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Reversible changes in protein phosphorylation during germinal vesicle breakdown and pronuclear formation in bovine oocytes *in vitro*

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Summary

This study examined the event of protein phosphorylation in bovine oocytes during germinal vesicle breakdown (GVBD) and formation of pronuclei following fertilisation *in vitro*. Immature oocytes were obtained from abattoir materials and cultured *in vitro*. The oocytes were labelled with [^{32}P]orthophosphate at 3 h intervals from 0 to 12 h following maturation in culture or from 3 to 18 h following insemination. One-dimensional gel electrophoresis indicated that levels of protein phosphorylation are low prior to GVBD. However, the levels of protein phosphorylation at approximately 40 kDa, 27 kDa, 23 kDa and 18 kDa increased substantially following GVBD and then decreased gradually as maturation in culture progressed. In contrast, the levels of protein phosphorylation increased gradually in the oocytes following pronucleus formation. Further, two-dimensional gel electrophoresis indicated that the protein at approximately 18 kDa reversibly changed in the oocytes during maturation and fertilisation. These results indicate that the reversible changes of this phosphoprotein may be related to either cell cycle transition or pronucleus formation during maturation and fertilisation in bovine oocytes.

Keywords: GVBD, Maturation, Oocyte, Pronucleus, Protein phosphorylation

Introduction

The reversible phosphorylation of proteins is now recognised to be a major mechanism for the control of intracellular events in eukaryotic cells. The relative concentration of phosphorylated and non-phosphorylated forms of protein substrates is controlled by two enzymes: a protein kinase and a protein phosphatase. Target proteins are phosphorylated at specific sites by one or more protein kinases, and these phosphates are removed by specific protein phosphatases. The importance of protein kinases in regulating cellular activities is underscored by the large number of protein kinase genes that are present in eukaryotic genomes (Hunter, 1995). There are also a large number of protein phosphatase genes, about half of which encode protein serine/threonine phosphatases and the other half protein tyrosine phosphatases (Wilson *et al*., 1994).

Obviously, protein phosphorylation and dephosphorylation play key roles in the regulation of meiotic maturation. The maturation-promoting factor (MPF) complex is a protein dimer composed of catalytic p34^{cdc2} serine/threonine kinase which is the homologue of the yeast cdc2/CDC28 protein kinase, and regulatory cyclin B subunits which are the homologue of the yeast cdc 13 gene product (Maller *et al*., 1977; Gautier *et al*., 1990; Nurse, 1990). Activation of MPF requires key side phosphorylation and dephosphorylation. The product of the c-*mos* proto-oncogene, a protein serine/threonine kinase known as Mos (Sagata *et al*., 1989; Pines & Hunter, 1990), and mitogen-activated protein kinase (MAP-kinase) (Watanabe *et al*., 1989), directly or indirectly modify MPF activity with the

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status of phosphorylation or dephosphorylation. Therefore, the phosphorylation cascade of Mos and MAP-kinase may play an important role in the meiotic and mitotic cell cycle from G2 to M-phase (Nebreda & Hunter, 1993; Dekel, 1996).

Oocytes and sperm contain histone variants, which serve the unique physiological requirements of oogenesis, spermatogenesis and early embryogenesis. Phosphorylation sites are typically located within variant DNA-binding regions of sperm and oocyte histones, indicating that their special functions are realised through the charge-altering effects of reversible histone phosphorylation (Norbury & Nurse, 1992). These indicate that histone kinases and phosphatases participate in the control of chromatin structure by altering histone–DNA interactions (Green & Piccia, 1985, 1989). Upon entering the oocyte cytoplasm at fertilisation, the sperm nucleus undergoes a dramatic morphological transformation as the male pronucleus is formed. The sperm nucleus is transformed into the male pronucleus through the interaction of oocyte cytoplasmic components with sperm chromatin (Perreault *et al*., 1984; Green *et al*., 1995). Molecular analysis indicates that the first step in the processing of the sperm nucleus is reduction in protamine disulfide bonds (Longo & Kunkle, 1978; Zirkin *et al*., 1985). The sperm-specific protamines are replaced by histone and non-histone proteins during pronuclear formation. Events related to pronuclear formation and subsequent DNA synthesis by the pronucleus appears to be related to the meiotic cycle of the oocytes. It has been suggested that several nuclear factors, which are necessary for inducing cytoplasmic changes and male pronucleus development following penetration of the oocyte, may be released during germinal vesicle breakdown (GVBD) of oocytes (Yanagimachi, 1978). The sperm pronucleus development factor (SPDF) may be synthesised as oocytes move from GVBD to metaphase-II (Thibault, 1977). Male pronucleus formation, however, is clearly dependent not only upon GVBD but also upon oocyte activation (Zirkin *et al*., 1985).

