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Phosphorylation of Elongation Factor-2 And Activity Of Ca^{2+}/Calmodulin-Dependent Protein Kinase III During The Cell Cycle

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Phosphorylation of the eukaryotic elongation factor 2 (eEF-2) blocks the elongation step of translation and stops overall protein synthesis. Although the overall rate of protein synthesis in mitosis reduces to 20% of that in S phase, it is unclear how the protein translation procedure is regulated during the cell cycle, especially in the stage of peptide elongation. To delineate the regulation of the elongation step through eEF-2 function, the changes in phosphorylation of eEF-2, and in activity of corresponding Ca^{2+}/calmodulin (CaM)-dependent protein kinase III (CaMK-III) during the cell cycle of NIH 3T3 cells, were determined. The *in vivo* level of phosphorylated eEF-2 showed an 80% and 40% increase in the cells arrested at G1 and M, respectively. The activity of CaMK-III also changed in a similar pattern, more than a 2-fold increase when arrested at G1 and M. The activity change of the kinase during one turn of the cell cycle also demonstrated the activation at G1 and M phases. The activity change of cAMP-dependent protein kinase (PKA) was reciprocal to that of CaMK-III. These results indicated: (1) the activity of CaMK-III was cell cycle-dependent and (2) the level of eEF-2 phosphorylation followed the kinase activity change. Therefore, the elongation step of protein synthesis might be cell cycle dependently regulated.

Keywords: CaMK-III, cell-cycle, eEF-2, phosphorylation, PKA, translation.

Introduction

Every cell generally passes through four phases during the cell cycle to reproduce and multiply: G1 (gap 1), S (synthesis), G2 (gap 2) and M (mitosis) (for a review, see Pardee *et al.*, 1978; Inoue 1981). The G1 is the phase in which cells either commit to undergo another round of DNA synthesis and continue to cycle; or to exit the cell cycle to enter the quiescent state referred to as G0. The S phase is a period of DNA synthesis and is followed by the G2 phase that prepares the cell for mitosis (M). These sequential events of the cell cycle are precisely controlled by the changes of external or internal signals such as nutrients, growth factors, cell size, the completion of genome replication, and the activity or content of various cell cycle specific proteins, such as cell cycle-dependent kinases and cyclins (Loden *et al.*, 1999).

One of the changes that occur in mammalian cells during the cell cycle is a decrease in the overall rate of protein synthesis in mitosis. For example, the protein synthesis of cultured HeLa and CHO cells in M phase is reduced to about 20-30% of the level of S phase cells (Fan and Pennman, 1970; Tarnowka and Baglioni, 1979). Protein synthesis is comprised of initiation, elongation, and termination steps. While there is substantial evidence to suggest that initiation is heavily regulated, and that an initiation block occurs in cells exposed to a variety of situations (*e.g.*, nutrient deprivation, heat shock, *etc.*; for a review, see Pain, 1986; Han, 1997), it has recently become evident that the elongation is a rate limiting step in overall protein synthesis. In particular, the discovery of a specific Ca^{2+}/CaM-dependent protein kinase that phosphorylates eEF-2, as well as the finding that the phosphorylated form of eEF-2 (pEF-2) was shown to be inactive in *in vitro* translation, suggest that protein synthesis rates are relative to intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) (Nairn and Palfrey, 1987; Ryazanov *et al.*, 1988).

Ca^{2+}/calmodulin-dependent protein kinase III (CaMK-III) was purified from rabbit reticulocytes and rat pancreas (Mitsui *et al.*, 1993; Redpath and Proud, 1993) and recently cloned (Redpath *et al.*, 1996). CaMK-III phosphorylates from one to a maximum of three threonine residues of eEF-2, depending on the phosphorylation condition (Redpath *et al.*, 1993). The regulation mechanism of CaMK-III activity is unknown, but being studied presently. The elongation factor-2 is a ubiquitous protein in mammalian cells with a molecular size of 100 kDa. Its physiological function is to catalyze the translocation of peptidyl-tRNA from the A- to P-site of the

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ribosome during each round of elongation (for a review, see Nygard and Nilson, 1991; Perentesis *et al.*, 1992). The eEF-2 is phosphorylated by CaMK-III with very high specificity and pEF-2 is unable to proceed the elongation step, which stops protein synthesis (Nairn *et al.*, 1985). The eEF-2 can also be ADP-ribosylated at the diphthamide residue by diphtheria and Pseudomonas toxins, which are also inactive for the translation (Robinson *et al.*, 1974).

In addition to the discovery of decreased protein synthesis at M, there is indirect evidence suggesting that the phosphorylation of eEF-2, and the activity of CaMK-III, is cell cycle dependent. Celis and his colleagues (1990), observed that most of eEF-2 appeared as two isoforms at G1 regarded as unphosphorylated forms, whereas it appeared as multiple isoforms (which are considered as phosphorylated forms) during M. In contrast, a separate study showed that the kinase activity for the phosphorylation of eEF-2 was the highest during S phase in Ehrlich ascites tumor cells; while the highest level of phosphatase activity toward phosphorylated eEF-2 was observed during M (Carlberg *et al.*, 1991). These two studies are thus contradictory: While the highest CaMK-III activity was found in early S phase, the highest level of phosphorylated eEF-2 was observed in M phase. In addition, neither group synchronized cells, and examined kinase activity *in vivo*, as well as *in vitro* phosphorylation of eEF-2 through the cell cycle in a systematic manner.

