing drug used to arrest cells at the prophase-metaphase boundary. Flow cytometry analysis measured 85% mitotic cells under the combined DTB/2me2 protocol, compared to 67% using the standard DTB protocol (Fig. 1B). This was also correlated with an increase in the levels of the mitotic marker phospho-histone H3 (P-H3, Fig. 1C). Polysomal profile analysis confirmed the presence of intact heavy polysomes in the DTB/2me2-arrested mitotic cells (Fig. 1D).

The DTB/2me2 synchronization protocol was then used to compare the amount of polysome-bound tRNA in non-synchronized and mitotic cells. To this end, cell extracts were fractionated on a sucrose gradient, followed by pooling of six consecutive fractions along the gradient. RNA was then extracted from the pooled fractions, separated by gel electrophoresis and probed with a [32P]-labeled DNA oligonucleotide specific to full-length lysyl-tRNA. Equivalent loading of ribosomal RNA was confirmed by methylene blue staining (data not shown). A prominent ~3.3-fold reduction in polysome-
associated lysyl-tRNA was observed in mitotic compared to non-synchronized cells (Fig. 2A). Similar results were obtained upon re-probing with $[^{32}P]$-labeled DNA oligonucleotide specific to arginyl-tRNA and glutamyl-tRNA (data not shown). Electrophoresis of tRNA samples from non-synchronized and mitotic cells under acidic conditions, which preserve aminoacylation, ruled out possible differences in the overall levels of charged aa-tRNA in mitotic cells (Fig. 2B). We speculated that the lower tRNA content in polysomal fractions of mitotic cells, i.e. lower tRNA:rRNA ratio, may be indicative of reduced tRNA delivery to mitotic polysomes, which is in agreement with a decrease in elongation rate. This finding suggests that reduced elongation rate may be the result of delayed cognate tRNA arrival to ribosomes due to possible regulation of the availability of eEF1A-tRNA complexes.

**Availability of eEF1A-tRNA complexes and association of eEF1A with polysomes decrease during mitosis**

To substantiate the hypothesis that the availability of eEF1A-tRNA complexes is regulated during mitosis, we first examined the level of polysome-bound eEF1A. HeLa cells stably expressing Flag-tagged recombinant eEF1A protein were generated with no detectable effects on cell cycle (data not shown). Non-synchronized and mitotic cells expressing Flag-eEF1A were analyzed for their polysomal profiles (Fig. 3A) followed by SDS-PAGE and immunoblot analysis of proteins extracted from each fraction of the gradient. This revealed a significant reduction in the proportion of polysome-bound eEF1A from 13.9% to 5.5% in non-synchronized and mitotic cells, respectively (Fig. 3B). To further confirm that the lower levels of polysome-associated tRNA and eEF1A (Fig. 2A and 3B) in mitotic cells are due to reduced availability of eEF1A-tRNA complexes, we next examined the interaction of eEF1A with tRNA. For this purpose, the amount of tRNA co-immunoprecipitated with Flag-eEF1A was compared in lysates of mitotic and non-synchronized cells. To monitor the efficiency of RNA extraction from both samples, 100 ng of low molecular-weight RNA ladder were added to equivalent amounts of each Flag-eEF1A immunoprecipitate (confirmed by immunoblotting using anti-Flag antibody, Fig. 3C, top panel) prior to the extraction procedure. Methylene blue staining of the extracted RNA marker following electrophoresis and blotting demonstrated similar extraction efficiency and loading from both Flag-eEF1A immunoprecipitates (Fig. 3C, second panel). Anti-Flag immunoprecipitates from HeLa cells not expressing Flag-eEF1A were used as a negative control (data not shown). The same membrane was then probed with a $[^{32}P]$-labeled DNA oligonucleotide specific to full-length lysyl-tRNA. The lysyl-tRNA signal obtained from eEF1A immunoprecipitate was considerably lower in mitotic compared to non-synchronized cells, representing a reduction of 70% in the amount of tRNA bound by eEF1A (Fig. 3C, bottom panel and bar graph). Similar results were obtained upon re-probing with a $[^{32}P]$-labeled DNA oligonucleotide specific to arginyl-tRNA (data not shown), confirming that the effect on eEF1A-tRNA complexes is not specific to lysyl-tRNA. Given that charged tRNA levels do not change in mitosis (Fig. 2B), this observation is consistent with decreased activity of eEF1A during mitosis.

