Mitogen-activated protein kinase during oocyte growth in the mouse.

by

Wafa Harrouk

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Department of Pathology
McGill University
Montreal, Quebec

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Harrouk, Wafa
Department of Pathology

M.Sc.

Mitogen - activated protein kinase during oocyte growth in the mouse

Abstract

Oogenesis is the developmental process that produces healthy and fertilizable ova. One crucial aspect of oogenesis is the acquisition by an oocyte of the capacity to execute the meiotic divisions which generate the haploid germ cell. Oocytes that can execute the meiotic divisions which occur during the process of meiotic maturation are said to be meiotically competent. Although the molecular aspects of meiotic competence are not known, there is evidence suggesting that the microtubular network may be involved. At metaphase, the microtubular network is responsible for assembling the spindle, an apparatus on which the chromosomes become aligned to be separated during meiotic divisions.

Previous reports have identified two species of mitogen-activated protein (MAP) kinase in fully grown, meiotically competent mouse oocytes. During meiotic maturation, MAP kinase becomes phosphorylated, activated as a kinase, and associated with the microtubule-organizing centers at the poles of the meiotic spindles.

In this study, the role of MAP kinase in the acquisition of meiotic competence in growing oocytes was investigated. The results presented in this thesis show that two species of MAP kinase, p42 and p44, are present in their unphosphorylated forms in oocytes as early as 5 days of age. At this age, oocytes are small and have not acquired the capacity to resume meiosis. They are referred to as meiotically incompetent. MAP kinase continues to be present throughout the growth phase and up to the acquisition of meiotic competence.

In growing mouse oocytes, a group of partially competent oocytes are abundant. Such oocytes arrest at metaphase I where they assemble a morphologically normal spindle. Immunoblotting results of partially competent

oocytes show that MAP kinase is present and becomes phosphorylated following culture as is indicated by the retarded mobility on the SDS gels.

Okadaic acid, an inhibitor of protein phosphatases 1 and 2A, induces incompetent oocytes to enter metaphase. These oocytes contain the slow migrating phosphorylated forms of p42 and p44, indicating that okadaic acid causes the phosphorylation of MAP kinase. A time course study shows that the okadaic acid-induced phosphorylation of MAP kinase occurs coincidentally with entry into metaphase in incompetent oocytes. In fully competent oocytes, this phosphorylation occurs after entry into metaphase. In addition, these oocytes do not assemble a spindle, indicating that phosphorylation of MAP kinase, although it may be necessary, is not a sufficient event to induce spindle formation.

The presence of MAP kinase in bovine oocytes is also investigated. Immunoblotting of bovine oocytes reveals the presence of three species of MAP kinase. In the immature oocyte, all three species are present in the unphosphorylated forms. Upon maturation, a partial phosphorylation of two species corresponding to p42 and p44 is observed. This phosphorylation seems to be complete by the end of meiotic maturation.

Harrouk, Wafa
Departement de Pathologie

M. Sc.

Protéine-activée par mitogéne kinase durant la croissance des ovocytes chez la souris

Abrégé

L'ovogenèse est le processus developmental qui a pour but de produire des ovules sains et fécondables. Parmi les aspects de l'ovogenèse est l'acquisition par l'ovule de la capacité de subir les divisions méiotiques qui vont rapporter l'état haploïde des cellules sexuelles. Les ovules qui sont capable d'accomplir des divisions méiotiques durant le processus appelé maturation méiotique, sont désignés d'être méiotiquement compétents. Malgré que les aspects moléculaires de la competence méiotique sont mal compris, il existe des preuves qui suggèrent la possibilité que le réseau microtubulaire pourrait y être impliqué. À metaphase, le réseau microtubulaire est responsable d'assembler le fuseau métaphasique, un appareil sur lequel les chromosomes seront alignés afin d'être séparés durant la division méiotique.

Fréalablement, des résultats ont identifié deux espèces de la protéine-activée par mitogéne (PAM) kinase dans les ovules à grandeur complet qui sont compétent méiotiquement chez la souris. Pendant la maturation, PAM kinase devient phosphorylé, activé tant que kinase, et s'associe avec les centres d'organization des microtubules au niveau des pôles des fuseaux méiotiques.

L'étude présentée dans la thèse a pour but de rechercher le rôle du PAM kinase dans l'acquisition de la compétence méiotique dans les ovocytes qui sont en train de croître et qui ne sont pas atteint leurs grandeurs maximals. Les résultats présentés là-dedans démontrent que le PAM kinase est présent sous sa forme nonphosphorylée dans les ovocytes des souris âgées d'aussi peu que 5 jours. À cet âge, les ovocytes sont petits et n'ont pas encore acquis la capacité d'amorcer la méiose. PAM kinase reste présent pendant la période de croissance, voire jusqu'à l'acquisition de la compétence méiotique. Parmi les ovocytes qui ont commencé à croître se trouve un groupe qui ne sera jamais

capable de compléter la maturation méiotique. PAM kinase se trouve sous la forme nonphosphorylée chez tels ovocytes. De plus, ces ovocytes qui arrêtent à metaphase I sont capable d'assembler des fuseaux qui ont l'air d'être morphologiquement normals.

L'acide okadaique, un inhibiteur des protéines phosphatases, induit les ovocytes incompétents à entrer en métaphase. De plus, ces ovocytes manifestent les formes plus lentes du p42 et p44 qui sont phosphorylées, ce qui indique que l'acide okadaique cause la phosphorylation du PAM kinase. L'induction de la maturation par l'acide okadaique ést précoce et coincide avec la phosphorylation de la PAM kinase contrairement aux ovocytes compétents òu la phosphorylation se produit suivant la maturation. Ces ovocytes n'assemblent pas des fuseaux métaphasiques, ce qui indique que même si la phosphorylation du PAM kinase n'est pas suffisant à induire la formation du fuseau métaphasique. Néanmoins, il reste à determiner s'il pourrait y être nécessaire.

Les ovocytes du bovin ont étè éxaminé pour la présence du PAM kinase. On a trouvé que trois espéces de cette protéine sont présentes dans les ovocytes prématurés sous sa forme nonphosphorylée. Une fois à métaphase, la forme nonphosphorylée des deux espéces, correspondant à p42 et p44, deviennent phosphorylées partiellement. Vers la fin de la maturation méiotique, seule la forme phosophorylée du PAM kinase se trouve dans les ovules du bovin.

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To the memory of my father .....

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Preface

Format of the Thesis

This thesis comprises a paper, which is included almost entirely in the form in which it was submitted for publication. The material that is present exclusively in the thesis consists of the detailed description of isolating oocytes from antral follicles, the light microscopy as well as the immunofluorescent analysis of mouse oocytes at different stages of meiotic maturation and Table 2 which describes the presence of partially competent oocytes. Results obtained using the bovine oocytes are only described in the thesis. The bovine samples were supplied by Dr. Lawrence Smith at CRRA, St. Hyacinthe, Québec.

The introduction, Chapter I, includes a general review of the relevant literature and the rationale behind investigating the presence of MAP kinase in small and growing mouse oocytes.

Chapter II has been submitted to *Developmental Biology*. The research described in this chapter was carried out under the supervision of Dr. H. Clarke of the Department of Obstetrics and Gynecology at McGill University. For this reason, Dr. Clarke's name appears as a coauthor for this paper. All experiments were performed by the candidate.

Chapter III describes a general discussion of the results obtained in Chapter II.

Chapter I
INTRODUCTION

1.1. From a primordial germ cell to a mature egg: An overview

At the time of birth the mammalian ovary contains the total number of oocytes that the female will have throughout her reproductive life. Prior to and during its residence in the ovary, an oocyte will undergo three major events: genesis, growth, and maturatior. Only if these events are executed properly will the development of the embryo be successful.

In mammalian species, the germ cells begin their development very early during embryogenesis. In the mouse embryo, the primordial germ cells (PGCs) are first seen between 7.5-8.5 day *post coitum* (p.c.) in the region of the allantois. Two days later, the PGCs start migrating towards the genital ridge of the presumptive gonads to colonize this area and to establish their coexistence with the resident somatic cells. For a few days after arrival at the gonadal ridge, the PGCs of females continue to proliferate until about day 13.5 p.c. where they undergo a last round of mitotic division before differentiating in a synchronous manner into meiotic cells, the primary oogonia. In this thesis, the term oogenesis will be used according to Wilson's definition (Wilson, 1925) where oogenesis extends beyond the mere formation of oogonia to include their migration to the gonadal ridge, growth, differentiation and the eventual formation of a mature fertilizable gamete. Once meiotic, primary oogonia undergo DNA synthesis to replicate their chromatin material and enter prophase where they undergo a number of changes at the chromosomal level. In about

two weeks, the meiocyte chromatin undergoes leptotene, zygotene, pachytene, and then arrests at the diplotene stage of the first meiotic prophase for a long period ranging from days to years depending on the length of the reproductive life span of the animal. This step usually referred to as the resting phase represents a block in the meiotic cell cycle of the oocyte (review: Albertini, 1992). Prior to and during their meiotic arrest, oogonia are active in RNA and protein synthesis which will be supportive of oocyte growth and metabolism later on during oogenesis.

Mouse oocytes reach the diplotene stage by day 5 *post partum* (p.p.) when the ovary is populated with thousands of small oocytes of 12-20 μm in diameter. Around the same time, primordial oocytes become surrounded by a few follicular (somatic) cells, which will form the follicular epithelium. For most oocytes and follicles, development is arrested at this stage. When oocytes from this category are released from their preantral follicles, they are incapable of resuming meiosis spontaneously, and are said to be meiotically incompetent (Szybek, 1972). In the mouse, around day 3 p.p., a group of follicle-enclosed oocytes, now termed primary follicles, are selected to start growing in size leaving behind the large pool of primordial follicles and their enclosed small oocytes at the resting stage (Pederson and Peters, 1968; Schultz and Wassarman, 1977). Within a period of two weeks, the primary oocyte increases in diameter from 15 μm as a primordial oocyte to 85 μm as a fully-grown mouse oocyte. This increase in diameter is proportional to that of the

oocyte volume, which represents a 300-fold increase, resulting in the formation of one of the largest cells in the body (Figure 1). During the growth period, the oocyte becomes engaged in an active biosynthesis process where it accumulates all the necessary components including proteins, messenger RNA, and ribosomes that will be needed throughout its growth, maturation, and until the embryonic genome is turned on at the 2-cell stage of embryogenesis (Review: Schultz, 1986).

Once fully grown, a prophase-arrested oocyte can reinitiate meiosis either in response to gonadotropin (Gates, 1971; Baker, 1972), or upon isolation from its surrounding follicular environment (Pincus and Enzmann, 1935; Edwards, 1965; Donahue, 1968; Szybek, 1972; Schultz and Wassarman, 1977). Thus, the oocyte will overcome another meiotic block under the appropriate conditions (Albertini, 1992).

In vivo, the increase in the level of follicle stimulating hormone (FSH) stimulates the follicle to undergo a number of changes (Baker, 1972). The granulosa cells start dividing mitotically, undergo mucification, and increase the synthesis and secretion of a high molecular weight proteoglycan hyaluronic acid (Yanagishita *et al.*, 1979) and tissue plasminogen activator (Gilula *et al*, 1978). The follicle starts accumulating fluid which forms the antrum in the preovulatory or Graafian follicles. Subsequently, the surge of plasma level of luteinizing hormone (LH) causes the ovulation of the oocyte. Upon the LH surge, the follicle undergoes what is known as the cumulus cell expansion

which leads to the eventual loss of the physical contact between the oocyte and its associated cumulus cells. The oocyte, in turn, undergoes a series of events on both the cytoplasmic and nuclear levels known collectively as meiotic maturation, thus overcoming a yet another block of meiotic arrest (Albertini, 1992).

During meiotic maturation, the nucleus or germinal vesicle (GV) breaks down and the nuclear envelope dissolves in the cytoplasm. The diffuse interphasic chromatin becomes condensed into metaphase chromosomes and assembles on an apparatus formed of microtubules known as the meiotic spindle. At this point, the oocyte reaches metaphase I of the first meiotic division. The spindle then moves to the periphery of the cytoplasm and extrudes one set of homologous chromosomes within the first polar body. The oocyte then arrests, and for the last time, at metaphase II in preparation for fertilization. It is at this stage that the oocyte is said to have completed meiotic maturation. The egg is then ovulated and passes into the oviduct where it either becomes fertilized or eventually degenerates. Upon fertilization, meiosis is resumed once more in the egg where the second meiotic division separates sister chromatids, and one set of chromosomes is expulsed from the cytoplasm in the second polar body. Finally, the haploid maternal and paternal pronuclei undergo DNA replication and, at mitosis, assemble on a single spindle which represents the embryonic genome from this point onwards (Figure 2).

In the absence of hormonal stimulation, fully grown mammalian oocytes undergo similar changes when they are mechanically isolated from their surrounding follicles. The initial observation describing the ability of oocytes to "spontaneously" resume meiosis has opened a wide field of research where a wealth of morphological changes have been well documented (Pincus and Enzmann, 1935). The capacity of an oocyte to undergo the meiotic maturation events is referred to hereafter as meiotic competence (Figure 3).

Among the oocytes that undergo maturation, a specific group can undergo GVBD but arrests at metaphase I failing to reach metaphase II (Szybek, 1972; Baker, 1972; Wickramasinghe *et al.*, 1991), referred to in the text as partially-competent oocytes. Based on this observation, it has been suggested that meiotic competence is acquired in a two-step process (Sorensen and Wassarman, 1976). The first step is achieved when GVBD takes place, followed by chromosome condensation and the assembly of the meiotic spindle. The second maturational step is involved with the progression from metaphase I to metaphase II when the first polar body is emitted.

1.2. The mouse oocyte as a model to study meiotic maturation

In this project, the mouse oocyte was used as a model to study specific aspects of oogenesis due to several experimental advantages. In the mouse ovary, a large group of oocytes initiates growth synchronously near the time

of birth. As a result, it is possible to trace the progression of development by looking at cohort oocytes from successive days following birth. As the oocytes grow, their mean diameter increases. Thus, populations of oocytes with increasing diameters can be obtained by isolation from juvenile mice of increasing ages. Another advantage of using the mammalian oocyte is the fact that oocyte growth occurs in the absence of cell division, a peculiar phenomenon that may be exploited to study aspects of growth.

Growing oocytes reach their full size around day 21 of age (Schultz and Wassarman, 1977), when they can be ovulated in response to gonadotropin (Gates, 1971). When prophase-arrested oocytes are isolated from their antral follicles in the absence of gonadotropin, they are capable of resuming meiosis as described above. Thus, physiologically occuring events could be mimicked under *in vitro* conditions where cohort oocytes can grow (Eppig, 1977) and resume meiosis in the absence of gonadotropin surge simply by releasing them from their surrounding follicles into the appropriate culture medium (Edwards, 1968; Szybek, 1972). Thus, oocytes can be analyzed at specific stages of the cell cycle and during progression from one stage to the next.

An important property of this model is the fact that mouse oocytes are transparent, so the oocyte can be easily classified as either a GV-stage, a GVBD-stage, or a metpahase II-stage under the light microscopy. This transparency also allows for immunofluorescent analysis of the whole oocyte where specific structures can be examined such as chromosomes and

microtubules.

Mouse oocytes, therefore, represent an ideal system to study the changes that take place during the growth and the acquisition of meiotic competence in mammalian species.

1.3. Oocyte growth during oogenesis

During its growth phase, the mammalian oocyte undergoes a number of well-documented changes all of which are dependent on the communication between the oocyte and its surrounding granulosa cells. The first wave of oocyte growth occurs following birth of the female where a large group of primary follicles becomes recruited (Pederson and Peters, 1968; Krarup *et al.*, 1969; Schultz and Wassarman, 1977). The selection to start growth is believed to be regulated within the ovary by some yet unknown mechanism (Peters *et al.*, 1973).

1.3.1. Size and growth

Prior to their selection to commence growth, resting primary oogonia are small in size ranging between 12-20 μm in diameter. In the mouse, within a period of two weeks, the oocyte reaches its full size of 80-85 μm in diameter. The first wave of oocytes which embark on the growth phase shortly after birth reach their full size around day 21 of age (Pedersen and Peters, 1968; Schultz,

1986).

1.3.2. Differentiation and growth

During oocyte growth, specific changes occur at the ultrastructural level in both nuclear and cytoplasmic components. In the cytoplasm, complex organelle reorganization occurs during this period. Mitochondria, Golgi complexes, and rough endoplasmic reticulum increase numerically and become dispersed throughout the oocyte (Szollosi, 1972). Moreover, centrosomal structures known as microtubule organizing centers (MTOCs) which are located near the cortex in small oocytes migrate to the perinuclear region in fully grown oocytes. Along with the changes in the centrosomes, some changes occur at the level of the microtubular network. Short microtubules present in the cytoplasm of a small oocyte are replaced by long and diffuse microtubules in the fully grown oocyte (Albertini, 1992; Wickramasinghe and Albertini, 1992). Concomitant with the cytoplasmic differentiation and as the oocyte nears its full size, nuclear and nucleolar reorganization take place where the diffuse chromatin becomes condensed around the nucleolus (Mattson and Albertini, 1990).

1.3.3. Protein synthesis during growth

To ensure that sufficient amounts of molecules are present at specific stages of oogenesis, growing oocytes rapidly synthesize and store

transcriptional and translational products that will be needed during subsequent development. In particular, a growing oocyte increases its protein synthesis rate to about 40 times that of a nongrowing counterpart (Schultz, 1986). Newly synthesized proteins such as glucose-6-phosphatase, lactate dehydrogenase (Mangia and Epstein, 1975; Schultz *et al.*, 1979), and *zona pellucida* proteins, *ZP*1, *ZP*2, and *ZP*3 (Bleil and Wassarman, 1980) accumulate throughout most of the growth period (Schultz *et al.*, 1979; Canipari *et al.*, 1979).