Inhibition of protein synthesis affects histone H1 kinase, but not chromosome condensation activity during the first meiotic division of the oocyte (Balakier & Tarkowski, 1980). It seems clear that protein synthesis is not required for the transition from metaphase II to the female pronucleus (Zhang & Masui, 1992; Moses & Masui, 1994; Moses & Kline, 1995; Kubelka *et al*., 1995; Hagemann *et al*., 1995). Inhibition of protein synthesis following sperm penetration shows that formation of the male pronucleus is not affected, suggesting that the newly synthesised proteins following fertilisation are not essential for male pronuclear formation (Chian & Sirard, 1996). In some species, it has been suggested that protein phosphorylation and dephosphorylation are involved during male pronuclear formation (Howlett & Bolton, 1985; Howlett, 1986; Reyes *et al*., 1991; Ding *et al*., 1992; Jung *et al*., 1993). The decondensation of sea urchin sperm chromatin following fertilisation is accompanied by specific protein phosphorylation (Poccia & Collas, 1997). It has been demonstrated that protein phosphorylation is essential for formation of the male pronucleus in bovine oocytes (Chian *et al*., 1999a). Although the formation of the female pronucleus is not affected by 6-dimethylaminopurine (6-DMAP), which is a protein kinase inhibitor, male pronucleus formation is completely inhibited by the presence of 6-DMAP. These results also suggest that formation of male and female pronuclei may involve different factors in the bovine zygote since they respond differently to the kinase modulations. The phosphorylation of protein complexes of 23 kDa and 18 kDa specifically increased with the formation of male and female pronuclei, suggesting that these phosphoproteins are related to formation of the male pronucleus in bovine oocytes (Chian *et al*., 1999b). However, it is unclear how these specific phosphoproteins change during oocyte maturation and fertilisation.

If there were specific reversible phosphoproteins during oocyte maturation and fertilisation, these specific phosphoproteins may be involved not only in the cell cycle transition of G2/M but also in male pronucleus formation. To clarify protein phosphorylation in oocytes following maturation and fertilisation will be useful for understanding mechanisms of cell cycle transition and reprogramming of the sperm genome in oocytes. The objective of the present study was to examine the profile of protein phosphorylation during oocyte maturation and fertilisation *in vitro* in order to determine which protein(s) are related to the transition of G2/M and formation of pronuclei following fertilisation.

Materials and methods

Maturation of oocytes *in vitro*

Ovaries from cycling or pregnant heifers or cows were removed within 30 min of slaughter and transported to the laboratory at 35 °C in 0.9% NaCl solution. Cumulus–oocyte complexes (COCs) were aspirated from 2–8 mm follicles with an 18 G needle attached to a 10 ml syringe, pooled and selected as described previously (Chian *et al*., 1994). The COCs were rapidly washed four times in HEPES buffered Tyrode's medium (TLH) (Bavister *et al*., 1983) supplemented with 10% heated (56 °C for 30 min) fetal bovine serum (FBS), 0.25 mM pyruvic acid and 50 μg/ml gentamicin. After washing, 10 COCs were cultured in 50 μl droplets of maturation medium (TC-199 medium

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supplemented with 10% FBS, 0.75 mIU/ml FSH and LH: Humegon, Organon Canada) under mineral oil. The COCs were cultured at 38.5 °C under an atmosphere of 5% CO_2 in air with high humidity. To examine the process of GVBD, the oocytes were fixed at 1 h intervals from 0 to 12 h following maturation in culture.

Sperm preparation and *in vitro* fertilisation

Frozen semen, pooled from five bulls, was donated by Alliance Boviteq (L'Alliance Boviteq, St-Hyacinthe, Quebec) and GENCOR (Guelph, Ontario). Straws of semen were thawed in a water bath (35 °C) for 30 s and processed by swim-up as described by Parrish *et al.* (1986). The sperm were then washed twice with modified Tyrode's albumin lactate pyruvate medium (Sp-TALP) used for sperm culture containing 6 mg/ml fatty acid-free bovine serum albumin (BSA; Sigma), 10 mM pyruvic acid and 50 µg/ml gentamicin.

After 24 h of maturation, the COCs were washed twice with fertilisation medium (modified Tyrode's medium: mTALP), and then five COCs were transferred into 50 µl drops of the fertilisation medium containing 2 µg/ml heparin (Sigma) under mineral oil at 38.5 °C in 5% CO_2 in air with high humidity (Parrish *et al.*, 1988). A final sperm concentration of 1×10^6 sperm/ml was used for insemination. To examine the time course of formation of male and female pronuclei, the oocytes were fixed at 1 h intervals from 3 to 18 h following insemination.

Fixation of oocytes

At the end of each culture period, the oocytes were fixed as described previously (Chian & Sirard, 1996). Briefly, the oocytes were mounted on slides with coverslips and then fixed with acetic acid/ethanol (1:3) solution for at least 24 h. Following fixation, the oocytes were stained with 1% orcein in 45% acetic acid and examined for evidence of oocyte GVBD and sperm nuclear change under a phase-contrast microscope.

Radiolabelling of oocytes

To determine the pattern of protein phosphorylation during maturation and fertilisation, the oocytes were transferred into 50 µl of mTALP+polyvinylpyrrolidone (PVP-40; Sigma) containing 400 µCi/ml [^{32}P]orthophosphate (Amersham, Arlington Heights, IL, USA) at 0, 3, 6 and 9 h following culture and at 3, 6, 9, 12 and 15 h following insemination labelled for 3 h, respectively. The oocytes were then washed twice with TLH containing 0.3% PVP and lysed with 60 µl sodium dodecyl sulfate (SDS) buffer. The samples were precipitated with acetone (99%), dried, and resuspended in 20 µl SDS-buffer, and then frozen at –80 °C until further use.

