Regulation of Translation During In Vitro Maturation of Bovine Oocytes: The Role of MAP Kinase, eIF4E (Cap Binding Protein) Phosphorylation, and eIF4E-BP1

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ABSTRACT

Meiotic maturation of mammalian oocytes (transition from prophase I to metaphase II) is accompanied by complex changes in the protein phosphorylation pattern. At least two major protein kinases are involved in these events; namely, cdc2 kinase and mitogen-activated protein (MAP) kinase, because the inhibition of these kinases arrests mammalian oocytes in the germinal vesicle (GV) stage. We show that during meiotic maturation of bovine oocytes, the translation initiation factor, eIF4E (the cap binding protein), gradually becomes phosphorylated. This substantial phosphorylation begins at the time of germinal vesicle breakdown (GVBD) and continues to the metaphase II stage. The onset of eIF4E phosphorylation occurs in parallel with a significant increase in overall protein synthesis. However, although eIF4E is nearly fully phosphorylated in metaphase II oocytes, protein synthesis reaches only basal levels at this stage, similar to that of prophase I oocytes, in which the factor remains unphosphorylated. We present evidence that a specific repressor of eIF4E, the binding protein 4E-BP1, is present and can be involved in preventing eIF4E function in metaphase II stage oocytes. Recently, two protein kinases, called Mnk1 and Mnk2, have been identified in somatic cells as eIF4E kinases, both of which are substrates of MAP kinase in vivo. In bovine oocytes, a specific inhibitor of cdk kinases, butyrolactone I, arrests oocytes in GV stage and prevents activation of both cdc2 and MAP kinase. Under these conditions, the phosphorylation of eIF4E is also blocked, and its function in initiation of translation is impaired. In contrast, PD 098059, a specific inhibitor of the MAP kinase activation pathway, which inhibits the MAP kinase kinase, called MEK function, leads only to a postponed GVBD, and a delay in MAP kinase and eIF4E phosphorylation. These results indicate that in bovine oocytes, 1) MAP kinase activation is only partially dependent on MEK kinase, 2) MAP kinase is involved in eIF4E phosphorylation, and 3) the abundance of fully phosphorylated eIF4E does not necessarily directly stimulate protein synthesis. A possible MEK kinase-independent pathway of MAP kinase phosphorylation and the role of 4E-BP1 in repressing translation in metaphase II oocytes are discussed.

INTRODUCTION

The development of mammalian oocytes can be roughly subdivided into two phases, in which they possess different properties. The period of oocyte growth is characterized by a relative constant pattern of gene expression together with the accumulation of macromolecules (e.g., RNAs and proteins). In contrast, during the short period of meiotic maturation (transition from prophase I to metaphase II) the morphological changes (germinal vesicle breakdown [GVBD], chromatin condensation, and spindle assembly) are accompanied by dramatic changes in the translation of specific mRNAs, as described in the mouse [1]. This phase is also associated with a poorly understood cascade of protein phosphorylation and dephosphorylation events [2].

Several maternal mRNAs are translationally dormant in growing oocytes and become activated during meiotic maturation [3–5]. The recruitment of mRNAs onto polysomes can be regulated on several levels. One evolutionarily conserved mechanism of activation is cytoplasmic polyadenylation of the transcripts [6–10]. Another mechanism is the phosphorylation and dephosphorylation of a number of different initiation factors and their regulators (i.e., eIF2, eIF2B, eIF3, eIF4E, and 4E-BP1) as well as ribosomal proteins [11–14].

The initiation step of translation (i.e., the binding of the small ribosomal subunit to the mRNA) seems to be the rate-limiting step in the cap-dependent translation [15, 16]. This crucial process is influenced by secondary and tertiary structures within the 5′ untranslated region of the mRNA and by the existence of the cap structure (m7GpppN), which is characteristic for all nuclear-encoded eukaryotic mRNAs [17]. This structure is recognized by the cap binding protein complex eIF4F, which consists of three subunits: eIF4A (an RNA helicase, which is responsible for unwinding of mRNA secondary structures), eIF4E (the cap binding protein itself), and p220 (eIF4G), a protein required to maintain the integrity of the complex [11, 16]. The interaction of eIF4F with repressed mRNA is absolutely necessary for an efficient initiation of translation [18]. Although direct evidence for the importance of eIF4E phosphorylation in the cap binding process is missing, many reports have shown a correlation between eIF4E phosphorylation and high translation rates (reviewed in [16, 19]). Therefore, the phosphorylation of eIF4E is directly linked to the mRNA recruitment from a stored pool to actively translated polyribosomes [20]. Moreover, during somatic cell cycle, the phosphorylation state of eIF4E is generally consistent with translation rates [21].