**eEF1D Ser133, a conserved CDK1 target site, is essential for reducing eEF1D interaction with its substrate eEF1A during mitosis**

To investigate the nature of the underlying regulatory mechanism leading to reduced availability of eEF1A-tRNA complexes, we analyzed the abundance of each of the four eEF1 subunits between the G1/S boundary and M-phase. To this end, total protein from HeLa cells harvested at various time points following release from DSB was subjected to immunoblot analysis using antibodies specific to eEF1 subunits and the mitotic marker phospho-histone H3 (P-H3), which peaked at 9 hours after block release (Fig. 4A). This experiment demonstrated that eEF1B2 and eEF1G do not exhibit any significant change in protein level or migration pattern on SDS-PAGE as the cells progress through the cell cycle and enter M-phase; eEF1A, however, showed a small and gradual increase in protein level towards M-phase. While we are unable to explain this increase at the current point, it does not seem to detract from our above conclusion regarding the reduced availability of eEF1A-tRNA complexes. On the contrary; levels of polysome-bound eEF1A and eEF1A-bound tRNA are lower in mitosis despite the apparent increase in total eEF1A levels.
In contrast to other eEF1 subunits, eEF1D was detected at all time points as two distinct bands that change in intensity around the M-phase (Fig. 4A). A clear shift of the lower to the upper band of eEF1D, indicative of a post-translational modification, was observed as cells entered mitosis. A similar shift in Xenopus laevis eEF1D was attributed to phosphorylation by CDK1 during metaphase (19-20, 28). It was therefore tempting to speculate that eEF1D activity may be directly or indirectly regulated by CDK1 during mitosis. To assess the involvement of this major mitotic kinase, we used roscovitine, a specific CDK1 inhibitor, to demonstrate that CDK1 activity in mitotic cells is necessary for the shift in migration pattern of eEF1D. In this in-lysate in-vitro experiment, we immunoprecipitated eEF1D from non-synchronized cells and incubated the immunoprecipitate with lysates from non-synchronized or mitotic cells with or without roscovitine, which prevented the shift in eEF1D migration (Fig. 4B). A similar experiment cannot be performed in-vivo as the use of roscovitine would not only prevent phosphorylation of CDK1 targets but also preclude entry of cells to mitosis.

Being one of the two catalytic subunits of the eEF1B complex, we hypothesized that CDK1 phosphorylation of eEF1D may negatively affect the interaction with its substrate eEF1A, leading to down-regulation of eEF1B ability to recycle eEF1A-GDP back to its active GTP-bound form. To examine this hypothesis, we first tested whether the change in migration pattern of eEF1D on SDS-PAGE is attributed to mitosis-specific modification on Thr147, Ser133, or both, since these two residues were identified as conserved consensus phosphorylation sites for CDK1 (29). We generated HeLa cell lines stably expressing Flag-tagged wild-type and mutant variants of eEF1D protein, in which Thr147 or Ser133 or both residues were replaced with alanine (T147A, S133A or T147A;S133A, respectively). We also generated a HeLa cell line stably expressing a phosphomimetic mutant eEF1D variant in which Ser133 was replaced with glutamic acid (S133E).

Human eEF1D was previously reported to undergo CDK1-dependent phosphorylation both in-vitro and in monkey epithelial cells on Ser133 (21); this is supported by multiple mass spectrometry experiments showing that mammalian eEF1D is phosphorylated on Ser133 during mitosis (30-32) and www.phosphosite.org). Our results, taken together with these previously-published reports, strongly implicate CDK1 phosphorylation on Ser133 as the modification responsible for the shift in eEF1D mobility on SDS-PAGE, although an indirect effect downstream of CDK1 cannot be ruled out.

