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ERK1/2 Map Kinase Metabolic Pathway is Responsible for Phosphorylation of Translation Initiation Factor eIF4E During In Vitro Maturation of Pig Oocytes

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ABSTRACT Eukaryotic initiation factor 4E (eIF4E) plays an important role in mRNA translation by binding the 5′-cap structure of the mRNA and facilitating the recruitment to the mRNA of other translation factors and the 40S ribosomal subunit. eIF4E undergoes regulated phosphorylation on Ser-209 and this phosphorylation is believed to be important for its binding to mRNA and to other initiation factors. The findings showing that the translation initiation factor eIF4E becomes gradually phosphorylated during in vitro maturation (IVM) of pig oocytes with a maximum in metaphase II (M II) stage oocytes have been documented by us recently (Ellederova et al., 2006). The aim of this work was to study in details the metabolic pathways involved in this process. Using inhibitors of cyclin-dependent kinases, Butyrolactone I (BL I) and protein phosphatases, okadaic acid (OA) we show that ERK1/2 MAP kinase pathway is involved in this phosphorylation. We also demonstrate that activation and phosphorylation of ERK1/2 MAP kinase and eIF4E is associated with the activating phosphorylation of Mnk1 kinase, one of the two main kinases phosphorylating eIF4E in somatic cells. *Mol. Reprod. Dev. 75: 309–317, 2008.*

Key Words: meiosis; translation initiation; eIF4E phosphorylation; MAP kinase

INTRODUCTION

Translational control of specific mRNAs is a widespread mechanism of gene regulation and contributes to diverse biological processes in many cell types. Also during the meiotic division of mammalian oocytes (so called oocyte maturation) protein synthesis plays an important role in controlling the progress of meiosis, since the regulation of gene expression on the level of transcription is highly diminished during this period.

Although some regulatory mechanisms exist during the elongation phase, translation initiation appears to be the rate limiting step in the overall process and is influenced by cis-acting elements located primarily in the mRNA 5′ and 3′ untranslated regions—UTRs (Hershey and Merrick, 2000). These regions are therefore targets of at least two regulatory mechanisms. On one hand, the translational activation of dormant mRNAs, stored during oogenesis seems to be controlled by de novo polyadenylation of the transcripts (Gebauer et al., 1994; Paynton and Bachvarova, 1994; Brevini-Gandolfi et al., 1999; Brevini et al., 2002). On the other hand, the phosphorylation/dephosphorylation changes of the number of different initiation factors and their regulators (such as eIF-2A, B, eIF-3, eIF-4F, or 4E-BP1) are associated with changes in the rate of translation (for review, see Hershey, 1991; Morley and Thomas, 1991).

The process of mRNA binding to ribosomes is mediated by a well-characterized protein complex eIF4F in conjunction with eIF4B (eukaryotic translation initiation factors 4F and 4B). eIF4F is the three-subunit complex that consists of eIF4A (an RNA helicase), eIF4E, the cap-binding protein, and eIF4G, a high molecular weight protein required for the integrity of the complex. Interaction of the cap-binding protein complex eIF4F with mRNA, followed by the unwinding of the RNA secondary structure by eIF4B, is thought to facilitate the attachment of the small 40S ribosomal subunit S6 to mRNA, which is required for scanning the appropriate AUG initiation codon to initiate protein synthesis (Mader et al., 1995).

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The activity of most of the factors that participate in mRNA-ribosome binding (including eIF4B, eIF4E, and eIF4G) is regulated under various circumstances, mostly by phosphorylation. The best studied and characterized is eIF4E, the cap-binding subunit of eIF4F. eIF4E is one of the main regulatory initiation factors, because it is present in limiting molar amounts in the cell, and as such serves as an attractive target for regulating translation. In fact, the amounts and/or activity of eIF4E are modulated at several levels: phosphorylation, translational repressors, and transcription (Sonenberg and Gingras, 1998).

EIF4E is phosphorylated in somatic cells in vivo on a single site at Ser209 (Joshi et al., 1995). The phosphorylated form has been often regarded as the active state of the protein, with ribosome-associated eIF4E enriched for the phosphorylated form. Many of earlier observations have indicated that increased levels of eIF4E phosphorylation correlate directly with the increased translational rates following mitogenic stimulation of mammalian cells (Morley and Pain, 1995; Haghighat and Sonenberg, 1997; Fraser et al., 1999b). Furthermore, phosphorylation of eIF4E has been reported to correlate with its increased binding to eIF4G (Morley, 1994, 1997; Proud and Denton, 1997; Kleijn et al., 1998) and, also, to increase its affinity for the cap structure in vitro (Minich et al., 1994). However, recently there have been contrasting observations suggesting that initiation of translation and eIF4F assembly does not require eIF4E phosphorylation (Saghir et al., 2001; Morley and Naegele, 2002; Scheper et al., 2002). Furthermore, biochemical studies by Scheper et al. (2002) have shown that phosphorylation of eIF4E actually reduces its binding to mRNA caps by promoting its rate of dissociation.

Phosphorylation of eIF4E in somatic cells is controlled by several signaling pathways via activation of mitogen-activated protein (MAP) kinases and MAPKAP kinases (Wang et al., 1998). Two kinases, which directly link MAP kinase and eIF4E phosphorylation, Mnk1 and Mnk2 (MAP kinase integrating kinases 1 and 2) have been described (Waskiewicz et al., 1997; Pyronet, 2000; Scheper et al., 2001). Both kinases can be activated in vitro by ERK (extracellular signal-regulated kinase) or p38 MAP kinases an in their active state they are both able to phosphorylate eIF4E in vitro at the physiological site (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997; Scheper et al., 1997). Phosphorylation of eIF4E by Mnks in vivo appears to be activated only when both molecules are associated with scaffolding eIF4G protein (Waskiewicz et al., 1999; Pyronnet, 2000). Little is known about eIF4E in mammalian oocytes. Except our recently published results (Ellederova et al., 2006), to our knowledge, there have been only two articles published concerning this subject, which show gradual phosphorylation of eIF4E during mouse (Gavin and Schorderet-Slatkine, 1997) and bovine (Tomek et al., 2002) oocyte maturation.