Therefore, it is important to investigate if CaMK-III activity is cell cycle-dependent. Also, there is a correlation between the kinase activity and the phosphorylation level of its substrate, eEF-2. For this purpose, NIH 3T3 cells were arrested at different phases of the cell cycle by various media or drugs, and then released from each arresting point for synchronization. In these conditions, the activity of the kinase, and the level of pEF-2, was measured to show that they were cell cycle dependent and were changed in a parallel manner during the cell cycle.

Materials and Methods

Materials Dulbeccos modified eagle medium (DMEM), Eagles minimum essential medium (EMEM), bovine calf serum (BCS), Hanks balanced salt solution (HBSS), antibiotics, hydorxyurea (HU), bisbenzimide Hoechst 33342 fluorochrome (H33342), nocodazole, Kemptide, calmodulin (CaM) from bovine brain, trypsin, and trypane blue were purchased from Sigma (St, Louis, USA). [^{3}H]-thymidine, [γ-^{32}P]-ATP, and carrier-free ^{32}Pi were obtained from Amersham (Arlington Hts., USA). All other chemicals were of the highest quality grade commercially available.

Cell culture NIH 3T3 cells were grown as monolayer culture in Dulbeccos modified Eagles medium (DMEM), supplemented with 10% (v/v) bovine calf serum (BCS) and antibiotics (penicillin at 100 units/ml and streptomycin at 50 μg/ml) at 37°C in an atmosphere of 5% CO_2.

Treatment of cells Exponentially grown subconfluent cells were treated with each type of culture media and/or drugs to examine CaMK-III activity and the phosphorylation of eEF-2 at designated points on the cell cycle. Each treatment, to arrest the cells at different phases of the cell cycle, was designed by extensive paper searches and substantial applications to the cells. (a) When cells grew to about 70% confluency they were incubated in serum-deficient DMEM (0.5% BCS) for 3 days (Tobey *et al.*, 1988). (b) The normal DMEM, supplemented with 10% BCS of exponentially growing cells, was changed to isoleucine-deficient (-Ile) Eagles minimum essential medium (EMEM) [containing low BCS (5%)] then the cells were cultured for 2 days (Tobey *et al.*, 1973). (c) The normally grown 70% confluent cells were incubated in DMEM, then supplemented with 10% BCS and 1 mM hydroxyurea for 16 h (Tobey *et al.*, 1988). (d) An inhibitor of DNA-topoisomerase II, H33342 (13.3 μM), was applied to the normal medium of exponentially growing cells and incubated for 17 h (Tobey *et al.*, 1990). (e) Cells growing in DMEM were treated with 1 μM nocodazole for 14 h (Brizuela *et al.*, 1988). At the end of each designated culture period, cell population was analyzed according to their phase in the cell cycle by flowcytometry (Park *et al.*, 1998a). For the release of cells from their arresting phase, the cells were washed by HBSS three times and then incubated in fresh DMEM, supplemented with 10% BCS. The viability was determined by a dye exclusion assay for every treatment of cells.

Protein extraction Cells from different treatments were washed with ice-cold TBS three times and harvested by scraping. After pelleting cells by low speed centrifugation (1,000×g, 3 min), the cells were resuspended in 5 volumes of an extraction buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02 mM leupeptin) and sonicated on ice for 10 sec. This homogenate was centrifuged at 100K×g for 30 min in order to obtain the soluble proteins. The aliquots of the soluble proteins were analyzed for pEF-2/eEF-2 contents and used as sources of kinase activity.

Protein kinase assays Protein kinase assays were performed using various time frames at 30°C (Park *et al.*, 1998b). Every assay was duplicated, and the results averaged from 2-4 separate experiments. CaMK-III activity was measured in a reaction mixture (final volume of 100 μl) containing 50 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10 μg/ml CaM, either 1 mM EGTA (minus Ca^{2+}) or 1.5 mM, (plus Ca^{2+}), 5 μg of soluble protein extract, and 5 μg of purified eEF-2. The mixture was incubated for 1 min and the reaction started by adding 20 μM [γ-^{32}P]-ATP (100-300 cpm/pmol). The phosphorylation reaction was stopped after 3 min incubation by addition of a SDS-containing stop solution. The phosphorylation of proteins was analyzed by SDS-PAGE and autoradiography. For the quantitation of eEF-2 phosphorylation, eEF-2 bands on the gels were localized by Coomassie blue staining and autoradiography, and excised from the dried gels. The incorporation of ^{32}Pi was measured by liquid scintillation spectrometry.