It is also of note that, while eEF1D is detected as two bands in cells synchronized by the DTB-release protocol (Fig. 4A), it is seen as a single slow-migrating band in cells synchronized by the DTB/2me2 protocol (Fig. 4D), consistent with a higher proportion of mitotic cells uniformly arrested at the end of prophase in the presence of 2me2. This also suggests that the entire population of eEF1D is modified on Ser133 during mitosis, and thus the proportion of eEF1D bands can serve as an additional marker for mitotic enrichment and efficiency of synchronization.

To establish whether the modification of eEF1D is correlated to a change in its interactions with eEF1A during mitosis, the above HeLa cell lines stably expressing wild-type or mutated variants of Flag-tagged eEF1D were used for co-immunoprecipitation experiments. Indeed, eEF1D modification during mitosis is correlated with a significant reduction of its binding to eEF1A, as only 52% of eEF1A was co-immunoprecipitated with Flag-eEF1D in mitotic compared to non-synchronized cells (Fig. 5A). However, the amount of eEF1A co-immunoprecipitated with Flag-eEF1D(T147A;S133A) was not lower in mitotic compared to non-synchronized cells (Fig. 5A). Whereas the Flag-eEF1D(T147A) variant was apparently able to undergo mitosis-specific modification along with decreased binding of eEF1A, the Flag-eEF1D(S133A) variant was apathetic to mitosis (Fig. 5B), confirming the functional significance of this putative
phosphorylation on Ser133, but not Thr147, to eEF1D-eEF1A interactions in human cells. Furthermore, the phosphomimetic S133E variant bound less eEF1A than S133A even in non-synchronized cells (Fig. 5C), giving further strength to the conclusion that the putative phosphorylation of eEF1D on Ser133 may have a negative effect on eEF1D-eEF1A interactions.

To determine whether the mitosis-specific reduction in eEF1A-eEF1D interactions occurs endogenously in cells that do not overexpress eEF1D, HeLa cells were subjected to double-labeling immunofluorescence experiments using antibodies specific to eEF1A and eEF1D. Representative double-labeled images of interphase and mitotic cells are shown in figure 5D. Pearson's coefficient of co-localization was calculated for 15 interphase and 15 mitotic cells, confirming the ~2-fold decline in eEF1A-eEF1D association during mitosis.