In somatic cells, a specific repressor of eIF4E function has been identified, the so-called binding protein, 4E-BP1 or PHAS I [14]. This protein is a member of a family of at least two other related proteins that bind in their unphosphorylated form to eIF4E and prevent eIF4E complex...
formation rather than the binding of eIF4E to the cap structure [22, 23]. It is not known whether 4E-B1 prefers binding to the phosphorylated form of eIF4E and whether it plays any role in regulating translation during meiotic maturation of oocytes.

During meiotic maturation of mammalian oocytes extensive changes in protein phosphorylation occur [24–26]. At least two major kinases or kinase cascades attend this process; namely, cdc2 kinase (cell division control kinase, also known as cyclin-dependent kinase, cdk1) and mitogen-activated protein (MAP) kinase, also known as extracellular-regulated kinase, ERK). Cdc2 kinase is the catalytic part of maturation promoting factor (MPF), and it is active only when associated with its regulatory subunit, cyclin B [27, 28]. The physiological targets for cdc2 and MAP kinase are known only in fragments.

Cdc2 can phosphorylate histone H1, at least in vitro [29, 30], and it also acts as a lamin kinase [31], which suggests its role in nuclear membrane disintegration. Its activation in bovine oocytes can be prevented by culturing the oocytes in the presence of butyrolactone I (BL I). This inhibitor arrests oocytes in the germinal vesicle (GV) stage without influencing chromosome condensation. BL I acts as a specific inhibitor of cdk kinases and has little direct effect on MAP kinase; however, when added in the beginning of maturation, BL I prevents MAP kinase activation indirectly, probably via a cdk-dependent pathway [32, 33].

MAP kinase is a part of a kinase cascade [34] in which MAP kinase is activated through p21ras/raf 1 and MAP kinase kinase (known as MEK). In bovine oocytes, MAP kinase becomes activated shortly before or concomitantly with cdc2 activation [32, 35]. In somatic cells, the MAP kinase pathway can be blocked by the synthetic inhibitor PD 098059 [36]. This substance prevents MEK phosphorylation by MEK kinase and raf 1. There have been few reports on the effect of PD 098059 during oocyte meiotic maturation, but it is likely that oocytes from different species vary in their sensitivity to this particular kinase inhibitor (unpublished data).

MAP kinase substrates are numerous; the best known in somatic cells are transcription factors and p90rsk [37–39]. Among others, two newly characterized protein kinases, called Mnk1 and Mnk2, have been identified as direct substrates of Erk1 and Erk2, as well as of p38 MAP kinases [40]. Recently, both Mnk1 and Mnk2 have been shown in somatic cells to phosphorylate eIF4E in vivo [41, 42]. Strong correlation between changes of MAP kinase activity and eIF4E phosphorylation has been also shown previously during maturation of mouse oocytes [43]. On the other hand, in starfish oocytes it has been recently documented that another protein kinase, protein kinase C-related kinase 2 (PRK2), phosphorylates eIF4E in vivo on Ser 209, the site that is supposed to be important for eIF4E biological activity [44].

In the present study we have analyzed the relationship between overall protein synthesis and the abundance and temporal changes of the phosphorylation state of eIF4E during meiotic maturation of bovine oocytes. We have used okadaic acid (OA), the phosphatase 1 and 2A inhibitor; the cdk kinases inhibitor BL I; and the specific inhibitor of MAP kinase cascade, PD 098059 [39] to elucidate the involvement of these kinases in meiotic maturation and eIF4E phosphorylation. Furthermore, we have analyzed the abundance and phosphorylation state of the specific repressor of eIF4E function, the binding protein 4E-BP1, and we discuss its role in repressing cap-dependent translation in metaphase II oocytes.

### MATERIALS AND METHODS

#### Materials

All chemicals were from Sigma (Taufkirchen, Germany) unless otherwise indicated.

#### Source and Collection of Oocytes

Bovine ovaries were obtained from a local slaughterhouse. Ovaries were transported to the laboratory in PBS in a thermos container at 30°C within 2 h of slaughter, and they were washed in fresh PBS immediately after arrival.