The cyclic AMP-dependent protein kinase (PKA) activity was measured in a reaction mixture (final volume of 100 μl)

containing 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 5 mM dithiothreitol, 150 μM of Kemptide, 4 μM cAMP and 5 μg of the soluble protein extract. The reaction was started by addition of 50 μM [γ-^{32}P]-ATP (100-200 cpm/pmol) and incubated for 5 min. After the incubation, 70 μl of each reaction mixture was applied to phosphocellulose membrane patch (1.5×2 cm) and immersed in 75 mM phosphoric acid. The residual radioactivity on the membrane was determined by liquid scintillation counting after intensive washing.

Analysis of *in vivo* phosphorylated eEF-2 In order to determine the *in vivo* proportion of pEF-2, cell extracts from different phases of the cell cycle were separated by SDS-PAGE, and then corresponding areas for eEF-2 or pEF-2 migration on duplicated gels (~100 kDa) were transferred onto nitrocellulose membrane. The membrane was then blocked with non-fat dried milk [5% in Tris-buffered saline (TBS) containing 1% Tween 20] and exposed to anti-pEF-2 antibody and anti eEF-2 antibody, separately. The specific binding of each antibody was detected by incubation with biotin-labeled Protein A. The proportion of pEF-2 was determined as [the band intensity of pEF-2 on western blot]/ [the band intensity of eEF-2 on Western blot].

[^{3}H]-thymidine incorporation Cells grown up to 70% confluency in 5 cm culture dishes was further incubated in serum-deficient DMEM. After 3 days of culture, cells were washed and cultured in fresh DMEM supplemented with 10% BCS. At this point, [^{3}H]-thymidine (0.2 μCi/ml) was added to label the cellular DNA. The radioactive culture medium was changed to a radioactivity-free one after 4 h. The medium was removed every 2 h and cells were washed 3 times with ice cold TBS. The washed cells were lysed on the dishes by 0.8 ml of TBS, containing 0.1% Triton X-100, for 15 min. To measure the DNA labeling, 50 μl of the total cell lysate was applied to scintillation cocktail and the radioactivity was counted.

Results

Treatment of cells In order to study the activity change of CaMK-III, and the phosphorylation level change of eEF-2 during the cell cycle, the cells were arrested at specific phases of the cell cycle by specific media or drugs, of which action mechanisms or targets were already known. In addition, these methods did not exert any direct effect on CaMK-III activity and the phosphorylation level of eEF-2, when examined in *in vitro* assay system (data not shown). At the end of each treatment, cells were analyzed by flowcytometry to determine the phase at which the majority of cells were arrested.

As shown in Table 1, serum-deprivation for 3 days effectively arrested most of the NIH 3T3 cells at G0 phase. The cells at G0 were determined by fluorescent dye staining of chromosome, measurement of DNA synthesis, and cell morphology. Cultivation of the cells in isoleucine-deficient EMEM, containing low BCS for 2 days, arrested 88% of cells at early G1 phase. This was known to be due to the limited availability of a nitrogen source (Tobey, 1973). This method was the mildest one because most of cells were viable after the designated incubation period. Hydroxyurea [an inhibitor of ribonucleotide reductase that regulates the level of deoxyribonucleotides (Tobey, 1988)] in the medium for 16 h arrested the cells at G1/S transition with relatively low efficiency (69%). It was observed that a relatively large amount of cell debris was floating in the medium. This was even seen after the shorter cultivation. It was likely that HU was cytotoxic, as reported earlier (Lerza *et al.*, 1999). Cells growing in the medium, supplemented with H33342, stopped growing (73%) at G2/M transit after 17 h. This drug was recently introduced as a cell cycle blocker for its ability to arrest cells at the specific phase of the cell cycle. A well-known anti-microtubule drug, nocodazole, arrested 81% of cells at M after 14 h incubation. However, its substantial efficiency would be higher because of the detachment of cells undergoing mitosis from their mother cells during the cell preparation. Enzyme assay, or protein quantitation in cells arrested at specific point, was normalized according to arresting efficiency.

Change of eEF-2 phosphorylation Since the phosphorylation of eEF-2 blocks the peptide elongation step in protein synthesis, the level of *in vivo* pEF-2 reflects the extent of the overall protein synthesis rate in cells. It would be interesting to examine whether the phosphorylation level of eEF-2 is changed at different phases in the cell cycle. This could provide evidence that may suggest that the change in eEF-2 phosphorylation level controls the protein synthesis as

Table 1. Condition of cell treatment and cell population arrested at a specific phase of the cell cycle

Treatment	Concentration	Culture period	Arresting phase	Cell population (%)
Serum deficient DMEM	0.5% BCS	3 days	G0	91.0±4.3
N-limited EMEM	minus Ile, 5% BCS	1 day	G1	88.1±6.1
Hydroxyurea	1 mM	12 h	G1/S	73.4±2.7
Hoechst33342	13.3 μM	17 h	G2/M	79.1±4.4
Nocodazole	1 μM	16 h	M	80.5±1.6

Cell population determined by flowcytometry indicates the proportion of cells arrested at an expected phase of the cell cycle. However, that arrested at G0 was determined by fluorescent dye staining of chromosomes, [^{3}H]-thymidine incorporation and morphological observation. All culture media were DMEM supplemented with 10% BCS otherwise indicated. N-limited EMEM, nitrogen source-limited EMEM.

