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CELLULAR SCAFFOLDS IN MAMMALIAN EGGS

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TABLE OF CONTENTS

1. ABSTRACT

Cellular scaffolds serve as structural components to which various elements of signal transduction pathways can be associated. The association of components on a scaffold can have several important functions, for example they can: 1) associate upstream regulatory components in a cascade that can increase the speed of response to a stimulus; 2) restrict access of substrates to enzymes associated with the scaffold; 3) permit cross talk between distinct signaling pathways, and; 4) aid in the establishment of cellular polarity. The conversion of the mammalian egg into the zygote requires many rapid alterations during a distinct time frame to mediate the biochemical and structural changes that occur. Cellular scaffolds provide a mechanism that can perform these rapid, highly orchestrated changes. They can permit interaction between distinct calcium-dependent pathways and also can provide a means for the calcium signal, that is initiated by fertilization, to act on calcium-independent pathways. This review considers various lines of evidence suggesting that in the mammalian egg, the meiotic spindle serves as a cellular scaffold that permits coordination among several signaling pathways essential for fertilization and the initiation of early development.

2. INTRODUCTION

To be converted into a zygote, the fertilization-competent egg must undergo a number of structural and biochemical changes directed by maternal factors spatially enriched and/or localized about the egg. These changes occur very rapidly as a consequence of fertilization and occur in the absence of gene transcription. This review considers the potential for regionally localized signaling pathways in mammalian eggs to mediate these rapid changes and mechanisms by which co-enriched elements may provide the opportunity for cross talk among signaling pathways involved in post-fertilization events.

The mouse egg, often used as a model for the understanding of eggs in all mammals, is a highly polarized cell (Figure 1). It contains the meiotic spindle at one site near the plasma membrane and there is an extensive actin network between the spindle and the overlying plasma membrane (1). In addition, the plasma membrane, in the region above the spindle, is relatively smooth and free from microvilli which encompass the remainder of the egg surface (2). Cortical granules which reside near the cell periphery also are excluded from this microvillar-free area (3).

The polarity in the egg suggests an intracellular heterogenity that could be established by cellular scaffolds. A number of reports (4, 5) have suggested that cellular scaffolds may be a molecular mechanism for establishing cellular polarity and these structures also serve to: 1) localize signaling pathways in the cell, 2) restrict access to substrates, and 3) provide for cross talk among signaling pathways.

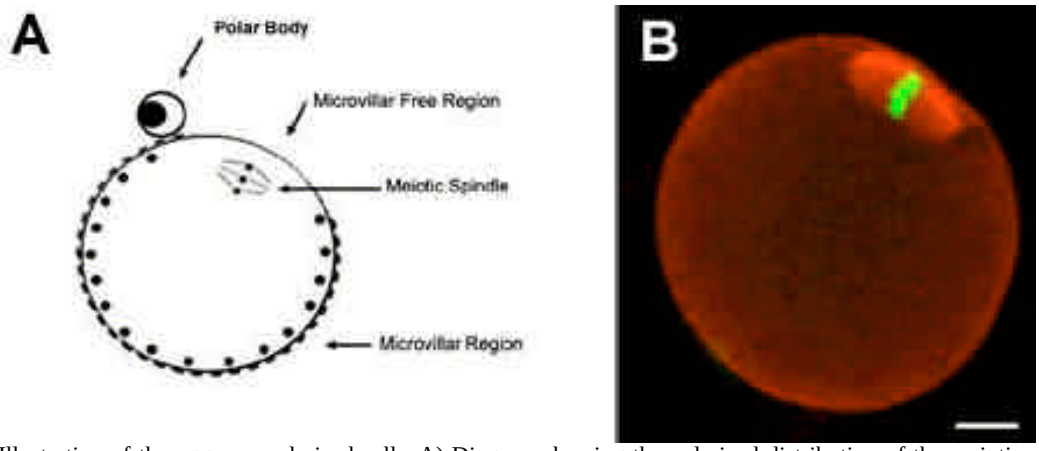

Figure 1. Illustration of the egg as a polarized cell. A) Diagram showing the polarized distribution of the meiotic spindle and chromosomes, cortical granules, microvilli and polar body. B) Optical section from a scanning laser confocal microscope of an egg arrested at meiotic metaphase II labeled with antibodies that bind to tubulin (red) and DAPI which binds to DNA (blue). Scale bar is 10 micrometers.