**Reduced binding of eEF1A to its GEF during mitosis is specific to eEF1A-eEF1D interaction**

To test whether it is eEF1A-eEF1D interactions that are specifically interrupted during mitosis, we next analyzed the binding capacity of eEF1A to each of the two catalytic subunits, eEF1D and eEF1B2. To this end, anti-Flag immunoprecipitates from non-synchronized and mitotic HeLa cells stably expressing Flag-eEF1A were subjected to immunoblot analysis using antibodies specific to eEF1D and eEF1B2. As expected, less (61%) eEF1D co-immunoprecipitated with Flag-eEF1A in mitotic compared to non-synchronized cells; however, in contrast to eEF1D, similar amounts of eEF1B2 co-immunoprecipitated with Flag-eEF1A in non-synchronized and mitotic cells (Fig. 6A). The reciprocal experiment, analyzing anti-Flag immunoprecipitates from HeLa cells stably expressing Flag-eEF1B2, confirmed that similar amounts of eEF1A are bound to the eEF1B2 catalytic subunit in non-synchronized and mitotic cells (Fig. 6B). These data reveal that eEF1D-eEF1A interactions are uniquely regulated during mitosis. We then reasoned that although eEF1A is not directly associated with the structural subunit eEF1G, indirect eEF1A-eEF1G interaction should be negatively affected during mitosis as a result of the decreased association of eEF1A with eEF1D. Co-immunoprecipitation experiments using HeLa cells stably expressing Flag-eEF1G demonstrated that only 52% eEF1A is co-immunoprecipitated with Flag-eEF1G from lysates of mitotic compared to non-synchronized cells (Fig. 6C). This is similar to the result obtained when Flag-eEF1D was immunoprecipitated (Fig. 5A), giving further support to the hypothesis that eEF1D-eEF1A interactions are specifically affected during mitosis. In contrast to the mitosis-specific decrease in eEF1D-mediated association of eEF1A with eEF1G, similar amounts of eEF1D and eEF1B2 were co-immunoprecipitated with Flag-eEF1G in non-synchronized and mitotic cells (Fig. 6C). This finding seems to indicate that, while eEF1A dissociates from eEF1D during mitosis, the catalytic exchange complex eEF1B consisting of eEF1G, eEF1B2 and eEF1D, remains intact (Figs. 5, 6). Reduced association of eEF1A with eEF1D may well lead to partial loss of eEF1B GEF activity, which is shared by the two catalytic subunits, eEF1D and eEF1B2. Such reduction may disrupt the temporal balance between eEF1A-GDP and eEF1A-GTP, leading to decreased eEF1A activity.

**Overexpression of eEF1D(S133A) negates the mitosis-specific reduction in eEF1A activity and destabilizes mitotic polysomes**

Based on the above data, we anticipated that if Ser133-dependent eEF1D modification is necessary for down-regulating translation elongation rate during mitosis by reducing the availability of eEF1A-tRNA complexes, then overexpression of the phosphorylation-null eEF1D(S133A) should prevent this decrease, leading to continued elongation and subsequently destabilization or disassembly of mitotic polysomes. Indeed, we found that overexpression of eEF1D(S133A) resulted in reduced stability of mitotic polysomes, which was correlated with comparable association of eEF1A with polysomes seen in HeLa cells not overexpressing eEF1D (Fig. 3B and 7). Therefore, this mutant may exert a dominant negative effect by abrogating the modification on Ser133 that is responsible for reduced availability of active eEF1A. Surprisingly, a similar though smaller effect was observed with wild-type eEF1D; compared to HeLa cells, HeLa overexpressing wild-type eEF1D showed some destabilization of mitotic polysomes, which was
correlated with a less drastic dissociation of eEF1A from mitotic polysomes. This may be attributed to the inability of CDK1 to fully phosphorylate the excess of eEF1D in cells overexpressing the wild-type protein. This observation confirms the regulatory role of eEF1D and its putative phosphorylation on Ser133 in managing translational elongation during mitosis.

**DISCUSSION**

**Regulation of translation elongation during mitosis**

The macromolecular complexity of eEF1B, its multiple putative phosphorylation sites, numerous cellular partners and aggregate data from yeast, *Xenopus laevis* oocytes and sea urchin embryos, served as the basis for the hypothesis that eEF1B plays an essential role in the control of gene expression in mammalian cells, particularly during mitosis (9). In the current study, we provide evidence for a regulatory role of eEF1D in translation elongation in mitotic human cells. We show for the first time that Ser133 residue of eEF1D, which is positioned within a conserved CDK1 phosphorylation motif, is essential for mitosis-specific reduction in eEF1D-eEF1A interaction. We also show that, during mitosis, less tRNA is bound to eEF1A and the association of both eEF1A and tRNA with polysomes is reduced. According to our proposed model, phosphorylation of eEF1D by CDK1 leads to reduced interaction of the catalytic subunit eEF1D with its substrate eEF1A-GDP, resulting in lower guanine nucleotide exchange rate by the eEF1B complex and subsequently lower level of active eEF1A-GTP during mitosis. Consequently, less eEF1A-GTP is available for binding and delivering aa-tRNA to mitotic ribosomes, leading to translational slow-down and transient stalling of elongating ribosomes. The stability of heavy polysomes despite the well-established inhibition of translation initiation during mitosis is in agreement with lack of ribosomal runoff due to ribosome stalling. We previously observed increased ribosome transit time in mitotic HeLa cells (5). This may be interpreted either as a global uniform decrease in elongation rate or as ongoing or elevated elongation by some polysomes coupled with slow-down or complete arrest of others. The data presented in the current study provides evidence for the negative effect of mitotic eEF1D modification on eEF1D-eEF1A interactions, while the eEF1B complex remains intact and active via the remaining eEF1B2 exchange function. This offers a basal level of eEF1A-GTP-aa-tRNA complexes that can promote translation to some extent, even during mitosis. Although first discovered in mitosis, this mechanism may also hold true for other biological conditions that require attenuation of global translation at the elongation level.

