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Calcium microdomains and cell cycle control

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Summary

The cell division cycle comprises successive rounds of genome replication and segregation that are never error-free. A complex signalling network chaperones cell cycle events to ensure that cell cycle progression does not occur until any errors detected are put right. The signalling network consists of cell cycle control proteins that are phosphorylated and dephosphorylated, synthesised and degraded interactively to generate a set of sensors and molecular switches that are thrown at appropriate times to permit or trigger cell cycle progression.

In early embryos, discrete calcium signals have been shown to be a key component of the molecular switch mechanism. In somatic cells in contrast, the participation of calcium signals in cell cycle control is far from clear.

Recent experiments in syncytial *Drosophila* embryos have shown that localised calcium signals in the nucleus and mitotic spindle can be detected. It appears that the nucleus comprises a calcium signalling microdomain bounded by endoplasmic reticulum that isolates the nucleus and spindle.

These findings offer a possible explanation for the apparent absence of calcium signals in somatic cells during mitosis.

Given the rate at which errors occur in DNA due to damage by oxidative stress and by UV and cosmic irradiation, it is remarkable that the fidelity of the genome is largely maintained. Cell proliferation increases the probability of error, since DNA polymerases are prone to make mistakes about once every million or so nucleotides, that is, 3000 potential errors in each replication cycle of the human genome. DNA repair mechanisms fix both random damage and replication error, so that the error per base pair is reduced to around one in 10^9 - 10^{10} , averaging out to around one error per replication cycle. The other danger associated with cell proliferation is the mis-segregation of chromosomes. Chromosome mis-segregation during mitosis alters gene dosage and exposes recessive alleles. DNA damage and chromosome mis-segregation are key events in embryonic abnormalities and the development of cancers.

Proliferating cells have mechanisms that ensure that cell division does not proceed if DNA damage has not been repaired or if chromosome alignment is imprecise. There appears to be an overarching control mechanism, illustrated by the identification of cell cycle checkpoints [1-3]. Progression through the cell division cycle is discontinuous, in the sense that the cell pauses prior to each cell cycle transition to check that everything necessary is in place before committing itself to proceeding further. Passage through a checkpoint might be expected to involve flicking a chemical switch. In all eukaryotic cells so far examined, passage through the pre-mitotic checkpoint leads to the activation of a mitotic kinase (Cdk1) that appears to be sufficient to trigger all the events necessary for entry into mitosis. Homologous Cdks are responsible for coordinating the onset of S-phase (DNA synthesis [4-6]. It seems very likely

that the major part of control of the cell division cycle can be explained in terms of pre- and post-translational regulation of this family of cell cycle kinases.

The focus of this review is the idea that calcium signals may contribute to the flicking of the chemical switch and that, in some circumstances, these calcium signals may be very localized, so localized in fact in some circumstances as to be undetectable.

Cell cycle calcium signals in echinoderm embryos

Cell cycle checkpoints have been identified just before mitosis and at metaphase in mitosis (Figure 1). If the idea that calcium signals flick switches during cell cycle progression is correct, then calcium signals should occur at these points in the cell cycle when fidelity of replication and precision of chromosome alignment is being monitored. In sea urchin embryos these calcium signals have been detected using fluorescent calcium indicator dyes [7-12]. The calcium transients are small in comparison to the more familiar calcium signals observed in muscle, in secretory cells and at fertilization [13]. An increase in the calcium-releasing messenger InsP_3 also occurs just before entry in mitosis and again at anaphase [10], consistent with a role for InsP_3 in generating the calcium signals.

Once detected, it is important to demonstrate that the cell cycle checkpoint-associated calcium transients are both necessary and sufficient for cell cycle progression. It has been shown in the sea urchin embryo that microinjection of calcium or InsP_3 can induce entry into mitosis [8, 9]. Microinjection of calcium chelators blocks entry into mitosis [8, 9, 11] and if a photolysable chelator is then destroyed with ultraviolet light, entry into mitosis occurs [11]. An analogous experiment has been made at the metaphase-anaphase transition during mitosis: heparin, an InsP_3 antagonist prevents chromosome segregation and photorelease of InsP_3 induces anaphase onset and chromosome segregation [12]. There is also evidence for the activation of downstream targets of calcium signals: activation of calmodulin has been shown around the nucleus just before entry into mitosis and on the mitotic spindle poles just before anaphase onset [14]; entry into mitosis is prevented by inhibitory peptides directed against calmodulin [14] and calmodulin regulated kinase II [15].

These data provide convincing evidence that calcium signals are associated with checkpoint-related cell cycle transitions and that they are both necessary and sufficient for progress through these cell cycle transitions in the early cell cycles of sea urchin embryos.

Entry into mitosis in starfish embryos is associated with a calcium transient and prevented by heparin injection [16].

Cell cycle calcium signals in vertebrate embryos

Calcium's involvement in mitosis has been studied in frog embryos and in a variety of mammalian embryos. Frog embryos are heavily pigmented and do not lend themselves to fluorescence detection methods. However, it has been reported that microinjection of heparin or calcium chelators [17, 18] prevent cell division in early frog embryos.