3. CELLULAR SCAFFOLDS AS A MECHANISM UNDERLYING CELLULAR POLARITY AND CO-LOCALIZATION OF FACTORS

Protein scaffolds in cells have been shown to be involved in both cell polarity (4) and co-localization of cellular factors (5, 6, 7). Scaffolds serve as a structure to which various components of a cell can be tethered, and often times elements required for the function of one or more signaling pathways are associated with these scaffolds. By restricting the location of the scaffold to specific parts of the cell, proteins and signaling pathways also can be restricted into certain areas of the cell (6). For example, the neuron is a highly polarized cell which contains different proteins near the axonal membranes and the dendritic membranes. Most notably the presynaptic and postsynaptic submembrane specializations of neurons contain a number extensive signaling pathways whose components are attached to scaffolds (8). In dendrites, the postsynaptic density serves as a scaffold that holds the upstream regulatory elements in the MAP Kinase pathway as well as MAP Kinase itself (9). In addition, this same scaffold holds other components that may interact with MAP Kinase such as CaM KII (9).

Restriction of the spatial distribution of the various scaffolds within cells can establish a polarized state for these cells (4). For example, in *Drosophila* expression of the *crumbs* gene occurs at the time cells of the embryo begin to express apical/basolateral polarity. The Crumbs protein is reported to be a scaffold protein and once it is present in cells, alters the distribution of other proteins and defines an apical boundary in these cells (10).

One of the best studied systems for the analysis of scaffold function involves the MAP Kinase pathway. The scaffold, originally identified in yeast, has been shown to bring many of the elements of the MAP Kinase signaling pathway together and homologous structures have been identified in mammalian cells (5, 11, 12). Such scaffolds could hold together both elements of a signaling pathway such as MAP Kinase and components from other pathways that might function in cross talk between two or more pathways. It has been suggested that clustering the components of a signaling pathway could have a number of advantages for the cells (4). Cellular scaffolds could: 1) increase the efficiency of reactions; 2) decrease the response time to a stimulus; 3) reduce cross talk with other pathways; 4) increase substrate specificity; 5) and as considered earlier, localize signaling pathways to specific parts of the cell.

Evidence suggests that microtubules themselves can serve as a specialized scaffold for various components of signaling pathways in mammalian cells. In particular, ERK 1 and ERK 2, the MAP Kinases present in the mouse egg, have been shown to associate with microtubules (13, 14, 15, 16). In another MAP Kinase pathway, that is the JNK pathway, elements of this pathway have been shown to bind directly to one of the molecular motor proteins that associates with microtubules (17). There are a number of potential advantages to using microtubules as a scaffold for attachment. Microtubules provide not only a large surface area in the cells for the binding of various proteins and other components, but also exhibit a distinct polarity both in interphase cells (from the plasma membrane to the perinuclear region) and in M-phase cells such as the fertilization-competent mouse egg arrested at meiotic metaphase II (18). If signaling molecules are associated with molecular motor proteins, as they are in the JNK pathway, they could be targeted along the microtubules to specific sites in the cell where other signal regulatory components are located and provide localized signals in the cell in association with cellular scaffolds. In this way, sites and heterogeneously distributed scaffolds within a cell could become enriched in components of different signaling pathways over time as molecular motors added or removed the components of multiple signaling pathways. Evidence presented in this review suggests that cellular scaffolds exist in mammalian eggs, as well as, how these scaffolds may act at the protein level to promote the rapid changes that occur as a result of fertilization.