1.3.4. Role of the follicle during growth

During the growth phase, the oocyte and its surrounding follicle grow coordinately through a well defined series of morphological stages (Pedersen and Peters, 1968). Follicle cells are thought to influence the growth of the oocyte by assuming a nutritional and informational role throughout the growth phase (Erickson, 1986).

In the small meiotically-incompetent oocyte, only a few follicular cells are attached to the oocyte. However, when the oocyte is selected to initiate the growth phase, the granulosa cells that surround the mammalian oocyte, also known as the cumulus oophorus, become an essential partner for the growth, development, and the eventual ovulation of the mature egg. First, the innermost layer of cumulus cells, the corona radiata, sends cytoplasmic processes through the intervening *zona pellucida* to contact the oolemma

(Zamboni, 1974). At some points of contact between these processes and the oocyte, gap junctions can occur through which the granulosa cells nourish the growing oocyte (Anderson and Albertini, 1976; Moor *et al.*, 1980). Among the factors that enter the oocyte through follicle cells are compounds such as energy sources, cyclic adenosine monophosphate (cAMP), and precursors for protein and phospholipid biosynthesis. Arrival of these factors to the oocyte depends on the maintenance of the gap junctions between the oocyte and the adjacent granulosa cells (Eppig, 1977). Upon disruption of this communication, oocyte growth is terminated (Canipari *et al.*, 1984).

1.3.5. Acquisition of meiotic competence during growth

Concomitant with reaching its full size, an important facet of oogenesis is manifested by the oocyte, namely the acquisition of meiotic competence. The ability of follicle-free oocytes to resume meiosis *in vitro* has permitted investigation of whether oocytes at different stages of growth can enter metaphase. It has been demonstrated that oocytes smaller than 60μm remain arrested at prophase I of meiosis when placed in culture. Slightly larger oocytes can enter metaphase but a significant number arrests prior to the completion of the first meiotic division. Oocytes larger than 80μm are able to complete maturation to metaphase II (Sorensen and Wassarman, 1976; Wickramasinghe *et al.*, 1991). The three groups of oocytes are meiotically classified as incompetent, partially competent, and fully competent,

respectively (Sorensen and Wassarman, 1976; Wickramasinghe *et al.*, 1991). The same terms will be used throughout this thesis. Oocytes isolated from mice younger than 14 days of age are incapable of resuming meiosis, and thus are termed meiotically incompetent. Mice between 14-16 days of age are enriched for the partially competent oocytes, as the majority of them arrest at metaphase I of meiosis. Mice older than 17 days of age contain oocytes which are almost fully grown and are fully competent (Szybek, 1972; Sorensen and Wassarman, 1976; Eppig, 1977; Bachvarova *et al.*, 1980). These results indicate that meiotic competence is acquired progressively among the growing population of oocytes.

1.3.6. c-*kit*

On the molecular level, a potential regulator of oocyte growth is a receptor-ligand complex. The receptor, termed c-*kit*, is a transmembrane tyrosine kinase receptor (Yarden *et al.*, 1987; Chabot *et al.*, 1988). c-*kit* is absent in prenatal ovaries, but present in ovaries after the time of birth (Paules *et al.*, 1989). It is specifically located on the surface of oocytes (Horie *et al.*, 1991) where it is believed to play a role in oocyte growth (Packer *et al.*, 1994). In contrast, c-*kit* is absent in granulosa cells (Horie *et al.*, 1991). The c-*kit* ligand, on the other hand, is present exclusively in granulosa cells (Horie *et al.*, 1991). Upon its secretion, the ligand is believed to interact with the c-*kit* receptor which is present on the oocyte surface (Manova *et al.*, 1993; Packer

et al., 1994). Histological studies of mutant mice lacking both the receptor and the ligand showed gonadal abnormalities starting at the 8-9.5 day embryo where the primordial germ cells had failed to increase in number leaving the ovary void of germ cells (Bennett, 1956; McCoshen and McCallion, 1975). The importance of this complex was also studied in the postnatal mouse ovary. The administration of an antibody against the c-*kit* receptor in follicles cultured *in vitro* resulted in the arrest of oocyte growth of the late fetal and neonatal ovarian oocytes. Moreover, when growing follicles were cultured in a medium enriched with the ligand, oocyte growth was dramatically enhanced (Packer *et al.*, 1994). These experiments have given the c-*kit*/ligand complex the role of a mediator of oocyte growth. The molecular target(s) of the ligand-stimulated c-*kit* receptor are not yet known. Identification of these targets would provide a further understanding of the growth phase of oogenesis.

1.4. Acquisition of meiotic competence

The *in vivo* developmental progress from an incompetent to a competent state in mouse oocytes has been suggested to involve a G2/M cell cycle transition (Wickramasinghe *et al.*, 1991; Wickramasinghe and Albertini, 1992). The G2/M transition is accompanied by a number of well characterized events especially on the morphological and the biochemical levels. However, little is known of the molecular nature of this process. On the morphological level, and under the appropriate *in vivo* or *in vitro* conditions, competent oocytes can

resume meiosis. This resumption consists of GVBD, the formation of the first meiotic spindle at metaphase I, the formation of the second meiotic spindle at metaphase II, and the arrest at the polar body stage as discussed earlier. The property of acquiring meiotic competence in growing oocytes has been linked to the age of the animal (Szybek, 1972; Sorensen and Wassarman, 1976; Wickramasinghe and Albertini, 1991). In turn, the age of the juvenile animal (In the mouse, before the age of 21-day p.p.) is correlated with the oocyte size in both mammals and frogs (mouse: Sorensen and Wassarman, 1976; Wickramasinghe *et al.*, 1991; pig: Tsafriri and Channing, 1975; frog: Sadler and Maller, 1983).

Another potential factor for acquiring meiotic competence is the interaction between the growing oocyte and its follicular microenvironment where the follicle plays an important role in preparation for meiotic maturation (Erickson, 1986).

In the following section, the above cited factors as well as relevant ultrastructural, biochemical, and whenever available molecular factors that seem to be involved in the acquisition of meiotic maturation will be covered.

1.4.1. Role of oocyte-follicle interaction

Two alternative hypotheses have been proposed for the role of follicle cells in the acquisition of meiotic competence in mammalian and amphibian oocytes. On the one hand, it has been proposed that the oocyte acquires

meiotic competence through an autonomous intracellular program which is independent of the cumulus cell association (Canipari *et al.*, 1984). On the other hand, under *in vivo* conditions, intercellular communication between the follicle cells and the oocyte is well regulated at the various stages of development in the resting, growing, and up to the ovulation of the mature egg (Buccione *et al.*, 1990; Eppig and Schroeder, 1989; Fagbohum and Downs, 1991). Gap junctions formed between the oocyte and its surrounding follicle early during the growth phase of the oocyte serve as a channel for transferring nutrients to the growing oocyte as discussed earlier.

The discrepancy in the literature for the role of the follicle cells during oogenesis could be attributed to the experimental conditions used to support either hypothesis. The "oocyte autonomous program" was based on results under *in vitro* conditions where oocytes were explanted from their follicular milieu. Hence, they initiated their intrinsic differentiative program in the absence of somatic cell input. When oocytes, on the other hand, were observed under *in vivo* conditions their development was found to be closely associated and dependent on their follicle cells. To resolve this difference, further work on the oocyte autonomous program needs to be conducted in order to eliminate either hypothesis.

1.4.1.1. Follicular factors promoting meiotic arrest

The hypothesis that the follicular microenvironment might play a role in the maintenance of the prophase-arrest of the oocyte was supported by two fundamental findings. The first came from the classical work of Pincus and Enzmann (1935) who showed that fully grown rabbit oocytes isolated from their follicles underwent meiotic maturation in the absence of gonadotropins. On the other hand, follicle-enclosed oocytes are incapable of resuming meiosis *in vitro* in the absence of gonadotropins (Tsafriri, 1978; Eppig, 1991). The specific origin of the inhibitory factor(s) was attributed to the granulosa cells (Sato *et al.*, 1982). Purines such as cAMP (Cho *et al.*, 1974; Dekel and Beers, 1978; Bornslaeger and Schultz, 1985; Bornslaeger *et al.*, 1986), hypoxanthine (Eppig *et al.*, 1985; Downs *et al.*, 1985; Eppig and Downs, 1987), guanosine (Hubbard and Terranova, 1982), phosphodiesterase (PDE) inhibitors such as isobutyl-1-methylxanthine (IBMX) (Bornslaeger *et al.*, 1984), and adenosine (Salustri *et al.*, 1985) all exert a reversible inhibitory effect on meiotic maturation. In addition, a low molecular weight peptide known as oocyte maturation inhibitor (OMI) (Tsafriri *et al.*, 1982), β-endorphin (O, 1990), Müllerian inhibiting substance (Takahashi *et al.*, 1986), and an atrial natriuretic peptide (Törnell *et al.*, 1990) have all been implicated in the maintenance of meiotic arrest. Due to their most documented involvement in meiotic arrest, only cAMP and calcium are discussed below.

A. cAMP

Evidence involving cAMP in regulating oocyte growth, resumption of meiotic maturation, and further egg development has been reported in many systems (*Xenopus laevis* : Maller and Krebs, 1980; *Rana pipiens*: Speaker and Butcher, 1977; sheep: Crosby *et al.*, 1985; mouse: Cho *et al.*, 1974; Wassarman *et al.*, 1976; Vivarelli *et al.*, 1983; rat: Magnusson and Hillensjo, 1977). When meiotically incompetent oocytes were cultured in the presence of dibutyryl cAMP (dbcAMP), a derivatized analog of cAMP, the percentage of those that acquired meiotic competence consequently increased in a dose-dependent manner (Chesnel *et al.*, 1994). This observation supports previous work which involves cAMP in a stimulatory role during the acquisition of meiotic competence in growing oocytes (Carroll *et al.*, 1991). Upon maturation, the level of cAMP in the oocyte decreases (Schultz *et al.*, 1983). Concomitantly, the level of cAMP in the follicular fluid and cumulus cells increases. Taken together, these results suggest that cAMP seems to promote the acquisition of meiotic competence in small and growing oocytes.

In contrast, cAMP promotes meiotic arrest in fully grown oocytes. When denuded, fully grown, dictyate-arrested mouse oocytes were cultured in the continuous presence of dbcAMP, they arrested at the GV stage (Wassarman *et al.*, 1976) even after a 16h culture period. Upon transfer into a cAMP-free medium, these oocytes were capable of resuming meiosis, demonstrating that this inhibitory effect is reversible. These results suggest that the G2/M

transition of the cell cycle in prophase-arrested oocytes is dependent on a decrease in the intracellular level of cAMP.

B. Calcium

Calcium in its ionic form (Ca^{+2}) is involved in cyclic nucleotide metabolism, protein phosphorylation, microtubule assembly, and calcium flux, all of which are involved in the acquisition of meiotic maturation (Sato and Koide, 1987).

Ca^{+2} seems to play several roles during oocyte growth, maturation, and ovulation of mammalian ova (De Felici and Siracusa, 1982; Bae et al., 1985). In small meiotically incompetent oocytes, Ca^{+2} is not required for the survival of cumulus-free oocytes cultured *in vitro* (De Felici and Siracusa, 1982). However, the presence of Ca^{+2} is essential for a number of events to occur as supported by the following findings. When cumulus-enclosed bovine oocytes were cultured in the absence of calcium and magnesium, they were blocked from resuming maturation and thus arrested at the GV stage (Liebfried and First, 1979). Furthermore, Ca^{+2} can overcome the inhibitory effect of dbcAMP in cumulus-free oocytes only when used at high concentrations as indicated by the following results. When mouse oocytes arrested at the GV stage with dbcAMP were cocultured in medium containing less than 1.7 mM Ca^{+2}, they did not undergo GVBD (Tombes *et al.*, 1992). However, when the extracellular calcium level was used at a concentration higher than 10 mM in cAMP-blocked

mouse oocytes, these latter were capable of resuming meiosis (Powers and Paleos, 1982). These results suggest that calcium and cAMP might be regulating meiotic maturation via a common pathway eventhough high Ca^{+2} promotes maturation whereas cAMP inhibits it.

Once maturation is initiated, external Ca^{+2} is important for preserving the normal chromosomes and spindle configurations between metaphase I and metaphase II, as well as for the formation of the first polar body (Tombes *et al.*, 1992). It has been shown that an increase in the intracellular level of Ca^{+2} is responsible for an increase in the percentage of oocytes that reach metaphase II of meiosis in the pig and mouse species (Paleos and Powers, 1981; Sato *et al.*, 1982; Tombes *et al.*, 1992). It is possible that partially competent oocytes, which arrest at metaphase I lack sufficient calcium to reach metaphase II.

Upon maturation, the survival of eggs *in vitro* depends on Ca^{+2}. Finally, Ca^{+2} is required for the initiation of development of the egg following fertilization (Whittingham and Siracusa, 1978; Kline and Kline, 1992). Thus, calcium plays a central role prior to, during, and following meiotic maturation.

1.4.2. Role of microtubular network

One of the hallmarks of meiotic competence is the reorganization of the cytoskeletal components of the oocytes. In particular, the microtubular structure of both the cumulus cells (Allworth and Albertini, 1993) and the

enclosed oocyte (Vandré *et al.*, 1984; Bornslaeger *et al.*, 1988) undergo major changes upon acquiring meiotic competence (Wickramasinghe *et al.*, 1991) and during meiotic maturation (Mattson and Albertini, 1990), as well as during embryonic mitosis (Schatten *et al.*, 1985).

The centrosomal structures, or centrioles, which are localized to the spindle poles during metaphase, are absent in several mammalian species starting at the pachytene stage of oogenesis. Instead, several microtubular foci, collectively known as microtubule organizing centers (MTOCs) are responsible for the nucleation of microtubular growth throughout the oocyte cell cycle (Szöllosi *et al.*, 1974). The G2/M transition of the cell cycle and meiotic competence have been linked to specific changes of MTOCs with respect to both the biochemical and physical configurations (Wickramasinghe *et al.*, 1991) Meiotically incompetent oocytes contain dephosphorylated MTOCs which are localized to the cortex of the cell. These MTOCs nucleate a diffuse and elaborate microtubular network which spreads throughout the cytoplasm. When the oocyte becomes competent to resume meiosis, the MTOCs migrate to the perinuclear region where they become phosphorylated and nucleate short microtubules (Centonze and Borisy, 1990). At this point, they are known to be activated in preparation for the formation of the spindle poles (Vandré *et al.*, 1990; Wickramasinghe *et al.*, 1991). This reorganization and phosphorylation of MTOCs during the acquisition of meiotic competence has been described in amphibians as well (Gard, 1991; Ohta *et al.*, 1993).

Once GVBD takes place and the chromosomes condense, MTOCs become localized to the spindle poles of the first and second meiotic divisions (Schatten *et al.*, 1985; Messinger and Albertini, 1991; Wickramasinghe *et al.*, 1991). The ultrastructural changes of the microtubules and the MTOCs within an oocyte are directly correlated with altered phosphorylation patterns of specific phosphoproteins (Maller *et al.*, 1977)

1.4.3. Role of protein phosphorylation

The decrease in oocyte cAMP at the time of GVBD leads to a decrease in cAMP-dependent protein kinase activity which, in turn, results in phosphorylation or dephosphorylation of a number of proteins (Bitensky and Gorman, 1973). Concomitantly, a three to five fold increase in cAMP-independent phosphoprotein synthesis becomes apparent (Bornslaeger *et al.*, 1986, 1988; Morgan *et al.*, 1989; Néant *et al.*, 1989). Protein phosphorylation is important during meiotic maturation since its inhibition in porcine oocytes that were matured *in vitro* inhibited both nuclear envelope breakdown and chromatin condensation (Jung *et al.*, 1993). Mediators of protein phosphorylation include factors such as Ca^{+2} which together with diacylglycerol (DAG) is known to activate protein kinase C (PKC). In frog oocytes, DAG levels increase transiently after exposure to maturation-inducing agents such as insulin, insulin-like growth factor-1, or progesterone (Chien *et al.*, 1991). DAG levels could be increased as well upon microinjection of v-ras into frog oocytes

(Lacal *et al.*, 1987). Increasing DAG levels either indirectly by the effect of the factors mentioned or alone causes GVBD to occur (Garcia de Herreros *et al.*, 1991). The mechanism of the action of GVBD could be mediated by the effect of DAG on increasing the level of PKC which in turn activates downstream kinases (Nishizoka, 1984· Mitchell *et al.*, 1989) thus leading to meiotic resumption marked by the occurrence of GVBD as seen in many species (*Chaetopterus*: Eckberg and Carroll, 1987; *Xenopus laevis*: Maller *et al.*, 1977; Karsenti *et al.*, 1987; Lohka *et al.*, 1987; mouse: Schultz *et al.*, 1983; Bornslaeger *et al.*, 1986; sheep: Crosby *et al.*, 1984; pig: Jung *et al.*, 1993). Such kinases include the cdc2 serine/threonine kinase which together with cyclin B forms the maturation promoting factor (MPF). Once active, MPF is responsible for a number of cytoplasmic and nuclear modifications during metaphase.

Other serine/threonine kinases that become active upon meiotic resumption include the p39 $^{c\text{-}mos}$ protein and mitogen activated protein (MAP) kinase (Rossomando *et al.*, 1989). Both of these kinases have been shown to play an important role in the microtubular dynamics that take place during metaphase.

In the following section, the role of MPF, c-*mos*, and MAP kinase in the acquisition of meiotic competence will be discussed.