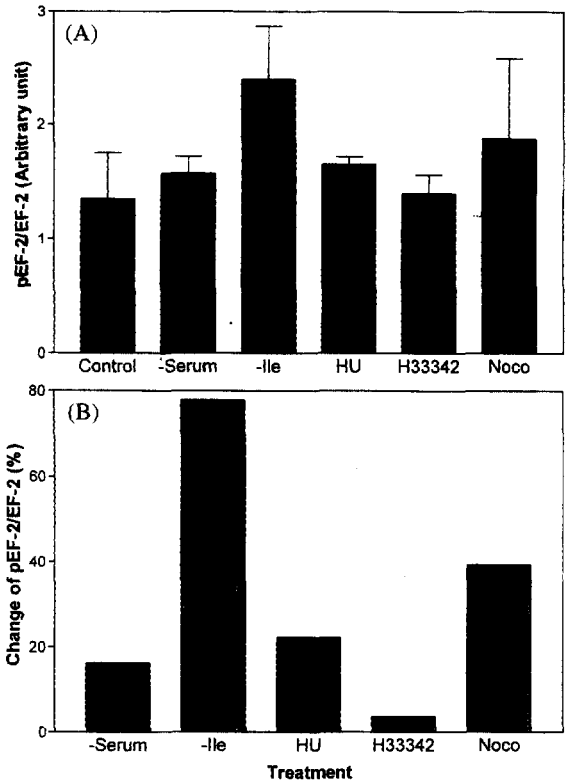

Fig. 1. The change of pEF-2 content from the cells treated with different media and drugs. Exponentially growing cells were treated as indicated in the Materials and Methods. The contents of total eEF-2 and pEF-2 were quantitated by antibodies specific to eEF-2 and pEF-2, respectively. The pEF-2 content was expressed [band intensity of pEF-2 on Western blot]/[band intensity of eEF-2 on Western blot] (A). The percentage of change of eEF-2 content was calculated as a net change of pEF-2 compared to that of control (B). Control, asynchronously growing normal cells; -Serum, treatment with serum deprived DMEM for 3 days; -Ile, treatment of isoleucine-deficient EMEM for 2 days; HU, hydroxyurea treatment for 16 h; H33342, Hoechst33342 treatment for 17 h; Noco, nocodazole treatment for 14 h.

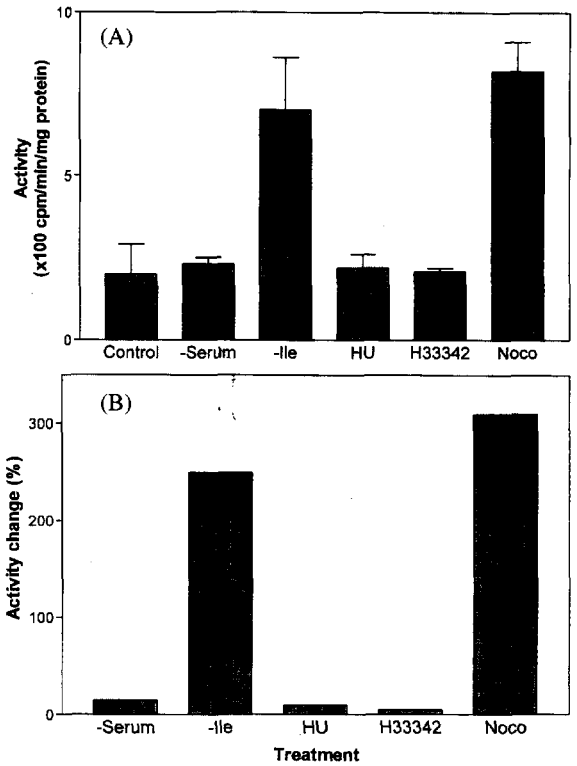

Fig. 2. The change of CaMK-III activity in the cells treated with different media and drugs. The kinase activity from treated cells was measured by use of [γ-^{32}P]-ATP and purified eEF-2. After analyzing phosphorylated proteins by SDS-PAGE, the pEF-2 band was excised and radioactivity was counted by liquid scintillation spectrometry. (A) Specific activity of CaMK-III after each treatment. (B) The net activity change of CaMK-III. Control, asynchronously growing normal cells; -Serum, treatment with a serum deprived DMEM for 3 days; -Ile, treatment of isoleucine-deficient EMEM for 2 days; HU, hydroxyurea treatment for 16 h; H33342, Hoechst33342 treatment for 17 h; Noco, nocodazole treatment for 14 h.