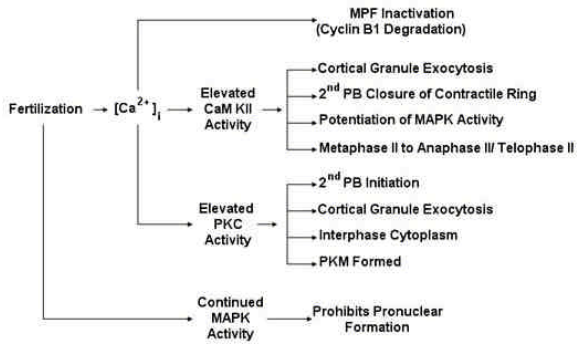

Figure 2. Diagram illustrating the pathways activated by the process of fertilization.

4. CONVERSION OF THE EGG TO THE ZYGOTE: A BRIEF OVERVIEW

The fertilization-competent egg is a specialized cell arrested at a specific point in its cell cycle progression through meiosis. The cell cycle arrest point differs in different classes of organisms. In the mouse, as in other mammals, the egg is arrested at meiotic metaphase II. The egg is a cell whose function is to "wait" for a short period of time and then to rapidly respond to binding and penetration by a sperm. In the absence of sperm penetration the egg will die, but if it is fertilized, an alternate pathway is initiated that is known as the program of early development. Early development initiates with a rapid series of changes at the biochemical and structural level that, as a part of the developmental program, converts the egg into the zygote.

In fertilization-competent eggs (i.e., eggs arrested at meiotic metaphase II) the chromatin is highly condensed in the form of chromosomes, and gene transcription does not occur in the egg from the time of cell cycle arrest through the first few hours after fertilization (19). Thus the extensive changes in biochemical and structural organization that rapidly occur over the first few hours post-fertilization are pre-programmed into the egg by the maternal supply of protein, mRNA, and other factors. To orchestrate such a major reorganization, these maternal components, including signaling factors, must be able to quickly propagate, amplify, modify, and terminate various signals. These signaling factors are likely to function in distinct temporal regimes and at specific spatial coordinates within the egg and may use cellular scaffolds to mediate these events.

In eggs, events that regulate the process of development are choreographed both temporally and spatially by the action of cytoplasmic signaling events. The illustration (Figure 2) indicates pathways that have been reported to be engaged as a result of the rise in intracellular-free calcium that accompanies mammalian fertilization and cell cycle resumption. Calcium acts at the top of a hierarchy of an extensive signaling cascade, and triggers more than one signaling pathway. In addition, in mammalian eggs, the initial calcium rise is followed by several hours during which the level of free calcium oscillates which could provide a means for continued activation of calcium-dependent kinases.

Many signaling events are rapidly engaged in the egg as a result of fertilization. One well studied calcium-dependent event involves inactivation of MPF (Figure 2). There is general agreement from many sources that the rise in intracellular-free calcium inactivates MPF by targeting cyclin B1 for degradation through a ubiquitin-dependent pathway (20, 21, 22). Proteasome-dependent degradation of cyclin has been demonstrated in rat oocytes at the metaphase I to anaphase I transition (23) and in lysates made from eggs of *Xenopus* and goldfish (24).

Once the fertilization-induced rise in intracellular free calcium occurs, other calcium-dependent pathways can be activated (Figure 2). Calcium/calmodulin-dependent protein kinase II (CaM KII) is one such kinase that is activated in mammalian eggs upon fertilization (25, 26, 27, 28) and this activation is dependent on the presence of both calmodulin and calcium (26). One study with mammalian eggs employed an inhibitor of calmodulin which (because calmodulin serves as a cofactor for CaM KII activation) should inhibit CaM KII. The results demonstrated that this delayed formation of the second polar body (29). This has been confirmed and extended by two other studies that used an inhibitor that acts on CaM KII (27, 30). Activation of CaM KII also is essential in the transition from metaphase II to anaphase II (26, 27). In addition, there is a report that inhibition of CaM KII blocks cortical granule exocytosis (27). Other studies have indicated that CaM KII potentiates the action of MAP Kinase (28).