#### Oocyte Maturation, and Treatment of Oocytes with BL I, PD 098059, and OA

The collected oocytes (from follicles with a diameter 3–5 mm, with compact layers of cumulus cells and evenly granulated cytoplasm) were washed twice in TCM 199 and matured according to the method of Torner et al. [30]. During maturation, oocytes were treated with BL I (100 μM final concentration), OA (2.5 μM final concentration), and PD 098059 (20 μM, 50 μM, and 100 μM final concentrations). The substances were dissolved in dimethyl sulfoxide (DMSO), and DMSO without inhibitor was added to the controls. The maturation medium contained 3 mg/ml BSA but not bovine serum [32]. The samples were analyzed at defined time points of 0, 6, 10, 14, 18, 20, and 24 h in control medium; 5 h for OA; 10 and 24 h for BL I and PD 098059 treatment, respectively. To determine the reversibility of the inhibition, maturation was performed for an additional 10 or 24 h without inhibitors in control medium.

#### Morphological Evaluation of Oocytes

After maturation, the cumulus cells were removed from the oocytes by treatment with 2.5 mg/ml of hyaluronidase for 2 min. For morphological analysis, oocytes were fixed with acetic alcohol (1:3 acetic acid:ethanol) and stained with aceto-orcein and classified according to stage (GV, GVBD, or metaphase II).

#### Measurement of Overall Protein Synthesis

In order to measure the overall protein synthesis, 50 μCi of [35S]methionine (Amersham, Freiburg, Germany) was added to the culture medium at different time points. Labeling time was 4 h (0 + 4 h, 6 + 4 h, 10 + 4 h, 14 + 4 h, and 20 + 4 h for in vitro maturation). Thereafter, denuded oocytes were lysed in hypotonic buffer and treated with 1 M NaOH containing 5% H2O2 for 10 min at 37°C. Proteins were precipitated with 50% trichloroacetic acid (TCA); final concentration 12.5%. Insoluble material was collected on glass microfiber filters (Whatman GF/C, Sigma), which were previously soaked for 30 min with 5% TCA containing 100 mM Na2HPO4, and washed extensively with 5% TCA (3 times in 20 ml). The filters were dried, transferred to a 5-ml scintillation cocktail, and the amount of radioactivity was determined in a liquid scintillation counter.

### SDS-PAGE and Native PAGE

For analysis of MAP kinase, 10 oocytes per time point were washed 5 times in protein-free PBS, lysed in 10 μL of 2× SDS sample buffer, denatured for 2 min at 95°C, and immediately loaded on the gel or stored at ~80°C until electrophoresis. Samples were then separated on 9% gels [45]. In the separation gel, the ratio of acrylamide to bisacrylamide was 100:1. For analysis of eIF4E and 4E-BP1, 50 and 100 oocytes per time point were used on 15% or 20% gels, respectively.

Native PAGE was performed on 15% gels with an acrylamide:bisacrylamide ratio of 100:3. For gel isoelectric focusing without stacking gel, SDS was entirely omitted in the gel, the sample buffer, and the running buffer. One hundred oocytes were analyzed for each lane.

### Vertical Slab Gel Isoelectric Focusing

For vertical slab gel isoelectric focusing (VSIEF), we used the method of OFarrell [47] with the following modifications. Oocytes (30 per time point for eIF4E, 100 per time point for 4E-BP1) were washed 5 times in...
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FIG. 1. Determination of overall protein synthesis and GVBD in the course of in vitro maturation. Oocytes were cultured for different times (0, 6, 10, 14, or 20 h; 10 oocytes per time point) following 4 h incubation with 50 μCi [35S]methionine. Incorporation of the labeled methionine into TCA-insoluble proteins is depicted (gray columns). In the parallel experiments, oocytes were matured without labeled methionine for morphological analysis (approximately 100 oocytes per time point). The occurrence of GVBD is shown (black columns). This experiment was repeated once and standard errors are shown.

FIG. 2. Analysis of the phosphorylation of MAP kinase during in vitro maturation by band-shift on Western blots. Oocytes (10 per time point) were matured for 0, 6, 10, 16, or 24 h; and extracts were separated by SDS-PAGE on 9% gels and analyzed by Western blotting with anti-ERK1 antibody.