The aim of this work was to study the metabolic pathways, which are involved in eIF4E phosphorylation during in vitro meiotic maturation of pig oocytes. We show that eIF4E becomes phosphorylated in M I stage oocytes and this phosphorylation remains constant until M II stage of oocyte maturation together with activation of ERK1/2 MAP kinase—Mnk1 pathway.

MATERIALS AND METHODS

Cultivation of Oocytes, and Treatment With CDK and Phosphatase Inhibitors

Pig oocytes were obtained from local slaughterhouse. Only oocytes surrounded by compact cumuli were used for the culture. Oocytes were cultured in TCM 199 medium (Sevac, Prague, Czech Republic) supplemented with 10% fetal calf serum (Bioveta, Ivanovice, Czech Republic), 100 ng porcine follicle stimulating hormone (Biogenesis, Poole, UK), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (both from Sigma, St. Louis, MO), at 38°C in an atmosphere of 5% CO_2. In the experiments using inhibitors, the oocytes were treated with Butyrolactone I (BL I) (100 μM final concentration, Funakoshi Co. Ltd., Tokyo, Japan), or okadaic acid (OA) (0.5 and 2.5 μM final concentration, Sigma). The samples were collected at 0 (GV), 28 (M I), and 45 (M II) hr during spontaneous in vitro oocyte maturation. Time intervals 0 (GV) and 28 (M I) were investigated when BL I alone was added to the culture in GV-stage oocytes at 0 hr. When BL I was applied together with OA in the same stage, the cultivation intervals were 4 and 8 hr. When BL I was used in late diakinesis (LD) and M I stages (time intervals 25 and 28 hr), oocytes were cultivated for additional 6 or 24 hr in the presence of the inhibitor.

Morphological Evaluation of Oocytes

At the end of culture, the oocytes were mechanically denuded and fixed in ethanol-acetic acid 3:1 (v/v). Staining was performed with 2% orcein in 50% aqueous-acetic acid 1% sodium citrate. The oocytes were observed with phase contrast NU Zeiss-Jena microscope (Germany).

Vertical Slab Gel Isoelectric Focusing (VSIEF)

The VSIEF was performed according to Maurides et al. (1989) in order to separate differently phosphorylated forms of eIF4E. Lysates prepared from oocytes (50 oocytes per sample) were lysed in 10 μl VSIEF sample buffer. This buffer contains 2% Ampholines (Amersham Biosciences, Uppsala, Sweden), pH 5–7, 9 M Urea, 5% β-mercaptoethanol, and 2% CHAPS. Lysates were separated on 4.5% polyacrylamide gels containing 9 M Urea, 2.5% Ampholines pH 5–7, and 2% CHAPS at 2 mA for 16 hr.

Immunoblotting

Oocyte lysates were separated by SDS–PAGE (Laemmli, 1970) or VSIEF. Then the proteins were transferred to Immobilon P membrane (Millipore, Bedford, MA) using a semidry blotting system (Biometra, Scheller Instruments, Prague, Czech Republic). Blots

were incubated with 10% Teleost gelatin (Sigma) or 5% low fat milk dissolved in 0.5% Tween-20, Tris-buffered saline, pH 7.4. (TTBS) The monoclonal anti eIF4E antibody (BD Transduction Laboratories, Franklin Lakes, NJ) and polyclonal anti-ERK1/2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:1,000 in TTBS. Anti-Mnk1 antibody (Santa Cruz Biotechnology) was diluted 1:500 in 5% low fat milk dissolved in TTBS. The polyclonal anti-phosphoSer209-eIF4E, anti-phosphoThr197/202-Mnk1, anti-phospho-Thr202/Tyr204 ERK1/2 MAPK, anti-p38 MAPK, and anti-phosphoThr180/Tyr182 p38 MAPK (all from Cell Signaling Technology, Beverly, MA) antibodies were all diluted 1:250 in 5% BSA in TTBS. The different forms of eIF4E, ERK1/2, p38 and Mnk1 proteins were visualized by ECL or ECL-plus chemiluminescent kit according to manufacturer's instructions (Amersham Biosciences).

MBP and Histone H1 Kinase Double Assay

CDK1 kinase and MAP kinase activities were measured in oocytes via their capacity to phosphorylate external substrates histone H1 and myelin basic protein (MBP), respectively, according to Motlík et al. (1996). At each time interval during the culture, 10 oocytes per sample were lysed in 5 μl homogenization buffer containing 40 mM MOPS, pH 7.2, inhibitors of phosphatases and proteases, by three rounds of freezing/thawing on dry ice. The kinase reaction was initiated by addition of 5 μl kinase buffer containing 10 mg/ml histone H1, and 5 mg/ml MBP, together with 0.4 mCi/ml [γ-^{32}P] ATP (Amersham Biosciences). The reaction was stopped after 30 min by the addition of SDS–PAGE sample buffer and boiling for 3 min. After electrophoresis on 15% SDS–PAGE gel, the gels were stained with Coomassie Blue R250, destained overnight, dried, and autoradiographed.

RESULTS

Morphological Changes During IVM of Pig Oocytes

In our culture system, GVBD in porcine oocytes initiates after 18–20 hr of culture, by 24 hr is finished and by 28 hr most of the oocytes reach M I. Between 30 and 34 hr the oocytes progress through anaphase I/telophase I stage and by 44–45 hr most of them reach M II stage (data not shown).