Protein Kinase C (PKC) acts downstream of the calcium signal and there have been several reports demonstrating this activation at a biochemical and/or immunocytochemical level in mouse and rat eggs (31, 32, 33, 34, 35) although the specific isotypes of PKC that act appear to differ depending on the study and species. Once activated, PKC appears to have several roles. Some reports have shown that PKC is involved with the initial formation of the second polar body (35, 36, 37). Gallicano *et al.* (36, 37) have shown that PKC participates in the initiation of the second polar body, but in the absence of other calcium-dependent signaling agents, that initiated polar body will be absorbed into the egg. Activation of PKC also causes cortical granule exocytosis to occur in mammalian eggs (34, 38, 39, 40, 41), however there is evidence to suggest that other calcium-dependent signaling pathways exist to stimulate cortical granule exocytosis (42, 43) since PKC inhibitors cannot block the fertilization-induced exocytosis of cortical granules. Activation of PKC has been found to drive the egg into an interphase state of the cell cycle as indicated by characteristics such as the formation of pronuclei, the Golgi apparatus, and the pattern of protein synthesis (31, 32, 44, 45, 46). Once activated, PKC subsequently is cleaved to form PKM and PKM can phosphorylate elements in the interior of the activated egg (47).

MAP Kinase is known to have important roles in formation of mammalian eggs and after egg activation (19).

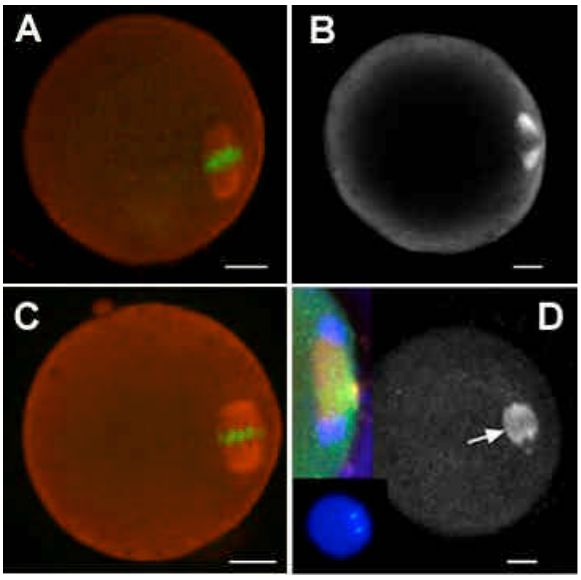

Figure 3. Scanning laser confocal images indicating the distribution of various forms of CaM KII and calmodulin. A) Optical section of an egg arrested at meiotic metaphase II shows the distribution of total CaM KII using an antibody that binds to both the total and active forms of the kinase (red). CaM KII is enriched and co-localized with the meiotic spindle. Chromosomes (blue) are stained with DAPI. B) Optical section of an egg detergent extracted 5 minutes after egg activation and stained with an antibody that binds to calmodulin (white). Calmodulin is bound to the meiotic spindle 5 minutes after egg activation. Chromosomes (blue) are stained with DAPI. C) Optical section of an egg 5 minutes after egg activation stained with an antibody that binds to the active form of CaM KII (red). Active CaM KII colocalizes with the meiotic spindle. Chromosomes (blue) are stained with DAPI. D) Total CaM KII present on the midzone microtubules 30 minutes after egg activation. Bottom inset shows the location of DNA in the same cell. Top inset is a triple labeled specimen. CaM KII is green, DNA is blue, tubulin is red, and co-localized CaM KII and tubulin is orange. Scale bar is 10 micrometers.

MAP Kinase requires phosphorylation on both tyrosine and threonine residues to become an active serine/threonine kinase (48) and MAP Kinase pathways are characterized by protein kinase cascades that contain at least two upstream kinases referred to as MAPK Kinase and MAPK Kinase Kinase. In various cell types several different MAP Kinase pathways have been identified functioning through different MAP Kinases, for example ERK 1/2, JNK/SAPK, p38, and ERK 5 (49, 50). In oocytes and eggs the ERK 1/2 pathway has been identified (28, 51, 52, 53, 54). In this pathway the upstream activator of MAP Kinase is MEK 1/2. Further upstream in this kinase cascade (i.e., the MAPK Kinase Kinase) in eggs is c-mos whereas in other cell types it is typically one of the Raf isotypes (50, 55, 56). As noted earlier, results from a number of reports indicate that the MAP Kinases and their upstream regulatory kinase cascades, are associated with a scaffolding system that serves to facilitate interactions among the kinases in the regulatory cascade (7).