Electrophoresis and autoradiography

One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 5% stacking gel and a 12% separating gel according to the method of Laemmli (1970). For the two-dimensional gels, the first-dimension gel was performed in acryl gel with Nonidet P40 and urea. The second dimension was performed on 12% SDS-PAGE. Twenty-five oocytes were put into each well for electrophoresis. All gels were soaked for 30 min in fixation solution of 5% glycerol/25% isopropanol/10% acetic acid, and then dried under vacuum at 80 °C. Autoradiography was carried out at –80 °C with intensifying screens.

Results

A time course of oocyte GVBD is shown in Fig. 1. All oocytes just after aspiration from follicles were at the germinal vesicle (GV) stage. A clear nucleolus was observed within the GV in some oocytes (Fig. 2*a*; 12% = 8/65). However, the nucleolus disappeared after 1 h of culture (Fig. 2*b*), and dispersed chromosomes gradually began to gather with further culture (Fig. 2*c*, *d*). GVBD was first observed 3 h after maturation in culture (Fig. 2*e*) and condensation of chromosomes (condensed GV) proceeded with further culture (Fig. 2*f*). All oocytes underwent GVBD after 10 h in culture.

A time course of the development of male and female pronuclei following insemination is shown in Fig. 3. The first evidence of formation of male and female pronuclei was observed 9 h after insemination and observed concurrently from 10 to 18 h post-insemination (Fig. 4).

The changes in protein phosphorylation in the oocytes occurred following maturation (Fig. 5*a*) and fertilisation (Fig. 5*b*). There were low levels of protein phosphorylation in the oocytes at the beginning of maturation (Fig. 5*a*, lane 1). However, overall protein phosphorylation increased following 3–6 h of culture (Fig. 5*a*, lane 2), and then the levels of protein phosphorylation at approximately 40 kDa, 27 kDa, 23 kDa and 18 kDa decreased gradually from 3 to 12 h following oocyte maturation in culture (Fig. 5*a*, lanes 2–4). Following fertilisation, the levels of phosphorylated protein at approximately 40 kDa, 27 kDa, 23 kDa and 18 kDa increased gradually from 6 to 18 h post-insemination (Fig. 5*b*, lanes 2–5).

Fig. 6 shows a two-dimensional profile of protein phosphorylation in the oocytes during GVBD. Referring to Fig. 5*a*, the points of phosphorylated protein at approximately 27 kDa and 18 kDa decreased following

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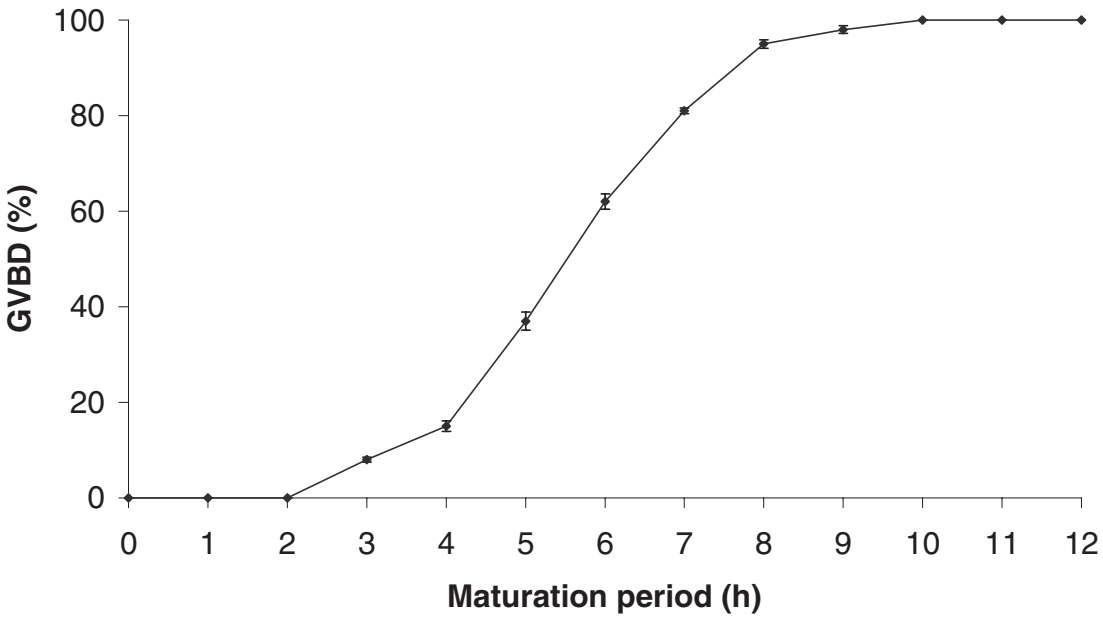


Figure 1 Time course of germinal vesicle breakdown (GVBD) of bovine oocytes following culture *in vitro* (total of 788 oocytes; three replicates).