**Evolution of a phosphorylation-dependent regulatory mechanism**

The evolutionary-conserved eEF1B2 subunit is thought to guarantee guanine nucleotide exchange on eEF1A, being the sole catalytic subunit in fungi. Therefore, the additional plant eEF1Bβ and metazoan eEF1D catalytic subunits have most likely evolved to promote specific regulatory functions. The presence of conserved phosphorylation sites on these extra subunits is attributed to the acquisition of a regulated function (20). Supporting this notion is the inability of plant eEF1Bβ subunit to complement a *Saccharomyces cerevisiae* mutant deleted for its single catalytic subunit, unless the sole consensus CDK1 phosphorylation site of eEF1Bβ was replaced by a nonphosphorylatable site (33). This supports our hypothesis that CDK1 phosphorylation of eEF1D down-regulates the GEF activity of human eEF1B complex during mitosis. Additional observations supporting the regulatory role of eEF1D are the dramatic reduction in translation efficiency of cellular mRNA, but not viral mRNA, due to the interactions of eEF1D with the lentivirus protein Tat and herpes simplex virus 1 (HSV-1) protein ICP0 (23,34). Interestingly, in HSV-infected cells, eEF1D is phosphorylated on Ser133 by a viral kinase that mimics CDK1 function (21). An attractive speculation is that modulation of eEF1D activity may not only lead to inhibition of global elongation but also generate conditions that permit elongation of specific sub-classes of cellular mRNAs, as it does for viral mRNAs in infected cells. Taken together with the current study, we conclude that eEF1D serves as an important regulatory component of translational elongation in mammalian cells.

In human fibroblasts, eEF1B is anchored to the endoplasmic reticulum (35) by the interaction of eEF1D with kinectin (36).
Disruption of eEF1D-kinectin interactions leads to specific inhibition of membrane protein expression and enhanced cytosolic protein synthesis, suggesting that eEF1B sub-cellular localization is important for translation regulation (37). In sea urchin embryos, just before nuclear membrane breakdown, eEF1D shifts to the nuclear envelope and concentrates as a ring around the nucleus and then as two large spheres around the mitotic spindle poles (38); although the effects of this spatial redistribution are not yet characterized, this may be another example of translation regulation by sub-cellular localization of eEF1B.

tRNA molecules are transferred by eEF1A from aminoacyl-tRNA synthetases (ARSs) to ribosomes and back to ARSs for aminoacylation in a closed-loop channel (39). Interestingly, ARSs were found to localize to the ER and cytoskeleton and to interact with eEF1 subunits (40). The reduced interaction of eEF1D with eEF1A during mitosis (this study), raises the question of whether the interaction of eEF1 with specific ARSs also changes in a manner dependent on eEF1D modification. Future experiments will verify the significance of CDK1-mediated eEF1D modification in mammalian cells to eEF1B sub-cellular localization and its role in the regulation of gene expression.

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**FOOTNOTES**

*Acknowledgements: We thank David Chetrit and Marcelo Ehrlich for help with confocal microscopy, Dalia Pinchasi for technical assistance, K.T. Jeang for pCMV-eEF1D plasmid and P.A. Coulomb for pGFP-eEF1G plasmid. This research was supported by THE ISRAEL SCIENCE FOUNDATION (grant No. 131/07).