A. Maturation Promoting Factor

The transition from interphase to metaphase during oocyte maturation is mediated by a cytoplasmic factor whose activity was first shown to reside in maturing amphibian eggs. This was first demonstrated by the pionneering work of Masui and Markert (Masui and Markert, 1971) who injected the cytoplasm of a *Rana pipiens* mature oocyte into an immature oocyte. As a result, the injected immature oocyte underwent GVBD and completed its meiotic maturation. This factor was named thereafter the maturation promoting factor (MPF). Since this original observation, MPF has been shown to be a universal metaphasic factor (Kishimoto *et al.*, 1982; Kishimoto, 1988) also referred to as the metaphase-promoting factor, and the M-phase factor (*Xenopus laevis*: Schorderet-Slatkine and Drury, 1973; Labbée *et al.*, 1988a; Erickson and Maller, 1989; mammals: Balakier, 1978; Fulka, 1983; Sorensen *et al.*, 1985; Lee *et al.*, 1988; fish: Dettlaf *et al.*, 1977; marine invertebrates: Kishimoto and Kanatani, 1976; Labbée *et al.*, 1988b; yeast: Weintraub *et al.*, 1982; mammalian culture cells: Sunkara *et al.*, 1979; Nelkin *et al.*, 1980).

MPF activity is first detected in the maturing oocytes of clams, sea urchins, starfish, frogs, and mice shortly before GVBD (Masui and Markert, 1971; Wasserman and Masui, 1976; Kishimoto and Kanatani, 1976; Kishimoto *et al.*, 1984; Sorensen *et al.*, 1985; Hashimoto and Kishimoto, 1988; Labée *et al.*, 1988b, 1989b; Choi *et al.*, 1991). This activity then increases and remains

high until the exit from the first meiotic division (metaphase I) when it declines shortly thereafter. MPF activity increases again at metaphase of the second meiotic division, and this increased level is maintained through metaphase II arrest in the oocyte until fertilization (Figure 4; Dorée *et al.*, 1983; Gerhart *et al.*, 1984). The rise of MPF activity at two peaks during meiotic maturation, namely at GVBD and at metaphase II, was found to be concomitant with two bursts of protein phosphorylation (Dorée *et al.*, 1983).

Upon investigating the molecular nature of MPF, it was found to be composed of two components (Labbée *et al.*, 1989b), one of which was first identified as the cell cycle gene *cdc2* in *Schizosaccharomyces pombe (S. pombe)*. This gene was initially found to code for a homologue of the *Xenopus* p34 subunit (Dunphy *et al.*, 1988; Gauthier *et al.*, 1988; Labbée *et al.*, 1989a). The $p34^{cdc2}$ is a serine/threonine kinase which is required for G2/M transition of the cell cycle manifested during meiotic resumption.

The other component of MPF, called cyclin, is the regulatory subunit responsible for the activation of the kinase (identified by Draetta *et al.*, 1989; Gauthier *et al.*, 1990). Cyclins are a family of proteins that accumulate during interphase of the cell cycle and are degraded during mitosis (Murray and Kirschner, 1989a, 1989b). This degradation inactivates the kinase activity of p34, and is responsible for the exit from mitosis (Figure 5, Evans *et al.*, 1983; Meijer *et al.*, 1989; Murray *et al.*, 1989). Upon comparison of cyclin abundance and MPF activity, a strong correlation was found between the

accumulation/destruction of cyclin, on the one hand, and the increase/decrease of MPF activity (Figure 6).

During interphase, $p34^{cdc2}$ becomes phosphorylated on a tyrosine and a threonine residue. Cyclin, on the other hand, is synthesized and accumulates throughout interphase. For MPF to become active at metaphase, both components of the heterodimer are modified by phosphorylation; $p34^{cdc2}$ is dephosphorylated on its tyrosine residue (Gauthier *et al.*, 1989) and cyclin is phosphorylated. Degradation of cyclin marks the exit from metaphase (Figure 5). Once activated, MPF is responsible for turning on a number of downstream kinases (Solomon *et al.*, 1992). Among the substrates for MPF are centrosomal components (Bailly *et al.*, 1989) and their associated microtubules (Verdé *et al.*, 1990), lamins, histone H1, nucleolin (Lewin, 1990), and MAP kinase (Kosako *et al.*, 1993), all of which may be involved in events leading to nuclear envelope breakdown, chromosome condensation and spindle formation (Lohka and Maller, 1985; Figure 7).

Nonetheless, it should be pointed out that cyclins alone are not sufficient for the activation of $p34^{cdc2}$. A family of *cdc25* gene products in *S. pombe* were found to positively regulate the activation of $p34^{cdc2}$ kinase (Russell and Nurse, 1986; Kumagi and Dunphy, 1992). Biochemical analysis has shown that the *cdc25* gene product is a phosphatase capable of dephosphorylating the tyrosine residue. This phosphorylation is crucial for the activation of $p34^{cdc2}$ (Gould *et al.*, 1990).

Other regulators of $p34^{cdc2}$ include two of the four major phosphatases in the cytosol of mammalian cells which dephosphorylate serine and threonine residues (Cohen *et al.*, 1990), referred to as phosphatases 1 and 2A (PP1 and PP2A). These phosphatases were first identified by genetic analysis of yeast and *Aspergillus* (Morris *et al.*, 1989). When PP1 and PP2A are inhibited by the drug, okadaic acid, $p34^{cdc2}$ becomes activated resulting in the entry to metaphase (Brautigan *et al.*, 1989). These phosphatases may be needed for the arrest of a given cell at interphase, and are overcome by an intracellular inhibitor at metaphase. The use of okadaic acid has been especially instrumental in understanding the molecular aspects of meiotic resumption in oocytes from several species. Such aspects include the involvement of PP1 and PP2A in the control of MPF activity in starfish (Picard *et al.*, 1989; 1991; Paulson *et al.*, 1994), *Xenopus laevis* (Goris *et al.*, 1989), mouse (Rime and Ozon, 1990; Alexandre *et al.*, 1991; Gavin *et al.*, 1991), bovine and porcine (Kalous *et al.*, 1993) oocytes. This control is manifested by an accelerated GVBD in meiotically competent oocytes (Kalous *et al.*, 1993). In addition, meiotically incompetent oocytes occuring either naturally as is the case of small oocytes (Gavin *et al.*, 1991), or under the effect of meiotic inhibitors are capable of resuming meiosis (Alexandre *et al.*, 1991) in the presence of okadaic acid.

B. Cytostatic factor

Another cytoplasmic component which is important in cell cycle regulation is termed cytostatic factor (CSF). CSF is a calcium-sensitive factor that was first described by Masui (Masui, 1974; Masui and Shibuya, 1987) as an activity that prevents exit of *Rana pipiens* oocytes from the metaphase II arrest. A similar activity is present in the metaphase II-arrested oocytes of the mouse (Kubiak *et al.*, 1993). CSF is unique to the germ cells (Goldman *et al.*, 1987). Upon investigating the activity of CSF, it was found that it first appears in the cytoplasm of a maturing oocyte at GVBD, remains high through meiotic maturation and disappears soon after fertilization (Meyerhof and Masui, 1977, 1979).

The site of action of CSF seems to be the cyclin component of MPF. The exit of an amphibian as well as a mammalian oocyte from metaphase II requires the degradation of cyclin B as was discussed in the previous section. However, in metaphase II-arrested oocytes the synthesis of cyclin B is continuous (Weber *et al.*, 1991) as well as its degradation (Kubiak *et al.*, 1993), resulting in a rapid turnover of CSF. Furthermore, the degradation of cyclin B occurs only in the presence of an intact metaphase spindle (Kubiak *et al.*, 1993). Taken together with the fact that CSF prevents the egg from exiting metaphase , this implicates CSF in the microtubular network where it may be monitoring the formation of a functional spindle prior to the exit from

metaphase by exerting its effect on the cyclin component of MPF.

C. *c-mos*

When the molecular nature of CSF was investigated, one factor seemed prominent, the c-*mos* proto-oncogene. c-*mos* encodes a serine/threonine protein kinase (Maxwell and Arlinghaus, 1985) of M_r 39 KDa, and is expressed at high levels in the germ cells of vertebrates (Goldman *et al.*, 1987; Mutter and Wolgemuth, 1987).

c-*mos* has been identified as a component of CSF based on the following results. When either $p39^{c\text{-}mos}$ mRNA or CSF-containing cytoplasm was microinjected into a *Xenopus* blastomere, this latter was arrested at mitotic metaphase (Sagata *et al.*, 1988). Furthermore, CSF activity can be depleted from metaphase II eggs with c-*mos* antibodies (Sagata *et al.*, 1989).

$p39^{c\text{-}mos}$ is absent in meiotically-incompetent oocytes (Paules *et al.*, 1989), and is required during meiotic maturation where it is necessary for the activation of MPF in meiosis I (Sagata *et al.*, 1988; O'Keefe *et al.*, 1989, 1991) and is also required after GVBD (Sagata *et al.*, 1988; 1989a). c-*mos* can, in the absence of any protein synthesis, induce GVBD and activate MPF in *Xenopus* oocytes. However, such treated oocytes cannot complete maturation to metaphase II (Yew *et al.*, 1992).

A substrate for c-*mos* is a tyrosine/threonine protein kinase, termed mitogen-activated protein kinase kinase, usually referred to as MEK, which is

directly responsible for the activation of a serine/threonine protein kinase, referred to as mitogen activated protein (MAP) kinase (Posada *et al.*, 1993; Nebrada and Hunt, 1993).

c-*mos* could exert an effect on the microtubular network either by activating MAP kinase (Figure 8), or directly since cellular localization experiments show that $p39^{c\text{-}mos}$ can associate with and phosphorylate tubulin (Zhou *et al.*, 1991). The association between $p39^{mos}$ and tubulin was shown by immunoprecipitation where the homodimer, β-tubulin, was preferentially coprecipitated with $p39^{mos}$. The ability to phosphorylate tubulin was demonstrated in a kinase assay where β-tubulin was the major phosphorylated product in a $p39^{mos}$-dependent immune complex kinase assay (Zhou *et al.*, 1991). This association was extended when c-*mos* was shown to be required for spindle function of mouse oocytes (Zhao *et al.*, 1991).

D. <u>Mitogen - activated protein kinase</u>

Prior to the knowledge of their kinase activity, mitogen-activated protein (MAP) kinases were known as microtubule-associated protein-2 (MAP-2) kinase (Jameson and Caplow, 1981). Upon the finding of their kinase activity in insulin-stimulated adipocytes (Sturgill and Ray, 1986), MAP kinases, also referred to as extracellular signal regulated kinases (ERK's), have been identified and characterized in many species ranging from the yeast to the human (*S. cerevisiae*: FU3S-KSS1, Cairns *et al.*, 1992; Courshesne *et al.*, 1989; Elion *et al.*, 1990; *S. pombe*: Spk1, Torres *et al.*, 1991; Byr1 and Byr2, Nadin-Davis

and Nasim, 1988; drosophila: Brunner *et al.*, 1994; *Xenopus*: Ahn *et al.*, 1991; Ferrell *et al.*, 1991; sea star: Sanghera *et al.*, 1990; murine: Verlhac *et al.*, 1993; Sobajima *et al.*, 1993; Gavin *et al.*, 1994; rabbit: Gregory *et al.*, 1989; human fibroblasts: Ek and Heldin, 1984; Pagès *et al.*, 1993) and in tissue culture cells (3T3 cell line: Kawakami *et al.*, 1991).

MAP kinase has been characterized (Hoshi *et al.*, 1989) and is capable of being active via a number of signal transduction pathways, including tyrosine kinases, G proteins (Lange-Carter *et al.*, 1993) and protein kinase C (Pelech and Krebs, 1987; Pelech and Sanghera, 1992). MAP kinase is responsible for activating other downstream factors and is itself regulated by upstream regulators. The family of MAP kinase includes members of 40-46 kDa isoforms with p42 and p44 as the most occuring forms among mammalian species. Both p42 and p44 require phosphorylation on tyrosine and threonine residues for maximal activation (Ray and Sturgill, 1988; Anderson *et al.*, 1990), and can undergo autophosphorylation on both tyrosine and threonine residues (Seger *et al.*, 1991). This autophosphorylation process, however, does not seem to be biologically important (Posada and Cooper, 1992).

Among MAP kinase targets are the ribosomal S6 kinase (Sturgill *et al.*, 1988; Sturgill and Wu, 1991), the nuclear transcription factors, c-*jun* and c-*fos* (Gille *et al.*, 1992), and a *Xenopus* p220 kDa microtubule-associated protein (Shiina *et al.*, 1992).

One of the most important features of MAP kinase, however, might

reside in their involvement in the control of the cell cycle where they respond to various external signals, usually mitogens, to participate in triggering the transition from G0 to G1 and from G2 to M phase (Cobb *et al.*, 1991; Boulton *et al.*, 1991; Thomas, 1992). In the G2/M transition of the cell cycle, MAP kinase might be involved in the reorganization of microtubules based on several findings conducted on murine and frog oocytes. MAP kinase is present in fully grown, G2-arrested oocytes of the frog (Gotoh *et al.*, 1991a; Ferrell *et al.*, 1991; Posada *et al.*, 1991), sea star (Pelech *et al.*, 1988), clam (Shibuya *et al.*, 1993), and mouse (Verlhac *et al.*, 1993; Sobajima *et al.*, 1993). When oocytes are induced to enter metaphase, both species p42 and p44 become phosphorylated thus rendering MAP kinase active as a kinase (Gotoh *et al.*, 1991).

In maturing mouse oocytes, MAP kinase is associated with the MTOCs (Verlhac *et al.*, 1993) which are localized at the spindle poles of metaphase I and II oocytes. MTOC's are known to nucleate microtubular assembly at metaphase (Schatten *et al.*, 1985). MAP kinase seems to play a role in the microtubular reorganization during the G2/M transition based on the following results. When purified M-phase activated MAP kinase from either *Xenopus* eggs or mammalian fibroblasts was added to interphase extracts from *Xenopus* oocytes, the interphasic microtubular network exhibited a transition to the metaphasic form. This transition was manifested by an increased nucleation of microtubules from the centrosomes. These microtubules were short and

dynamic as is seen usually at metaphase (Gotoh *et al.*, 1991).

The effect of activated MAP kinase on the microtubular network seems to be initiated by the activation of MPF which, in turn, activates MAP kinase (Gotoh *et al.*, 1991; Ohta *et al.*, 1993; Figure 9). To determine the effect of these two kinases, MPF and MAP kinase, on the microtubular network, the following experiment was designed. The ability of centrosomes to nucleate microtubules in the presence of either a purified $p34^{cdc2}$/cyclin B complex or MAP kinase was compared. The results show that the increase in microtubular network nucleated from centrosomes was greatly enhanced in the presence of the MPF complex but not in the presence of MAP kinase (Ohta *et al.*, 1993). However, since MAP kinase is activated downstream of MPF, the increased effect of microtubular nucleation manifested upon the addition of the MPF complex could be due to a cooperation between the action of both MPF and MAP kinase.

To learn more about the involvement of MAP kinase in the microtubular network rearrangement during meiotic maturation, a possible place to study this effect could be in cells that are incapable of resuming meiosis, thus incapable of entering metaphase, under either *in vivo* or *in vitro* conditions. Such a system is naturally found in the meiotically incompetent and partially competent oocytes. I have taken advantage of such naturally-arrested cells to study further the role of MAP kinase in the acquisition of meiotic competence as well as during meiotic maturation of mammalian oocytes.

1.5. Summary

During mammalian oogenesis, the acquisition of meiotic competence is of central importance for the conversion of an oocyte into a fertilizable ova. Understanding the details of this phenomenon could provide insight into cell cycle regulation which regulates the reproductive life of an organism.

To dissect further this multifaceted event, I studied the expression of MAP kinase in the mouse oocyte due to its possible involvement in initiating the cell cycle in meiotically-arrested oocytes. My objective was to document the changes, if any, of the pattern of MAP kinase among oocytes of various meiotic potential, namely incompetent, partially-competent, and fully-competent oocytes. These three groups could be obtained by targeting a specific age in the juvenile mouse which shows a good synchrony during growth *in vivo* and during culture *in vitro* as well. In the following chapter, I will describe the experimental evidence for the presence of a key regulator in a number of signal transduction pathways, MAP kinase, in incompetent, partially-competent, and fully-competent mouse oocytes. Based on previous results which showed that MAP kinase becomes activated as a kinase following phosphorylation, I will describe evidence regarding the phosphorylation patterns of the two species, p42 and p44, in all three classes of oocytes. In addition, experiments done with okadaic acid, a specific phosphatase inhibitor of both PP1 and 2A, will be described. Taken together, these results should help elucidate the mechanism underlying the action of this kinase during the G2/M transition of the cell cycle.