Changes in eIF4E Expression and Phosphorylation During IVM of Pig Oocytes in Correlation With Changes of ERK1/2 and p38 MAP Kinase, CDK1 Kinase, and Mnk1 Kinase Activities

The data showing eIF4E expression and phosphorylation during in vitro maturation (IVM) of pig oocytes have been already published by us recently (Ellederova et al., 2006). However, we present these data here in order to document the temporal correlation of eIF4E phosphorylation with changes in ERK1/2 MAP kinase and Mnk1 kinase activities.

Figure 1A shows that expression of eIF4E does not significantly change during IVM of pig oocytes. On the other hand, phosphorylation of eIF4E changes substantially during this period: it starts to be phosphorylated at the time of GVBD (after 24 hr of culture), reaches a maximum in M I stage (28 hr of culture) and remains elevated up to M II stage (44 hr of culture). This has been documented both by SDS–PAGE followed by Western blotting with anti-phoshoSer209-eIF4E antibody (Fig. 1B), and by VSIEF followed by Western blotting with anti eIF4E antibody (Fig. 1C). Using the VSIEF method, we have been able to detect four bands of eIF4E—one basic form, which represents the unphosphorylated eIF4E, and three more acidic forms. The middle one from these three acidic forms represents eIF4E phosphorylated on Ser209 as documented in Figure 1J, showing the separation of total oocyte GV, M I and M II lysates by VSIEF and their subsequent probing with either anti eIF4E, or anti-phoshoSer209-eIF4E antibodies. The method of VSIEF combined with Western blotting with eIF4E antibody has been used by us in the majority of experiments in order to perform more detailed analysis of changes in eIF4E phosphorylation, since this method does not require large numbers of oocytes for sample preparation.

In order to elucidate the possible involvement of the two major M-phase kinases CDK1 and ERK1/2 MAP in regulation of eIF4E phosphorylation, we have measured changes in the activities of these kinases in pig oocyte lysates during IVM. As seen from Figure 1D (showing the result of double kinase assay for CDK1 and MAP kinase using histone H1 and MBP as external substrates) both kinases become activated approximately at the time of GVBD—at 24 hr, reaching a maximum at MI stage at 28 hr. MAP kinase activity remains high and stable until M II stage at 45 hr, while CDK1 kinase activity temporarily drops down at 32 hr, the approximate time of Anaphase I/Telophase I stage. The changes of MAP kinase activity are also shown in Figure 1E, representing the result of Western blot analysis of oocyte lysates with anti-MAP kinase antibody (both anti-ERK1 and anti-ERK2 forms). Partial activation of both ERK1 and ERK2, documented as shift to higher molecular weight due to the phosphorylation, can be seen after 24 hr, while full activation occurs after 28 hr and remains at this level up to 45 hr. This result has been further confirmed by using anti-phosphoThr202/Tyr204 ERK1/2 MAPK antibody (Fig. 1F).

In somatic cells, eIF4E is phosphorylated by Mnk kinases, which themselves are activated by number of MAP kinases, including p38 MAP kinase, ERK1 and ERK2 kinases (Wang et al., 1998). We have investigated the putative involvement of p38 MAP kinase in eIF4E phosphorylation pathway, as well as the changes of Mnk1 kinase activity and their correlation with eIF4E phosphorylation. In Figure 1G–H we show that while the expression of p38 MAP kinase is slightly increased in later stages of pig oocyte maturation (after 20 hr of culture), its phosphorylation (and activation) rather decreases during this period. This suggests that p38

MAP kinase is not involved in eIF4E phosphorylation in pig oocytes. On the other hand activity of Mnk1 kinase (measured by its phosphorylation on Thr197/202) gradually increases during pig oocyte IVM starting at the time of GVBD (24 hr of culture) and reaching the maximum in M II stage oocytes (44 hr of culture), showing a high correlation with activation of ERK1/2 MAP kinase and eIF4E phosphorylation (Fig. 1I).

Requirement of MAP Kinase Pathway Activation for eIF4E Phosphorylation

Increased phosphorylation of eIF4E has been shown during IVM of mouse (Gavin and Schorderet-Slatkine, 1997) and bovine (Tomek et al., 2002) oocytes; the latter authors also show partial correlation of ERK1/2 MAP kinase activity and eIF4E phosphorylation.

To elucidate the role of ERK1/2 MAP kinase in phosphorylation of eIF4E during IVM of pig oocytes we have used a specific inhibitor of cyclin-dependent kinases, Butyrolactone I, which in specific conditions does not influence ERK1/2 MAP kinase activity in pig oocytes. When applied in a concentration of 100 μM (previously described by us as the most effective concentration during IVM of pig oocytes—Kubelka et al., 2002a) in the beginning of maturation in GV-stage oocytes, for 28 hr, BL I totally inhibits CDK1 kinase activity, but it also prevents ERK1/2 MAP kinase activation (Fig. 2B,C; Kubelka et al., 2002a). In such treated oocytes no eIF4E phosphorylation occurs (Fig. 2A). However, when BL I was applied to early or late MI stage oocytes (after 25 or 28 hr of culture, respectively), which posses highly active CDK1 kinase and ERK1/2 MAP kinase, the activity of CDK1 kinase was inhibited, but that of ERK1/2 MAP kinase remained high for at least 6 hr; longer treatment with BL I (24 hr) deteriorated also ERK1/2 MAP kinase activity (Fig. 4B,C). The specific inhibition of CDK1 kinase in M I pig oocytes has been shown by us previously (Kubelka et al., 2002a,b), however, some CDK1 kinase activity can be seen in kinase assay of such treated oocytes (Fig. 3B, lanes 6, 8). This virtuous contradiction is most likely due to the fact that BL I inhibits the kinase activity by occupying the ATP-binding site; BL I does not

