Effect of serum and hydrogen peroxide on the Ca^{2+} /calmodulin-dependent phosphorylation of eukaryotic elongation factor 2(eEF-2) in Chinese hamster ovary cells

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Abbreviations: eEF-2, eukaryotic elongation factor 2; CaM, calmodulin; CHO, Chinese hamster ovary

Abstract

Eukaryotic elongation factor eEF-2 mediates regulatory steps important for the overall regulation of mRNA translation in mammalian cells and is activated by variety of cellular conditions and factors. In this study, eEF-2 specific, Ca^{2+} /CaM-dependent protein kinase III (CaM PK III), also called eEF-2 kinase, was examined under oxidative stress and cell proliferation state using CHO cells. The eEF-2 kinase activity was determined in the kinase buffer containing Ca^{2+} and CaM in the presence of eEF-2 and [γ - ^{32}P] ATP. The eEF-2 kinase activity in cell lysates was completely dependent upon Ca^{2+} and CaM. Phosphorylation of eEF-2 was clearly identified in proliferating cells, but not detectable in CHO cells arrested in their growth by serum deprivation. The content of the eEF-2 protein, however, was equivalent in both cells. Using a phosphorylation state-specific antibody, we show that oxidant such as H_2O_2 , which triggers a large influx of Ca^{2+} , dramatically enhances the phosphorylation of eEF-2. In addition, H_2O_2 -induced eEF-2 phosphorylation is dependent on Ca^{2+} and CaM, but independent of protein kinase C. In addition, okadaic acid inhibits phosphoprotein phosphatase 2A(PP2A)-mediated eEF-2 dephosphorylation. These results may provide a possible link between the elevation of intracellular Ca^{2+} and cell division and suggest that phosphorylation of eEF-2 is sensitive cellular reflex on stimuli that induces intracellular Ca^{2+} flux.

Keywords: Ca^{2+} , CaM, eEF-2, eEF-2 kinase

Introduction

The regulation of protein synthesis in eukaryotes is integrated with the translation process and other metabolic pathways of the cell, *i.e.* altered translation rates occur during mitosis, upon nutrient starvation or oxidant stress, and following treatment with hormones or growth factors. The process of mRNA translation takes place by three distinct phases termed (peptide-chain) initiation, elongation, and termination. The acute regulation of mRNA translation depends on changes in the activity of initiation factors or elongation factors, which is also commonly mediated by alteration in their states of phosphorylation (Hershey *et al.*, 1996). Elongation factor 2 (eEF-2), one of two elongation factors involved in the elongation phase of translation in higher eukaryotes, facilitates translocation of peptidyl-tRNA from the ribosomal A site to the P site. Phosphorylation of eEF-2 reduces the affinity of the factor for the ribosome, thereby decreasing the rate of protein synthesis (Nilsson and Nygard, 1991). The translational activity of the phosphorylated eEF-2 can be restored by the action of phosphoprotein phosphatase 2A and 2C (PP2A and PP2C, respectively) (Redpath and Proud, 1990). eEF-2 is phosphorylated by a specific eEF-2 kinase previously also known as Ca^{2+} and calmodulin-dependent protein kinase III, on threonine residues (Calberg *et al.*, 1991). Calmodulin mediates many of the intracellular effects of Ca^{2+} (Park, 2001), and the eEF-2 kinase is entirely dependent on Ca^{2+} /CaM for activity in mammalian tissues. Increased phosphorylation of eEF-2 occurs in several types of cells in response to stimuli, which elevates cellular Ca^{2+} levels or stimulates cellular proliferation (Enkemann *et al.*, 1999). Decreased kinase activity is seen in the presence of drugs that increase the concentration of cAMP (Hovland *et al.*, 1999). Previous study has demonstrated that oxidants such as hydrogen peroxide (H_2O_2) stimulate several early growth response events such as the stimulation of protein tyrosine phosphorylation (Rao, 1997). Modification of the redox thiol status of the cytosol could also contribute to the wide-ranging effects of H_2O_2 . In particular, H_2O_2 and oxidized glutathione has been shown to potentiate the spontaneous release of Ca^{2+} from inositol triphosphate-sensitive Ca^{2+} stores (Missiaen *et al.*, 1991). Increased intracellular Ca^{2+} is also associated with phosphorylation of eEF-2 and inhibition of protein synthesis (Marin *et al.*, 1997).