Immunoblotting

Proteins were transferred from the gels to Immobilon P, polyvinylidene difluoride membranes (Millipore, Eschborn Germany) according to the method of Towbin et al. [50] using a semidry blotting apparatus. Transfer was performed at 1 mA per cm2 for 1 h. Blots for MAP kinase and eIF4E were saturated in 10% Teleost gelatin (Sigma) in 0.1% Tween 20 in Tris-buffered saline pH 7.4 (TTBS). After each incubation, blots were washed extensively with TTBS. ERK-1 (MAP kinase) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:1500 in TTBS and blots were incubated for 1.5 h. The secondary anti-rabbit, horseradish peroxidase-linked antibody (Santa Cruz) was diluted 1:5000 in TTBS, and blots were incubated for 1 h. All incubations were performed at room temperature.

eIF4E antibody (BD Transduction Laboratories, Lexington, KY) was diluted 1:1000 in TTBS and incubated overnight at 4°C under permanent agitation. The secondary anti-mouse horseradish peroxidase (HRP)-linked immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was diluted 1:10000 in TTBS. The incubation time was 2.5 h at room temperature.

To detect 4E-BP1, antibody raised in rabbits against pig 4E-BP1 sequence was used. This antibody, which cross-reacts with bovine 4E-BP1 protein, was prepared by immunizing rabbits with the purified His-tag fusion protein (unpublished results) and by purifying the resulting serum by ammonium sulphate precipitation and by protein A agarose. The blots for 4E-BP1 were blocked with 2.5% fat-free dry milk (Roht, Karlsruhe, Germany) in TTBS for 2 h. The 4E-BP1 antisera were diluted 1:2000 in TTBS with 50 mM Tris pH 8, 10 mM MgCl2, 2 mM benzamidine, 40 μg/ml aprotinin, and 40 μg/ml aprotinin) was added to each sample, and the samples were subjected to 3 rounds of thawing and freezing on ice. After the final thawing the tubes were briefly vortexed and centrifuged at 10,000 x g for 15 sec. The kinase reaction was initiated by adding 5 μl of buffer B (100 mM MOPS pH 7.2, 20 mM para-nitrophenyl phosphate, 40 mM beta-glycerophosphate, 10 mM EGTA, 0.2 mM EDTA, 2 mM diithiothreitol [DTT], 0.2 mM Na2VO4, 2 mM benzamidine, 40 μg/ml leupeptin, and 40 μg/ml aprotinin) was added to each sample, and the samples were incubated for 2.5 h at room temperature.

RESULTS

Overall Protein Synthesis, MAP Kinase and cdc2 Kinase Activities, and eIF4E Phosphorylation During Meiotic Maturation

To characterize the relationship between translation rates and the activities of MAP kinase, cdc2 kinase, and eIF4E phosphorylation during meiotic maturation, we first analyzed their temporary changes during the course of maturation. We found that GVBD, which was completed in our culture system at 10 h after the beginning of maturation, was accompanied by a pronounced increase in overall protein synthesis as determined by the incorporation of [35S]methionine in trichloroacetic acid-insoluble proteins (Fig. 1). At the latest point, after 18 h, translation declined and reached basal levels at metaphase II (20–24 h matu-
ylation, the translation rate decreases and shows only basal translation, which was similar to the GV stage. The phosphorylation of MAP kinase as seen by band-shift on Western blots (Fig. 2) started in accordance with the onset of translation after 10 h of maturation, but was continuous up to metaphase II, when it was fully phosphorylated. The activity of MAP kinase during in vitro maturation is shown in Figure 3A, and was determined by its ability to phosphorylate the external substrate, MBP, in an in vitro kinase assay. In the same assay the pattern of cdc2 kinase activation is shown using histone H1 as an external substrate (Fig. 3A). These kinase assay results, which characterize the activities of these particular kinases, correspond to a more precise analysis by Torner et al. [30]. Those authors show that cdc2 kinase and MAP kinase of bovine oocytes show stable activities during meiotic maturation, starting slightly before GVBD.

To determine the abundance of eIF4E, we separated oocyte extracts by SDS-PAGE and performed Western blots with a monoclonal eIF4E antibody. The result (Fig. 4A) shows eIF4E as a single band, present from GV stage to metaphase II stage without significant differences in its concentration.

Changes in the phosphorylation state of eIF4E could be observed when the oocyte extracts were separated by VSIEF (Fig. 4, B and C). In GV stage, the factor was unphosphorylated. The increase in phosphorylation began at the time of GVBD and occurred in parallel with cdc2 kinase and MAP kinase activation until metaphase II stage, when eIF4E was fully phosphorylated. Furthermore, treatment of oocytes for 5 h with OA (Fig. 4B), which has been shown earlier to promote activation of cdc2 kinase and MAP kinase [32, 51] also resulted in premature phosphorylation of eIF4E, comparable to that in oocytes after 24 h of maturation in control medium. Treatment of the extracts with alkaline phosphatase before separation also confirmed that the upper band represented the phosphorylated form of eIF4E (Fig. 4C).