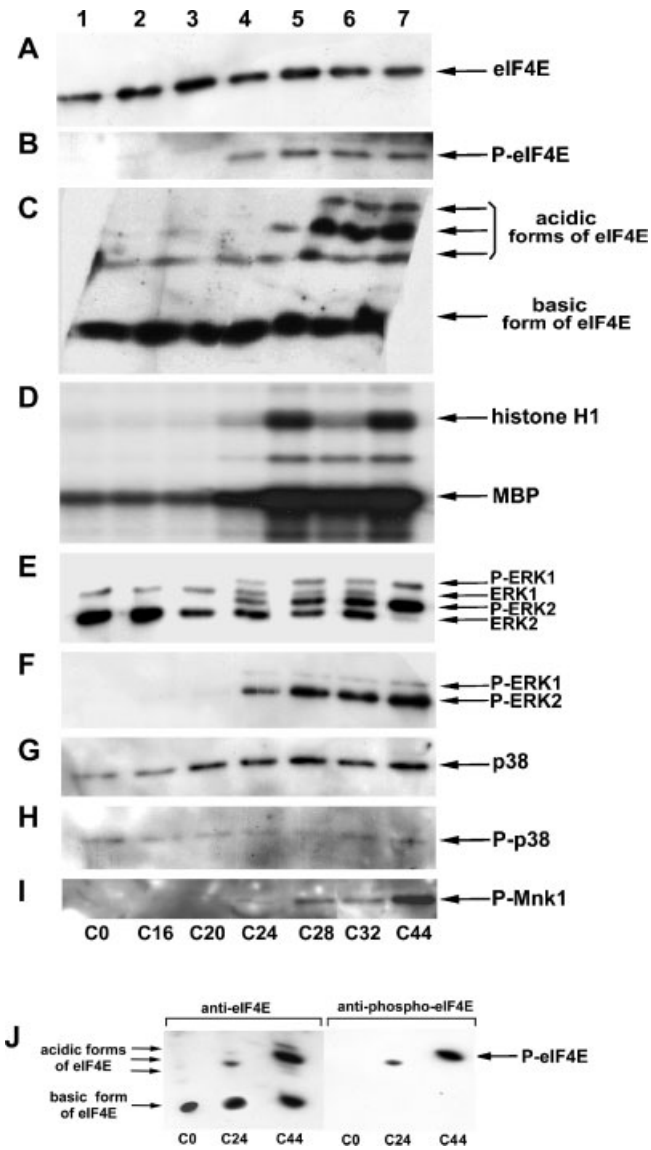

Fig. 1. EIF4E phosphorylation correlates with ERK1/2 MAP kinase (but not p 38 MAP kinase or CDK1 kinase) activity and Mnk1 kinase activity during in vitro maturation of pig oocytes. **A**: The lysates from 20 oocytes of each sample cultivated in control medium (C) during IVM were collected at indicated time intervals (hours). The abundance of eIF4E (24 kDa) was determined by Western blotting using monoclonal anti eIF4E antibody. **B**: Phosphorylation state of eIF4E during in vitro maturation was verified by Western blot analysis. The lysates from 200 oocytes were loaded on SDS–PAGE and eIF4E phosphorylation was visualized using phospho Ser209 eIF4E specific antibody. **C**: Vertical slab gel isoelectric focusing was used to resolve the forms of eIF4E carrying different charges. Lysates equal to 50 oocytes cultured in control medium (C) and collected at indicated time intervals (hours) were loaded to each lane. **D**: The lysates from 10 oocytes for each sample were used for histone H1 and MBP double kinase assay. Phosphorylation of substrate proteins was visualized by autoradiography. **E**: Changes in phosphorylation status of ERK1/2 MAP kinase were detected by mobility shift in SDS–PAGE separation of lysates from 10 oocytes using anti ERK1/2 MAP kinase antibody. **F**: Changes in phosphorylation status of ERK1/2 MAP kinase were further proved by Western blotting after SDS–PAGE separation of lysates from 100 oocytes using anti phosphoThr202/Tyr204 ERK1/2 MAP kinase antibody. **G**: The expression of p38 MAP kinase during pig oocyte IVM was determined by Western blotting with anti p38 MAP kinase antibody in lysates from 100 oocytes separated by SDS–PAGE. **H**: Changes in phosphorylation status of p38 MAP kinase were detected by Western blotting after SDS–PAGE separation of lysates from 100 oocytes using anti phosphoThr180/Tyr182 p38 MAPK kinase antibody. **I**: Changes in phosphorylation status of Mnk1 kinase were detected by Western blotting after SDS–PAGE separation of lysates from 100 oocytes using anti phosphoThr197/202-Mnk1 kinase antibody. **J**: Phosphorylation state of eIF4E during IVM of pig oocytes was verified by Western blot analysis. The lysates from 200 oocytes collected at indicated time intervals were loaded on VSIEF gels. At first eIF4E phosphorylation was visualized using anti phosphoSer209 eIF4E antibody (right panel), and then anti eIF4E antibody was used (left panel). Results are representative of those obtained in three separate experiments. The data showing eIF4E expression and phosphorylation during IVM of pig oocytes (A,C,J) have been already published by us recently (Elledérova et al., 2006). However, we present these data here in order to document the temporal correlation of eIF4E phosphorylation with changes in ERK1/2 MAP kinase and Mnk1 kinase activities.