In this report we have studied regulation of the eEF-2 kinase activity in Chinese hamster ovary cells treated with various stimuli including H_2O_2 to influence intracellular Ca^{2+} concentration. In addition, this study was to determine whether induction of cell division by serum was linked to the activation of eEF-2 kinase.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM), antibiotic-antimycotic mixture, and trypsin-EDTA were purchased from Gibco/BRL Life Technologies, Inc (Gaithersburg, MD). Sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) were obtained from Boehringer Mannheim. [γ - ^{32}P] ATP (6,000 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). Okadaic acid was from Calbiochem, diacyl ethylene glycol (DAEG) was from Avanti Polar Lipids, Inc., fetal bovine serum (FBS) was from Hyclone, and ECL Western blotting detection kit was from Amersham Pharmacia Biotech. Hydrogen peroxide (H_2O_2), phosphoprotein phosphatase 2A (PP2A), trifluoperazine (TFP), Nutrient mixture F-12 HAM, and all other chemicals were from Sigma.

Cell culture

HeLa cells, Vero cells, and NIH3T3 cells were grown in DMEM containing 25 mM D-glucose, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.25 $\mu\text{g}/\text{ml}$ amphotericin B and 10%(v/v) FBS in a humidified 5% CO_2 incubator at 37°C. Chinese hamster ovary(CHO)-K1 cells were grown in Nutrient mixture F-12 supplemented with 10% FBS and same concentrations of antibiotic-antimycotic mixture described above. Serum deprivation was carried out by lowering the concentration of FBS from 10% to 0.5% for the period of 24 h prior to specific treatment.

Preparation of cell lysates

CHO cells were plated onto 60-mm dishes and grown in Nutrient mixture F-12 containing 10% FBS. At 70-80% confluence, cells were growth-arrested by incubating for 24 h in Nutrient mixture F-12 containing 0.5% FBS. Growth-arrested CHO cells were treated with and without various concentrations of H_2O_2 , TFP, DAEG, or EGTA for 30 min at 37°C. In the case of time course experiments, CHO cells were treated with and without 200 μM H_2O_2 , for the indicated times at 37°C. After treatments, medium was removed, and cells were rinsed with ice-cold phosphate-buffered saline (PBS) and collected with a rubber policeman, suspended in 0.5 ml of suspension buffer (10 mM Tris/HCl, pH 7.5, and 2 mM MgCl_2) and centrifuged at 2,700 g at 4°C for 5 min. Then they were freeze-thawed, suspended in 20 μl of suspension buffer containing 1% Triton X-100, 5 mM DTT, and 1 mM

phenylmethylsulfonyl fluoride (PMSF), vortexed, and then stood for 10 min on ice. The cell lysates were cleared by centrifugation at 15,000 g at 4°C for 10 min. Cell lysates containing equal amounts of protein from control and each treatment were used for SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Purification of eEF-2 protein

Purification of eEF-2 from chicken embryo was carried out according to Kim *et al.* (1991) with some modification. The peak fractions of eEF-2 obtained from DEAE-Sephacel column chromatography were dialyzed against 50 mM potassium phosphate buffer, pH 6.7, containing 10% glycerol, and applied to CM-Sephadex C-50 column. Finally, gel filtration chromatography was done using Ultrogel AcA-44 in 50 mM Tris/HCl, pH 7.5, 5 mM MgCl_2 , 100 mM KCl, 10 mM β -mercaptoethanol, and 25% glycerol. eEF-2 activity was assayed by ADP-ribosylation with diphtheria toxin in the presence of [^{32}P] NAD(Kang *et al.*, 1994).

Production of antibodies against total or phosphorylated eEF-2

The serum that specifically recognizes the phosphorylated form of eEF-2 was obtained by immunization of rabbits with a peptide encompassing eEF-2 phosphorylation sites [GETRFT(P)DTRK]. This peptide was synthesized, and the threonine at the position corresponding to Thr 56 in the native protein was phosphorylated chemically (Czernik *et al.*, 1995). The serum recognizing total eEF-2 was obtained by immunization of rabbits with a peptide derived from the same region of the protein [ARAGETRFTDTRKD] (Alirezai, *et al.*, 2001).