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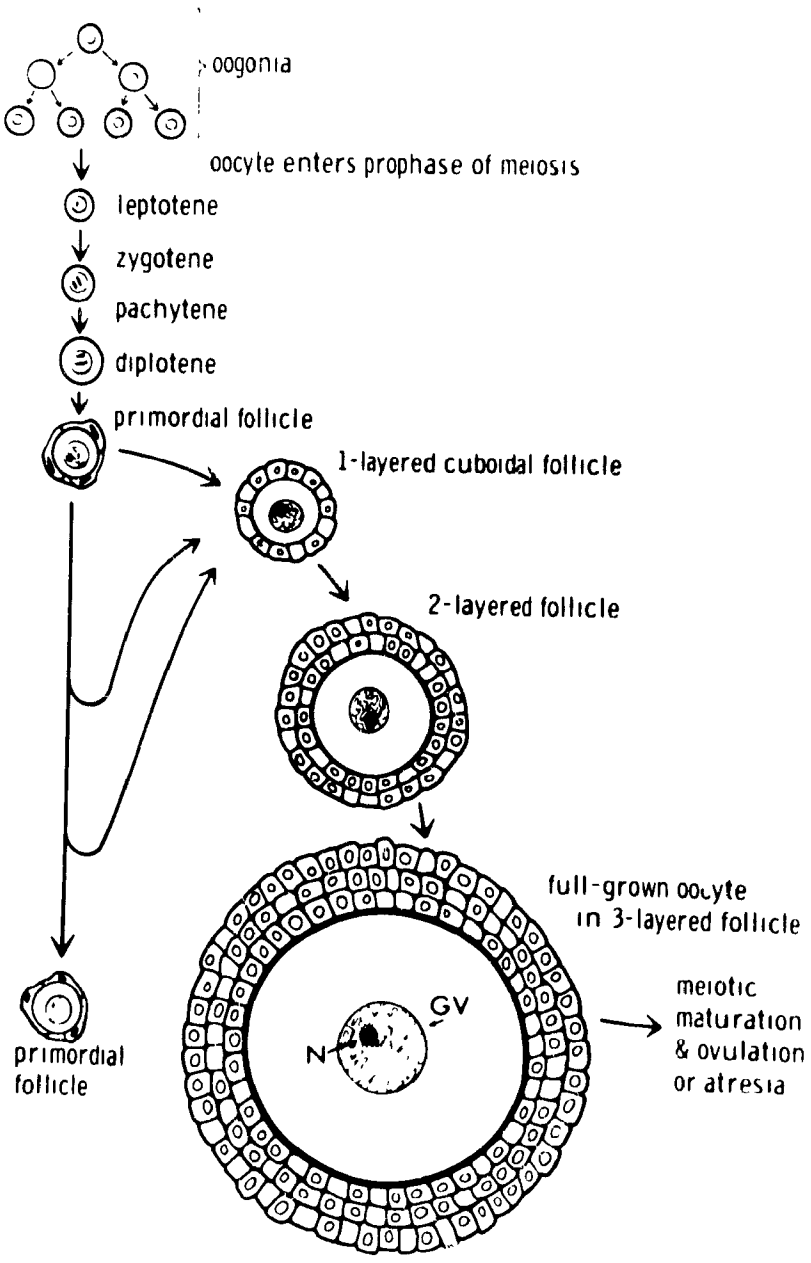
oogonia
oocyte enters prophase of meiosis
leptotene
zygotene
pachytene
diplotene
primordial follicle
1-layered cuboidal follicle
2-layered follicle
full-grown oocyte
in 3-layered follicle
GV
N
meiotic
maturation
& ovulation
or atresia
primordial
follicle

Figure 1. A schematic representation of the development of mammalian oocytes from oogonia to fully grown oocytes. In the fetal life of the female, the oogonia progress through meiotic prophase in a relatively synchronous pattern and arrest at the diplotene stage of meiosis shortly after birth. Diplotene-arrested oocytes either stay in a one-layered follicle, or they initiate their growth phase. During its growth phase, an oocyte grows in size where it accumulates all the necessary components that will be used during its maturation and post-fertilization development. Once fully grown, a follicle-enclosed oocyte could either become ovulated in preparation for fertilization or it undergoes degeneration (From Bachvarova, 1985).

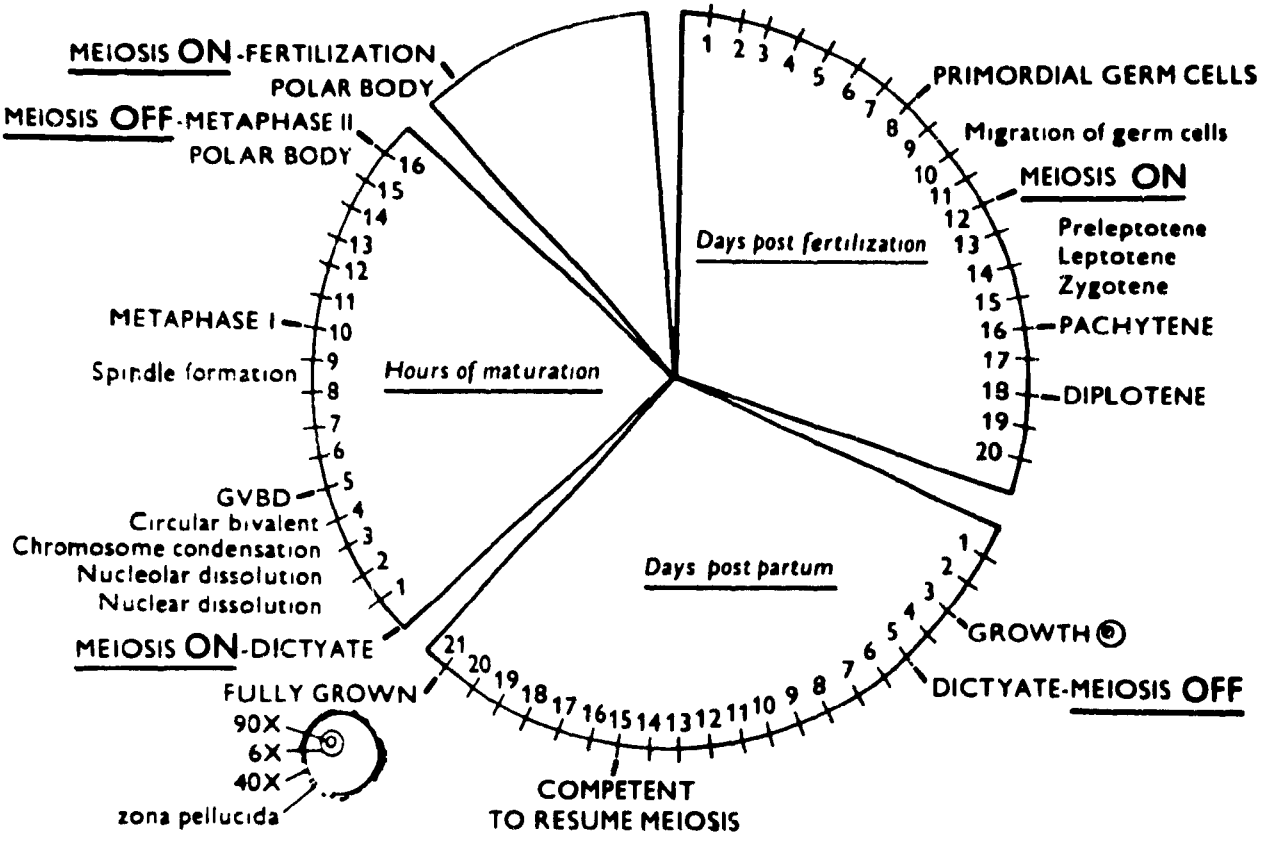
MEIOSIS ON-FERTILIZATION
POLAR BODY
MEIOSIS OFF-METAPHASE II
POLAR BODY
METAPHASE I
Spindle formation
GVBD
Circular bivalent
Chromosome condensation
Nucleolar dissolution
Nuclear dissolution
MEIOSIS ON-DICTYATE
FULLY GROWN
90X
6X
40X
zona pellucida
Hours of maturation
PRIMORDIAL GERM CELLS
Migration of germ cells
MEIOSIS ON
Preleptotene
Leptotene
Zygotene
PACHYTENE
DIPLOTENE
Days post fertilization
Days post partum
GROWTH
DICTYATE-MEIOSIS OFF
COMPETENT
TO RESUME MEIOSIS

Figure 2. Diagrammatic representation of two aspects of oogenesis, meiosis and growth, in the life cycle of the mouse (From Schultz and Wassarman, 1977).

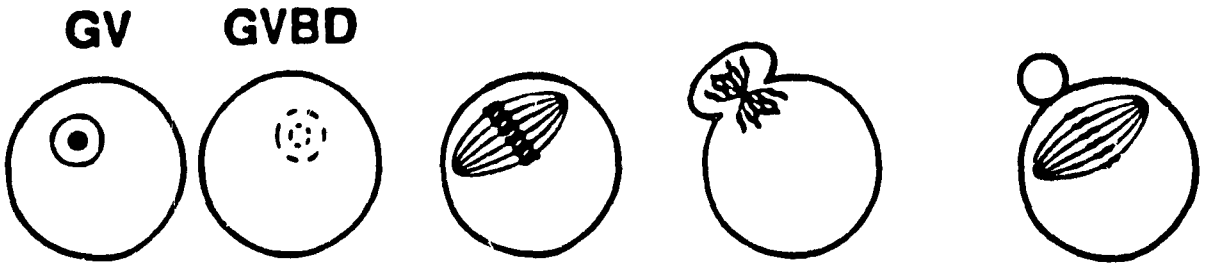
GV
GVBD
Metaphase 1
Telophase 1
Metaphase 2

Figure 3. Diagrammatic representation of the stages of oocyte meiotic maturation. When a fully grown, prophase-arrested oocyte is exposed to a maturation-inducing environment, it undergoes germinal vesicle breakdown (GVBD), where the nuclear envelope breaks down and the homologous chromosomes assemble on the first meiotic spindle at metaphase I. The oocyte then undergoes telophase I without cell division. Finally, sister chromatids are separated at metaphase II where the first polar body is emitted from the oocyte. At the end of meiotic maturation, the oocyte contains the diploid chromosomal content (From Wickramasinghe and Albertini, 1993).

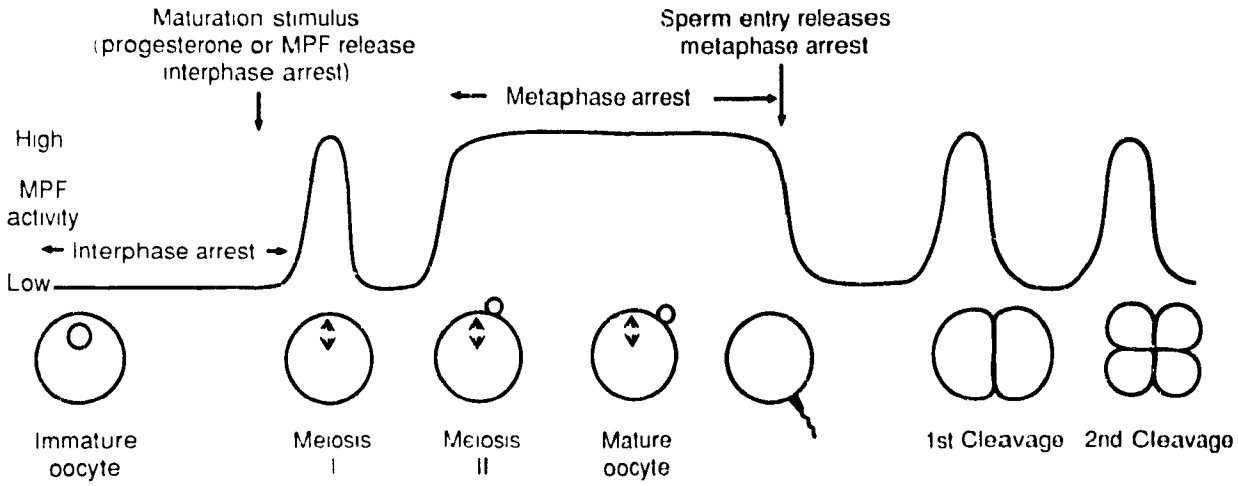
Maturation stimulus
(progesterone or MPF release
interphase arrest)
Sperm entry releases
metaphase arrest
Metaphase arrest
High
MPF
activity
Interphase arrest
Low
Immature
oocyte
Meiosis
I
Meiosis
II
Mature
oocyte
1st Cleavage
2nd Cleavage

Figure 4. Fluctuation of maturation promoting factor (MPF) activity during the early frog life cycle. MPF level is low in a fully grown, immature *Xenopus* oocyte. Upon stimulation to resume meiosis, the oocyte undergoes GVBD and the level of the oocyte MPF increases dramatically at metaphase I of meiosis after which it decreases. At metaphase II of meiosis, MPF level increases and stays up by the effect of cytostatic factor until the time of fertilization where it drops again. This fluctuation in activity continues with every cell division of the early embryo (From Murrray and Kirschner, 1989b).

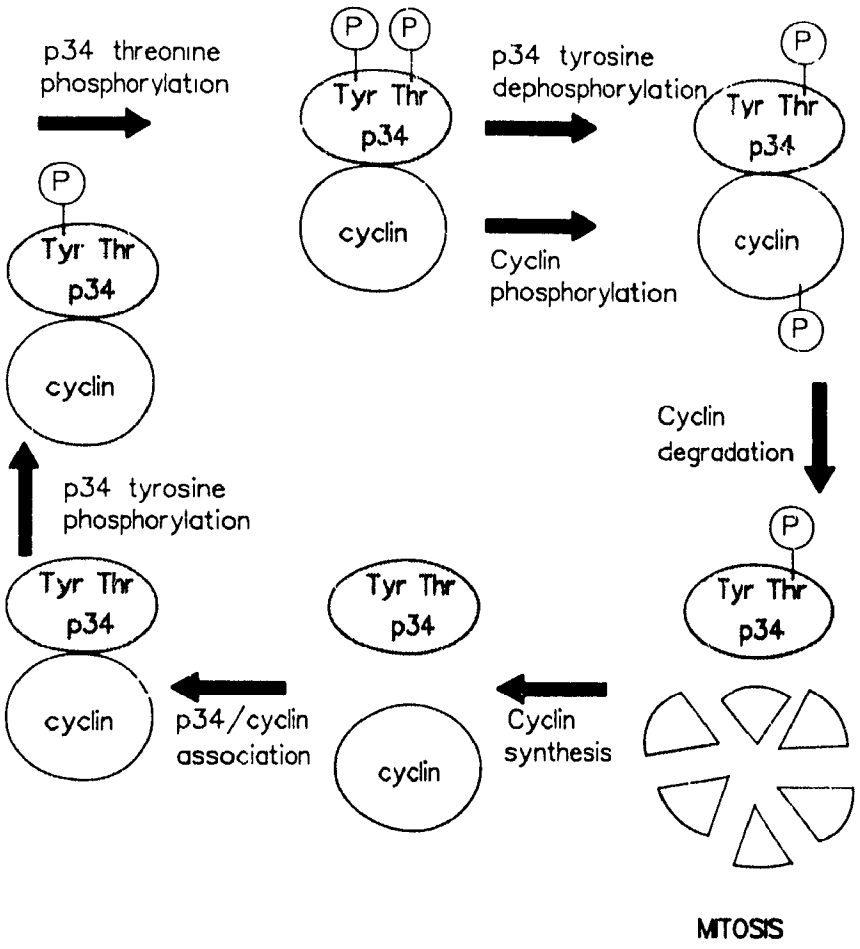
p34 threonine phosphorylation
P
P
Tyr Thr p34
cyclin
p34 tyrosine dephosphorylation
Tyr Thr p34
cyclin
P
Cyclin phosphorylation
P
Tyr Thr p34
cyclin
Cyclin degradation
p34 tyrosine phosphorylation
P
Tyr Thr p34
Tyr Thr p34
cyclin
p34/cyclin association
Tyr Thr p34
cyclin
Cyclin synthesis
MITOSIS

Figure 5. The cycle of association between the two components of MPF, p34 and cyclin in the G2/M transition. In interphase, cyclin B is synthesized and accumulates in the cell and p34 becomes phosphorylated on a tyrosine residue. Prior to metaphase, p34 becomes phosphorylated on a threonine residue. At metaphase, p34 is dephosphorylated on the tyrosine residue whereas cyclin becomes phosphorylated. To exit mitosis, a proteolytic degradation of cyclin is required (From Lewin, 1990).

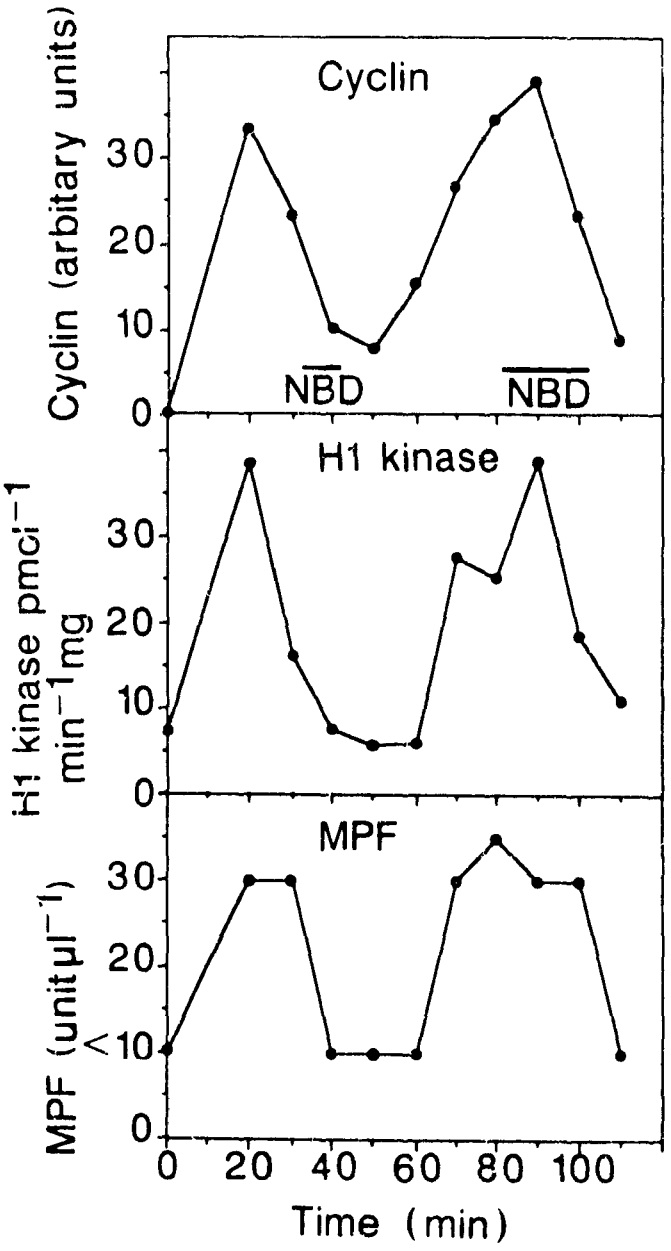
Cyclin
Cyclin (arbitary units)
30
20
10
0
NBD
NBD
H1 kinase
H1 kinase pmci^{-1} min^{-1}mg
30
20
10
0
MPF
MPF (unit µl^{-1})
30
20
<10
0
0 20 40 60 80 100
Time (min)

Figure 6. The fluctuation in cyclin abundance relation to the activity of MPF and H1 kinase. Upon addition of 5 μg/ml of sea urchin cyclin mRNA to an mRNA-dependent extract, the activity of cyclin in the extract fluctuates at the same time periods as did both MPF and H1 kinase activity. The activity of all three parameters dropped as nuclear envelope breakdown (NBD) occured at metaphase. This activity then increases during the rest of the cell cycle (From Murray and Kirschner, 1989a).