In summary, this first experiment showed that the increase of translation at the time of GVBD (10 h maturation) starts together with the activation of cdc2 and MAP kinase and together with the phosphorylation of eIF4E. But in contrast to the ongoing kinase activities and eIF4E phosphorylation, the translation rate decreases and shows only basal levels in metaphase II, similar to that in GV stage.

**Effect of BL I or PD 098059 Treatment on MAP Kinase and cdc2 Kinase Activities, and eIF4E Phosphorylation During Meiotic Maturation**

To investigate the pathways leading to phosphorylation of eIF4E we supplemented the maturation medium with the cdc2 kinase inhibitor, BL I. The rationale for this treatment was the ability of BL I to block MAP kinase activation in a reversible manner. This effect of BL I is supposed to be indirect, because only cdc2 kinase inhibition, but not MAP kinase inhibition, could be observed when BL I was added to an in vitro kinase assay [32].

In accordance to previously published data, in our results, BL I arrested oocytes at the GV stage (data not shown) and prevented MAP kinase phosphorylation, and therefore, its activation (Fig. 5A) [32]. Maturation of oocytes in the presence of BL I (100 μM final concentration) completely prevented eIF4E phosphorylation when BL I was added to a maturation medium containing 0.025 μM OA. Lane D shows the result from bovine hepatocytes as a control. B) Extracts from oocytes (same culture times and amount as in A) were separated by VSIEF and analyzed for eIF4E as described. Lane OA shows the oocytes treated with OA (5 μM okadaic acid). Lane C shows the results from bovine hepatocytes.

In summary, this first experiment showed that the increase of translation at the time of GVBD (10 h maturation) starts together with the activation of cdc2 and MAP kinase and together with the phosphorylation of eIF4E. But in contrast to the ongoing kinase activities and eIF4E phosphorylation, the translation rate decreases and shows only basal levels in metaphase II, similar to that in GV stage.
FIG. 5. Influence of BL I or PD 098059 on MAP kinase and eIF4E phosphorylation. Oocytes were cultivated for 10 or 24 h with 100 μM BL I or 20 μM PD 098059. To estimate whether the inhibition is reversible, maturation for an additional 10 or 24 h was performed without the inhibitor. Extracts were separated by SDS-PAGE for MAP kinase analysis (A and C) and by VSEIF for eIF4E phosphorylation analysis (B and D).

was used for 10 or 24 h of maturation. This effect was fully reversible, as was shown by cultivating the oocytes for an additional 10 or 24 h without inhibitor (Fig. 5B).

In contrast to this finding, an effect of the inhibitor of MEK activation PD 098059 (20 μM final concentration) could be observed only at early stages of maturation. Both MAP kinase and eIF4E phosphorylation were affected only during the first 10 h of culture. In the further course of maturation, MAP kinase and eIF4E became phosphorylated with a delay of approximately 4 h (Fig. 5, C and D). Similar results were also obtained when higher concentrations of PD 098059 (50 μM and 100 μM) were used (data not shown).

To verify these results, we performed an in vitro double-kinase assay. The oocytes were matured for different times in the presence of 50 μM PD 098059, and the oocyte extracts were analyzed for cdc2 kinase and MAP kinase activity by their ability to phosphorylate the external substrates, histone H1 and MBP. In comparison to the control oocytes, in which both cdc2 kinase and MAP kinase began activation approximately at the same time, after 10 h of maturation (Fig. 3A), the onset of MAP kinase activation in PD 098059-treated samples was postponed (Fig. 3B). However, cdc2 kinase also became affected by this treatment.

These biochemical data are supported by cytological analyses elucidating morphological changes in response to PD 098059 treatment. In control samples (Fig. 3C), the oocytes reached GVBD stage after 10 h of culture. In contrast, oocytes remained mainly in GV stage after 10 h of treatment with PD 098059 (although a smaller proportion of oocytes reached GVBD stage). However, even in the presence of the inhibitor, the oocytes reached metaphase II stage after 24 h (Fig. 3D).