Determination of eEF-2 kinase activity

For determination of the eEF-2 kinase activity, cell homogenate was incubated with reaction buffer (50 mM Hepes, pH 7.6, 1.5 mM CaCl_2 , 1.0 mM DTT, 0.25 mM Na_3VO_4 , 1.0 mM MgCl_2 , and 1.5 mM MnCl_2) containing 1 μmol of purified eEF-2 in the presence of 0.25 mM [γ - ^{32}P]ATP. The reaction mixtures were incubated at 37°C for 10 min, and terminated by the addition of 10 μl of 5 X Laemmli sample buffer containing 0.2 M Tris, pH 6.8, 6% SDS, 30% glycerol, and 15% β -mercaptoethanol. Samples were boiled for 5 min and proteins were separated by a 8% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 and dried using a Hoefer Scientific gel drier. X-ray film (Fuji Super RX) was exposed at -70°C using an intensifying screen. The autoradiograms were analyzed with GS-710 Calibrated Imaging Densitometer (Bio-Rad).

Analysis of eEF-2 phosphorylation by sequential immunoblotting

Phosphorylation of eEF-2 was analyzed by sequential

immunoblotting, first with an antibody that specifically recognizes eEF-2 phosphorylated on Thr 56 (1 : 1,000 dilution), and then with an antibody that recognizes total eEF-2 (Alirezai, *et al.*, 2001). Immunoreactive bands were detected with a horseradish peroxidase-coupled secondary antibody using an enhanced chemiluminescence (ECL) method.

Miscellaneous

Total protein was determined according to the method of Bradford (1976) using Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

Results

Various cells such as HeLa, CHO, Vero, and NIH3T3 cells and tissues from rat and chicken were screened for the eEF-2 kinase activity. Cell and tissue extracts were obtained as described in Materials and Methods. Figure 1 demonstrates that the highest activity of eEF-2 kinase was observed in CHO cells, followed by chicken embryo and NIH3T3 cells. Since CHO cells had the greatest specific activity of the kinase, we used the cells for further study.

Ca^{2+} has been implicated to be the main factor responsible for the activation of eEF-2 kinase (Mitsui *et al.*, 1993; Redpath and Proud, 1993). Autoradiography of the phosphorylated proteins from CHO cell homogenates separated by SDS-PAGE showed. A strong correlation between the increase in cytosolic free Ca^{2+} concentration and the phosphorylation of eEF-2 was observed in CHO cells. In the absence of Ca^{2+} (Figure 2A, lane 1), phosphorylation of the eEF-2 protein was not detectable. Ca^{2+} alone stimulated phosphorylation of

the substrate (Figure 2A, lane 2), which was maintained to the same level in the presence of calmodulin (Figure 2A, lane 3). To determine whether the phosphorylation of eEF-2 in CHO cells was related to the state of cell proliferation, we measured the *in vitro* phosphorylation of eEF-2 in confluent CHO cells. The experiments shown on Figure 2A were carried out using cells in logarithmic growth. However, the Ca^{2+} /CaM-dependent phosphorylation of eEF-2 was lost in CHO cells in their resting state (Figure 2B).

Serum deprivation markedly decreased the Ca^{2+} /CaM-dependent phosphorylation of eEF-2 compared to that of control cells (Figure 3). The addition of serum to the previously serum-deprived CHO cells for 48 h restored the Ca^{2+} /CaM-dependent phosphorylation of

Figure 1. Specific activity of the eEF-2 kinase in various cell or tissue extracts. Fifty μg homogenates were incubated in the kinase buffer containing 1 μmol eEF-2 in the presence of 0.25 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reaction mixtures were incubated at 37°C for 10 min and subjected to a SDS-polyacrylamide gel electrophoresis followed by autoradiography. eEF-2 protein (95 kDa) is indicated by an arrow.

Figure 2. Ca^{2+} /CaM-dependent phosphorylation of eEF-2 in logarithmic growth and plateau phase CHO cells. (A) CHO cell extracts harvested in logarithmic growth were phosphorylated in an assay mixture containing 1 mM EGTA, 1.5 mM CaCl_2 or 1.5 mM CaCl_2 plus 2 μM calmodulin. Labeled proteins were detected by autoradiography (upper panel). After the decay of the radioactive signal, the same filter was subjected to a Western blotting (W/B) with an antiserum against eEF-2 (bottom panel). (B) Homogenates of CHO cells in plateau phase of growth were phosphorylated (upper panel) or subjected to Western blotting with eEF-2 antibody (bottom panel).