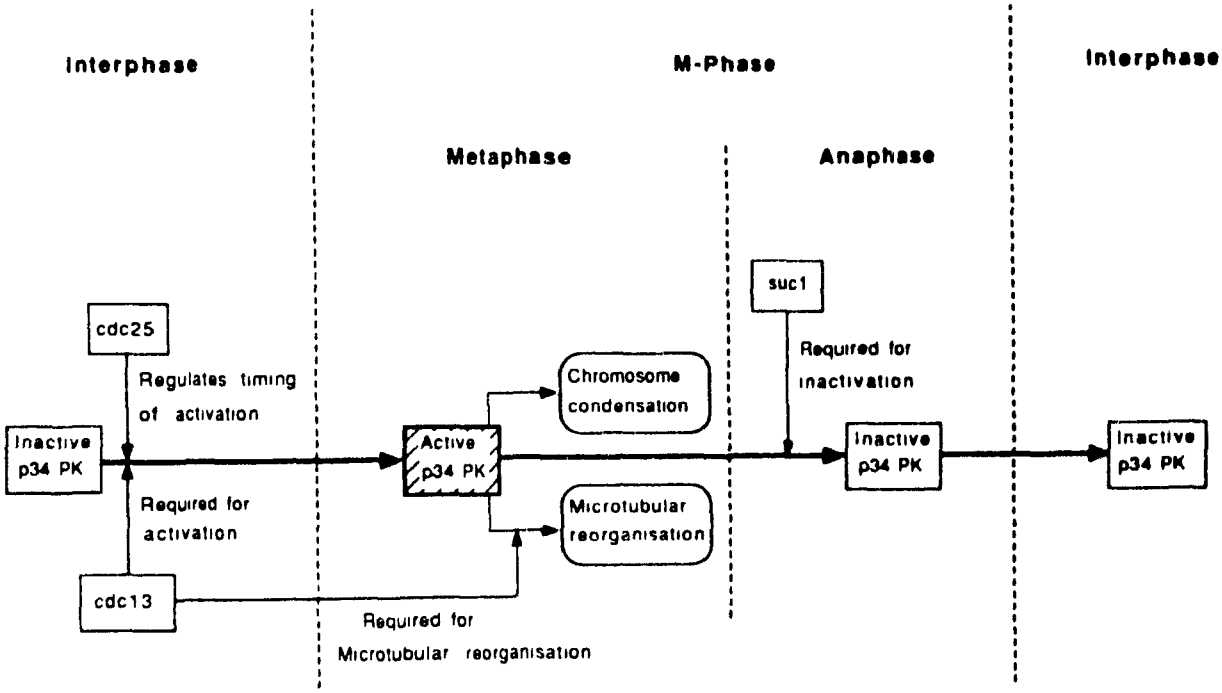
Interphase
M-Phase
Interphase
Metaphase
Anaphase
cdc25
Regulates timing
of activation
Inactive
p34 PK
Required for
activation
cdc13
Active
p34 PK
Chromosome
condensation
Microtubular
reorganisation
Required for
Microtubular reorganisation
suc1
Required for
inactivation
Inactive
p34 PK
Inactive
p34 PK

Figure 7. Regulation of $p34^{cdc2}$ protein kinase during the G2/M transition of the cell cycle of the fission yeast. Entry into mitosis follows the activation of $p34^{cdc2}$ protein kinase and requires both *cdc*25$^+$ and *cdc*13$^+$. Exit from mitosis, however, follows the kinase inactivation and requires *suc*1$^+$. *cdc*13$^+$ is also required for the reorganization of microtubular cytoskeleton leading to the generation of the mitotic spindle (From Moreno *et al.*, 1989).

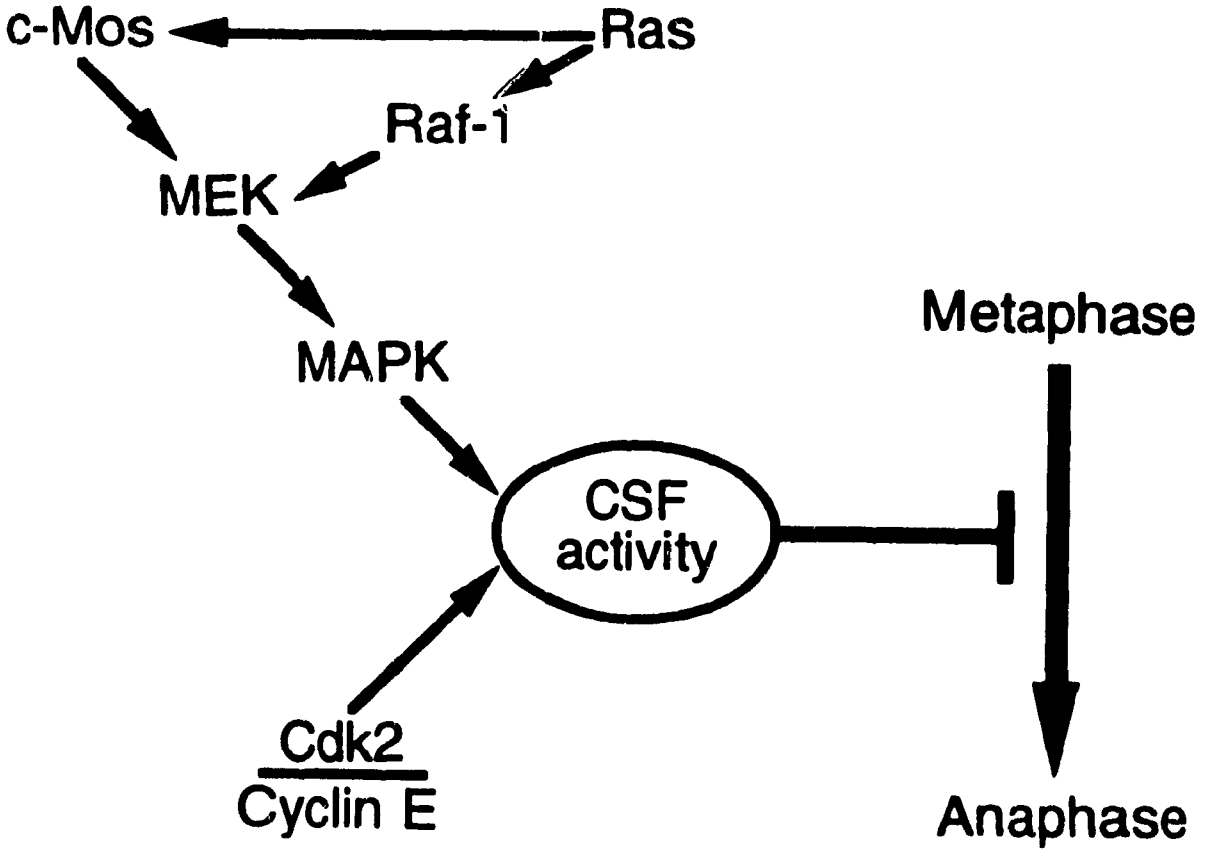
c-Mos
Ras
Raf-1
MEK
MAPK
Metaphase
CSF
activity
Cdk2
Cyclin E
Anaphase

Figure 8. Model for the mechanism involving c-*mos* and MAP kinase in the metaphase arrest through the effect on cytostatic factor (CSF). Exit from metaphase requires the inactivation of CSF. The CSF arrest induced by c-*mos* is mediated by the activation of MAP kinase. CSF activation can also be mediated through the Ras pathway. Ras can induce the activation of MAP kinase either by the c-*mos* effect or by activating Raf. In either case, both MAP kinase and MPF cooperate in forming CSF. CSF is responsible for blocking the metaphase to anaphase transition in metaphase II eggs (From Haccard *et al.*, 1993).

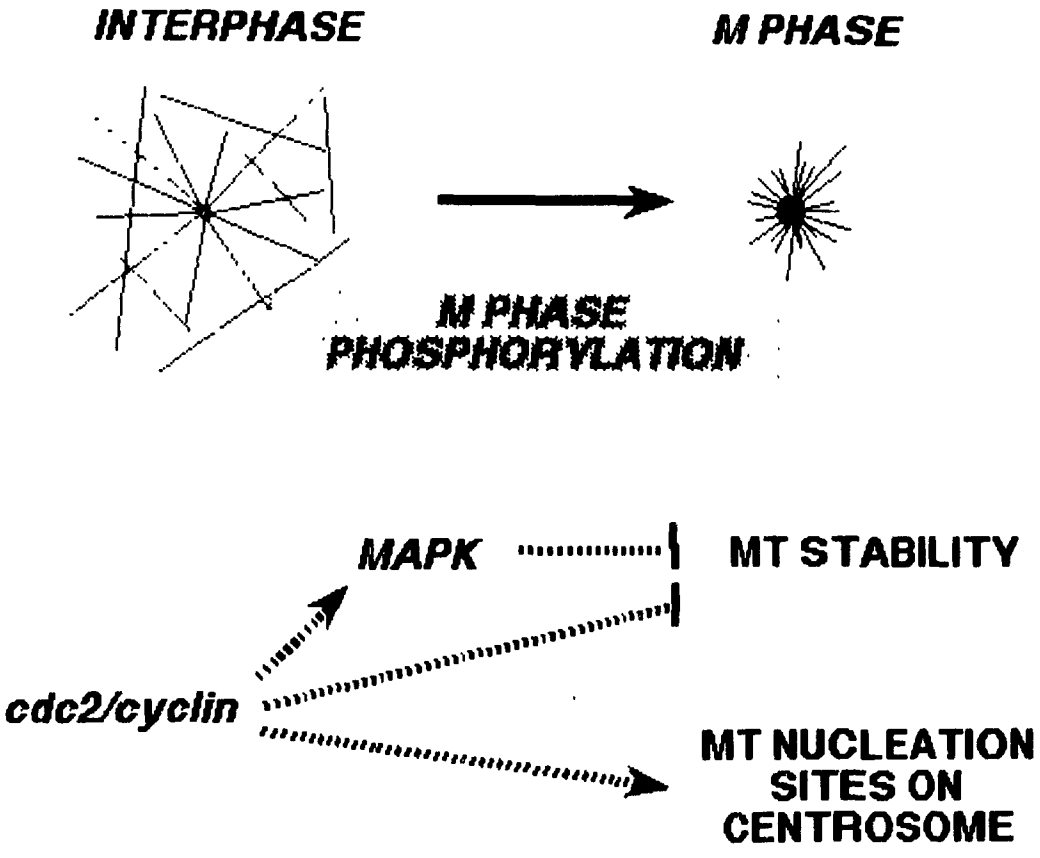
INTERPHASE
M PHASE
M PHASE
PHOSPHORYLATION
MAPK
MT STABILITY
cdc2/cyclin
MT NUCLEATION
SITES ON
CENTROSOME

Figure 9. A schematic representation of the opposite effects of protein phosphorylation on microtubule assembly and microtubule organizing centers (MTOCs) activity. The activation of MPF at metaphase results in a number of phosphorylation processes among which is the activation of a downstream kinase, MAP kinase. When active, MAP kinase decreases the stability of microtubules, thus favoring the dynamic configuration of metaphase. In turn, active MPF might be responsible for regulating directly the nucleation of metaphasic microtubules through its effect on activating MTOCs (From Ohta *et al.*, 1993).

CHAPTER II

MITOGEN-ACTIVATED PROTEIN (MAP) KINASE DURING THE ACQUISITION OF MEIOTIC COMPETENCE BY GROWING OOCYTES OF THE MOUSE

Wafa Harrouk and Hugh J. Clarke

ABSTRACT

During the growth phase of oogenesis, oocytes acquire the ability to undergo meiotic maturation. Although the molecular basis of this meiotic competence is unknown, specific differences in microtubular organization exist between incompetent and competent mammalian oocytes. Mitogen-activated protein (MAP) kinase has been implicated in microtubular regulation and is present in fully grown competent oocytes of mice, suggesting a possible role for this protein in the acquisition of meiotic competence. We report that the MAP kinase species, $p42^{ERK2}$ and $p44^{ERK1}$, were detectable by immunoblotting in incompetent oocytes at the early stages of oocyte growth and throughout subsequent growth and acquisition of competence. In partially competent oocytes, which can enter metaphase but cannot complete the first meiotic division, both $p42^{ERK2}$ and $p44^{ERK1}$ became phosphorylated, as judged by retarded electrophoretic mobility, and a morphologically normal spindle was assembled. In incompetent oocytes, which cannot enter metaphase, $p42^{ERK2}$ and $p44^{ERK1}$ remained non-phosphorylated. When these oocytes were treated with okadaic acid, an inhibitor of protein phosphatases 1 and 2A, they entered metaphase and the slow-migrating phosphorylated forms of $p42^{ERK2}$ and $p44^{ERK1}$ were observed. These phosphorylated forms appeared more rapidly, relative to the time of germinal vesicle breakdown, than during maturation of fully competent oocytes. These results suggest that the acquisition of meiotic

competence during oocyte growth is not linked to the *de novo* appearance of $p42^{ERK2}$ or $p44^{ERK1}$, that the failure of partially competent oocytes to complete meiosis I reflects a defect acting downstream or independently of MAP kinase phosphorylation, and that meiotically incompetent oocytes contain both okadaic acid-sensitive phosphatases that directly or indirectly maintain $p42^{ERK2}$ and $p44^{ERK1}$ in non-phosphorylated state and kinases that can generate the phosphorylated forms when these phosphatases are inhibited.

INTRODUCTION

During mammalian embryogenesis, oogonia proliferate mitotically until a few days after they have colonized the genital ridge, then enter the meiotic cell cycle and progress to the diplotene stage of prophase I where the cycle becomes arrested. Beginning shortly after birth and continuing throughout reproductive life, groups of diplotene-arrested oocytes then enter a growth phase during which the diameter increases from about 12 μm to about 80 μm, representing a 300-fold volume increase. Following gonadotrophic stimulation of the follicle, fully grown oocytes are released from cell cycle arrest and undergo meiotic maturation. During maturation, oocytes enter metaphase, complete the first meiotic division, and become arrested at metaphase of the second meiotic division (Schultz, 1986; Wassarman, 1988).

When fully grown oocytes are removed from the follicular environment and placed in culture, meiotic maturation occurs in the absence of gonadotropins. Such *in vitro*-matured oocytes are normal, as judged by their ability after fertilization to give rise to live animals (Eppig and Schroeder, 1989). The phenomenon of *in vitro* maturation has been exploited to investigate whether non-fully grown oocytes possess the capacity to undergo meiotic maturation (Sorensen and Wassarman, 1976; Albertini, 1992). In the mouse, oocytes smaller than 60 μm in diameter remain arrested at prophase I when

placed in culture. These oocytes are defined as meiotically incompetent. Slightly larger oocytes enter metaphase but become arrested prior to completion of the first meiotic division, and are termed partially competent. Oocytes larger than about 70 μm are able to complete maturation to metaphase II *in vitro*, and are termed fully competent (Sorensen and Wassarman, 1976; Albertini, 1992). These results indicate that meiotic competence, which represents the ability to undergo meiotic maturation, is progressively acquired during oocyte growth.

The acquisition of meiotic competence by growing oocytes appears to be controlled by both an interaction with the surrounding cumulus granulosa cells and an autonomous intracellular program (Canipari *et al.*, 1984; Chesnel *et al.*, 1994). The role of the cumulus cells in promoting competence does not require gap junctional communication between the two cell types (Bachvarova *et al.*, 1980; Canipari *et al.*, 1984), suggesting this influence is mediated through secreted molecules. The oocyte-autonomous events leading to competence are largely unknown. Several proteins, including tubulin, LDH, and histone H4, accumulate in growing oocytes (Schultz *et al.*, 1979; Wassarman and Mrozak, 1981; Roller *et al.*, 1989). As well, a 28-kD protein has recently been identified whose synthesis is linked to the acquisition of competence (Chesnel *et al.*, 1994). Few other major qualitative changes in the pattern of protein synthesis occur during this time (Schultz *et al.*, 1979; Schultz, 1986;

Wassarman, 1988). Elevation of intracellular cyclic AMP also promotes the acquisition of competence (Chesnel *et al.*, 1994).

Several observations suggest that changes in microtubular morphology or activity may play an important role in the acquisition of meiotic competence (van Blerkom, 1991; Albertini, 1992). Incompetent oocytes contain non-phosphorylated microtubule-organizing centres (MTOCs) and an interphasic network of microtubules radiating from the MTOCs. By contrast, prophase-arrested competent oocytes contain phosphorylated MTOCs which nucleate much shorter microtubules that are perinuclear in location (Wickramasinghe *et al.*, 1991; Wickramasinghe and Albertini, 1992). These changes may be regulated in part by the product of the *c-mos* proto-oncogene, $p39^{c\text{-}mos}$, which is required during oocyte maturation in amphibians (Sagata *et al.*, 1988, 1989) and mice (Paules *et al.*, 1989; O'Keefe *et al.*, 1989, 1991). $p39^{c\text{-}mos}$ can associate with and phosphorylate tubulin (Zhou *et al.*, 1991), and is required for spindle function in mouse oocytes (Zhao *et al.*, 1991). $p39^{c\text{-}mos}$ is not synthesized by meiotically incompetent oocytes (Paules *et al.*, 1989).