Mouse embryos exhibit large calcium transients during mitosis [19-23] and entry into mitosis is blocked by acetoxymethyl ester calcium chelators that become active once they have been hydrolysed within the embryo [19, 20]. At first sight, these observations offer evidence that calcium is essential for mitosis entry in mouse embryos, but in fact the situation is more complex.

Large mitotic calcium transients are not detected in all mouse embryos [19, 21-23]. Parthenotes (that is, eggs activated artificially without sperm) lack mitotic calcium transients

[20, 24]. The large mitotic calcium transients in mouse embryos can be explained by the release during mitosis of a phospholipase C released from the nucleus *after* the dissolution of the nuclear envelope that accompanies and marks entry into mitosis [24, 25]. In addition, very high concentrations of calcium chelators, higher than those shown to block mitotic events in echinoderms, abolish the mitotic transients but do not effect entry into mitosis [26]. Downregulation of InsP_3 receptors using Adenophostin A also abrogates the calcium transients but does not affect mitosis [26]. These data imply that in mouse the mitotic calcium signals are an epiphenomenon unrelated to cell cycle control.

Yet it has also been shown that photorelease of caged InsP_3 advances mitosis in mouse embryos [26], just as it does in sea urchin embryos [9]. Even after receptor downregulation, a small InsP_3 response persists [26] and though the large calcium transients are abolished by calcium chelators, a small transient comparable in size to those seen in sea urchin embryos occurs at the time of mitosis entry [26].

Cell cycle calcium signals in early deuterostome embryos

The deuterostome lineage includes sea urchins, starfish and vertebrates. There is clear evidence of the causal significance of cell cycle calcium transients in the early embryos of sea urchin, starfish and frog, but the evidence in mammals is equivocal.

Cell cycle calcium signals in somatic cells

Calcium signals in non-embryonic cells during mitosis were first detected in plant cells. A calcium signal at the mitotic spindle poles was detected in plant endosperm [27]. This was followed by reports of a substantial calcium transient at anaphase [28] and of localized calcium increases at the spindle pole or in the cytoplasm surrounding the mitotic spindle in mammalian cells in culture [29]. An anaphase calcium transient was also reported in plant stamen hair cells [30].

Two functional studies report the effects of suppressing mitotic calcium transients in mammalian cells in culture. Removal of serum from the culture medium abolished the mitotic transients in Swiss 3T3 cells without affecting mitotic progression [31, 32], as did removal of extracellular calcium [31]. Permeant acetoxymethyl ester calcium chelators also led to loss of the calcium transients without interfering with mitosis [32]. In one of the reports, microinjection of BAPTA to chelate calcium led to a block of mitosis entry [32], while in the other, microinjection EGTA and BAPTA were only effective in blocking mitotic progression in calcium-free media [31]. Emptying internal calcium stores by addition of the calcium ionophore ionomycin in calcium free medium halted mitosis [32]. Release of caged calcium by photolysis could induce premature entry into mitosis and, if release during mitosis, alteration in chromosome morphology consistent with chromosome separation [32]. Both studies also reported that mitosis-associated calcium transients could not be detected in other fibroblast and epithelial cell lines [31, 32] but concluded that calcium was essential for mitotic progression.

Downstream targets of calcium signals have also been shown to operate during mitosis in somatic cells. Knockdown of calmodulin in a mouse cell line led to marked delays in cell cycle progression, including mitosis [33]. An *Aspergillus* cdc25 phosphatase homologue, nimT was shown to be dependent on calcium and calmodulin for entry into mitosis [34]. Cdc25 activation leads to stimulation of Cdk1 mitotic kinase and mitosis entry. CaM Kinase II has been shown to phosphorylate Cdc25 in HeLa cells in culture [35]. Evidence that calmodulin plays a key role in mitosis has been extensively reviewed [36].

Calcium and cell cycle control

The contribution of calcium signals to cell cycle control has been the subject of several review articles [13, 36-39]. There is general agreement that calcium signals and calcium targets are implicated in cell cycle progression and also in contrast that calcium signals cannot always be detected in cells undergoing mitosis.

The picture that emerges from the exposition thus far is that there is functional evidence for the involvement of calcium signals in mitotic progression, but that measurements of intracellular calcium do not universally support the idea of essential calcium signals during mitosis. The large mitotic calcium transients observed in mouse oocytes and in somatic cells do not correlate well with mitotic progression, as there are a number of observations that indicate that mitosis can take place in their absence. These considerations have led to the idea that the mitotic calcium signals themselves may be cryptic and local in many instances and that the larger transients may represent amplification of these small and localized calcium signals under some experimental conditions [20, 32, 38, 40, 41]. These authors speculate that the calcium signals that control mitotic progression may occur in calcium microdomains so localized as to be undetectable in global calcium images.

Calcium microdomains

This volume is dedicated to a discussion of calcium signaling microdomains in many contexts and the subject has also been recently comprehensively reviewed [42]. Calcium microdomains can be created by calcium fluxes across both plasma and endoplasmic reticulum (ER) membranes. Calcium fluxes during mitosis originate from internal stores and are mediated by the InsP_3 receptor [10, 17, 18], suggesting that they involve the endoplasmic reticulum. A combination of InsP_3 receptor clustering and the specialized architecture of the ER in different cell types can generate cytoplasmic calcium microdomains. [42, 43]. Nothing is known about receptor clustering in the ER in dividing cells, but its architecture changes markedly during mitosis.