These findings indicate that eIF4E is phosphorylated via the MAP kinase pathway, and that during bovine oocyte maturation, the activation of MAP kinase is only partially dependent on MEK activation. Furthermore, that PD 098059 also induces a delay in cdc2 kinase activation suggests that MAP kinase is involved in cdc2 kinase activation (see Discussion).

FIG. 6. Analysis of bovine oocytes for the presence of 4E-BP1. Extracts of oocytes (100 per lane) were separated by SDS-PAGE on 18% gels and analyzed by Western blotting with 4E-BP1 antiserum. A predominant band at about M, 18,000 is shown.

FIG. 7. Analysis of eIF4E/4E-BP1 complex formation during meiotic maturation by native-PAGE. A) Extracts of oocytes (100 per lane) were separated on 15% native gels and probed for 4E-BP1 or eIF4E by Western blotting. The position of the 4E-BP1/eIF4E complex and of free 4E-BP1 is shown. B) Estimation of the optical density of the obtained bands. The optical density from scanned films was evaluated by Scanalytics One-Dscan software. For anti-4E-BP1, the difference of the relative amount of free 4E-BP1 (black column) and eIF4E/4E-BP1 complex (white column) was determined. For anti-eIF4E, the amount of complex form is depicted. C) Oocytes were matured for 10 h, extracts were separated by SDS-PAGE on 18% gels (30 oocytes per lane), and analyzed by Western blotting for eIF4E (left lane) or 4E-BP1 (right lane). This picture illustrates the differences compared with native-PAGE.
Abundance of 4E-BP1 and elf4E/4E-BP1 Complex Formation During Meiotic Maturation and 4E-BP1 Phosphorylation State

To resolve the contradiction of our results showing that elf4E is fully phosphorylated in metaphase II-stage oocytes, but that overall protein synthesis reaches basal levels, such as in GV-stage oocytes in which the factor is unphosphorylated, we analyzed oocytes for the presence of a specific repressor of elf4E function. Therefore, we cloned 4E-BP1 and produced antisera against this antigen in rabbits, which we used in further experiments.

Western blots with this antisera showed a band in the range of $M_r$ 18,000 (Figs. 6 and 7C, second line). The same values for 4E-BP1 were given by other researchers [14]. This protein was present from GV stage to metaphase II stage.

Because the elf4E and 4E-BP1 antibodies proved to be not suitable for immunoprecipitation analyses, we decided to perform native PAGE to show elf4E/4E-BP1 complex formation in the course of meiotic maturation. We found that in all stages, a predominant band with a size of approximately $M_r$ 40,000 was recognized both by elf4E and 4E-BP1 antibodies (Fig. 7A). This was in accordance with the calculated molecular weight of the elf4E/4E-BP1 complex. Additional, smaller bands were detected by anti-4E-BP1. These bands should represent “free 4E-BP1.” In contrast to this, no unbound elf4E could be observed when anti-elf4E antibody was used (see Discussion). We estimated the amount of the elf4E/BP1 complex and the relative amount of bound and unbound 4E-BP1 by measuring the optical density of the obtained bands. We found that after 24 h the elf4E/BP1 complex (as obtained using anti-elf4E antibody) was predominant compared with 10 h of maturation (Fig. 7B). In addition, we obtained higher values for elf4E/4E-BP1 after 24 h of maturation in contrast to 10 h, when we compared the relative amounts of free 4E-BP1 with the complex form (as obtained using anti 4E-BP1 antibody). Figure 7C shows single bands of elf4E (first lane) and 4E-BP1 (second lane) as obtained after SDS-PAGE, and illustrates the differences to native PAGE.

Figure 8 shows the phosphorylation state of 4E-BP1 at 0, 10, and 24 h of in vitro maturation as obtained by Western blotting after VSIEF. Two distinct bands could be recognized on the gel. The unphosphorylated 4E-BP1 (lower band) did not bind to elf4E [52], but it could be seen that at 10 h of maturation, a smaller amount of 4E-BP1 remained unphosphorylated when compared to that at 0 and 24 h. We interpret these results as a strong hint that 4E-BP1 is involved in repressing translation in metaphase II oocytes, although elf4E is found only in its phosphorylated form at this stage of development (see Discussion).