Another potential regulator of microtubular activity in oocytes is mitogen-activated protein (MAP) kinase. This serine/threonine kinase is present in oocytes of many invertebrate and vertebrate species and, when maturing oocytes enter metaphase, it becomes phosphorylated and active as a kinase (Verlhac *et al.*, 1994). Phosphorylation appears to be mediated through $p39^{c\text{-}}$

mos (Nebreda and Hunt, 1993; Posada *et al.*, 1993). Activated MAP kinase phosphorylates a microtubule-associated protein present in frog eggs (Shiina *et al.*, 1992) and when added to cell-free extracts prepared from frog eggs causes both the growth rate and the steady-state length of microtubules to decrease (Gotoh *et al.*, 1991). In fully grown oocytes of the mouse, two species of MAP kinase are present, $p42^{ERK2}$ and $p44^{ERK1}$ (Sobajima *et al.*, 1993; Verlhac *et al.*, 1993, 1994; Gavin *et al.*, 1994) and, in maturing oocytes, MAP kinase is associated with the MTOCs (Verlhac *et al.*, 1993) that nucleate microtubule assembly at metaphase (Maro *et al.*, 1985; Schatten *et al.*, 1985). Based on the links between altered microtubules and meiotic competence, and between microtubules and MAP kinase, we investigated whether changes in the abundance or phosphorylation of MAP kinase occurred as growing oocytes acquired meiotic competence.

MATERIALS AND METHODS

Collection and culture of oocytes

CD-1 mice (originally obtained from Charles River, Canada) were used in all experiments. To obtain immature oocytes from females aged between 5 and 12 days, the ovaries were placed in phosphate-buffered saline (PBS) devoid of calcium and magnesium, shredded into several pieces, and transferred into PBS containing 0.1% collagenase IV (Gibco, Canada), 0.1% DNase (Gibco, Canada). The fragments were agitated using a mechanical shaker for 30-45 minutes at 37°C, following which the preparation was transferred into a petri dish and the enzymatic solution was diluted with a Hepes-buffered minimum essential medium (MEM-H) modified as described (Schroeder and Eppig, 1984) in order to arrest further digestion. Immature oocytes recognized by the presence of the germinal vesicle (GV) were collected under a dissecting microscope using a hand-pulled glass pipette. They were either cultured in modified MEM supplemented with 0.3% bovine albumin serum (BSA, fraction V, Sigma) in an atmosphere of 5% CO_2 in air, or lysed immediately for electrophoresis.

Growing and fully grown immature oocytes were obtained from females aged between 13-30 days by puncturing medium and large size follicles as described previously (Harrouk & Clarke, 1993). Typically, two mice were used in an experiment. Following sacrifice, the ovaries were dissected and placed

in minimum essential medium (MEM) buffered using Hepes (pH 7.4) and containing sodium pyruvate (25μM), antibiotics and bovine serum albumin (BSA, 3μg/ml, fraction V, Sigma) (Schroeder and Eppig, 1984). After removing the adhering fat and blood, ovaries were shredded into small fragments, which were examined individually for the presence of medium and large size follicles. Using forceps and a 30G½ needle, these follicles were punctured to release the immature oocytes, which were easily recognized under the dissecting microscope by the presence of the germinal vesicle. In a typical experiment, the yield was about 100 healthy immature oocytes. After several washes to remove any attached cumulus cells, the immature oocytes were transferred using a mouth-controlled drawn glass pipet into a plastic dish (Nunc) containing MEM buffered using $NaHCO_3$ and supplemented with fetal bovine serum. The medium was covered with paraffin oil and incubated at 37°C in an atmosphere of 5% CO_2 in air.

Depending on the experiment to be carried out, oocytes were either cultured for 3h to allow germinal vesicle breakdown (GVBD) to occur and only those that underwent GVBD following 3h of culture were cultured overnight to complete maturation. GV oocytes were either lysed or fixed following their isolation immediately.

Immunoblotting

Oocytes at the appropriate experimental stage were lysed in a gel loading buffer (Harlow and Lane, 1988) keeping the number of oocytes per μl approximately constant, heated at 85°C for 10 minutes, chilled on ice for 10 minutes, centrifuged, and stored at -70°C until use. The proteins in the samples were separated by electrophoresis in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Harlow and Lane, 1988) for 1 h at 200 volts, electrically transferred to nitrocellulose membranes (Schleicher & Schuell, pore size 0.5 μm) for 1.5 h, 80 volts, at 4°C. The blot was left to dry overnight and the gel was stained to verify the success of the transfer. All immunoblotting steps from blocking the membrane onwards were carried out 0at room temperature with gentle agitation. The membrane was blocked by soaking for 2h in a 3% powdered skim milk in 10 mM Tris (pH 7.5), 140 mM NaCl (TBS). The primary antibody anti-MAP kinase 691, (Santa Cruz Biotech, California) was diluted 1:500 in blocking buffer, applied to the blot and agitated overnight. Following three washes of 15 minutes each in TBS containing 0.1% Tween-20 (TBST), a biotinylated secondary antibody (Jackson Immunoresearch Laboratories) was applied to the blot at a dilution of 1:200 in TBST, 3% BSA. After 3 washes in TBST, the membrane was incubated in streptavidin-conjugated alkaline phosphatase (Jackson Immunoresearch Laboratories) diluted

1:500 in TBST, 3% BSA. After 3 washes in TBST, the bound antibody was revealed using the NBT/BCIP reagents diluted in appropriate buffer (Harlow & Lane, 1988).

Immunofluorescence

Oocytes were denuded of their zonae pellucidae by exposing them briefly to acidified (pH 2.5) Tyrode's medium. Once the zonae had disappeared, the oocytes were washed several times in MEM-H and then fixed in a freshly prepared solution of 2% paraformaldehyde in PBS for 15 minutes at room temperature. The fixed oocytes were then permeabilized using 0.5% Triton-X 100 in PBS for 15 minutes, following which they were blocked for 15 minutes in a solution containing PBS, 3% BSA, 10% goat serum, 0.1% Tween-20. The primary antibody (mouse anti-α-tubulin, Cedarlane Laboratories), diluted at 1:5000 in the blocking buffer, was applied for 3 h at room temperature. After 3 washes of 15 minutes each in blocking buffer, the oocytes were then transferred into an FITC-labeled anti-(mouse IgG) secondary antibody (Jackson Immunoresearch Laboratories) diluted at 1:100 in blocking solution, and containing the DNA stain, DAPI (1 μg/ml). Following 2 washes in the blocking buffer, the oocytes were transferred into a drop of Moviol (Hoechst) on siliconized microscope slides. The oocytes, which tend to float to the surface of the Moviol drop, were then picked up using the micropipette, moved down

to the bottom, and gently covered with a glass coverslip. Preparations were examined using a Leitz Laborlux S microscope equipped with ultraviolet and fluorescein filters. Photographs were taken using Kodak TMAX 100.

Drugs

Okadaic acid (Gibco, Canada) was prepared as a stock solution of 100 μM and used at a final concentration of 1 μM.

RESULTS

Meiotic competence of growing oocytes

During the reproductive life of the mouse, only a small number of oocytes are growing at a particular time, so it is difficult to collect large numbers of oocytes at different stages of growth. Shortly after birth, however, a large group of oocytes initiate and progress synchronously through growth and acquisition of meiotic competence (Sorensen and Wassarman, 1976; Albertini, 1992). Oocytes collected from mice up to 12 days of age are meiotically incompetent. Oocytes obtained from slightly older mice are partially competent, being able to undergo GVBD but not to complete the first meiotic division in culture. Many oocytes from mice older than 18 days are able to complete maturation to metaphase II in culture. Thus, ovaries harvested from prepuberal mice at different ages provide a convenient source of oocytes at specific stages of growth and meiotic competence.

To test the relationship between mouse age and oocyte meiotic competence in our colony, growing oocytes were collected either by enzymatic digestion of ovaries of 5- to 12-day mice or by puncture of ovarian follicles of 13- to 30-day-old mice. The oocytes were incubated for 18 h, and then classified into one of 3 categories which are easily distinguished under the dissecting microscope: Germinal vesicle (GV) stage, where the nucleus is

prominently observed; germinal vesicle breakdown (GVBD) stage, where the nucleus is no longer visible; and metaphase II, where the first polar body is seen protruded from the plasma membrane (Figure 1).

The results (Table 1) show that the majority of oocytes isolated from mice younger than 14 days of age remained arrested at the GV stage in culture, indicating that they were meiotically incompetent. The small percentage of oocytes isolated from the 13-day animals that underwent GVBD in culture were larger in diameter than the rest of the oocytes in this group (data not shown). Mice between 14 and 16 days of age contained all three types of oocytes. In particular, these mice contained relatively large numbers of partially competent oocytes, which underwent GVBD but failed to complete meiosis I (Table 2). Finally, most oocytes obtained from mice older than 17 days were able to reach metaphase II in culture. These results confirm previous reports (Sorensen and Wassarman, 1976) and demonstrate that a population of oocytes undergoes a transition from a meiotically incompetent state in mice younger than 14 days to a partially competent state at 14-16 days and become fully competent by 17 days of age.

Expression of MAP kinase in meiotically incompetent and competent oocytes

Having established that relatively homogeneous populations of oocytes at different stages of meiotic competence can be obtained from mice during the

first three weeks after birth, we examined the abundance of MAP kinase in these oocytes. One hundred oocytes at the GV-stage were collected from 5, 8, 10, 15, and 18-day mice. One hundred oocytes either at GV or GVBD stage were collected from 20-day mice. Lysates were prepared, subjected to SDS-PAGE, electrically tranferred onto nitrocellulose membranes, and immunoblotted using an affinity-purified antibody raised against subdomain XI of the rat *ERK1* gene product (Boulton and Cobb, 1991).

Two doublets of approximate M_r 42 and 44 kDa were present in oocytes of 20-day mice (Figure 2). Based on previous results from our laboratory (Verlhac *et al.*, 1993) and others (Sobajima *et al.*, 1993; Gavin *et al.*, 1994), these represent MAP kinase encoded by the mouse *ERK2* and *ERK1* genes, respectively, and will be referred to as $p42^{ERK2}$ and $p44^{ERK1}$. The slower migrating form of each doublet represents the phosphorylated protein, while the faster migrating form is the non-phosphorylated protein (Posada and Cooper, 1992; Verlhac *et al.*, 1993, 1994). The presence of both phosphorylated and non-phosphorylated $p42^{ERK2}$ and $p44^{ERK1}$ in the 20-day sample is due to the fact that it contained both GV- and GVBD-stage oocytes.

Oocytes collected from mice between 5 and 18 days also contained immunoreactive species of approximate M_r 42 and 44 kDa. As no other immunoreactive species were regularly detected, we conclude that these represent $p42^{ERK2}$ and $p44^{ERK1}$. All oocytes in these samples were at the GV-

stage and only the fast-migrating, non-phosphorylated forms of the proteins were present. Comparison of the intensity of staining between lanes, which each contained the same number of oocytes, suggests that the amount of $p42^{ERK2}$ and $p44^{ERK1}$ increased during oocyte growth. As noted in the Discussion, total protein content also increases during growth. These results clearly indicate that $p42^{ERK2}$ and $p44^{ERK1}$ are present beginning early during oocyte growth, and several days before growing oocytes acquire partial or full meiotic competence.

Phosphorylation of MAP kinase and spindle formation in partially competent oocytes

As discussed above, growing oocytes pass through a stage of partial meiotic competence, when they can enter metaphase but cannot complete meiosis I. These oocytes assemble a spindle, indicating that the point of arrest lies at the metaphase-anaphase transition (Sorensen and Wassarman, 1976; Wickramasinghe *et al.*, 1991). The results of the previous section indicated that the inability of partially competent oocytes to complete meiosis I was not correlated with an absence of MAP kinase. We next examined whether MAP kinase became phosphorylated in metaphase I-arrested oocytes.

Oocytes were collected from 14- to 16-day mice, when the partially competent oocytes are most abundant (Table 1, Table 2). They were incubated

for 6, 9, or 18 h and then separated into groups according to their stage of meiotic maturation. A portion of each group was fixed for immunofluorescent analysis of spindle configuration, while the remainder was used for immunoblotting.

Oocytes examined after 6 h or 9 h of incubation had entered metaphase as is shown by immunofluorescence (Figure 3) and contained predominantly the slow-migrating, phosphorylated forms of $p42^{ERK2}$ and $p44^{ERK1}$ (Figure 4A, lanes 2, 3). Based on the results shown in Table 1, these samples contained a mixture of partially competent oocytes, which would arrest at metaphase I, and fully competent oocytes which would progress to metaphase II. These could not be morphologically distinguished at this time. After 18 h of incubation, however, the partially competent oocytes could be identified by their failure to emit the first polar body. Immunofluorescent analysis confirmed that these contained a well-formed spindle (Figure 4B). These oocytes, as well as those that progressed to metaphase II, contained predominantly the phosphorylated forms of $p42^{ERK2}$ and $p44^{ERK1}$ (Figure 4A, lanes 4 & 5). These results confirmed that partially competent oocytes become arrested at metaphase I of maturation and showed that MAP kinase became phosphorylated in these oocytes.

Okadaic acid-induced phosphorylation of MAP kinase in incompetent oocytes

To further investigate the relationship between MAP kinase and meiotic

competence, we examined whether $p42^{ERK2}$ and $p44^{ERK1}$ could become phosphorylated in meiotically incompetent oocytes. Incompetent and prophase-blocked oocytes can be induced to enter metaphase by exposure to okadaic acid, an inhibitor of type 1 and type 2A phosphatases (Rime & Ozon, 1990; Alexandre *et al.*, 1991; Gavin *et al.*, 1991; Schwartz & Schultz, 1991). We examined whether MAP kinase became phosphorylated following okadaic acid treatment of incompetent oocytes.

Oocytes were collected by follicular puncture from 14- to 16-day mice and incubated for 9 h. Those that remained at the GV stage during this period were defined as meiotically incompetent. One portion was lysed immediately for immunoblotting and the other portion was incubated for an additional 9 h in the presence of 1 μM okadaic acid. By the end of this second incubation, all of the oocytes had undergone GVBD, although none had formed a polar body. Immunoblotting revealed that the GV-arrested oocytes collected after 9 h incubation contained the fast-migrating, non-phosphorylated forms of $p42^{ERK2}$ and $p44^{ERK1}$, whereas the okadaic acid-treated oocytes contained the slow-migrating, phosphorylated forms (Figure 5A, lanes 1, 2).

To examine more closely the kinetics of the okadaic acid-induced mobility shift, the following experiment was performed. Oocytes were isolated from 14- to 16-day mice, incubated for 3 h, and those that remained at the GV stage were selected. These oocytes were incubated in medium containing okadaic

acid and checked every 30 minutes for GVBD. Under these conditions, GVBD occurred in 48% of the oocytes after 2 h of incubation (data not shown). These oocytes displayed certain morphological abnormalities, particularly loss of spherical shape (data not shown). Aliquots of these oocytes were collected for immunoblot analysis at 2, 4, 6, and 18h after the start of the drug treatment.

As shown in Figure 5B (lanes 1, 2), the incompetent oocytes that underwent GVBD during 2 h exposure to okadaic acid contained both the fast-migrating and the slow-migrating forms of $p42^{ERK2}$ and $p44^{ERK1}$. By contrast, oocytes that remained at the GV stage in the presence of the drug contained only the fast-migrating, non-phosphorylated forms. Oocytes that underwent okadaic acid-induced GVBD contained both fast- and slow-migrating forms of MAP kinase after 4, 6, and 9 h of incubation. These results indicate that when incompetent oocytes are exposed to okadaic acid, a portion of $p42^{ERK2}$ and $p44^{ERK1}$ becomes phosphorylated within 2 h.

Immunofluorescent analysis using anti-α-tubulin of the incompetent oocytes exposed to okadaic acid for 15 h indicated that no spindle was assembled (Figure 5C), which is consistent with previous reports (Rime & Ozon, 1990; Alexandre *et al.*, 1991). The chromatin was condensed and clumped into several aggregates.

Expression of MAP kinase in meiotically competent bovine oocytes

In order to test whether bovine oocytes contain similar species of MAP kinase as seen in mouse oocytes, fifty GV bovine oocytes and forty GV mouse oocytes were lysed, separated on SDS gels, followed by immunoblotting using the 691 antibody described above.

Three separate bands were detected in the bovine sample. The two top bands migrated at the same level as p42 and p44 of the mouse sample, suggesting that MAP kinase is present in the bovine oocyte and has the same molecular weight as in the mouse oocyte. A third band which is absent in the mouse oocyte was also detected in the bovine oocyte. This band migrated faster than the other two and corresponds to a protein of approximately 40 kDa (Figure 6A). All three bands represented the unphosphorylated species of bovine MAP kinase.

To look at the phosphorylation pattern of bovine MAP kinase, fifty oocytes were lysed at three time points of maturation, 0h, 12h, and 18h which represent the GV, metaphase I, and metaphase II stages, respectively. Samples were subjected to SDS-PAGE, electrically transferred onto nitrocellulose membranes, and immunoblotted using the 691 antibody described above. All three bands were detected in each lane at each time point. In addition, the two upper bands, presumably the p42 and p44 isoforms, contained doublets where each isoform had both the phosphorylated and the unphosphorylated forms of

the protein as judged by the slower mobility shift on the gel. In addition, a shift in the p42 band from the unphosphorylated, fast migrating form in the metaphase I sample (Figure 6B, lane 2), to the phosphorylated, slow migrating form in the metaphase II sample (Figure 6B, lane 3) is observed. In contrast to the mouse oocyte where the switch occurs in both species at the same time, the bovine forms of both p42 and p44 are switched from the unphosphorylated to the phosphorylated during the progression from metaphase I to metaphase II. This observation might have some involvement in the stepwise process of meiotic maturation in the bovine oocyte.