In mammalian eggs, MAP Kinase activity remains elevated for several hours after fertilization and/or egg activation and may function to prevent nuclear membrane assembly of the male and female pronuclei (21, 57). Results from studies with mammalian eggs do not provide much evidence to suggest that MAP Kinase activity is influenced by the level of intracellular free calcium. However, in other systems, most notably the neuron, there is evidence for cross talk among MAP Kinase and calcium signaling pathways (6). In addition, some recent results using mouse eggs suggests that upregulation of CaM KII can potentiate the activity of MAP Kinase after egg activation (28).

5. SIGNALING FACTORS ASSOCIATED WITH STRUCTURAL ELEMENTS IN THE EGG/ZYGOTE

5.1.Calcium/calmodulin-dependent protein kinase II and the metaphase/anaphase transition

In experiments in which the level of free calcium and PKC activity were manipulated it was noted that a normal transit from metaphase II to anaphase II did not occur when PKC was activated, while the level of free calcium was experimentally clamped low (36, 37). This suggested that calcium-dependent enzymes other than PKC may be acting in this transit. Investigators have hypothesized that calcium/calmodulin-dependent protein kinase II (CaM KII) is the signaling agent responsible for the normal chromosomal transit. This was proposed because CaM KII was reported to be involved in the transit to interphase in somatic cells (58) and eggs (20, 25, 59). A more recent study by Johnson *et al.* (26) differed from these previous studies in that this study conducted both immunocytochemical, as well as biochemical, analyses during mammalian egg activation, and the experiments were designed to test for a causal role of CaM KII, rather than simply reporting on a correlation. The immunocytochemical studies revealed that CaM KII was enriched around the area of the meiotic spindle (Figure 3a). When eggs were detergent extracted, a process which removes soluble components and leaves the cytoskeleton and elements bound to the cytoskeleton, it was observed that CaM KII was colocalized with the microtubules of the meiotic spindle (26) in the metaphase II egg and after continuation of meiosis. However, the presence of CaM KII did not by itself demonstrate that the kinase was active, as the kinase requires calcium and calmodulin as co-factors for activation. The investigators also mapped the distribution of calmodulin at increasing time intervals after egg activation (induced by a single transient rise in free calcium). It was noted that calmodulin became tightly associated with the CaM KII on the detergent-resistant meiotic spindle five minutes after the egg was activated (Figure 3b), and was absent by 15 minutes after egg activation. This is the appropriate time interval for binding of calmodulin if it were associated with calcium and if activation of CaM KII were involved with the transit from metaphase II to anaphase II, and in fact CaM KII on the spindle became active five minutes after egg activation

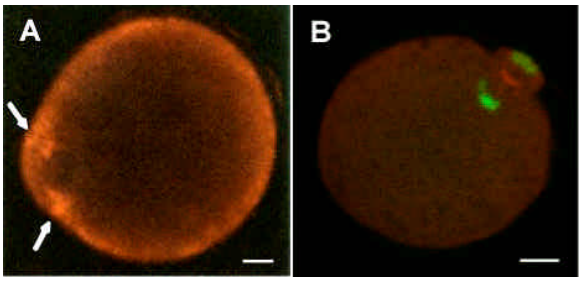

Figure 4. Scanning laser confocal images illustrating formation of the second polar body. A) Optical section showing the distribution of PKC during initiation of the second polar body demonstrated by the distribution of the PKC reporter dye Rim-1. Arrows point to the enrichment of PKC along the sites of the polar body as its formation is initiated. B) Optical section showing distribution of active CaM KII (red) at the contractile ring separating the second polar body from the egg 55 minutes after egg activation. DNA (green) is stained with DAPI. Scale bar is 10 micrometers.