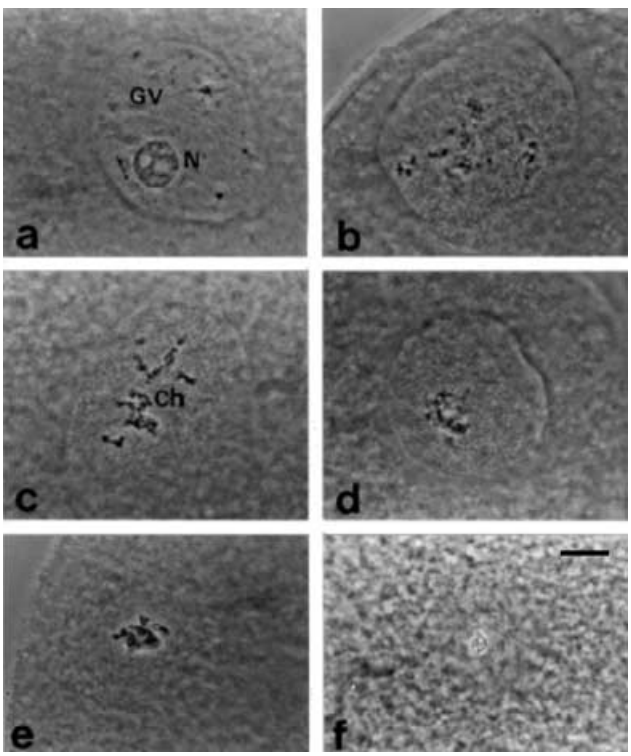


Figure 2 The process of germinal vesicle breakdown (GVBD) of bovine oocytes cultured *in vitro*. (*a*) Germinal vesicle (GV) in an oocyte after aspiration from its follicle. One clear nucleolus (N) is visible. (*b*) GV in an oocyte 1 h after culture; the nucleolus has already disappeared. (*c*) GV in an oocyte 2 h after culture; the chromosomes (Ch) are beginning to gather. (*d*) GV in an oocyte 3 h after culture; chromosome condensation proceeds. (*e*) An oocyte 3 h after culture; GVBD has occurred. (*f*) An oocyte 6 h after culture; the chromosomes are condensed. Scale bar represents 5 μm.

oocyte GVBD (Fig. 6*a–d*). As shown in Fig. 7, the point of protein phosphorylation at approximately 18 kDa increased following fertilisation. In contrast to Fig. 6, the point of protein phosphorylation at approximately 27 kDa is missing following fertilisation. However, the point of phosphoproteins at approximately 15 kDa is absent during oocyte maturation in Fig. 6. Taking Figs. 5–7 together, there is indication that the point of protein phosphorylation at approximately 18 kDa appears reversibly changed following oocyte maturation and fertilisation.

Discussion

The present study demonstrates that reversible changes in phosphoproteins occur during maturation and pronuclear formation in bovine oocytes. The morphological changes of GVBD in bovine oocytes observed in the present study are similar to those in previous reports (Motlik *et al.*, 1978; Hyttel *et al.*, 1986; De Loos *et al.*, 1989). Although it has been reported that a visible nucleolus is not typically observed at the GV stage of bovine oocytes (Motlik *et al.*, 1978), the results of the present study indicate that 12% of oocytes had clearly observed nucleoli within the GV after aspiration from the follicles (Fig. 2). It seems that the disappearance of nucleoli and the gathering of chromosomes are typical phenomena before GVBD, which were observed in most oocytes following 3 h of culture.

Protein phosphorylation plays a major role in the regulation of cell growth and division (Oelgeschläger,

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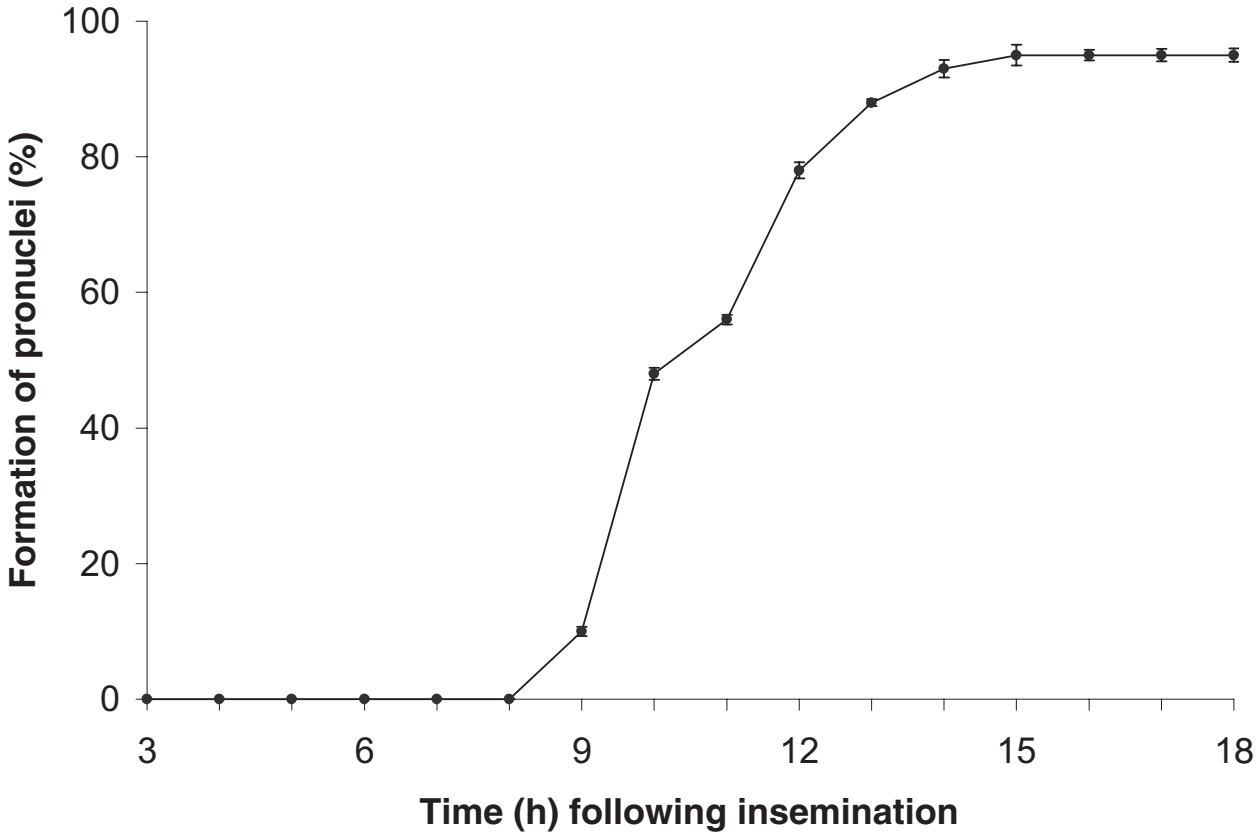


