ABUNDANCE OF POLY (A) BINDING PROTEIN IN BOVINE OOCYTES DURING IN VITRO MATURATION¹

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Abstract: To improve the conditions for in vitro production of embryos in animal husbandry it can be helpful to obtain deeper insights into regulatory mechanisms which are directing maturation of oocytes on a molecular level. Recent observations have shown that protein synthesis in mature oocytes (metaphase II stage) is impaired. Although oocytes possess a stockpile of mRNA at this stage of development, the translation of these mRNAs is repressed. It has been shown that essential components of cap dependent regulation of translation (eIF4E, 4E-BP1) potentially could attend in this process. More recent investigation suggest that these factors probably not alone modulate translation rates during in vitro maturation of bovine oocytes. Therefore in the present study the abundance and potential modifications of another regulator of translation, namely the poly (A)-binding protein was analysed. This protein was shown to bind to mRNAs poly-(A)tails and link them to the cap-binding complex eIF4F. By this mechanism, cap dependent translation should by synergetically stimulated. In the present study, we have analysed the abundance of poly (A) binding protein in the course of meiotic maturation and we have elucidated potential expression of isoforms or post translational modifications of this protein by 2D-Gelelectrophoresis.

Key words: bovine oocyte, in vitro maturation (IVM), poly (A)-binding protein PABP.

Introduction

In vitro production of embryos (IVP) in animal husbandry have received great attention and support in the last two decades. The IVP technology is well established in the bovine and it is increasingly used in practice.

IVP includes oocyte in vitro maturation (IVM), in vitro fertilization (IVF) and the development of the fertilized oocyte to the blastocyste stage (embryo cultivation) as well as embryo transfer. Each of these steps has to be completed successfully to obtained viable embryos and high pregnancy rates. The expected rate of transferable blastocysts after oocyte maturation, fertilization and embryo culture in vitro reaches 30 to 40%. It is believed that the low yield has one reason in sub-optimal in vitro maturation condition of oocytes.

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Fully grown oocytes are arrested in follicle in germinal vesicle stage (GV stage). Oocytes released from their follicle mature spontaneously when they are transferred into a suitable culture medium. During the short period of meiotic in vitro maturation (in bovine 24 hours) morphological changes occur: After chromatin condensation, the nuclear envelope becomes disintegrated and germinal vesicle breakdown (GVBD) takes place (Süss et al., 1988).

![Diagram of 3'-poly(A) and proteins eIF4F, eIF4E, eIF4G, eIF4A, and AUG]

Adapted from Mangus, et al., 2003

Function of PABP in stimulating the initiation of translation

_Slika 1. Stimulacija translacije sa približenim krajevima iRNK. PABP interaguje sa eIF4E, komponentom kapica-vezujućeg kompleksa eIF4F. To omogućava vezivanje male subadnince ribozoma u blizini 5' kraja iRNK. Translacija se stimuliše. Verovatno je približavanje krajeva iRNK sprečeno postranslacionalnim modifikacijama (fosforilacijom) PABP. U tom slučaju translacija se zaustavlja._

The meiotic spindle is formed, chromosomes were separated and the polar body is excluded. Finally oocytes reach metaphase II (M II) where they are arrested again. This phase is first accompanied by dramatic decreases in transcriptional activity (Tomek et al., 2002a). After chromatin condensation, transcription is generally impaired. Second, the pool of accumulated mRNA is translationally inactive in M II. The reason for this effect seems to be the inactivation of the translational machinery,
especially by the impaired function of initiation factors of translation (cap binding complex eIF4F) and probably also by the reduced action of poly(A)-binding proteins (PABP) (Tomek et al., 2002b, Smiljaković et al., 2003, Tarun et al., 1997).