Figure 3. Effect of serum on the phosphorylation of eEF-2 in serum-deprived CHO cells. CHO cells were plated and grown in F-12 media containing 10% FBS. After a 24 h attachment period, monolayers were washed and maintained in serum-free F-12 media for 48 h followed by refeeding of serum for 24, 36 or 48 h (lane 3-5). Media in control plates contained 10% FBS (lane 1). Cell homogenates were phosphorylated in the presence of 1.5 mM CaCl_2 and 2 μM calmodulin. Endogenous proteins were separated by SDS-PAGE and phosphoproteins were detected by autoradiography (A, upper panel), and the same filter was treated with eEF-2 antibody as described in Figure 2 (A, bottom panel). The data shown are representative of results obtained from three experiments, each performed on different sets of cultured CHO cells. Phosphorylation of eEF-2 was measured by counting the radioactivity in autoradiogram and total amount of eEF-2 was obtained from densitometric analysis of immunoblotting experiments. The ratio between the phosphorylated form and the total amount of eEF-2 in control plate was defined as 100% value (B).

eEF-2 to control level (Figure 3A, upper panel). Kinetic recovery of serum-deprived cells to phosphorylated eEF-2 upon addition of 10% serum showed 45% at 24 h, 88% at 36 h and 100% at 48 h to that level of control cells (Figure 3B). The levels of eEF-2 proteins during the experiments appeared to remain unaltered as indicated by the immunoblots of eEF-2 proteins in the five cell populations (Figure 3A, bottom panel).

To evaluate the phosphorylation state of eEF-2 in

response to oxidative stress, growth-arrested CHO cells were treated with and without various concentrations of H_2O_2 for 30 min, and eEF-2 phosphorylation was analyzed by sequential immunoblotting, first with an antibody that specifically recognizes eEF-2 phosphorylated on Thr 56, and then with an antibody that recognizes eEF-2 independent of its phosphorylation state. H_2O_2 induced eEF-2 phosphorylation in a concentration-dependent manner with a near maximum effect of a 3-

Figure 4. H_2O_2 induces phosphorylation of eEF-2 in a dose- and time-dependent manner. CHO cells were left untreated or treated for 30 min with different concentrations of H_2O_2 (A) or for the indicated times with 200 μM H_2O_2 (B). Immunoblotting was performed with two antibodies, one that recognizes eEF-2 phosphorylated (phospho eEF-2) at Thr 56 (upper panel) and one that recognizes eEF-2 independent of its phosphorylation state (total eEF-2) (middle panel). Immunoreactive bands were detected and quantified as in Figure 3 (lower panel). The ratio between the phosphorylated form and the total amount of eEF-2 under basal conditions was defined as 1 arbitrary unit. The data shown are representative of results obtained from three experiments, each performed on different sets of cultured CHO cells.

Figure 5. Ca^{2+} /CaM kinase antagonist trifluoperazine (TFP) and Ca^{2+} chelator, but not protein kinase C inhibitor diacyl ethylene glycol (DAEG) inhibit H_2O_2 -induced eEF-2 phosphorylation. Growth-arrested CHO cells were treated with and without H_2O_2 (200 μM), TFP (25 μM), EGTA (10 mM) or DAEG (200 μM) for 30 min, and the cell lysates were prepared as described in Materials and Methods. Kinase inhibitor or Ca^{2+} chelator was added 30 min prior to the addition of H_2O_2 . Cell lysates containing equal amounts of protein from control and each treatments were analyzed by immunoblotting with phospho eEF-2 antibody.

fold increase at 200 μM (Figure 4A, upper and lower panel). Although H_2O_2 at 400 μM was found to be more effective in the stimulation of eEF-2 phosphorylation (3.3-fold), we have used non-toxic 200 μM concentration in the subsequent experiments (Rao and Berk, 1992). H_2O_2 induced eEF-2 phosphorylation in a time-dependent manner (Figure 4B, upper and lower panel). After 5 min of H_2O_2 (200 μM) treatment, eEF-2 phosphorylation was increased 2.2-fold as compared with control and the increase in eEF-2 phosphorylation continued at least up to 60 min. The same blots stained with eEF-2 antibody showed no significant changes were observed in eEF-2 levels between control and H_2O_2 treated cells (the middle panel of Figure 4A and 4B).