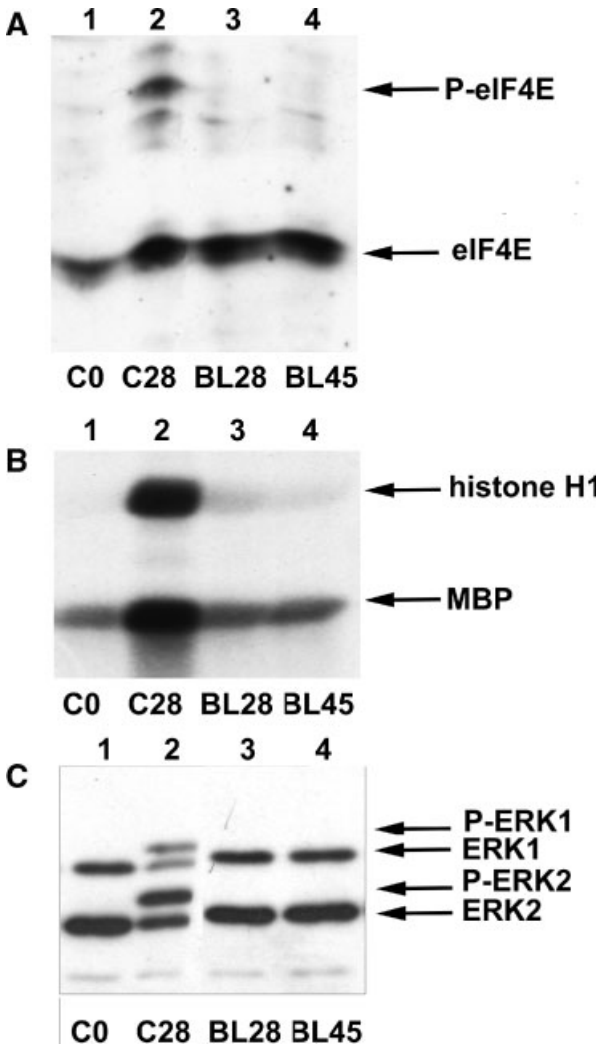

Fig. 2. The phosphorylation of eIF4E and activation of CDK1 and ERK1/2 MAP kinases do not occur in the presence of Butyrolactone I. **A**: Lysates equal to 50 oocytes for each sample were collected at 0 and 28 hr of IVM in the control medium (C) or in the presence of 100 μM Butyrolactone I (BL) and analyzed by vertical slab gel isoelectric focusing. Acidic forms of eIF4E (including the phosphorylated eIF4E) were observed 28 hr following cultivation in control medium, but they were not present when oocytes were cultivated with BL. **B**: The lysates from 10 oocytes for each sample were used for histone H1 and MBP double kinase assay. The activities of CDK1 and MAP kinases, revealed by phosphorylation of substrate proteins, were increased 28 hr following cultivation in control medium, but they remained low in the presence of BL. **C**: The samples from 10 oocytes were analyzed by SDS–PAGE. Mobility shift associated with phosphorylation of ERK1/2 MAP kinase after 28 hr of control IVM was not detected when oocytes were cultivated in the presence of BL. Representative example of three independent experiments with similar results is shown.

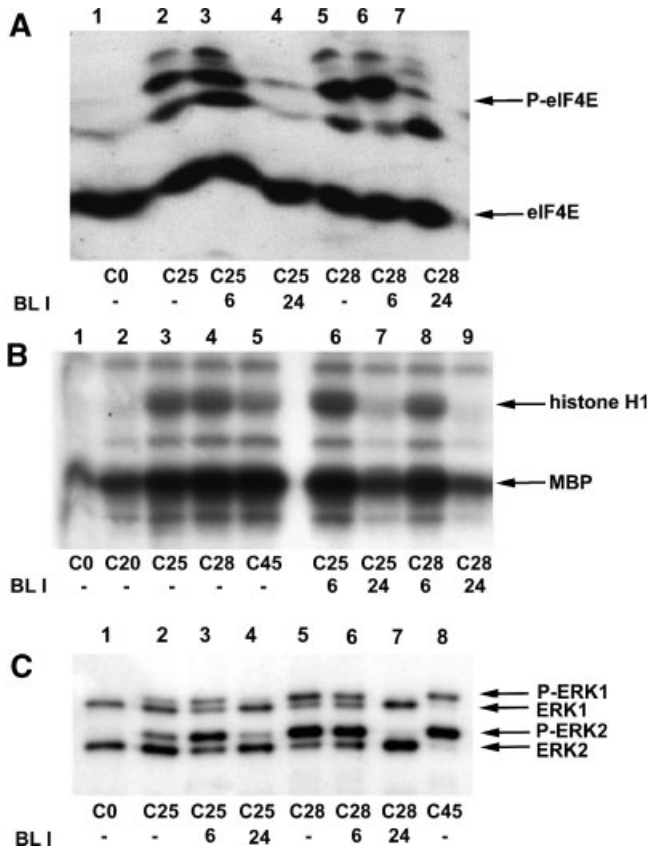

Fig. 3. Activity of ERK1/2 MAP kinase but not CDK1 kinase closely correlates with the phosphorylation of eIF4E when Butyrolactone I is applied to oocytes in early/late MI stage of in vitro maturation. **A**: The oocytes were cultured in control medium (C) for 25 or 28 hr (early/late MI oocytes). Then 100 μM Butyrolactone I (BL) was added and oocytes were cultivated for additional 6 or 24 hr of culture. Lysates from 50 oocytes were prepared at the indicated time intervals and resolved by vertical slab gel isoelectric focusing and analyzed for differentially charged forms of eIF4E by Western blotting. **B**: Oocytes were cultivated in control medium (C) for 25 or 28 hr (GVBD/MI stage oocytes) and then for further 6 or 24 hr in the presence of Butyrolactone I (BL). Thereafter, histone H1 and MBP double kinase assay was performed in lysates prepared from 10 oocytes. The lysates were separated by SDS–PAGE and the phosphorylated substrates were visualized by autoradiography. **C**: Phosphorylation status of ERK1/2 MAP kinase was detected by mobility shift in SDS–PAGE. Separation of lysates from 10 oocytes collected at time intervals as indicated above was followed by specific anti-MAP kinase immunoblotting. Representative example of at least three independent experiments is shown.