To give a contribution for solving this problem we first investigate the abundance of PABP during IVM. It is known from investigation in somatic cells that the binding of PABP is, at least in somatic cells involved not only in increasing the stability of mRNA (Bernstein et al, 1989) but also in the initiation of translation. The most sophisticated model of this process describes the formation of a circularisation of mRNA ends where the PABP bound at the 3′-poly (A) tail interacts with the component eIF4G of the cap binding complex eIF4F (composed of eIF4E, eIF4G and eIF4A) located at the 5′-cap structure of the mRNA (Fig. 1). The formation of this structure is necessary to remove secondary structures from the mRNA and therefore it stimulates the binding of the small ribosomal subunit mRNA (Tarun and Sachs, 1995). The consequence of this process are enhanced translation rates. Until now it is not fully understood how this events were regulated. In detail it is not clear if posttranslational modifications like phosphorylation of the PABP is essential. Up to date only one article has shown unequivocally that PABP function could be regulated by phosphorylation (Le et al., 2000). Therefore it is of high interest to investigate if phosphorylation of PABP is involved in modulation gene expression during oocyte maturation on translational level.

**Materials and Methods**

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 2h of slaughter, and they were washed in fresh PBS immediately after arrival. Ovaries were cut in small pieces, and oocytes were collected.

*Oocyte Maturation.* The collected oocytes (from follicles with diameter 3-5 mm, with compact layers of cumulus cells and evenly granulated cytoplasm) were washed twice in PBS and matured 0, 10 and 24 hours according to the method of Torner et al., (2001). Before analyses the cumulus was removed by treatment with hyaluronidase.

*SDS-PAGE.* For analysis of PABP, 10 oocytes per time point (0h, 10h, 24h of IVM) were washed 5 times in protein-free PBS, lysed in 10μl of 2x SDS sample buffer, denatured for 2 min at 95 C, and immediately loaded on the gel. Samples were then separated on 12,5%gels. In the separation gel the ratio of acrylamide to bisacrylamide was 1:100 (method according to Laemmli, 1970).

*2D-Electrophoresis.* 200 oocytes per time point (0, 10, 24 h of IVM) in rehydration buffer (8M urea, 2,5 % CHAPS, IPG Buffer 0,5% (v/v), 0,002% bromphenol blue) were loaded on the IPG strip (immobilized pH gradient 6-11) and proteins were separated in first dimension electrophoresis according to their isoelectric
points (pI) (method according to Gorg et al., 1998, Westermeier et al., 1983). After IPG strip equilibration (50mMTris-HCl, pH 8.8, 6M urea, 30%glycerol, 2%SDS, bromphenol blue, with DTT and iodoacetamide) second step of 2D electrophoresis that separates proteins according to their molecular weight was performed on 12,5 % SDS slab gels.

**Immunoblotting.** Proteins were transferred from the gels to Immobilon P, polyvinylidene difluoride membranes (Milipore, Eschborn, Germany) using the semidry blotting apparatus at 1 mA per 1 cm² for 1h, according to the method of Towbin et al., (1970). Blots for PABP were saturated in 5% fat free dry milk in 0.1%Tween 20 in Tris-buffered saline pH7.4 (TTBS). After each incubation, blots were washed extensively with TTBS.

The blots for PABP were incubated with 1:1000 diluted antibodies (Santa Cruz Biotechnology) in 5% milk, overnight, at 4°C. The secondary antibody was HRP-labelled donkey anti goat, diluted 1:2000 in TTBS, and incubated with blots 1,5h, at room temperature. The reactive bands were detected by chemiluminescence reaction using a ECL or ECL-plus kit (Amersham) and visualised on X-ray films (Kodak) according to manufactures instruction.

![Image of protein bands](image)

**Figure 2.** Immunodetection of PABP during in vitro maturation of bovine oocytes (left panel), and immunodetection of MAP kinase, ERK1 (right panel). After separation by SDS-PAGE, proteins from oocyte cell extracts obtained after 0, 10 and 24 h of IVM were analysed by Western blotting for the abundance of PABP. The progress of the maturation process was confirmed by reprobing the blot for analysing the phosphorylation of MAPK by band shift.