Figure 3 Time course of male and female pronucleus formation following *in vitro* fertilisation with frozen-thawed sperm (total of 724 oocytes; three replicates). Only oocytes penetrated by a single sperm were recorded.

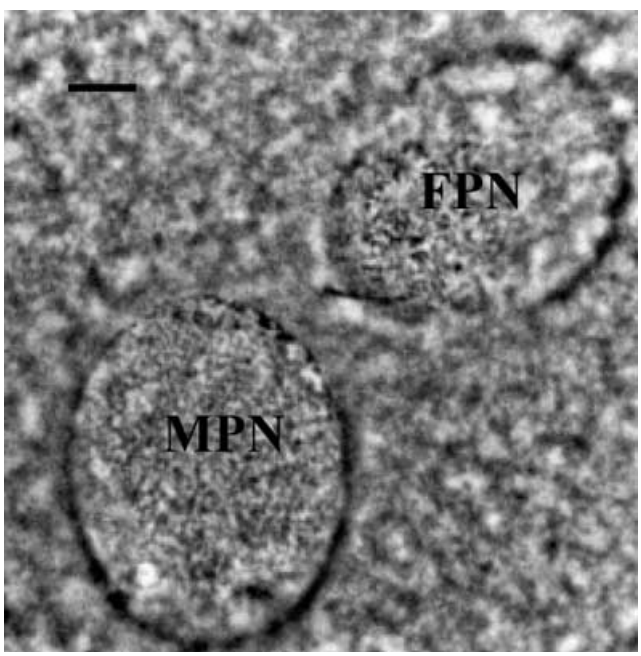


Figure 4 An oocyte fertilised 18 h after insemination showing male and female pronuclei (MPN, FPN) in the cytoplasm. Scale bar represents 5 μm.

2002). Results from studies with amphibian eggs indicate that protein phosphorylation plays an important role in controlling nuclear envelope assembly/breakdown and chromosome condensation/decondensation in cell-free extracts (Lohka & Maller, 1987). It has been reported that protein phosphorylation is increased at the time of GVBD in sheep oocytes (Crosby *et al*., 1984). Although it has been described that the specific changes in protein phosphorylation are programmed during bovine oocyte maturation, the exact function of these phosphoproteins during oocyte maturation is still largely unknown (Kastrop *et al*., 1990). The first GVBD was observed 3 h following incubation and it concurrently occurred from 3 to 10 h post-incubation (Fig. 1). Although less phosphorylation of proteins occurred during the oocytes in the first 3 h of culture, the levels of protein phosphorylation increased considerably 3 h after culture (Fig. 5*a*). The predominant increase in levels of protein phosphorylation at approximately 40 kDa, 27 kDa, 23 kDa and 18 kDa occurred from 3 to 6 h following maturation in culture. Apparently, the phosphorylation of these protein complexes accompanies oocyte GVBD.

During oocyte maturation, MPF directly regulates oocyte progression from the diplotene stage to