impair the dephosphorylation and activation of CDK1 kinase (Kitagawa et al., 1993, 1994). The lysates used for CDK1 kinase assay do not contain added BL I and therefore the activated kinase can phosphorylate the external substrate, histone H1. After 25 or 28 hr of culture, eIF4E was already phosphorylated at high levels and this phosphorylation did not change even after 6 hr of BL I treatment (Fig. 3A). Only after 24 hr of BL I treatment eIF4E phosphorylation decreased to levels similar to those in GV-stage oocytes. This decrease corresponds tightly to the decrease of the activity of ERK1/2 MAP kinase (Fig. 3B,C).

In another experiment GV-stage pig oocytes were treated for 4 or 8 hr with BL I in combination with OA a potent inhibitor of type 1 and 2A phosphatases. This inhibitor has been shown previously to activate prematurely MAP kinase in mammalian oocytes. Two concentrations of OA were used, 0.5 or 2.5 μM, because the lower one is believed to be more specific for phosphatase 2A. CDK1 kinase can be prematurely activated by OA

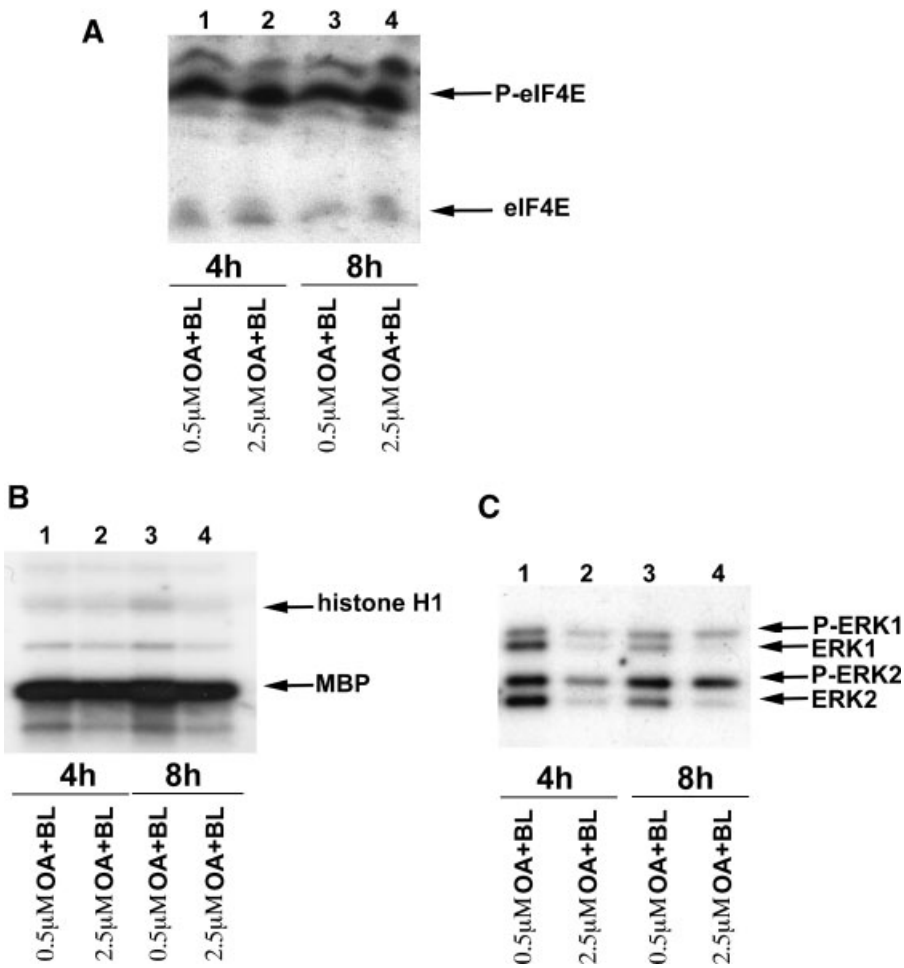

Fig. 4. Simultaneous treatment of pig oocytes in GV stage with okadaic acid and Butyrolactone I induces ERK1/2 MAP kinase activation and eIF4E phosphorylation. **A**: GV oocytes cultivated in the presence of 100 μM Butyrolactone I (BL) and 0.5 or 2.5 μM okadaic acid (OA) were collected and lysed after 4 and 8 hr of culture. Lysates equal to 50 oocytes for each sample were analyzed by vertical slab gel isoelectric focusing and eIF4E was detected by immunoblotting. **B**: The oocytes were cultured under the conditions mentioned above and lysates from 10 oocytes were used in histone H1 and MBP double kinase assay. Phosphorylation of substrate proteins was visualized by autoradiography. **C**: Phosphorylation status of ERK1 and ERK2 MAP kinase was detected by mobility shift after SDS–PAGE separation. Lysates from 10 oocytes cultured in the presence of okadaic acid (OA) and Butyrolactone I (BL) were prepared after 4 and 8 hr of culture. Band shift of phosphorylated MAP kinases ERK1 and ERK2 was detected by western blotting. Representative example of three independent experiments with similar results is shown.