DISCUSSION

It is well-established that as mammalian oocytes progress through the growth phase of development they acquire the ability to undergo meiotic maturation (Sorensen and Wassarman, 1976; Albertini, 1992), but the molecular basis of this meiotic competence is unknown. We investigated whether the acquisition of competence might be linked to changes in the quantity or phosphorylation pattern of the two major MAP kinase species present in mouse oocytes, $p42^{ERK2}$ and $p44^{ERK1}$. To this end, we collected oocytes from mice aged between 5 days and 21 days post-partum, when a large population of oocytes progress synchronously through growth. Oocytes obtained from mice up to 13 days of age were meiotically incompetent, whereas as those obtained from older mice were partially or fully competent, consistent with the previous results. Nevertheless, both MAP kinase species were detectable in oocytes obtained from mice as young as 5 days of age. These results indicate that the acquisition of competence is not associated with the *de novo* appearance of $p42^{ERK2}$ or $p44^{ERK1}$.

During oocyte growth, the quantity of $p42^{ERK2}$ and $p44^{ERK1}$ increased as judged by the intensity of staining in immunoblots. The amount of each relative to the other appeared to remain constant during growth, which suggests that their synthesis is co-ordinately controlled. As increases in the quantity of

several other proteins and in the total protein content also occur during growth (Schultz, 1986; Wassarman, 1988), the present results therefore do not indicate whether the concentration of $p42^{ERK2}$ and $p44^{ERK1}$ change during growth. It is possible that changes in the concentration of $p42^{ERK2}$ or $p44^{ERK1}$, which would not be detected by our methods, are critical for meiotic competence. It may be more likely, however, that these proteins accumulate steadily during oocyte growth but do not directly regulate meiotic competence.

Previous results (Sorensen and Wassarman, 1976; Wickramasinghe and Albertini, 1992), confirmed in the present study, showed that meiotic competence is acquired in two stages: first, the ability to enter metaphase and second, the ability to complete the first meiotic division. We observed that $p42^{ERK2}$ and $p44^{ERK1}$ became phosphorylated in partially competent oocytes, which can enter metaphase but cannot complete meiosis I. This resembles the situtation in fully competent oocytes, where $p42^{ERK2}$ and $p44^{ERK1}$ become phosphorylated during maturation by means of a process requiring protein synthesis (Verlhac *et al.*, 1993; Gavin *et al.*, 1994). These results considered together imply that partially competent oocytes possess the capacity to synthesize those proteins required to generate phosphorylated $p42^{ERK2}$ and $p44^{ERK1}$. The nature of these proteins is unknown, but could include upstream activators of MAP kinase such as MEK, Raf, or MEK kinase (Roberts, 1992; Lange-Carter *et al.*, 1993). We conclude that the failure of partially competent

oocytes to complete meiosis I is not due to an inability to generate phosphorylated MAP kinase, but rather to a defect acting downstream or independently.

It should be noted, however, that the partially competent oocytes were not examined until after 18 h of culture. As phosphorylation of $p42^{ERK2}$ and $p44^{ERK1}$ normally occur after 2 to 4 h of culture (Sobajima *et al.*, 1993; Verlhac *et al.*, 1993; Gavin *et al.*, 1994), it is possible that this event is delayed in partially competent oocytes. This is difficult to test, because partially competent oocytes cannot be identified until about 12 h of culture when fully competent oocytes emit the first polar body. If phosphorylation were delayed in partially competent oocytes, this might disrupt the synchrony of timing between MAP kinase phosphorylation and another event required for meiosis I.

We also observed that the $p42^{ERK2}$ and $p44^{ERK1}$ in meiotically incompetent oocytes could become phosphorylated, as judged by retarded electrophoretic mobility, when these oocytes were treated with the inhibitor of protein phosphatase types 1 and 2A, okadaic acid. This drug also induces meiotically incompetent oocytes to enter metaphase (our results, Gavin *et al.*, 1991; Chesnel *et al.*, 1994) and activates MAP kinase in fully competent oocytes (Gavin *et al.*, 1994), extracts prepared from oocytes (Nebreda and Hunt, 1993), and quiescent fibroblasts (Gotoh *et al.*, 1990). Two inferences may be drawn

from the observation that phosphorylated forms of p42^{ERK2} and p44^{ERK1} were observed in the okadaic acid-treated incompetent oocytes. First, incompetent oocytes apparently possess type 1 or type 2A phosphatases whose activity is required to maintain MAP kinase in a non-phosphorylated state. Second, they also possess protein kinases that generate phosphorylated MAP kinase when the phosphatase activity is inhibited. One possibility is that both kinase and phosphatase act directly on p42^{ERK2} and p44^{ERK1}, the predominance of the non-phosphorylated form being due to relatively higher activity of the phosphatase. Phosphatases specific for MAP kinase have recently been identified (Keyse and Emslie, 1992; Charles *et al.*, 1992; Sun et al, 1993; Zheng and Guan, 1993; Ward *et al.*, 1994). However, these dual-specific threonine/tyrosine phosphatases may not be sensitive to okadaic acid, which does not inhibit tyrosine phosphatase (Hardie *et al.*, 1991), thus making it unlikely that the drug acts on a MAP kinase-specific phosphatase. Alternatively, one of the components of the MAP kinase phosphorylation pathway, such as a MAP kinase kinase (Gómez and Cohen, 1991), may be kept inactive in incompetent oocytes by an okadaic acid-sensitive phosphatase.

Although p42^{ERK2} and p44^{ERK1} became phosphorylated in meiotically incompetent oocytes when phosphatase activity was inhibited, this may not reflect the natural mechanism that operates during meiotic maturation of competent oocytes. We observed that okadaic acid-induced phosphorylation

of $p42^{ERK2}$ and $p44^{ERK1}$ had occurred by 2 h of culture (see also Gavin *et al.*, 1994). By contrast, their phosphorylation during meiotic maturation requires between 2 and 4 h of culture (Verlhac *et al.*, 1993). Also, okadaic acid-induced phosphorylation can precede entry into metaphase (Gavin *et al.*, 1994), whereas phosphorylation during mammalian meiotic maturation can occur only after entry into metaphase (Sobajima *et al.*, 1993; Verlhac *et al.*, 1993). Similarly, when $p21^{ras}$ is injected into immature Xenopus oocytes, MAP kinase is activated more rapidly, relative to the activation of maturation-promoting factor, than during progesterone-induced maturation (Nebreda *et al.*, 1993). Perhaps okadaic acid and $p21^{ras}$ are able to activate MAP kinase through a pathway that is different from that used during physiological maturation.

Our results indicate that the acquisition of meiotic competence is not directly linked to substantial changes in the quantity or phosphorylation patterns of MAP kinase. This suggests that the differences in microtubular morphology between incompetent and competent oocytes are not directly regulated by MAP kinase. To address whether MAP kinase is required for the microtubular rearrangements during meiotic maturation, it will be necessary to eliminate this activity from meiotically competent oocytes. Additionally, however, it is possible that MAP kinase plays a role in oocyte growth. Oocyte growth requires products of the somatic cells that surround the oocyte (Buccione *et al.*, 1990; Eppig, 1991). Among these is the c-*kit* ligand (Manova

et al., 1993), which could interact with the c-*kit* receptors present in the oocyte (Horie *et al.*, 1991; Keshet *et al.*, 1991), and recent evidence suggests a direct role for c-*kit* ligand in stimulating oocyte growth (Packer *et al.*, 1994). MAP kinase is a component of the pathway by which ligand-stimulated c-*kit*, like other tyrosine kinase receptors, transmits a signal to the cell nucleus (Pelech and Sanghera, 1992). Given that $p42^{ERK2}$ and $p44^{ERK1}$ were present in oocytes at all stages of growth examined, MAP kinase may be required for the large and rapid increase in cell size that occurs during the growth phase. This hypothesis could be tested by depleting $p42^{ERK2}$ and $p44^{ERK1}$ in growing oocytes.

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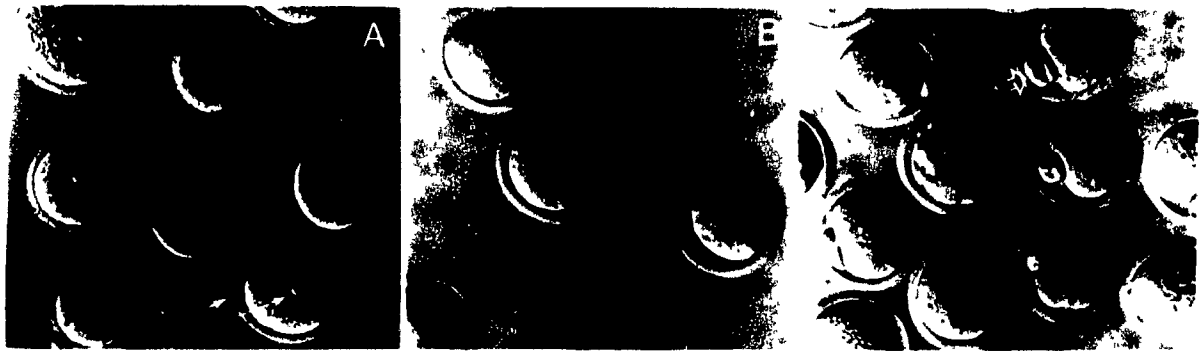

A
B

Figure 1. Photomicrographs of fully grown mouse oocytes collected from antral follicles and cultured *in vitro*. A, B, C, oocytes at the dictyate, metaphase I, and metaphase II stages, respectively. GV, germinal vesicle, ZP, *zona pellucida*, PB, polar body. Bar = 10 mm.

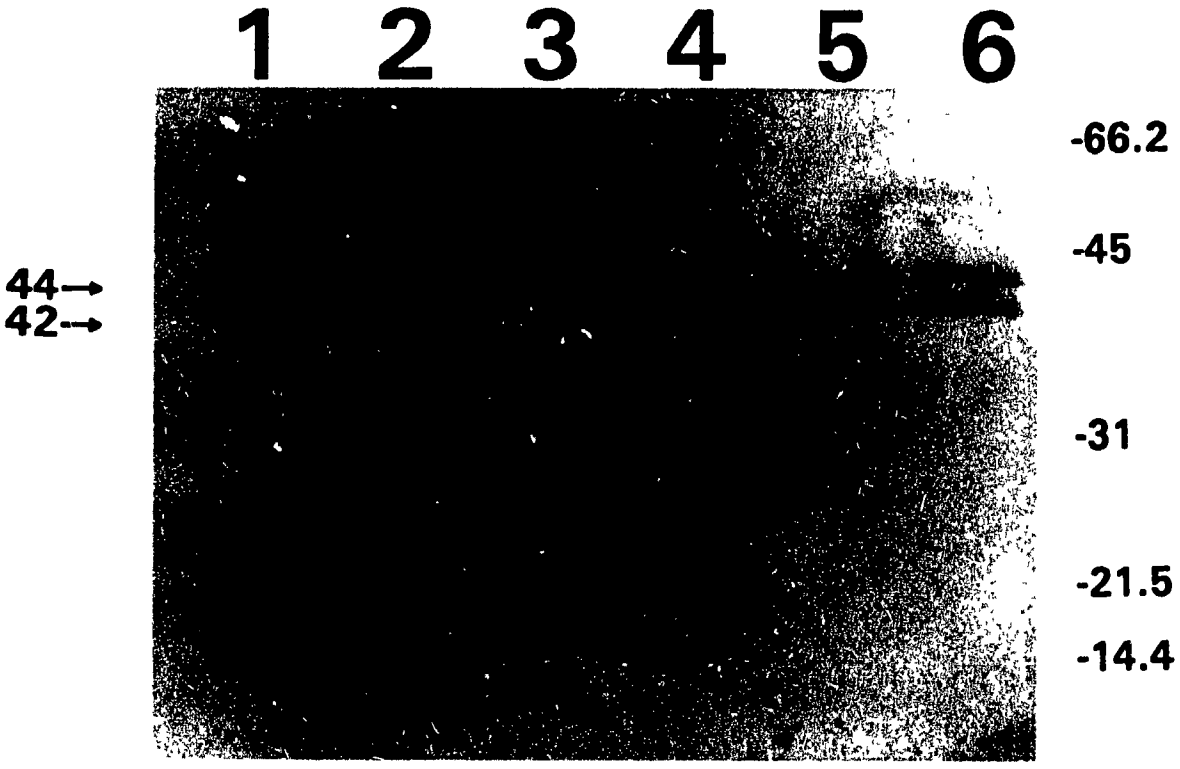

1 2 3 4 5 6
-66.2
-45
44→
42→
-31
-21.5
-14.4

Figure 2. $p42^{ERK2}$ and $p44^{ERK1}$ in meiotically incompetent and competent mouse oocytes. Ovarian oocytes were collected from mice of different ages and immunoblotted using antibody 691 raised against subdomain XI of the rat ERK1 gene product. All oocytes were at the GV-stage, except the 20-day group which contains a mix of GV- and GVBD-stage oocytes. Each lane contains 100 oocytes. Lane 1, 20-day mice. Lane 2, 18-day mice. Lane 3, 15-day mice. Lane 4, 10-day mice. Lane 5, 8-day mice. Lane 6, 5-day mice.

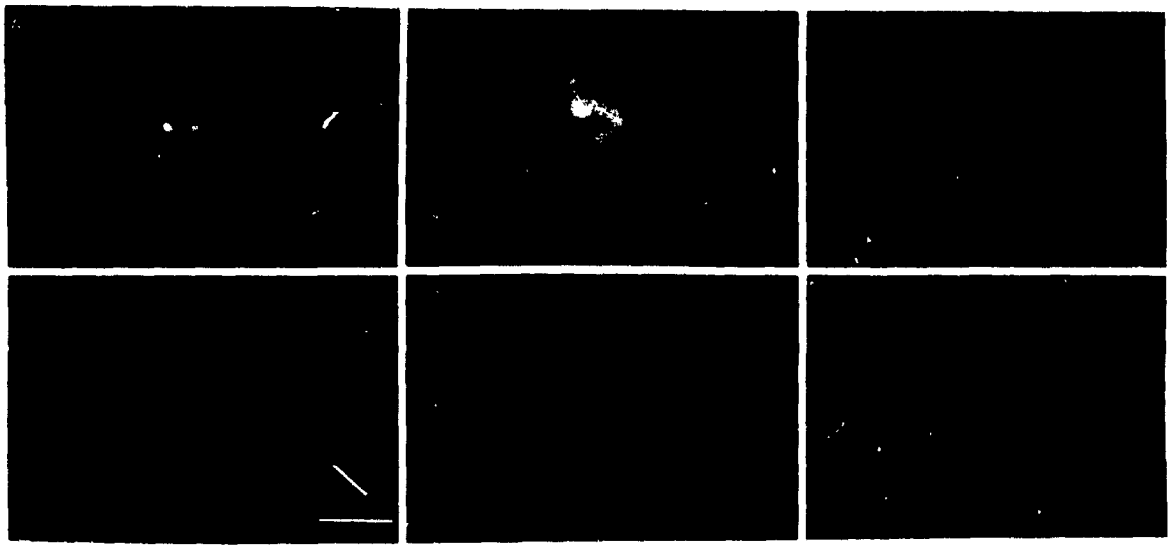
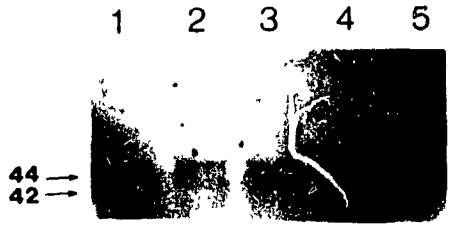


Figure 3. Immunofluorescent analysis of the mouse oocytes during given stages of meiotic maturation. Oocytes cultured for 18h in MEM were stained with Hoechst (A, B, C) and reacted with anti-tubulin antibodies (A', B', C') at these stages: A, A', an oocyte that arrested at the GV stage. B, B', an oocyte that underwent GVBD but failed to complete maturation, termed partially-competent. C, C', an oocyte that completed maturation and arrested at metaphase II. Bar = 10 mm.