cells proceed the cell cycle. The soluble proteins from the cells arrested at different phases were separated by SDS-PAGE and then total eEF-2 and *in vivo* phosphorylated form of eEF-2 were quantitated by antibodies specific to each form. The general profile of pEF-2 content change showed a distinct pattern (Fig. 1A). The proportion of pEF-2 to total eEF-2 increased in the cells treated by Ile-deprivation and nocodazole that arrested cells at G1 and M, respectively. The G1-arrested cells showed approximately a net 80% increase of pEF-2 proportion (Fig. 1B). Nocodazole treated cells, which were arrested at M, demonstrated net 40% increase of pEF-2. These results indicated that the cells at early G1 or M phase had at least 40-80% less content of active eEF-2 compared to asynchronized control cells. Since the pEF-2 is inactive for peptide elongation, the protein synthesis rate at early G1 and M might be decreased. The cells treated by H33342 showed no change, whereas serum-deprivation and hydroxyurea treatment increased the pEF-2 content about 20%. Therefore, it was likely that there was a relatively large extent of decrease in elongation activity at early G1 and M; whereas there was no change in elongation activity at G2/M, or there was only a small extent of decrease at G0 and G1/S.

Activity change of CaMK-III Since the level of eEF-2 phosphorylation was different at each phase of cell cycle, it was necessary to investigate whether the activity of corresponding kinase for eEF-2 phosphorylation was also cell cycle-dependent. For this purpose, the activity of CaMK-III of cells from each arrested point was also determined (Fig. 2A). When the cells were growing in a serum-deficient medium for 3 days, there was no change in the kinase activity. Similar results were obtained when the cells were cultured in the media supplemented with hydroxyurea and H33342, respectively. As serum deprivation caused cells to enter G0,

and hydorxyurea and H33342 arrested cells at G1/S and G2/M, respectively, CaMK-III was not activated at these points. However, the cells grown in Ile-deficient medium showed a 2.5-fold increase in the enzyme activity (Fig. 2B). Nocodazole treatment also increased the kinase activity up to 3-fold. These results indicated that the activity of CaMK-III at early G1 and M phases was increased more than 2-fold.

The kinase activity in nocodazole-treated cells was more potent than isoleucine-depleted cells, whereas the phosphorylation level of EF-2 was higher in isoleucine-depleted cells than nocodazole-treated cells (Fig.1 and Fig. 2). This is likely due to the different activity level of phosphoprotein phosphatases in M. Since mitosis dynamically changes cellular structure, it requires the activation of more protein kinases and phosphoprotein phosphatase than G1. This kind of kinase/phosphatase activation might generate new equilibrium of kinases and phosphatases. Elongation factor 2 was highly specific to kinase (only to CaMK-III), whereas pEF-2 was not specific to phosphatase (data not shown). Therefore, pEF-2 in M could be exposed to more phosphatases.

The changing patterns of CaMK-III activity, and pEF-2 content, seemed correlated. The cells arrested at G1 showed an increase in the kinase activity and in the phosphorylation of eEF-2. Mitotic cells also demonstrated an increase in the kinase activity and in eEF-2 phosphorylation. Other phases, such as G0, G1/S, and G2/M, showed little or no change in the kinase activity and eEF-2 phosphorylation. However, the extent of activation of the enzyme, and that of eEF-2 phosphorylation, were different. CaMK-III was activated at least 250% at both phases of G1 and M, whereas phosphorylation of eEF-2 was increased 40-80% at the same phases.

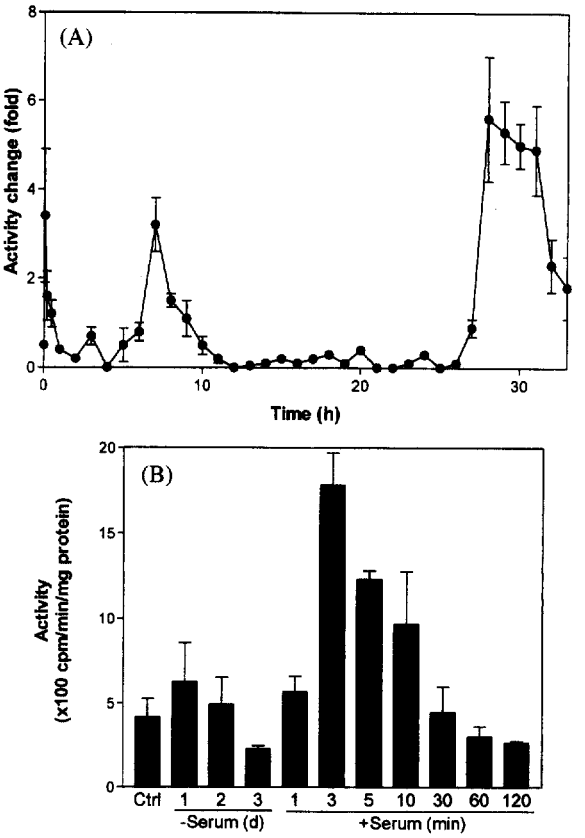

Fig. 3. Activity change of CaMK-III in the cells released from G0 by serum replenishment (A). The activity was measured at one-hour intervals, except at the very early stage in which the activity was measured at 3, 5, and 30 min. (B) CaMK-III activity change during serum deprivation and very early stage of serum replenishment. -Serum, serum deprivation; +Serum, serum replenishment.