(Kalous et al., 1993), however, here we have documented that in the simultaneous presence of BL I its activity in situ is inhibited. The results show the activation of ERK1/2 MAP kinase already after 4 hr of BL I/OA treatment, and even more pronounced after 8 hr, while CDK1 kinase activity remained low in such treated oocytes (Fig. 4B,C). The activation of ERK1/2 MAP kinase appeared to be also dependent on OA concentration. Even with the lower dose of OA, the activity of ERK1/2 MAP kinase after 8 hr treatment was significantly high. In this experimental scheme the phosphorylation of eIF4E is also reflected by the pattern of ERK1/2 MAP kinase activation and the OA/BL I treatment (even the low OA concentration for 4 hr) induced increased eIF4E phosphorylation, with a maximum after 8 hr treatment with high OA concentration.

The results of these experiments suggested that ERK1/2 MAP kinase pathway, rather than CDK1 kinase pathway is involved in eIF4E phosphorylation during maturation of pig oocytes.

Correlation of Mnk1 Kinase Activity With ERK1/2 MAP Kinase Activity and eIF4E Phosphorylation in BL- and OA-Treated Pig Oocytes

The results presented in Figure 1 show high correlation of Mnk1 kinase activity with ERK1/2 MAP kinase activity and eIF4E phosphorylation during pig oocytes IVM in control conditions. In order to further confirm the involvement of Mnk1 kinase in eIF4E phosphorylation, we have measured its activity also in lysates from oocytes treated with inhibitors, BL I and OA. Figure 5 documents that while the expression of Mnk1 kinase remains nearly constant during control pig oocyte IVM, its activity (measured by phosphorylation on Thr197/202) increases substantially in M I and, especially in M II oocytes. Figure 6 (upper panel) shows that the

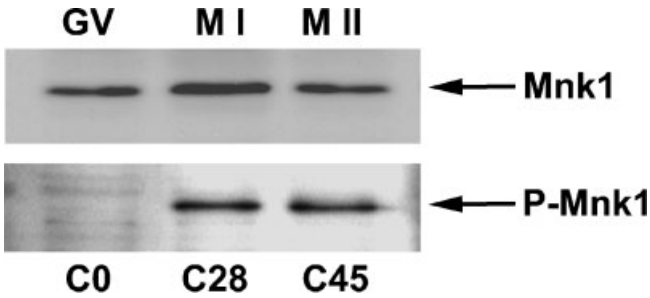

Fig. 5. Mnk1 kinase becomes phosphorylated in M I and M II stages of pig oocyte in vitro maturation. The lysates from 200 oocytes for each sample were separated by SDS–PAGE and expression of Mnk1 kinase was determined by Western blotting using anti Mnk1 antibody (upper panel). The lysates from 100 oocytes for each sample were separated by SDS–PAGE and phosphorylation of Mnk1 kinase was detected by Western blotting using antibody specific to phosphorylated Thr197/202 position in Mnk1 (lower panel). Results are representative of those obtained in four separate experiments.

treatment of pig oocytes with the inhibitors does not influence Mnk1 kinase expression (partial decrease can be seen only after 28 hr treatment with BL I). On the other hand, while treatment of early, or late M I oocytes (25 or 28 hr of culture, respectively) with BL I results only in slight decrease of Mnk1 kinase activity, the treatment of these oocytes with BL I for 24 hr leads to complete inactivation of Mnk1. Furthermore, the treatment of oocytes with combination of BL I and OA for 8 hr leads to premature activation of Mnk1 kinase in these oocytes (Fig. 6, lower panel).

Taken together, our results show that activation of Mnk1 kinase in pig oocytes is highly correlated with the activity of ERK1/2 MAP kinase (but not p38 MAP kinase or CDK1 kinase), as well as with eIF4E phosphorylation both in control conditions and in oocytes treated with BL I and OA. These findings therefore suggest that phosphorylation of eIF4E during IVM of pig oocytes is regulated by ERK1/2 MAP kinase, most likely via activation of Mnk1 kinase.

DISCUSSION

In this study, we have investigated the pathways responsible for phosphorylation of translation initiation

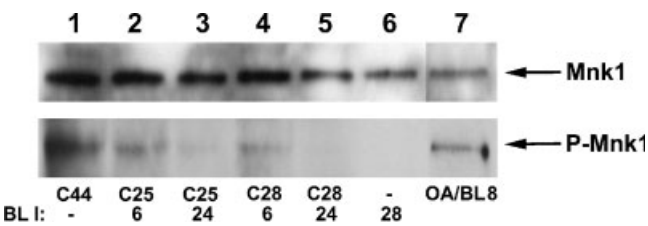

Fig. 6. Mnk1 phosphorylation in oocytes treated with Butyrolactone I and okadaic acid. The lysates from 200 oocytes for each sample were separated by SDS–PAGE and expression of Mnk1 kinase was determined by Western blotting using anti Mnk1 antibody (upper panel). The lysates from 100 oocytes for each sample were separated by SDS–PAGE and phosphorylation of Mnk1 kinase was detected by Western blotting using antibody specific to phosphorylated Thr197/202 position in Mnk1 (lower panel). Representative example from two experiments is presented.

factor eIF4E during IVM of pig oocytes. Our previous work (Ellederova et al., 2006) has shown that eIF4E becomes gradually phosphorylated during IVM and similar results have been also obtained earlier in mouse (Gavin et al., 1997) and bovine (Tomek et al., 2002) oocytes. To describe the changes of eIF4E phosphorylation in pig oocytes under different culture conditions, the method of VSIEF combined with Western blotting with eIF4E antibody has been used in majority of experiments. This method allowed us to perform more detailed analysis of eIF4E phosphorylation, as the number of oocytes needed for experiments was substantially lower than that for experiments using anti-phospho(Ser-209) eIF4E antibody. Using the VSIEF method we have been able to detect three highly acidic forms of eIF4E starting at GVBD stage onwards, as opposed to the basic eIF4E form, which was present during the whole period of maturation. The middle one of the acidic forms represents eIF4E phosphorylated on Ser-209, the identity of the other two bands is not clear. Although eIF4E has been suggested to be phosphorylated in vivo on a single site (Ser-209—Joshi et al., 1995), two other sites have been described earlier as putative phosphorylation sites—Ser-53 and Thr-210 (Rychlik et al., 1987; Makkinje et al., 1995). The additional acidic forms of eIF4E might then represent phosphorylation on these sites or, alternatively, another posttranslational modifications of the eIF4E protein.