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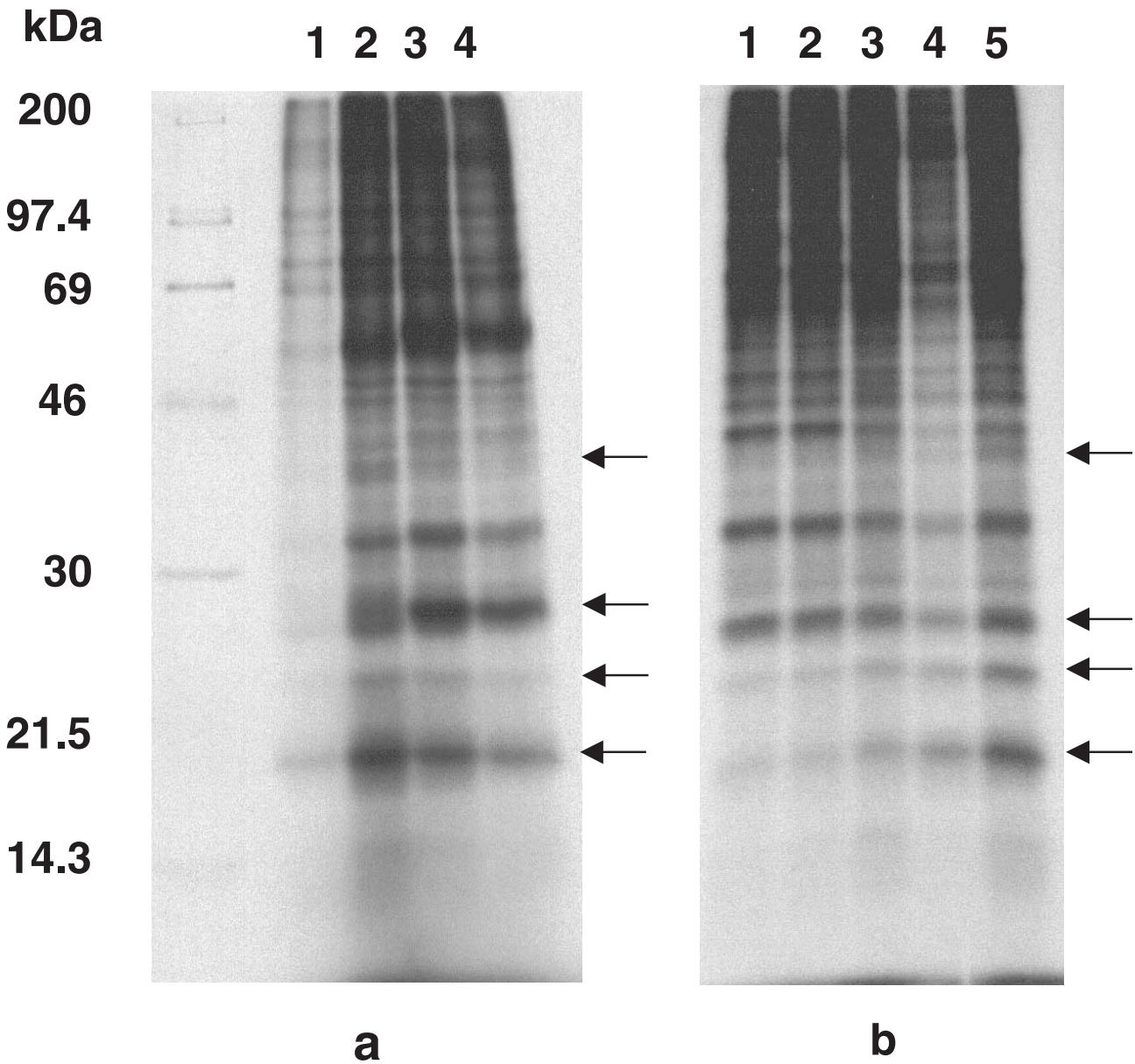
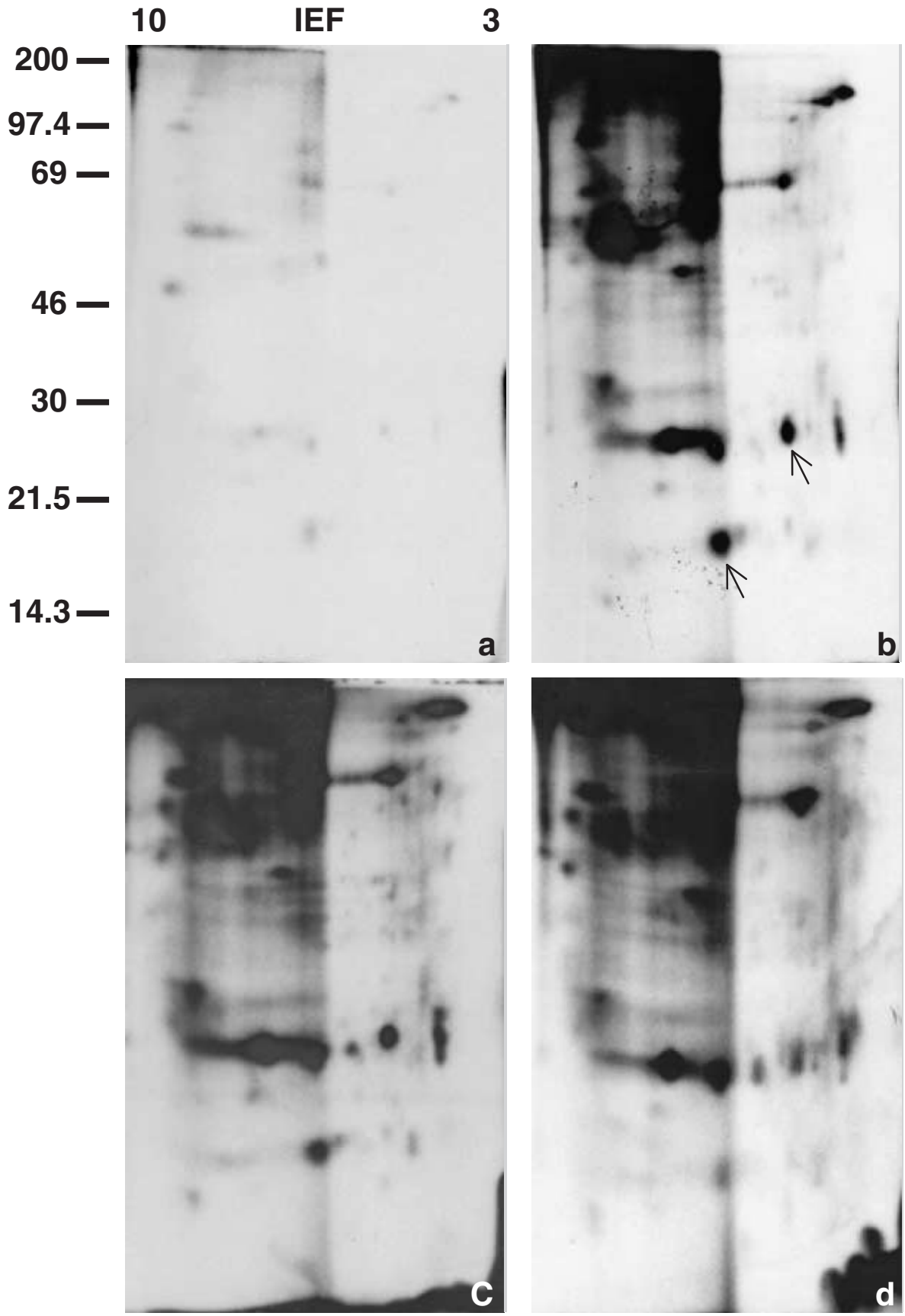