The possible role of Ca^{2+} /CaM kinases and protein kinase C on the H_2O_2 -induced eEF-2 phosphorylation was also examined. As shown in Figure 5, trifluoperazine (25 μM), a specific antagonist of Ca^{2+} /CaM kinases, completely inhibited H_2O_2 -induced eEF-2 phosphorylation. Ca^{2+} /CaM kinases require Ca^{2+} for their activity. In order to examine the role of Ca^{2+} in H_2O_2 -induced eEF-2 phosphorylation, growth-arrested CHO cells were treated with H_2O_2 and EGTA (10 mM) and the degree of phosphorylation was measured by immunoblotting using

phospho eEF-2 antibody. EGTA significantly inhibited H_2O_2 -induced eEF-2 phosphorylation. In contrast, diacyl ethylene glycol (200 μM), a potent and specific inhibitor of protein kinase C, did not affect H_2O_2 -induced eEF-2 phosphorylation.

Activation of eEF-2 by its dephosphorylation might be one crucial process in the regulation of protein biosynthesis. Therefore, the nature of the respective protein phosphatase is of great interest. Treatment of PP2A to the CHO cell extract induced an increase in the dephosphorylation of eEF-2 (Figure 6, lane 2). However, PP2A-catalyzed eEF-2 dephosphorylation in the cell lysates could be inhibited completely by 30 and 300 nM okadaic acid, by adding simultaneously with PP2A to the cell-free system (Figure 6, lane 3 and 4).

Discussion

Protein synthesis consumes a significant proportion of the available energy of eukaryotic cells. Rates of protein synthesis are regulated, thereby integrating the translation process with other metabolic pathways of the cell. In many of these cases, translation rates change within minutes of the inductive events and are readily reversed, suggesting that specific activities of the protein synthesis machinery rather than changes in the cellular concentration of any of its component is regulated. Covalent modification of proteins by phosphorylation is known to control many metabolic pathways and may likewise regulate rates of translation (Hershey *et al.*, 1996). Protein synthesis also shows a cell cycle-dependent variation. During mitosis the rate of translation is decreased compared to that observed during the interphase (Celis *et al.*, 1990). The reduction has been attributed to an inhibition at the initiation step in protein synthesis, but a reduced elongation rate has also been suggested (Celis *et al.*, 1990). The latter suggestion was based on the observation that the cellular content of phospho eEF-2, a translationally inactive form of the factor (Carlberg, 1990), increases during mitosis (Celis *et al.*, 1990).

Eukaryotic elongation factor 2 is phosphorylated by a specific eEF-2 kinase (Ryazanov *et al.*, 1988). The phos-

Figure 6. Okadaic acid inhibits phosphoprotein phosphatase 2A (PP2A)-mediated eEF-2 dephosphorylation. Homogenates of CHO cells were phosphorylated with 1 μmol eEF-2 in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 37°C for 10 min as in Figure 1. Phosphorylated proteins were incubated at 37°C for 15 min with buffer alone, with PP2A or PP2A plus okadaic acid (OA) at the indicated concentrations and separated by SDS-PAGE followed by autoradiography. eEF-2 is indicated by an arrow.

phorylation of the eEF-2 protein is Ca^{2+} /CaM dependent. While little phosphorylation of eEF-2 was detected in CHO cells in the absence of Ca^{2+} , both Ca^{2+} and CaM stimulated the phosphorylation of this substrate. The ability of Ca^{2+} alone to stimulate the phosphorylation of eEF-2 is probably due to the presence of endogenous CaM in the homogenates. The CaM dependence of this reaction was confirmed by the ability of trifluoperazine to inhibit phosphorylation. On the other hand, the protein level of eEF-2 was nearly identical irrespective of the addition of Ca^{2+} and/or calmodulin. The activity of eEF-2 kinase is physiologically regulated (Bagaglio, *et al.*, 1993). For example, certain mitogens such as epidermal growth factor, vasopressin, and bradykinin stimulate the phosphorylation of eEF-2 in human fibroblasts (Palfrey, *et al.*, 1987). In addition, the phosphorylation of eEF-2 is increased in rat glioblasts when they are stimulated by glia maturation factor, which promotes cell growth and differentiation in normal glioblasts through an increase in intracellular Ca^{2+} (Okumura *et al.*, 1982). The current studies also suggest that the phosphorylation of eEF-2 is related to cellular proliferation, since it was markedly decreased in nonproliferating CHO cells in plateau phase of growth irrespective of the addition of Ca^{2+} and/or CaM compared to that observed in proliferating cell populations in logarithmic growth.