(Figure 3c). While these events were induced by a single calcium transient in the egg, rather than the fertilization-induced calcium spike followed by several hours where the free calcium level oscillates, it is important to note that once CaM KII is activated it can autophosphorylate (60, 61) and as a consequence can maintain its state of activation after the calmodulin disassociates from the CaM KII.

The correlative results described in the previous paragraph suggested that CaM KII was involved in the transit into anaphase II, but do not demonstrate a causal role for CaM KII. To determine whether activation of CaM KII caused this event, CaM KII inhibitors were applied to eggs. The results demonstrated that inhibition of CaM KII blocked the transit into anaphase II when eggs were activated by calcium ionophore as the chromosomes remained arrested at meiotic metaphase II. In contrast, inhibitors to PKC, or other kinases, did not block the transit into anaphase II. As a further test of the causal nature of CaM KII, eggs were permeabilized and calcium and calmodulin at ratio of 4 to1 were flushed into the eggs, along with an ATP regenerating system. (It was not necessary to add CaM KII to the permeabilized system because CaM KII remained tightly associated with the meiotic spindle even after permeabilization.) Under these conditions the chromosomes transited from metaphase II into anaphase II and later acquired a telophase configuration. Inhibitors to CaM KII, but not inhibitors to other kinases including PKC, blocked the chromosomal transit in the permeabilized system. Moreover, it was demonstrated that the ability to regulate chromosomal transit into anaphase II was dependent on the ratio of calcium to calmodulin; when the ratio is 4 to1, the transit into anaphase occurs at the same rate as in a living egg. A lower level of calcium reduces the efficiency of the transit, and in the absence of calcium, the chromosomes remain arrested at metaphase II. To confirm that CaM KII was active at this time the investigators employed a biochemical assay to assess the level of activity of CaM KII (26). The biochemical assay for CaM KII activity confirmed that CaM KII increased above its basal level immediately after egg activation. Subsequently, its activity tapered off until about 55 minutes after egg activation when CaM KII activity again increased, a time when the contractile ring of the second polar body begins to constrict.

The studies above demonstrate the possibility that microtubules of the meiotic spindle serve as a cellular scaffold to immobilize CaM KII. When appropriate co-factors associate with the scaffold-associated CaM KII, the kinase becomes active and initiates a process that alters the scaffold itself, in such a way that it triggers the shift from metaphase to anaphase. In addition, CaM KII on the scaffold (i.e., spindle) is reorganized by the change in microtubule organization that accompanies the transition to anaphase, as the midzone microtubules form, so that the CaM KII can participate in subsequent cellular events.

5.2. CaM KII and the midzone microtubules

Midzone microtubules have been frequently studied in somatic cells (62, 63), but they can exist in all cells, including eggs, released from M-phase. During anaphase, midzone microtubules form from the interpolar microtubules that collect between the separating sets of chromosomes. In somatic cells, the midzone microtubules acquire a collection of proteins thought to aid in the formation of the contractile ring (64, 65). In the mouse egg, the midzone microtubules contain an enrichment of CaM KII (Figure 3d) and in late telophase II appear to deposit the kinase at the contractile ring 45- 55 minutes after egg activation when the contractile ring begins to close. This provides an example of how one cellular structure that carries out an initial function (i.e., the meiotic spindle used for chromosome separation) can be structurally remodeled to aid in a different function (i.e., production of the second polar body). Production of the second polar body also demonstrates how two signaling pathways may interact to cause a change in cell function and structure (Figure 4). In this case, it appears that PKC initiates the formation of a second polar body (Figure 4a; 32) and CaM KII completes the formation of the second polar body by aiding in the assembly or constriction of the contractile ring (Figure 4b; 26).