The abbreviations used are: CDK1, cyclin-dependent kinase 1; DTB, double thymidine block; eEF, eukaryotic translation elongation factor; EF, prokaryotic translation elongation factor; ER, endoplasmic reticulum; M, mitosis; NS, non-synchronized cells; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SEM, standard error of the mean; 2me2, 2-Methoxyestradiol.
FIGURE 1. Polysomes remain stable during mitosis. (A) HeLa cells, either non-synchronized or synchronized to mitosis using nocodazole or release from double thymidine block (DTB release), as indicated, were analyzed for their polysomal profile. 80S and polysomes are indicated. (B) HeLa cells, either non-synchronized or synchronized to mitosis by release from DTB or by release from DTB followed by treatment with 2-methoxyestradiol (DTB/2me2), as described in Materials and Methods, were stained for DNA content with propidium iodide (PI) followed by flow cytometry analysis and (C) subjected to immunoblot analysis using antibodies specific to the mitotic marker phospho-histone H3 (P-H3) and β-actin. (D) Mitotic (DTB/2me2) HeLa cells were analyzed for polysomal profile. 80S and polysomes are indicated.

FIGURE 2. Less tRNA is associated with mitotic polysomes. (A) Non-synchronized (NS) and mitotic (M) HeLa cells were analyzed for their polysomal profile. Free RNA, ribosomal subunits and polysomes are indicated. RNA was extracted from six consecutive pooled fractions along the gradient, and resolved on an 8 M UREA-12% polyacrylamide gel followed by staining with Methylene blue to verify equal loading. The RNA was then analyzed by northern blot hybridization with [32P]-labeled DNA oligonucleotide corresponding to lysyl-tRNA. Shown is a representative of three independent experiments. Intensity of Lysyl-tRNA signal in polysomes relative to total tRNA was quantified by densitometry. The bar chart shows the mean ± SEM of polysome-associated tRNA in mitotic relative to non-synchronized cells. (B) tRNA aminoacylation is not reduced during mitosis. RNA was extracted from NS and M HeLa cells under acidic conditions (pH=4.0) and tRNA was deacylated by base treatment (pH=9.5) for 30 min at 37°C. Treated and untreated samples were then resolved on an acidic 8 M UREA-15% polyacrylamide gel (pH=5.0) and stained with ethidium bromide. Aminoacylated-tRNAs (charged-tRNA) and non-aminoacylated tRNAs (tRNA) are indicated.

FIGURE 3. eEF1A is depleted from polysomes and binds less tRNA during mitosis. (A) Non-synchronized (NS) and mitotic (M) HeLa cells stably expressing Flag-tagged eEF1A were analyzed for polysomal profile. 80S and polysomes are indicated. (B) Total protein extracted from each of 22 sucrose gradient fractions was resolved on a 10% SDS-PAGE followed by immunoblot analysis using anti-Flag antibody. Shown in percents is the fraction of polysome-bound to total eEF1A intensity. The data represent one of two independent experiments. (C) Flag-tagged eEF1A was immunoprecipitated from NS and M HeLa cells using anti-Flag antibody. RNA was extracted from the immunoprecipitates by Trizol reagent and spiked with low-molecular weight RNA ladder to monitor extraction efficiency. RNA was then resolved on an 8 M UREA-12% polyacrylamide gel, followed by blotting and hybridization with [32P]-labeled DNA oligonucleotide corresponding to lysyl-tRNA (bottom panel). Equivalent amounts of Flag-eEF1A immunoprecipitates were confirmed by immunoblotting with anti-Flag antibody (top panel); equivalent loading of extracted RNA was confirmed by Methylene blue staining (middle panel). Shown is a representative of three independent experiments. Intensity of Flag-eEF1A and lysyl-tRNA signals was quantified by densitometry. The bar chart shows the mean ± SEM of eEF1A-bound lysyl-tRNA in mitotic relative to non-synchronized cells.