DISCUSSION

Fully grown bovine oocytes mature spontaneously when they are transferred from their follicles to a suitable culture medium. In the course of meiotic maturation the two major M-phase kinases, cdc2 kinase and MAP kinase, become activated, as reported in a number of different species [29, 30, 43, 53, 54]. In this study we show that the inhibition of these kinases prevents phosphorylation of translation initiation factor elf4E, which under normal culture conditions, starts at the time of GVBD and persists until metaphase II. Furthermore, we demonstrate that elf4E phosphorylation is accompanied by a remarkable increase of protein synthesis at the time of GVBD-metaphase I transition, whereas in metaphase II stage, oocyte protein synthesis declines to a level similar to that of GV-stage oocytes, even though elf4E is fully phosphorylated at this time.

Recently, two novel protein kinases, called Mnk1 and Mnk2 (i.e., mitogen-activated protein kinase signal-integrating kinases 1 and 2) [40, 41] have been shown to be the main kinases in somatic cells that phosphorylate elf4E in vivo [21, 41, 42]. Both Mnk1 and Mnk2 are themselves activated, as reported in a number of different species [29, 30, 43, 53, 54]. In the present paper we intended to show that similar mechanisms of elf4E phosphorylation by a MAP kinase-dependent pathway also functions in bovine oocytes during maturation.

We have demonstrated a strong correlation between elf4E phosphorylation and MAP kinase activation in the following situations. First, during culture of oocytes in control medium, elf4E starts to become phosphorylated after 10 h (i.e., approximately at the time of GVBD), then the maximum phosphorylation is achieved after 16–20 h, which is maintained until metaphase II stage. The same timing has been detected for MAP kinase activation: it starts to become activated (phosphorylated) approximately at the same time as cdc2 kinase (i.e., after 10 h of culture). After reaching a maximum at 16 h, MAP kinase activity stays stable until metaphase II stage, whereas cdc2 kinase activity temporarily decreases at the time of anaphase/telophase I stage at 18 h, followed by another increase at metaphase II stage ([30, 32] and present data).

Second, no elf4E phosphorylation was observed when the oocytes were cultured in the presence of the cdk kinase inhibitor, BL I. Under such conditions, both cdc2 kinase and MAP kinase activation was prevented in vivo: these findings regarding the activities of cdc2 kinase and MAP kinase have already been reported [32]. These authors proved that the BL I effect on MAP kinase was indirect, because MAP kinase activation was not inhibited in extracts obtained from metaphase II oocytes, which possess high MAP kinase activity, to which BL I was added to in vitro kinase assays. The authors concluded that in bovine oocytes, activation of MAP kinase must somehow be linked to cdc2 kinase activation. This assumption is in agreement with other findings showing that in oocytes from numerous mammalian species, cdc2 kinase is activated slightly before the activation of MAP kinase [32, 35, 55, 56].

Third, when bovine oocytes were treated with PD 098059, which specifically blocks phosphorylation and activation of MEK, a kinase upstream of MAP kinase [36,
decrease gradually until metaphase II to levels similar to maximum in metaphase II oocytes. Translation rates grow after 10 h of maturation, and then it grows steadily to reach eIF4E becomes phosphorylated just at the time of GVBD, can be only partially seen in bovine oocytes, although phosphorylation and translation rates was observed, this effect comes active before full activation of MAP kinase. As shown by Palmer et al. [60], no activity of MAP kinase, nor eIF4E phosphorylation, could be seen after 10 h of treatment and, in addition, GVBD was inhibited. However, after 14 h, the oocytes reached GVBD stage, MAP kinase became activated (to a level similar to that in control oocytes after 10 h of culture), and it remained at this level until metaphase II. At the same time, the phosphorylation of eIF4E protein could be detected with high correlation to MAP kinase activity. These results document that PD 098059 treatment only postpones GVBD and MAP kinase activation, as well as eIF4E phosphorylation, and the oocytes reach metaphase II after 24 h of maturation in a considerable amount, also in the presence of this particular inhibitor.

The most straightforward explanation for this incomplete MAP kinase inhibition by PD 098059 is the possibility that a proportion of MEK protein is already phosphorylated in GV-stage oocytes. It is known that MAP kinase is activated by MEK, a dual specificity tyrosine/threonine kinase [58]. MEK itself is activated as a result of the activity of an upstream Raf1 kinase, which is induced by Ras type G proteins. However, Raf1 is not the only MEK activator, because other protein kinases, including c-mos and MEK kinase, can activate MEK [59]. The MEK inhibitor PD 098059 binds to the inactive forms of MEK and inhibits its phosphorylation and activation by upstream activators MEK kinase and Raf1 [36, 57]. However, PD 098059 does not influence MEK that has already been phosphorylated, so we suggest that a small proportion of activated MEK that is present in GV oocytes causes gradual phosphorylation and activation of MAP kinase, although it is delayed by treatment with the inhibitor.