Activity change of CaMK-III during one turn of the cell cycle Forcing cells arrested at a specific phase of the cell cycle by alteration of external environments was not a physiologically intact state. The activity of CaMK-III, and phosphorylation of eEF-2 in arrested cells may not reflect those in native cells. Therefore, it was necessary to study the change of CaMK-III activity and eEF-2 phosphorylation in the native and synchronous cells. For this purpose, the cells were cultured in a serum-deficient medium for 3 days and serum replenishment was followed. Since the cells were arrested at a quiescent state, application of serum to the medium caused the cells to re-enter the cell cycle. All of the cells proceeded the cell cycle synchronously, since each cell entered the cell cycle from the same start line. As shown in Fig. 3A, the replenishment of serum to the G0-arrested cells activated CaMK-III three times during the 33 h of culture. The first activation in a rapid and brief mode was seen right after the serum application, the second one was observed at 7 h point, and the last one was at 28 h point. The first two activation peaks showed about a 3-fold increase in the activity; whereas the last activation demonstrated 6-fold increase. After 6 hours of serum supplement, CaMK-III activity began to increase and reached its highest activity after 7 h. This increased kinase activity returned to basal level after 10 h. Afterward, there was no evident change of the activity until 27 h. The kinase activity increased abruptly after 27 h and the maximal activity was 6-fold higher than basal level.

Because there was no report demonstrating how the kinase activity was changed during serum-deprivation, and the very early stage of serum replenishment, the kinase activity change was scrutinized (Fig. 3B). The kinase activity increased up to 50% on the first day of serum-deprivation and decreased down to 40% of control as the treatment was continued for 3 days. However, the replenishment of serum on the third day activated the kinase up to 3.5-fold in 3 min. The fast and brief serum activation was terminated within 30 min. The activation of CaMK-III under this condition was a distinct process.

As cells could be synchronized when they were released from the arresting point, it was possible to determine the time required to reach each phase. Therefore, the activity of CaMK-III was measured in the cells released from the early G1-arrest, which did not show a lag period to restart the cycle

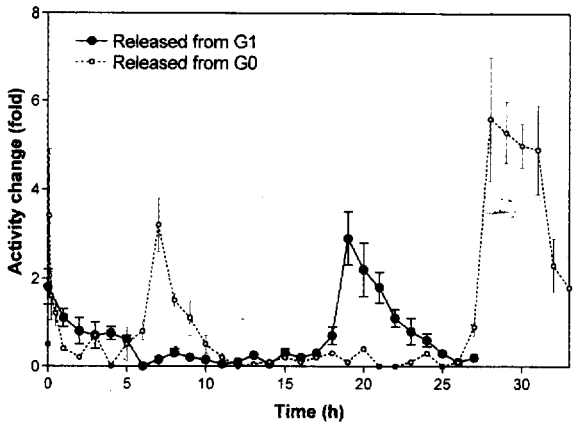

Fig. 4. The activity change of CaMK-III in the cells released from early G1. The kinase activity was measured in the cells released from G1 by changing culture medium from Ile-deficient EMEM to DMEM supplemented with 10% BCS (closed circle). The activity change profile of G0-released cells, shown in Fig. 3, was co-plotted for a comparison (open circle). The activity was measured every hour.

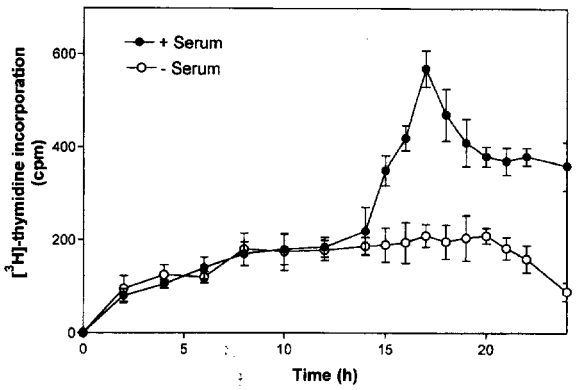

Fig. 5. Labeling of DNA with [^{3}H]-thymidine in the cells released from G0. After cells were cultured in serum deficient DMEM for 3 days, serum was replenished when [^{3}H]-thymidine was applied to the medium (+Serum). The same treatment, without serum replenishment, was carried out as control (-Serum). The radioactive medium was changed to non-radioactive one after 4 h. The incorporation of radioactivity to DNA was measured at every 2-hours by liquid scintillation spectrometry.

(Fig. 4). The general profile of activity change was similar to that from G0-release (open circle), except there was less of an activity change. The G1 arrested cells had already activated CaMK-III (closed circle). However, the activity returned to the basal level slowly as time passed. The kinase started to be activated 17 h after the release and reached the highest activity in 2 h. The cells, showing the activation of the kinase in the both G0- and G1-release experiments, were analyzed by flowcytometry to determine which phase the cells were proceeding. The activity peak of 6-8 h points from G0-release experiment turned out to be the kinase activity at early G1 phase. The activity peak of 27-29 h points from G0-release, and that of 18-20 h points from G1-release, was at M phase. From these two separate experiments, the lag period from the G0 to early G1 could be calculated as about 8 h. M phase was about 19 h apart from early G1. Therefore, if the mitotic period were 2-3 h, as in other cell lines, the generation time of NIH 3T3 cells would be 21-22 h. This coincided with a previous study, which reported that the generation time of fibroblast cells was 22-24 h (Zocchi *et al.*, 1998).