5.3. Interaction between CaM KII and MAP Kinase

The meiotic spindle also contains an enrichment of MAP Kinase as well as CaM KII (Figure 5). Multiple mammalian MAP Kinase pathways have been reported, and in the mouse egg ERK1 and ERK 2 (p44 and p42 respectively) have been identified (28, 51, 52, 53, 54). Using antibodies to the active form of these kinases, it has been demonstrated that the active forms of both CaM KII and MAP Kinase co-localize on the meiotic spindle (28). In this case, the spindle microtubules appear to serve as a scaffold that provides for potential interactions between two independent signaling pathways (i.e., CaM KII and MAP Kinase). In mouse eggs there is evidence to suggest that these two pathways interact as activation of CaM KII, caused by fertilization, has been shown to potentiate the activity of MAP Kinase (28). This report does not suggest

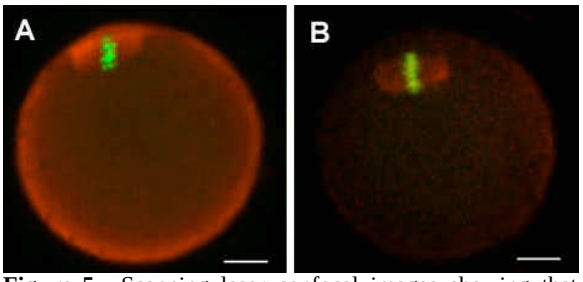

Figure 5. Scanning laser confocal images showing that both CaM KII and MAP Kinase associate with the meiotic spindle in eggs arrested at meiotic metaphase II. A) Total CaM KII (red) on the spindle. B) Total MAP Kinase (red) on the spindle. DNA (green) is stained with DAPI. Scale bar is 10 micrometers.

that CaM KII activates MAP Kinase, which occurs by phosphorylation on both tyrosine and threonine residues through a cascade of up stream kinases (i.e., MEK, etc.), but rather proposes that the stability of MAP Kinase is influenced when CaM KII also is in an active state. In this case the meiotic spindle again appears to be serving as a scaffold and may function to mediate interactions between two distinct signaling pathways, that is MAP Kinase and CaM KII.

6. CONCLUSION

The mouse egg contains a prominent scaffold, the meiotic spindle, which serves to orchestrate the action of many signaling pathways in a spatially and temporally regulated fashion as well as to provide a physical mechanism through which different signaling pathways can interact. If molecular motors are involved with the movement of specific signaling components, as has been reported for some elements of the MAP Kinase pathway (17), then there is an underlying system for movement of components of signaling pathways to discrete sites within the cell. This also could provide a system for establishing a spatial organization that can lead to enrichment or localization of signaling pathways in regions of the egg or zygote. The temporal changes that occur in signaling pathways during the post-fertilization events would also be regulated because the major scaffold, the microtubules of the meiotic spindle, undergo time-dependent changes after fertilization.

The large number of studies examining cellular scaffolds makes it is reasonable to consider that this fundamental system of organization also occurs within the egg, and also may assist in guiding development. Cellular scaffolds are not restricted simply to the localization of kinases. For example, results from investigations have reported that even while active kinase elements are activated on the meiotic spindle, their specific phosphatase counterparts are spatially arranged at the appropriate levels to be promptly utilized at the required time of dephosphorylation (66, 67).

Realizing the potential of the cytoskeleton to act in the regulation of signal transduction events in the cell introduces new possibilities for studying signaling pathways. Perhaps studies should not concentrate only on kinase interaction but also on the scaffolding factors that arrange them or the mechanisms which arrange the scaffolds. For example, improper signaling may be caused by incorrect cytoskeletal alignment and not simply dysfunction occurring at the level of the enzyme.

An egg undergoing meiotic maturation may strategically control its development by assembling on its scaffold an array of signaling elements. In cooperation with feedback pathways and checkpoints, the egg could regulate each stage of its development by a communication network at the protein level including major events such as germinal vesicle disassembly, cortical granule exocytosis and perhaps even polar body formation. Maternal factors have an important role in the early post-fertilization events and the maternal cellular scaffolds can serve as a mechanism to provide the spatial organization and interaction among signaling pathways necessary for formation of a functional zygote. A better understanding of the system of scaffolds and anchor proteins in eggs and zygotes may provide many essential insights into the regulation of the complex processes that orchestrate early development.

7. ACKNOWLEDGMENT

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