In mammalian somatic cells it has been shown that phosphorylation of eIF4E occurs via multiple signaling pathways (Mendez et al., 1997; Wang et al., 1998; Gingras et al., 1999). Two protein kinases, Mnk1 and Mnk2, phosphorylate eIF4E at the physiological site both in vitro and in vivo (Pyronnet et al., 1999; Waskiewicz et al., 1999; Scheper et al., 2001) and are believed to be the eIF4E kinases. Both Mnk1 and Mnk2 can be activated by phosphorylation by the mitogen-activated extracellular signal-regulated kinases (Erks) and the stress- and cytokine-activated p38 MAP kinase pathways (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997; Scheper et al., 2001), although there are important differences in their in vivo activities. While Mnk1 displays a low level of activity, which is greatly enhanced by treatment of cells with agents that activate either the Erk or the p38 MAP kinase pathways (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997; Wang et al., 1998), Mnk2 has high basal activity, which is not enhanced further by agents that activate Erk/p38 pathways (Scheper et al., 2001). These enzymes (Mnk1 and Mnk2) also associate with eIF4G in vivo; this mode of recruitment is thought to ensure that eIF4E is phosphorylated only as a part of eIF4F complex (Pyronnet et al., 1999; Waskiewicz et al., 1999; Morino et al., 2000; Pyronnet, 2000; Scheper et al., 2002). Two novel Mnks have been identified recently (Mnk1b and Mnk2b), which are splice variants of the original Mnk1 and Mnk2 and which are both localized in the nucleus. Mnk1b has high basal activity, similarly as Mnk2 and both Mnk1b and Mnk2b lack C-terminal region containing MAP kinase binding site and as such are much less

readily activated by MAP kinases (Slentz-Kesler et al., 2000; Scheper et al., 2003; O'Loghlen et al., 2004).

The data presented here are in good agreement with those published on somatic cells (see above). Although Mnk2 is present in pig oocytes, its expression, as well as its activity does not change during IVM (data not shown). Similarly, the expression of Mnk1 is stable during pig oocyte maturation, however, its activity (measured by its phosphorylation on the activatory site—Thr-177/202) changes substantially: it is low in GV-stage oocytes, starts to increase approximately at the time of GVBD, reaches the maximum in M I and then remains constant until M II. Activation of Mnk1 tightly correlates with eIF4E phosphorylation both in oocytes cultured in control conditions and in oocytes cultured in the presence of the inhibitors, BL I and OA. These two inhibitors and their combinations have been used in this study in order to specify also, which of the two major M-phase kinases, which become activated during pig oocyte maturation (CDK1 kinase and MAP kinase), might be involved in phosphorylation (and activation) of Mnk1 kinase. Our results have shown that ERK1/2 MAP kinase activity, but not CDK1 or p38 MAP kinase activity, is tightly correlated and precedes phosphorylation of Mnk1 kinase in pig oocytes. Furthermore, the activity of Mnk1 also tightly correlates with eIF4E phosphorylation both in oocytes cultured in control conditions and in oocytes cultured in the presence of the inhibitors. The obtained data suggest that ERK1/2 (but not p38) MAP kinase pathway (most likely via Mnk1 kinase activation) is involved in eIF4E phosphorylation during IVM of pig oocytes.

The functional significance of eIF4E phosphorylation has been subject of a considerable interest. Numerous studies have shown earlier high correlation of changes in eIF4E phosphorylation and protein synthesis under variety of conditions (Joshi-Barve et al., 1990; Lamphear and Panniers, 1990; Pain, 1996; Morley, 1997). Phosphorylation of eIF4E has been also reported to increase its binding to the cap (Minich et al., 1994), and to correlate with its increased binding to eIF4G (Morley, 1994, 1997; Proud and Denton, 1997; Kleijn et al., 1998). However, more recent reports indicate that phosphorylation of eIF4E is not a prerequisite for complex formation with eIF4G (Ohlmann et al., 1997), and that it is not required for translation (Morley and Naegele, 2002). Also, report of Scheper et al. (2002) even shows reduction of phosphorylated eIF4E affinity for capped mRNA. This suggests that phosphorylation of eIF4E is not absolutely required for eIF4E function. However, phosphorylation of eIF4E may still modulate a number of protein–protein interaction that can affect the multiprotein 43S or 48S complex, especially taking into account a number of translation initiation factors and other interacting proteins involved in this process. Another possible role of eIF4E phosphorylation suggested by Scheper and Proud (2002) might be the "reprogramming" of the translation machinery by the release of the eIF4F complex to promote the chance of under-represented mRNAs for ribosome binding. These findings are in good agreement with the results of the study recently published by us (Ellederova et al., 2006) showing that while eIF4E becomes phosphorylated during IVM of pig oocytes, the overall protein synthesis rates drop down during this period, although translation of some mRNAs is increasing.

Taken together, the results of the presented study show high correlation of ERK1/2 MAP kinase, as well as Mnk1 kinase activation with eIF4E phosphorylation, suggesting that ERK1/2 (but not p38) MAP kinase signaling pathway is involved in the process of eIF4E phosphorylation, most likely via Mnk1. Our results, for the first time document the expression and activation of Mnk1 kinase in mammalian oocytes and, also, suggest an explanation for the possible function of ERK1/2 MAP kinase activation during early stages of mammalian oocyte maturation.

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