For further confirmation of the time required to reach a specific point on the cell cycle, [^{3}H]-thymidine incorporation was measured in the cell from the very beginning of G0 release (Fig. 5). The radioactivity started to increase 15 h after the release. It reached the maximum at 17 h, then reduced to half of the maximum level and stayed as plateau. Since the DNA synthesis reflected S phase, it took at least 17 h from the G0 to S phase. When 7-9 h of lag from G0 release was subtracted, early S phase was about 9 h apart from early G1. These results showed that the calculated period of each phase, from three separate experiments, was matched to each other.

PKA activity change in the cells from different phases It

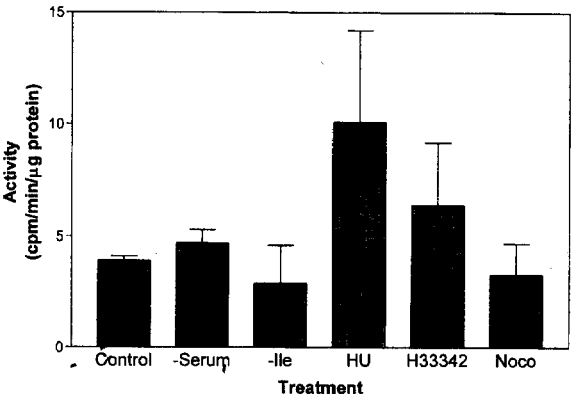

Fig. 6. The activity change of PKA in the cells treated with different media and drugs. Exponentially growing cells were treated as indicated in the Materials and Methods. PKA activity was measured in the soluble proteins from cells treated with different media and drugs. Control, asynchronously growing normal cells; -Serum, treatment with a serum deprived DMEM for 3 days; -Ile, treatment of isoleucine-deficient EMEM for 2 days; HU, hydroxyurea treatment for 16 h; H33342, Hoechst33342 treatment for 17 h; Noco, nocodazole treatment for 14 h.

was known that CaMK-III was phosphorylated *in vitro* and *in vivo* by the catalytic subunits of PKA and the treatment raising the cAMP level in cells, respectively (Nairn *et al.*, 1987; Mitsui *et al.*, 1993). Therefore, it was examined whether PKA activity was correlated to CaMK-III activity at different phases of the cell cycle (Fig. 6). The activity of PKA was increased 2-fold at hydroxyurea treated G1/S arresting cells; whereas that at early G1 was decreased below the basal level. PKA activity was almost unchanged at M, and increased 42% and 24% at G2/M and G0, respectively. These results were

relevant to the activity change of CaMK-III at corresponding points of the cell cycle. PKA activity was low at G1 and M, and CaMK-III activity was high at the same phases. This indicated a possibility that CaMK-III might be regulated reciprocally by the activity of PKA during the cell cycle. That is, PKA was inactivated or inactive at G1 and M, respectively. At these phases, CaMK-III had less chance to be phosphorylated by PKA than at the other phases, indicating its higher activity. Therefore, relatively activated CaMK-III could phosphorylate more eEF-2 at G1 and M. This sequence of reactions resulted in a decrease of protein synthesis in M and G1, as previously reported (Fan and Pennman, 1970).

Discussion

Cell cycle is an orderly series of events that every cell must execute to reproduce and multiply at specific times during its life span. Therefore, every physiological procedure should work exactly according to its time schedule on the cell cycle. It is already well known that the overall rate of protein synthesis at mitosis is decreased 80% in non-mitotic cells (Fan and Pennman, 1970; Tarnowka and Baglioni, 1979). However, it is still unclear how the protein synthesis is regulated during the cell cycle. If protein synthesis is cell cycle dependent, its component machinery is also cell cycle dependent.

The idea that the phosphorylation state of eEF-2 is cell cycle-dependent is supported by the changes of Ca^{2+} and CaM levels during the cell cycle. Since eEF-2 phosphorylation exhibits a strict dependence on Ca^{2+}/CaM (Nairn *et al.*, 1987; Mitsui *et al.*, 1993), the activity of eEF-2 may well be regulated by Ca^{2+} and CaM levels *in vivo*. There is a large body of data suggesting that the $[Ca^{2+}]_i$ and CaM levels during the cell cycle (for a review, see Lu and Means, 1993) $[Ca^{2+}]_i$ is transiently increased at G1/S, G2/M and during M phase, which is parallel to the change of CaM level. These temporal changes of the Ca^{2+}/CaM level are essential for cells to execute each round of the cell cycle. If cells fail to increase Ca^{2+}/CaM transiently at certain specific times in the cell cycle, they are arrested at a critical point in the cell cycle (Sasaki and Hidaka, 1982; Kao *et al.*, 1990).