**Slika 2.** Imunodetekcija PABP za vreme in vitro sazrevanja jajnih čelija goveda (slika levo) i imunodetekcija MAP kinaze, ERK1 (slika desno). Nakon razdvajanja na SDS-PAGE, proteini iz ekstrakata jajnih čelija nakon 0, 10 i 24 sata in vitro sazrevanja (IVM) su analizirani Western blotting-om na prisustvo PABP. Tok procesa sazrevanja jajnih čelija je potvrđen ponovnom analizom blot-ova za fosforiliranost MAPK pomoću položaja tračica na gelu.
Results

Analysis of oocyte extracts for the abundance of PABP by 1D-SDS-PAGE.

Oocytes were matured for different times (0, 10, 24 hours) to reach GV, GVBD-M I and M II stage. After 1D electrophoresis and immunoblotting with anti PABP we have made the following observations at the different maturation stages (Fig. 2). In GV stage there is one main band of 70 kDa. This result is highly similar to this obtained by analysing PABP from post-mitochondrial supernatants of bovine hepatocytes (lane PMS of the left blot). In GVBD and MII stage several new bands appear. In metaphase II the most complex protein pattern was observed. If this increase in complexity reflects the expression of isoforms of PABP during IVM or is caused by posttranslational modifications of PABP already present in GV stage remains to be analysed.

To investigate the progress of IVM, immunoblotting for MAP kinase (ERK1) has been also performed by reprobing the PABP-blot for MAPK, which gradually becomes phosphorylated during IVM. After 0h IVM, ERK 1 shows a single band, which represents the unphos- phorylated form of ERK1. At 10h an additional band appeared indicating that MAPK becomes phosphorylated. Finally after 24h IVM, most of ERK1 is phosphorylated. This analysis confirms that the maturation for analysing PABP works as expected.

![Image of SDS-PAGE gel showing PABP bands at different stages of IVM](image-url)

*Figure 3. Immunodetection of PABP after 0h, 10 and 24 hours of IVM after 2D-gel electrophoresis. After maturation oocytes extracts were separated in the first dimension by isoelectric focusing according to their isoelectric points. In the second dimension the samples were separated by SDS-PAGE. PABP was detected by Western blotting on PVDF membranes.*

*Slika 3. Imunodetekcija PABP nakon 0, 10 i 24 sata in vitro sazrevanja jajnih čelija goveda na 2D-elektroforezi. Nakon sazrevanja jajnih čelija ekstrakti su razdvojeni po jednoj dimenziji pomoću izoelektričnog fokusiranja prema svojim izoelektričnim tačkama. U drugoj dimenziji uzorci su razdvojeni na SDS-PAGE. PABP je detektovan pomoću Western-blotting-a na PVDF membranama.*
Analysis of oocyte extracts for the abundance of PABP by 2D-Gelelectrophoresis

The results obtained from 2D analysis of PABP are depicted in Fig. 3. After 0h IVM several spots in the range of 70 kDa could be detected (lower arrow left panel Fig. 3; three major spots and additionally three or four weaker spots). Additionally, at least two spots with a slightly higher molecular weight could be detected (upper arrow).

After 10 hours of maturation (middle panel) it seems that the lower size spots obtained at 0 h IVM were shifted to higher molecular weight (upper arrow). This is concluded by the fact that the abundance of the smaller spots decrease (lower arrow) while the larger spots increase. Additionally a moderate shift to a more acidic pl was observed.

Finally at M II stage after 24 hours of IVM a more complex protein pattern can be observed (Fig. 3, right panel). The upper bands (lower arrow in this image) shift even to a more acidic pl compared to those obtained after 0 or 10 hours of IVM. Additionally several distinct spots with higher molecular weights appear.