Figure 5 One-dimensional SDS–12% polyacrylamide gel electrophoresis of [^{32}P]orthophosphate-labelled proteins. Molecular weight (range 14 300–200 000) is the migration of [^{14}C]methylated proteins. (*a*) Incorporation of [^{32}P]orthophosphate into phosphoproteins from 0 to 12 hours post-culture (hpc) for oocyte maturation. Lane 1, 0–3 hpc; lane 2, 3–6 hpc; lane 3, 6–9 hpc; lane 4, 9–12 hpc. (*b*) Protein phosphorylation of oocytes after fertilisation was labelled at 3 h intervals from 3 to 18 hours post-insemination (hpi). Lane 1, 3–6 hpi; lane 2, 6–9 hpi; lane 3, 9–12 hpi; lane 4, 12–15 hpi; lane 5, 15–18 hpi. Arrows point to the changes described in the text. Each lane represents 25 oocytes. The experiment was performed twice with similar results.

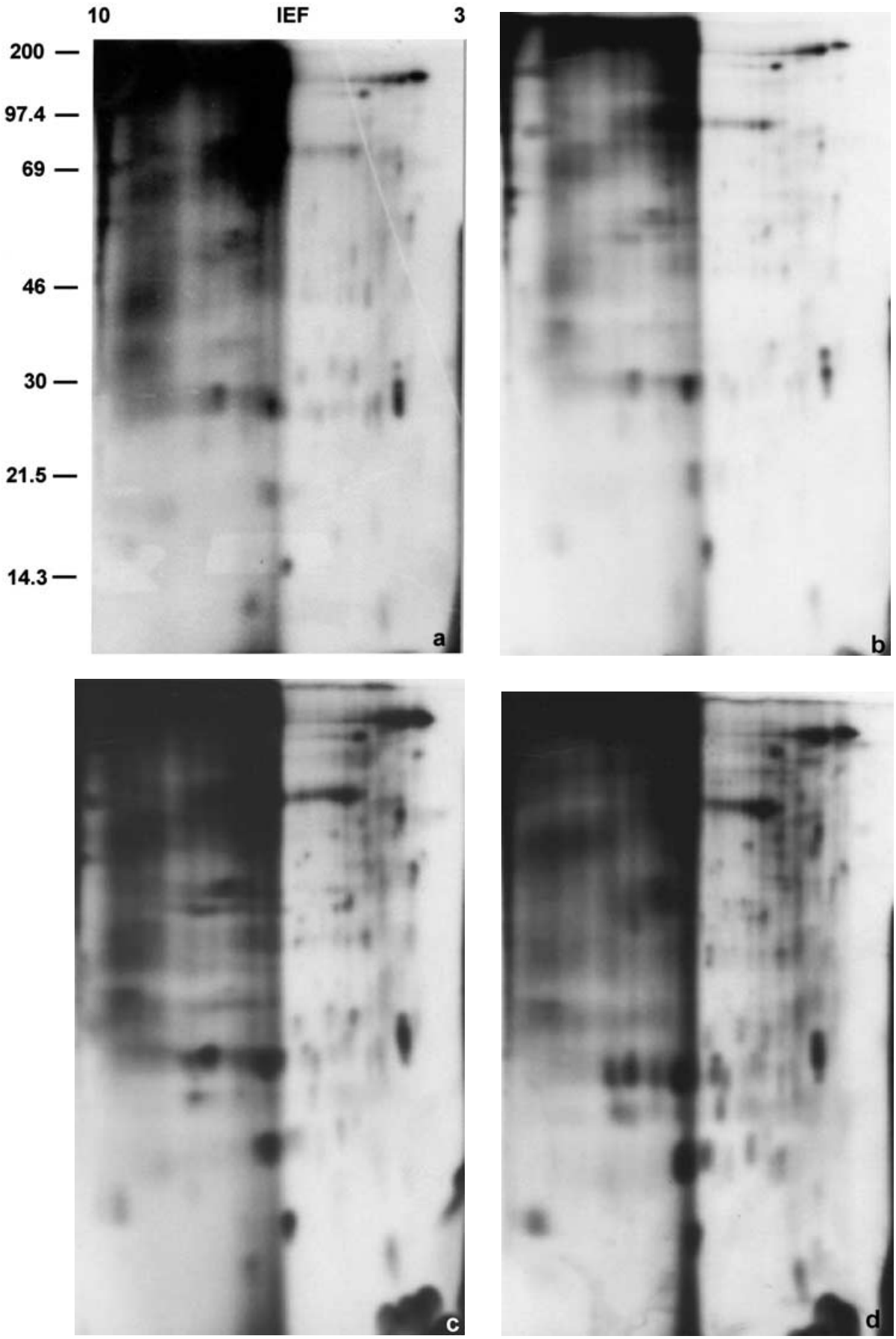
metaphase II. When MPF activity is first detected, an increase in protein phosphorylation occurs (Maller *et al*., 1977; Belle *et al*., 1978; 1979; Boyer *et al*., 1986). A tremendous increase in phosphoproteins occurs shortly before GVBD and in association with the activity of MPF in *Xenopus* oocytes, indicating that MPF is involved in oocyte GVBD (Maller & Smith, 1985). Preparations of 3000-fold purified MPF contain two major components – a 34 kDa protein and a 45 kDa protein – and exhibit considerable kinase activity *in vitro*