It could be noticed, however, that aside from postponing MAP kinase activation and GVBD, PD 098059 also causes a delay in the activation of cdc2 kinase. This finding suggests that MAP kinase is somehow involved in the activation of cdc2 kinase. There is growing evidence obtained from Xenopus oocytes that an appropriate link exists between these two kinases. As shown by Palmer et al. [60], a MAP kinase substrate, p90 rsk, phosphorylates the C-terminus of Myt1 (a kinase that inhibits cdc2) and down-regulates its inhibitory activity on cdc2. Other authors [61] have also recently shown that low MAP kinase activity in Xenopus oocytes leads to cyclin B degradation and cdc2 kinase inactivation. To the contrary, data from mammalian oocytes show that MAP kinase becomes activated around the time of cdc2 kinase activation or slightly later, as in cattle [32, 35], or even after GVBD, as in mice [62] or goats [63]. However, a possible explanation for the PD 098059 effect on cdc2 kinase in mammalian species is that low levels of MAP kinase present in GV oocytes are sufficient for initial activation of cdc2 kinase, which then becomes active before full activation of MAP kinase.

It has been reported that phosphorylation enhances eIF4E activity, and that only the phosphorylated form of eIF4E is present in the 48S mRNA ribosome complex in somatic cells [64, 65]. In this study we document that eIF4E phosphorylation also occurs during meiotic maturation of bovine oocytes. But in contrast to somatic cells, in which a strong, positive correlation between eIF4E phosphorylation and translation rates was observed, this effect can be only partially seen in bovine oocytes, although eIF4E becomes phosphorylated just at the time of GVBD, after 10 h of maturation, and then it grows steadily to reach maximum in metaphase II oocytes. Translation rates grow sharply at the time of GVBD, but after metaphase I they decrease gradually until metaphase II to levels similar to those found in GV-stage oocytes. Similar results have been reported earlier in mouse oocytes [66] and such a decrease in protein synthesis in metaphase II oocytes could be explained by the presence of some repressors of eIF4E function. Three such repressors, called 4E-BP1, 4E-BP2, and 4E-BP3, which are structurally related, have been described in somatic cells [14, 22, 67]. These proteins specifically inhibit eIF4E-dependent translation initiation by competing with eIF4G for binding to eIF4E and preventing the formation of the eIF4F complex [23]. The binding of 4E-BPs is controlled by their phosphorylation state; the underphosphorylated forms of 4E-BPs interact with eIF4E, whereas the hyperphosphorylated forms do not [14, 22, 68].

In the present study we document that 4E-BP1 protein is present in bovine oocytes from the GV stage to metaphase II. Moreover, our results show that 4E-BP1 forms a complex with eIF4E. Because we did not find “free eIF4E” at any stage of maturation we conclude that this factor is always bound to the cap structure or to 4E-BP1. This suspicion is supported by the recently published data showing that even 4E-BP1 binding to eIF4E does not interfere with eIF4E binding to the cap structure [16], but it does not allow binding of eIF4E to eIF4G and eIF4F complex formation. Our results also show that 4E-BP1 does not discriminate between the phosphorylated and nonphosphorylated forms of eIF4E, suggesting that the binding should be regulated exclusively by the 4E-BP1 phosphorylation state. Furthermore, when measuring the phosphorylation state of 4E-BP1 by VSIIEF, we have found that a significantly higher amount of 4E-BP1 is unphosphorylated at 24 h of maturation compared with 10 h. This result suggests an enhanced eIF4E/4E-BP1 complex formation in metaphase II oocytes, and could explain decreased translation rates in this stage. From the above-mentioned data we conclude that 4E-BP1 is likely to be involved in repressing cap-dependent translation in metaphase II bovine oocytes, although the involvement of the two other repressors, 4E-BP2 and 4E-BP3, cannot be excluded.

Taken together, our results indicate that 1) the translation initiation factor, eIF4E, and its binding protein, 4E-BP1, are both present in bovine oocytes, and their amounts do not change significantly during meiotic maturation; 2) phosphorylation of eIF4E during maturation of bovine oocytes is closely correlated with activation of MAP kinase; and 3) cap-dependent translation in these oocytes is regulated not only by the phosphorylation state of eIF4E, but also by its binding to 4E-BP1 repressor, which may cause the decrease in the translation rates in metaphase II oocytes.

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