In order to investigate the cell cycle dependency of eEF-2 phosphorylation and CaMK-III activity, it was important to arrest cell reversibly at various points in the cell cycle. Five methods to arrest cells were selected by extensive paper searches and applications to cell culture. These methods were quite effective in arresting cells at G0, G1, G1/S, G2/M, and M, respectively. The methods showed a 82% average ability to arrest cells at designated points. Hydroxyurea and H33342 were cytotoxic when they were incubated for longer periods. The advantage of these methods was their reversibility. Each medium, or drug, was easily removed from the culture medium and cells could restart the cell cycle synchronously from where they were arrested. Therefore, it provided good tools to study the activity change of CaMK-III in synchronized cells.

The change in the eEF-2 phosphorylation level was cell cycle-dependent. It was increased at G1 and M. A similar change was observed in the activity of CaMK-III. However, both the phosphorylation and the activity were low at G1/S and G2/M. Since the phosphorylation of eEF-2 blocks the elongation step, early G1 and M phases may carry out a low level of protein synthesis. This analysis was supported by early reports showing that the Ca^{2+}/CaM level increased transiently at similar phases (Arnoult *et al.*, 1988; Zimmer and Van Eldik, 1988). When cells enter the G1 or M, there was a rapid increase in Ca^{2+} and CaM levels. The increase in Ca^{2+}/CaM activated CaMK-III and, in turn, the activated kinase phosphorylated more eEF-2. As the contents of pEF-2 increased, the elongation step in translation started to be blocked. This event eventually led to the inhibition of overall protein synthesis. The phosphorylation of eEF-2 by CaMK-III was specific during the cell cycle. When the *in vivo* phosphorylation change of the soluble proteins at specific points on the cell cycle was measured, it increased 25% at G1, as well as at M, (data not shown) where the pEF-2 level also increased. However, those at G0, G1/S, and G2/M were unchanged, or decreased to a large extent.

The change of CaMK-III activity during serum-deprivation was investigated for the first time in this present study. It showed a biphasic pattern, activation and inactivation during 3 days of serum-deprivation. It was likely that the cells had to slow down, or shut down most of protein synthesis machinery after serum deprivation in order to reserve energy, and to maintain a minimal level of protein synthesis. As a result, cells from minimal protein synthesis contained less total protein than cells growing in serum-sufficient medium. This might explain why the CaMK-III activity decreased 50% below the basal activity. Another unique aspect of serum-deprivation treatment was a very rapid and transient activation of the kinase after serum replenishment, as already shown in a previous study (Palfrey *et al.*, 1987). Although it is still unclear what the physiological meaning of this kind of huge transient activation is, there is an acceptable explanation for it. When cells are quiescent, they maximally minimize energy consumption by shutting down protein synthesis. When the condition is restored, in order to make it good for growth, cells should turn on the protein synthesizing machinery. At this moment cells reprogram or restart the machinery by stopping currently on-going protein synthesis. The activation of CaMK-III and thus the phosphorylation of eEF-2 is one of possible ways to do this.

The activity of CaMK-III seemed to be regulated by PKA, because CaMK-III could be down-regulated in PC12 cells by nerve growth factor or agents that increase cAMP levels (Nairn *et al.*, 1987). The purified CaMK-III could be phosphorylated by PKA, and the phosphorylated CaMK-III showed Ca^{2+}-independent activity that reached to about 40% of Ca^{2+}-activated CaMK-III activity (Mitsui *et al.*, 1993). Similar results were observed in isoproterenol-treated adipocytes (Diggle *et al.*, 1998) and IPC-81 cells (Hovland *et*

al., 1999). They especially showed that the Ca^{2+}-independent activity of CaMK-III played a role to phosphorylate eEF-2 and consequently to inhibit protein synthesis. These observations were physiologically important in two aspects. First, they provided an indirect evidence for the close relationship between CaMK-III and PKA *in vivo*. Second, Ca^{2+}-independent activity of CaMK-III could last longer than the time span of Ca^{2+} signaling. Since the Ca^{2+} signaling in the cell is very rapid (usually within 1 min), CaMK-III activity is also short-lived. If this is the case, there would not be enough time to complete the signal transduction through CaMK-III, because the protein synthesis is a process requiring a relatively longer time period. In the present study, the activity change of PKA at various points on the cell cycle showed an opposite pattern to that of CaMK-III. This was another evidence for the direct phosphorylation of CaMK-III by PKA *in vivo*.

Altogether, the regulation of protein synthesis in the elongation step during the cell cycle can be speculated as follows. The intracellular Ca^{2+} and CaM level increases as cells approach G1 or M resulting in the activation of CaMK-III. The activated CaMK-III phosphorylates eEF-2, and in turn pEF-2 slows down or shuts down the peptide elongation. This kind of sequential reaction eventually leads to overall inhibition of cellular protein synthesis. PKA may play a role as a negative regulator of CaMK-III when the cell cycle proceeds, or certain external signals arrive.

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