Figure 6 Two-dimensional gel electrophoresis of [^{32}P]orthophosphate-labelled proteins. Molecular weight (range 14 300–200 000) is the migration of [^{14}C]methylated proteins. The phosphorylated proteins of the oocytes were first separated on IEF acryl gel with an ampholine range of 3–10, and secondly separated on 12% SDS-PAGE. The proteins phosphorylated in the oocytes were labeled at 3 h intervals from 0 h to 12 h following culture *in vitro*: (*a*) 0–6 hours post-culture (hpc); (*b*) 3–6 hpc; (*c*) 6–9 hpc; (*d*) 9–12 hpc. The arrows indicate the changes described in the text. Each lane represents 25 oocytes. The experiment was performed twice with similar results.

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Figure 7 Two-dimensional gel electrophoresis of [^{32}P]orthophosphate-labelled proteins. Molecular weight (range 14 300–200 000) is migration of [^{14}C]methylated proteins. The phosphorylated proteins of the oocytes were first separated on IEF acryl gel with an ampholine range of 3–10, and secondly separated on 12% SDS-PAGE. The proteins phosphorylated in the oocytes were labeled at 3 h intervals from 3 to 18 h following insemination *in vitro*: (*a*) 3–6 hours post-insemination (hpi); (*b*) 6–9 hpi; (*c*) 9–12 hpi; (*d*) 12–15 hpi; (*e*) 15–18 hpi. The arrows indicate the changes described in the text. Each lane represents 25 oocytes. The experiment was performed twice with similar results.

(Lohka *et al*., 1988). Activation of MPF requires key side phosphorylation and dephosphorylation. It is known that the product of the c-*mos* proto-oncogene, a protein serine/threonine kinase known as Mos (Sagata *et al*., 1989; Watanabe *et al*., 1989), and MAP kinase (Nebreda & Hunter, 1993) directly or indirectly modify MPF activity with the status of phosphorylation or dephosphorylation. Therefore, the phosphorylated proteins at approximately 40 kDa, 27 kDa, 23 kDa and 18 kDa in the oocytes observed in the present study seem not to be MPF itself. Thus these phosphoproteins may play a role in the activation of MPF by putative substrates. These phosphoproteins could be unknown protein kinases or phosphatases, because there are a large number of protein kinase genes and protein phosphatase genes in oocytes. These phosphoproteins may play an important role in the meiotic cell cycle from G2 to M-phase. The role of the phosphorylation cascade of these proteins in the meiotic cell cycle needs to be further verified.

The sperm nucleus is transformed into the male pronucleus through the interaction of oocyte cytoplasmic components. At the time of pronucleus formation in mouse (Howlett & Bolton, 1985; Howlett, 1986), porcine (Ding *et al*., 1992) and bovine (Chian *et al*., 1999*a*) oocytes, protein phosphorylation occurs. The first evidence of formation of male and female pronuclei was observed 9 h following insemination and observed concurrently from 10 to 18 h of post-insemination (Fig. 3). This finding is similar to our previous report (Chian *et al*., 1999*b*). Our previous results also indicate that the levels of protein phosphorylation at approximately 40 kDa, 23 kDa and 18 kDa in bovine oocytes increase gradually following fertilisation and parthenogenetic activation and that there is a difference in the level of protein phosphorylation at 18 kDa between fertilised and activated oocytes, suggesting that this phosphoprotein may be related to the formation of the male pronucleus in bovine oocytes (Chian *et al*., 1999*b*). Taken together the phosphoproteins results following oocyte maturation and fertilisation indicate that the point of protein phosphorylation at approximately 27 kDa may be mainly related to oocyte GVBD. Interestingly, the point of protein phosphorylation at approximately 18 kDa is reversibly changed during GVBD and following formation of pronuclei in bovine oocytes (Figs. 6, 7). This indicates that the reversible protein phosphorylation at approximately 18 kDa in bovine oocytes may play an important role in the meiotic cell cycle from G2 to M-phase or pronucleus formation during oocyte maturation and fertilisation.

In conclusion, the results of the present study indicate that reversible changes in phosphoproteins occur during oocyte maturation and fertilisation, suggesting that this reversible change in phosphoproteins may play a key role in either the cell cycle transition or pronucleus formation during maturation and fertilisation in bovine oocytes.

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