FIGURE 4. Level and migration pattern of eEF1 subunits. (A) HeLa cells were synchronized to G1/S boundary using a double-thymidine block (DTB) and harvested at the indicated time points following release from the block. Forty µg of total protein at each time point were subjected to immunoblot analysis using antibodies specific to the indicated proteins. (B) eEF1D was immunoprecipitated from non-synchronized HeLa cells expressing Flag-tagged wild-type (WT) eEF1D. The sepharose beads-associated Flag-eEF1D was then incubated with buffer alone or with lysate from non-synchronized (NS) or mitotic (M) HeLa cells either in the absence or presence of Roscovitine, a specific CDK1 inhibitor. The beads-associated Flag-eEF1D was then subjected to immunoblot analysis using anti-Flag antibody. (C) Total protein from HeLa cells stably expressing Flag-tagged eEF1D wild-type (WT) and T147A, S133A, T147A;S133A and S133E was subjected to immunoblot analysis using anti-Flag antibody. (D) Total protein from non-synchronized (NS) and mitotic (DTB/2me2) HeLa cells was subjected to immunoblot analysis using anti-phospho-H3 (P-H3) and anti-eEF1D antibodies.
FIGURE 5. eEF1D variants and their interaction with eEF1A. (A) A fraction of total protein used for immunoprecipitation (input) and the anti-Flag immunoprecipitates (IP Flag) from non-synchronized (NS) and mitotic (M) HeLa cells expressing Flag-tagged wild-type (WT) and T147A:S133A mutant eEF1D were immunoblotted using antibodies specific to eEF1A. (B) Similar to (A), only with HeLa cells expressing Flag-tagged T147A or S133A mutant variants of eEF1D, respectively. (C) Similar to (A), only with HeLa cells expressing Flag-tagged S133E mutant variant of eEF1D. (A) to (C) represent one of three independent experiments. The intensity of bands from all experiments was quantified by densitometry. Quantitative data is represented as mean ± SEM. (D) Representative single-plane images (x100 magnification) of fixed non-synchronized HeLa cells stained with anti-eEF1D and anti-eEF1A taken using a spinning disc confocal microscope. Pearson’s coefficients for the colocalization of eEF1D and eEF1A in 15 interphase and 15 mitotic cells is shown. Data are represented as mean ± SEM.

FIGURE 6. Mitosis-specific reduced binding of eEF1A to its GEF is specific to eEF1A-eEF1D interaction. A fraction of total protein used for immunoprecipitation (input) and the anti-Flag immunoprecipitates (IP Flag) from non-synchronized (NS) and mitotic (M) HeLa cells stably expressing Flag-eEF1A (A) or Flag-eEF1B2 (B) or Flag-eEF1G (C) were immunoblotted for the indicated proteins. Images represent one of three or four independent experiments. The intensity of bands from all experiments was quantified by densitometry. Quantitative data is represented as mean ± SEM.

FIGURE 7. Overexpression of wild-type and S133A mutant of eEF1D reverse mitotic depletion of eEF1A from polysomes. Non-synchronized (NS) and mitotic (M) HeLa cells or HeLa cells expressing either Flag-tagged wild-type (WT) or S133A eEF1D were analyzed for their polysomal profile. Shown is a representative of two independent experiments. For each profile, the area under the curve of polysomal RNA (P) peaks and sub-polysomes (SP) peaks (containing free RNA, 40S and 60S ribosomal subunits) was calculated. P/(SP+P) for NS and M cells is presented as mean ± SEM. For each profile, fractions containing polysomes (P) or sub-polysomal (SP) were pooled, followed by total protein extraction from each pool. 10% of the SP samples or 100% of the P samples were resolved on a 10% SDS-PAGE followed by immunoblot analysis using anti-eEF1A and anti-PABP antibody.
Figure 1