Discussion

The results presented in this study show that the pattern of the poly(A) binding protein changes in the course of meiotic maturation of bovine oocytes. While in the GV stage one main band can be detected by 1D-SDS-PAGE, the pattern became more complex in the GVBD-M I and in M II stage. A more precise distribution of the abundance of PABP could be obtained by analysing the oocyte extracts by 2D-gelelectrophoresis. With this method not only a shift to higher molecular weight but also differences in the pl could be found during IVM. If this differences represent changes in the phosphorylation state or in the expression of isoforms remains to be investigated.

Taking into account that the PABP is involved in the regulation of initiation of translation, this finding can be important with regard to translation rates during IVM. It has been shown recently that translation in bovine oocytes significantly change during IVM (Tomek et al., 2002). In GV stage, basal rates were observed. This can be due to a lack of the phosphorylation of the cap binding protein eIF4E. Concomitantly with the onset of phosphorylation of eIF4E at the time of GVBD, translation increased. Finally, in M II stage, where eIF4E was fully phosphorylated only low translation rates were observed, comparable to those found in GV stage oocytes where the factor is unphosphorylated. These results indicate that beside eIF4E phosphorylation other factors must be involved in modulating translation during IVM. It can be speculated that one of these factors is PABP. Wakiyama et al., (2000) has shown that such a mechanism including PABP is essential for the regulation of translation during maturation of progesterone-induces Xenopus oocyte maturation.
Investigation by others have described isoforms of PABP in several species, tissues or cell types (Mangus et al., 2003, Le et al., 2000, Kleene et al., 1998, Kleene et al., 1994.). On the other hand, phosphorylation of PABP has only been reported so far in yeast and wheat (Le et al., 2000). These authors showed that the interaction of PABP with the poly(A) tail of the mRNA or with its partner proteins depends on the phosphorylation state of PABP. In this context, the most straightforward hypothesis to explain PABP function in translational control during IVM would be that a similar phosphorylation mechanism exists also in bovine oocytes. This assumption include differences in the phosphorylation of PABP during meiotic maturation which impair the circularisation of the mRNA in M II stage and in such a way repress translation at this stage of development.

Until now, direct evidence for the existence of the described mechanism is missing. More precise analysis are necessary to establish the phosphoprotein character of PABP. Additionally also the abundance of isoforms of PABP has to be considered and furthermore the existence of specific binding proteins (PABP interacting proteins, Paips) which have been described to modulate PABP function in somatic cells (Khaleghpour et al, 2002, Craig et al., 1998) have to be elucidated in bovine oocytes.

KOLIČINA POLI (A) VEZUJUĆEG PROTEINA U OOCITIMA GOVEDA ZA VREME IN VITRO SAZREVANJA

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Rezime

Za poboljšanje uslova in vitro proizvodnje embriona u stočarstvu, značajno je imati bolji uvid u regulatorne mehanizme koji upravljaju sazrevanjem jajnih čelija na molekularnom nivou. Skošanja istraživanja pokazuju da je sinteza proteina u zrelim jajnim čelijama (metafaza II) usporena ili zaustavljena. Iako jajne čelije sadrže visok nivo iRNA na tom stupnju, translacija sa iRNA je zaustavljena. Pokazano je da osnovne komponente u "kapica"-zavisnoj translaciji mogu uticati na ovaj proces. Istraživanja su pokazala da ovi faktori ne regulišu stopu translacije sami za vreme in vitro sazrevanja jajnih čelija. Stoga je u ovom radu urađena analiza rasprostranjenosti i mogućih promena na jednom drugom regulatoru translacije, poli (A) vezujući protein. Ovaj protein se vezuje za poli (A) kraj iRNA a takođe je veza poli (A) kraja sa "kapica"- vezujućim kompleksom eIF4F. Ovim mehanizmom, "kapica" zavisna translacija se sinergistički pospešuje. U ovom radu analizirali smo zastupljenost poli (A) vezujućeg proteina za vreme mejotičkog sazrevanja i dali uvid u moguću ekspresiju izoformi ili posttranslacionih modifikacija ovog proteina pomoću 2D elektroforeze.

Ključne reči: oocit goveda, in vitro sazреванje, poli(A) vezujući protein.
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References