A



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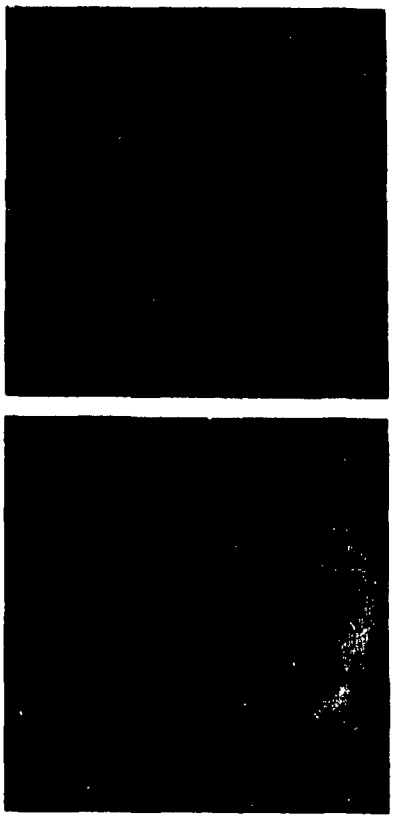
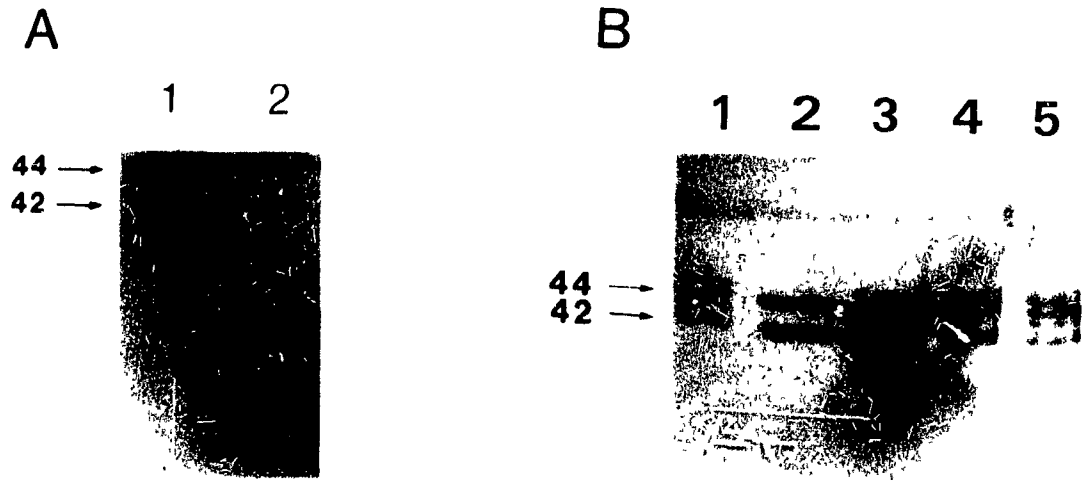


Figure 4. Phosphorylation of $p42^{ERK2}$ and $p44^{ERK1}$ and spindle formation in partially competent oocytes. (A). Immunoblot. Ovarian oocytes were collected from 14- to 16-day mice, incubated for the indicated period of time, and immunoblotted using antibody 691. Each lane contains 50 oocytes. Lane 1, 18 h, all oocytes contained a GV. Lane 2, 6 h, all oocytes had undergone GVBD. Lane 3, 9 h, all oocytes had undergone GVBD. Lane 4, 18 h, all oocytes had undergone GVBD. Lane 5, 18 h, all oocytes had emitted the first polar body. (B). Immunofluorescence. Ovarian oocytes were incubated for 18 h and those that underwent GVBD but did not emit a polar body were fixed and stained using an anti-α-tubulin antibody (bottom). Chromosomes were stained using DAPI (top). Bar = 8 mm.


A
1
2
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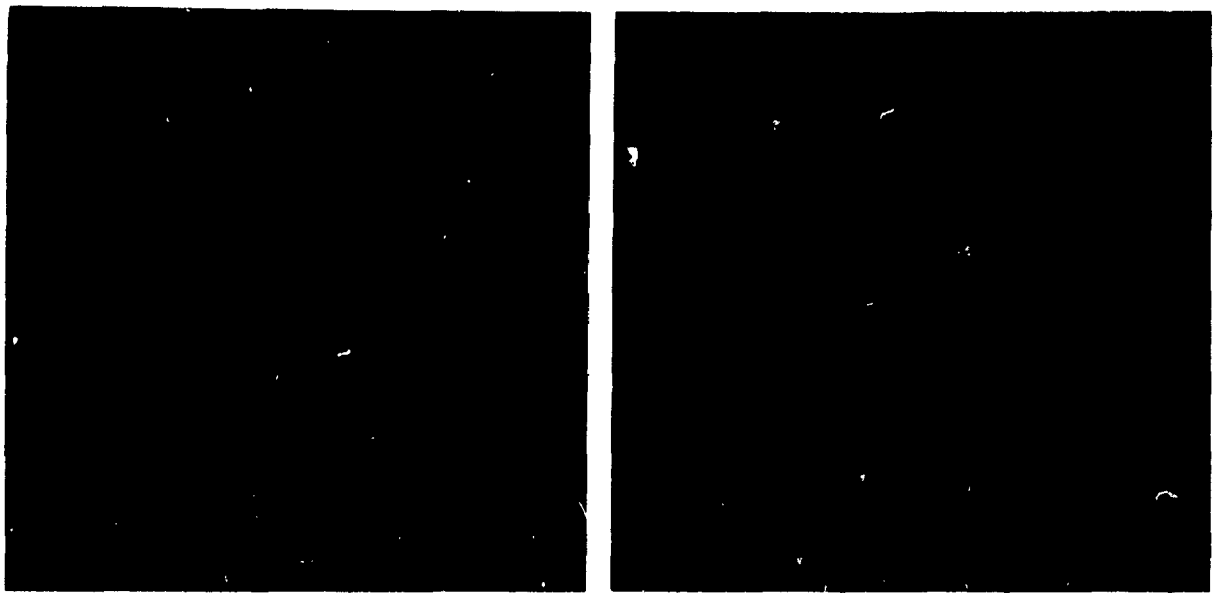

Figure 5. Effect of okadaic acid on meiotically incompetent oocytes. (A). Phosphorylation of $p42^{ERK2}$ and $p44^{ERK1}$. Ovarian oocytes were collected from 14- to 16-day mice, incubated for 9 h, and those that remained at the GV stage were selected. One portion was prepared for electrophoresis (left lane); all oocytes contained a GV. The other portion was incubated in the presence of 1 μM okadaic acid for 9 h and then prepared for electrophoresis (right lane); all oocytes had undergone GVBD. Immunoblotting was performed using antibody 691. Each lane contains 50 oocytes. (B). Timing of phosphorylation. Ovarian oocytes were collected as before and incubated for 3 h. Those that remained at the GV stage were exposed to 1 μM okadaic acid for the indicated length of time and immunoblotted using antibody 691. All oocytes in lanes 1, 3, 4, 5 underwent GVBD; all oocytes in lane 2 remained at the GV-stage. Lane 1, 2 h. Lane 2, 2 h. Lane 3, 4 h. Lane 4, 6 h. Lane 5, 9 h. Each lane contains 50 oocytes. (C). Immunofluorescence. Ovarian oocytes were incubated for 3 h and those that remained at the GV stage were incubated for an additional 15 h in the presence of 1 μM okadaic acid, fixed and stained using an anti-α-tubulin antibody (right). Chromosomes were stained using DAPI (left).

A

B

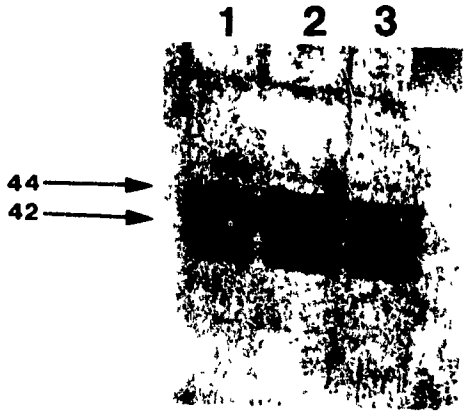

Figure 6. $p42^{ERK2}$ and $p44^{ERK1}$ in meiotically competent bovine oocytes. A, a sample of 40 fully grown mouse oocytes (Lane 1) and 50 fully grown bovine oocytes (Lane 2) were immunoblotted using antibody 691. In both samples, oocytes were at the GV stage. B, Phosphorylation of p42 and p44 in meiotically competent bovine oocytes. Each lane contained 50 oocytes. Bovine oocytes were lysed at 0, 12, and 24h following culture in MEM corresponding to GV, metaphase I, and metaphase II stages of meiotic maturation, respectively. Lane 1, all oocytes were at the GV stage. Lane 2, all oocytes had undergone GVBD. Lane 3, all oocytes had emitted a polar body.

TABLE 1

THE EXTENT OF MEIOTIC MATURATION IN GROWING MOUSE OOCYTES IN RELATION TO THE AGE OF THE MOUSE.

Age (Days)	Total number of eggs	GV (%)	GVBD (%)	PB I (%)
10	106	100	0	0
13	32	94	6	0
14	110	55	16	29
15	100	31	42	27
16	100	41	7	52
17	166	18	2	80
18	100	8	6	86

TABLE 2

PARTIALLY-COMPETENT OOCYTES IN GROWING MOUSE OOCYTES.

Duration of culture (h)	Total number of GVBD oocytes	Metaphase I (%)	Metaphase II (%)
9	45	87	13
20	64	16	84

CHAPTER III

General Discussion

1. Summary of experimental findings

In this thesis, I have described experiments done using oocytes from the CD-1 mouse strain to investigate some of the changes that may be relevant in elucidating the mechanism underlying the acquisition of meiotic competence in mammalian species.

1.1. Acquisition of meiotic competence during oocyte growth

The mammalian ovary contains at any given time during the life of the female a mixed population of follicle-enclosed oocytes in different stages of development. Basically, two major groups inhabit the ovary, the small nongrowing group of oocytes and those that have initiated their growth phase. The total number of oocytes in the ovary is the largest at the time of birth and it decreases thereafter with age. This decrease in the number of oocytes is partly due to the flow of a group of oocytes which start their growth phase synchronously (Pedersen, 1968). In the mouse, oocytes which start their growth phase shortly after birth reach their full size in about two weeks.

Around the time they reach their full size, oocytes acquire the capacity to undergo meiotic maturation when exposed to a maturation-inducing environment (Sorensen and Wassarman, 1976; Schultz and Wassarman, 1977).

Previous reports have linked the acquisition of meiotic competence to the age of the mouse from which oocytes were isolated. The age of the mouse is directly linked to the size of the growing oocyte which, in turn, is a factor in the acquisition of meiotic competence (Sorensen and Wassarman, 1976, Wickramasinghe *et al.*, 1991).

To establish the synchrony of growth and meiotic competence among oocytes that will be used in answering the proposed questions, the extent of meiotic maturation of oocytes isolated at increasing ages of the juvenile mouse was studied in our breeding colony. This was shown by culturing follicle-free oocytes in culture medium where competent oocytes underwent GVBD within 3h of culture, and assembled their first meiotic spindle 6h later. Within 4h, the second meiotic spindle formed and oocytes completed their meiotic maturation.

Following a culture period of 18h, oocytes obtained from mice younger than 13 days of age were incapable of undergoing GVBD, and are thus said to be meiotically incompetent. Oocytes isolated from mice in the age groups of 14-16 days, were capable of undergoing GVBD but contained a subgroup of oocytes that arrest following the formation of first meiotic spindle and are termed partially competent oocytes. Most oocytes isolated from 17 days or older mice were capable of completing their maturation and extruding their first polar body. Such oocytes are referred to as fully competent.

These results confirmed previous reports and established the presence of a group of oocytes that, during their growth phase, acquire the ability to undergo maturation in a progressive manner in the juvenile mouse.

1.2. Expression of MAP kinase in growing oocytes

Among the features of a growing oocyte is the accumulation of materials that will be needed for fertilization and the early stages of embryogenesis. Such materials include proteins which become expressed after the oocyte starts its growth phase. Among these proteins are the *zona pellucidae* proteins, lactate dehydrogenase and others that are still unidentified.

Previous reports have shown the presence of two species of mitogen-activated protein (MAP) kinase in fully grown mouse oocytes, namely p42 and p44. Upon meiotic resumption, both species become phosphorylated, MAP kinase becomes active as witnessed by its ability to phosphorylate myelin basic protein, and is localized to the MTOCs on both meiotic spindles of meiosis as seen by immunofluorescence (Verlhac *et al.*, 1993).

However, in small and growing oocytes which are incapable of entering metaphase under the appropriate conditions, neither the presence of MAP kinase nor its phosphorylation state has been reported.

Using the western blotting technique, both species of MAP kinase, p42 and p44, were found to be present in oocytes isolated from mice as early as 5

days of age. At this stage, oocytes have just started their growth phase and are incompetent to resume meiosis. As expected, only the nonphosphorylated species of p42 and p44 were detected in oocytes collected from incompetent oocytes. This falls in agreement with previous results where the phosphorylated species of p42 and p44 are not detected in GV-arrested oocytes.

That MAP kinase is present very early during the life of a female eliminates the possibility that MAP kinase belongs to the group of proteins that become expressed only after the oocyte has started its growing phase in preparation for the acquisition of meiotic competence. Alternatively, due to its presence at an early stage of the oocyte development, it would be interesting to hypothesize that MAP kinase is an essential protein in the growth process or such a related event. This hypothesis could be tested by depleting the stock of p42 and p44 from the oocyte and documenting any changes that occur at the level of oocyte growth, maturation or at a later stage of embryonic development.

1.3. MAP kinase in partially competent oocytes

The acquisition of meiotic competence in growing oocytes has been reported to occur in a stepwise process where the first step is acquired when oocytes undergo GVBD and reach metaphase I of meiosis I. The second step

is accomplished when oocytes complete maturation and arrest at metaphase II of meiosis awaiting either fertilization or parthenogenetic activation (Sorensen and Wassarman, 1976). This process seems to be related to the age of the mouse as growing oocytes isolated from mice between 14-16 days of age are enriched for those oocytes that are capable of undergoing GVBD but arrest at metaphase I thus failing to complete maturation. The majority of oocytes isolated from mice 17 days of age or older are capable of completing meiotic maturation. The results shown in the previous section on the extent of meiotic maturation are in accordance with this hypothesis.

Nevertheless, the molecular components regulating the program of partial meiotic competence exhibited by metaphase I-arrested oocytes are not known. One molecule that might be involved in this regulation is MAP kinase which was compared in oocytes with full meiotic competence and those with partial competence. Parameters that were tested in the two groups were the presence or absence of MAP kinase, the phosphorylation pattern of both species of MAP kinase, namely p42 and p44, and the microtubular and chromosomal configurations.

Upon comparing the pattern of expression of MAP kinase in partially competent and fully competent oocytes, the two species, p42 and p44, were both detected in both groups.

Using the retarded mobility shift as an assay to test for the

phosphorylated species, partially competent oocytes were found to contain the phosphorylated, slow-migrating species of the protein similar to metaphase II oocytes. The finding that both species of MAP kinase are present and phosphorylated in metaphase I-arrested oocytes eliminates the implication of MAP kinase in their failure of complete maturation.

Immunofluorescent results comparing the chromosomal and microtubular structures of partially and fully competent oocytes did not reveal any major deficiencies at least at the morphological level. Partially competent oocytes which failed to form a polar body following an 18h culture period contained bivalent chromosomes situated on the equator of a seemingly normal spindle.

Due to the fact that metaphase I-arrested oocytes cannot be identified any earlier than 12h of culture, it is plausible that these oocytes were slower in either their phosphorylation process or in the assembly of their spindle. Alternatively, MAP kinase in metaphase I-arrested oocytes might be regulated differently by upstream kinases or is itself regulating downstream substrates in a different fashion than is usually the situation in fully competent oocytes. To test these hypotheses requires a fine dissection of the upstream regulators and downstream substrates of MAP kinase in a given signal transduction pathway.

Meiotic maturation can be arrested in fully grown, competent mouse oocytes by increasing the levels of PKC and PKA (Alexandre *et al.*, 1991). However, exposure of such oocytes to okadaic acid, a phosphatase inhibitor, releases them from their meiotic arrest. Meiotically incompetent oocytes are naturally arrested at the diplotene stage of meiosis. In this thesis, the capacity of meiotically incompetent oocytes to enter metaphase was tested using okadaic acid. Such oocytes are those that cannot enter metaphase following their release from their surrounding follicles even after a 9h of culture period. In the presence of 1μM okadaic acid, incompetent oocytes underwent GVBD within 9h following their culture. When the oocyte proteins were separated on SDS gels and immunoblotted for MAP kinase, both species of MAP kinase, p42 and p44, were found to be phosphorylated as manifested by their slower mobility shift than the nonphosphorylated forms present in meiotically-arrested oocytes. This finding imply that the machinery required for the phosphorylation of MAP kinase is present in incompetent oocytes but, probably for some developmentally- regulated purposes, is put on hold by some factor(s). A good candidate for such a factor is the two phosphatases, 1 and 2A, which are inhibited by the effect of okadaic acid.

PP1 and PP2A might be regulating either the phosphorylation step of MAP kinase directly or acting upstream on some regulator of MAP kinase along the MAP kinase pathway. Once activated, such a regulator would cause the

MAP kinase directly or acting upstream on some regulator of MAP kinase along the MAP kinase pathway. Once activated, such a regulator would cause the phosphorylation of MAP kinase. Endorsing such a hypothesis requires testing the effect of okadaic acid on the various kinases along the MAP kinase pathway and documenting any changes in their phosphorylation patterns.

The effect of okadaic acid on the morphology of treated oocytes was assessed. When the minimal concentration of the drug was used, 1μM, some changes were observed as treated oocytes lost their spherical shape and became irregularly shaped. Immunofluorescent analysis of treated oocytes showed that those oocytes which had undergone GVBD contained condensed chromosomes that were never capable of forming separate bivalents as is usually the case in normally-occuring GVBD. On the other hand, the microtubular network was deeply disrupted, as no microtubules were detected by immunofluorescence in such treated oocytes. This implies that the drug has a double effect on the maturation state of the oocyte. On the positive side, okadaic acid can induce GVBD in otherwise incompetent oocytes. On the other hand, however, the drug acts negatively on the formation of metaphase spindle which might suggest the involvement of phosphatases 1 and 2A in the normal assembly of spindle microtubules. The clumped configuration of chromosomes in okadaic acid treated oocytes could be due to a direct effect of the drug on the chromosomes. The other possibility might be due to the absence of a

metaphasic network represented by the spindle which plays a role in separating condensing chromosomes into the homologous bivalents.

A time course experiment showed that the minimum time required for okadaic acid to drive incompetent oocytes into maturation was 2h. Immunoblotting results however, do not show a complete shift in the nonphosphorylated species of p42 and p44 into the phosphorylated forms as all four forms of MAP kinase were detected, p42, phosphorylated p42, p44, and phosphorylated p44 following the three time points examined ,2, 4, and 6h in the presence of the drug.

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