A

Non-synchronized (NS)  Mitotic cells (DTB release)  Mitotic cells (Nocodazole)

OD260

80S Polysomes  80S Polysomes  80S Polysomes

Light  Heavy  Light  Heavy  Light  Heavy

B

NS  DTB release  DTB/2me2

Cell number

2N  4N  2N  4N  2N  4N

7%  67%  85%

C

NS  DTB-release  DTB/2me2

P-H3

β-actin

D

DTB/2me2

OD260

80S Polysomes

Light  Heavy
Figure 2
Figure 3
Figure 4

A

| Time (h) following DTB release | NS | 0 | 3 | 6 | 7 | 8 | 9 | 11 |
|--------------------------------|----|---|---|---|---|---|---|----|
| P-H3                          |    |   |   |   |   |   |   |    |
| eEF1A                         |    |   |   |   |   |   |   |    |
| eEF1B2                        |    |   |   |   |   |   |   |    |
| eEF1D                         |    |   |   |   |   |   |   |    |
| eEF1G                         |    |   |   |   |   |   |   |    |

B

| Lysate | NS | M | M | + |
|--------|----|---|---|---|
| Roscovitine |    |    |    | + |
| eEF1D  |    |    |    |    |

C

| P-eEF1D | eEF1D |
|---------|-------|
| WT      |       |
| T147A   |       |
| S133A   |       |
| T147A-S133A | |
| S133E   |       |

D

| P-H3 | eEF1D |
|------|-------|
| NS   |       |
| DTB2me2 |     |
Figure 5

A) Table showing the 1A/1D ratio for Flag-eEF1D WT and Flag-eEF1D T147A;S133A.

B) Table showing the 1A/1D ratio for Flag-eEF1D T147A and Flag-eEF1D S133A.

C) Table showing the 1A/1D ratio for Flag-eEF1D S133A and Flag-eEF1D S133E.

D) Images showing the eEF1A and eEF1D distributions under NS and M conditions, with a graph showing the Pearson's coefficient (R^2).
Figure 6

A

|                     | Flag-eEF1A |
|---------------------|------------|
|                     | NS         | M          |
| 5% Input            | +          | +          |
| IP Flag             | +          | +          |
| Flag-eEF1A          | +          | +          |
| eEF1D               | +          | +          |
| 1D/1A ratio         | 100%       | 61±20%     |
| eEF1B2              | +          | +          |
| 1B2/1A ratio        | 100%       | 99±1%      |

B

|                     | Flag-eEF1B2 |
|---------------------|------------|
|                     | NS         | M          |
| 5% Input            | +          | +          |
| IP Flag             | +          | +          |
| Flag-eEF1B2         | +          | +          |
| eEF1A               | +          | +          |
| 1A/1B2 ratio        | 100%       | 98±9%      |

C

|                     | Flag-eEF1G |
|---------------------|------------|
|                     | NS         | M          |
| 5% Input            | +          | +          |
| IP Flag             | +          | +          |
| Flag-eEF1G          | +          | +          |
| eEF1A               | +          | +          |
| 1A/1G ratio         | 100%       | 52±9%      |
| eEF1B2              | +          | +          |
| 1B2/1G ratio        | 100%       | 103±6%     |
| eEF1D               | +          | +          |
| 1D/1G ratio         | 100%       | 105±24%    |
Figure 7

A

HeLa

NS
80S
M
P/SP+P:
NS: 0.5±0.02
M: 0.5±0.05

P = Polysomes

eEF1D WT

NS
80S
M
P/SP+P:
NS: 0.5±0.03
M: 0.3±0.01

eEF1D S133A

NS
80S
M
P/SP+P:
NS: 0.5±0.06
M: 0.2±0.02

OD260

Light

Hoavy

Polysomes

Polysomes

Polysomes

NS

M

SP

P

SP

P

SP

P

SP

P

SP = Sub-Polysomes

PABP

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