To determine whether Ca^{2+} /CaM-dependent phosphorylation of eEF-2 is linked, in general, to cellular division, we studied the phosphorylation of eEF-2 in proliferating and growth-arrested CHO cells. Phosphorylation of eEF-2 was not detectable in CHO cells arrested in their growth by serum deprivation. The re-addition of serum to cultures of CHO cells, which had been growth-arrested by deprivation of serum for 48 h, increased the phosphorylation of eEF-2. In fact, by 48 h the levels of eEF-2 phosphorylation returned to that of control. Thus, it appears that serum can regulate the activity of eEF-2 kinase. The activation of eEF-2 kinase in quiescent cells exposed to serum suggests a link between transient inhibition of protein synthesis and the induction of cellular division (Bagaglio and Hait, 1994). Recent observations have raised interest in this possibility. For example, the transcription of proto-oncogenes, such as *c-fos*, *c-jun*, and *c-myc*, in quiescent cells, can be induced not only by mitogens but also by protein synthesis inhibitors, such as cycloheximide and puromycin (Greenberg *et al.*, 1986). Like these drugs, the phosphorylation of eEF-2 inhibits translation (Ryazanov and Davydova, 1989). Therefore, upon mitogenic stimulation by serum, eEF-2 kinase is activated, and then subsequently phosphorylates eEF-2.

In this study, we have shown that transient exposure of CHO cells to H_2O_2 markedly increases the phosphorylation of eEF-2. This effect was in a time-dependent (5–60 min), and concentration-dependent manner (25–400 μM). We also found that, in the absence of extracellular

Ca^{2+} , H_2O_2 treatment resulted in the mobilization of intracellular Ca^{2+} (data not shown). In addition, H_2O_2 -induced eEF-2 phosphorylation is dependent on Ca^{2+} and CaM, but independent of protein kinase C. The interesting observation made in the present study is that trifluoperazine, an antagonist of Ca^{2+} /CaM kinases, completely blocked H_2O_2 -induced eEF-2 phosphorylation. This result suggests that Ca^{2+} /CaM kinases are important in oxidant-induced eEF-2 phosphorylation, at least in CHO cells. These findings along with those of Alirezaei *et al.* (2001) clearly provide evidence for the ability of oxidants, particularly H_2O_2 , to regulate translation machinery.

Rapid and transient phosphorylation of eEF-2 is observed after application of mitogens to cultured cells (Palfrey *et al.*, 1987). The role of this eEF-2 phosphorylation is rather mysterious, especially since phosphorylated eEF-2 was shown to be inactive in the elongation process (Ryazanov *et al.*, 1988). Thus, dephosphorylation of eEF-2 should be expected to follow the brief pulse of phosphorylation and to be essential for stimulation of protein synthesis (Redpath *et al.*, 1993). Here we show that phosphoprotein phosphatase 2A is much more efficient in dephosphorylating eEF-2 than that of phosphoprotein phosphatase 1 (data not shown). It has been demonstrated that okadaic acid inhibited various types of protein phosphatases to a different extent (Bialojan and Takai, 1988; Gliksman *et al.*, 1992). According to this report, 10 nM okadaic acid caused more than 80% suppression of PP2A activity, but almost no suppression of PP1C activity with phosphorylase *a* or myosin light chain as substrates. In our hands, 30 nM okadaic acid caused complete suppression of PP2A activity.

In summary, the present study demonstrates that eEF-2 kinase activity in CHO cells are regulated by mitogen such as serum and oxidants like H_2O_2 to influence intracellular Ca^{2+} mobilization.

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