Architecture of the endoplasmic reticulum during mitosis

In most cell types the ER clusters around the nucleus as mitosis entry approaches and becomes intimately associated with the mitotic spindle as it forms [44-46]. Figure 2 illustrates these events in one-celled sea urchin embryos. ER first accumulates around the centrosomes just before the nuclear membrane is dissolved as the nucleus enters mitosis. It then invades the nucleus, densely filling each half-spindle at metaphase. At anaphase, it segregates with the separating spindle poles and in early telophase engulfs the separating chromosomes and forms around the midbody. It remains concentrated around the chromosomes as they decondense to form the daughter nuclei. The main active component of the mitotic spindle is microtubules. It is well known that microtubules form a close association with ER [47]. The dynamic redistribution of ER is most likely mediated by microtubule motors [48-51]. ER-associated membrane has been shown to sequester and release calcium [52] and preventing calcium sequestration in living cells by microinjection of an antibody inhibitory to the calcium pump causes failure of mitosis due to depolymerization of spindle microtubules [53]. This last observation suggests that tight control of calcium levels in the spindle may be very important.

The very close associations between ER and elements of the nucleus and spindle and the denseness of ER in the spindle are certainly suggestive of an environment in which calcium may be under very tight spatial control in calcium microdomains, but there is little direct evidence. It is known in sea urchin embryos that the calcium transient that precedes mitosis entry is often confined to a small perinuclear region [11], consistent with a microdomain

within the perinuclear ER. It has also been observed, again in sea urchin embryos, that activation of calmodulin occurs very locally during mitosis, first in the perinuclear region and then at the spindle poles [14]. That this local activation occurs despite global cytoplasmic calcium increases [11-13] again suggests that highly localized calcium microdomains may be controlling the activation of calmodulin.

As will be apparent from our earlier discussion of mitotic calcium signals, it has not yet proved possible to detect calcium signals from these putative microdomains, with one exception [11]. However, support for the idea that spindle-associated ER may generate calcium microdomains controlled separately from bulk cytoplasmic calcium has come from experiments in *Drosophila* embryos.

Architecture of the endoplasmic reticulum during mitosis in *Drosophila* embryos

Nuclear divisions in early *Drosophila* embryos occur in the same cytoplasm. At around cycle 8, most nuclei migrate to the embryo cortex to lie just beneath the plasma membrane (Figures 3,4) and so can be readily imaged using confocal microscopy. An unusual feature of these syncytial nuclei is that ER remains outside the spindle during mitosis [54]. It is now known that the nuclear envelope breaks down during entry into mitosis in these embryos, but depolymerization of nuclear lamins is delayed which may prevent the ER from invading the mitotic spindle [54]. Figure 5 shows the disposition of ER and microtubules during mitosis in these syncytial embryos. As mitosis approaches, ER becomes concentrated around the nucleus and developing spindle poles, but does not enter the nucleus after nuclear envelope breakdown, becoming increasingly concentrated in the pole region. At telophase, ER invades the spindle to surround the chromosomes and mid body. Thus in these nuclei, an ER-bounded calcium microdomain can be visualized using confocal microscopy.

Cell cycle calcium signalling is necessary for progression of the nuclear division cycle in syncytial *Drosophila* embryos

Experiments with recombinant proteins that sequester InsP_3 (Figure 6) as well as with antagonists such as heparin and Xestospongins C and calcium chelators demonstrate that InsP_3 -generated calcium signals are essential both for entry into mitosis and for the metaphase-anaphase transition that marks exit from mitosis [55, 56]. Functionally, then, calcium signaling during mitosis conforms to the model developed from work on embryos and in somatic cells.

Cell cycle calcium signals in syncytial *Drosophila* embryos

The most striking calcium signal in *Drosophila* embryos is a cortical calcium increase in step with the nuclear division cycle [55]. However, the peak of the calcium signal synchronizes with interphase/cytokinesis, not mitosis. This observation is at odds with the finding that functionally, calcium controls mitosis entry and mitosis exit. The paradox is resolved with the observation that the nuclear calcium microdomain harbours the calcium signals that control mitosis entry and mitosis exit [55, 56]. Calcium concentrations in the nucleus and mitotic spindle do not track bulk cortical calcium concentrations, but are controlled separately; calcium concentrations within the microdomain never rise to levels measured in the embryo cortex.

Conclusion

There are indications that the calcium transients that control mitosis may in some circumstances remain cryptic, perhaps because they are confined to microdomains of very limited sub-micron dimensions. Support for this notion is provided from experiments in *Drosophila* embryos that, by lucky chance, have a mitotic calcium microdomain that encompasses the entire nucleus and spindle. Measurements of calcium concentrations within this mitotic microdomain are feasible and demonstrate that calcium signals within the mitotic microdomain are controlled separately from bulk cortical calcium. These observations demonstrate the existence of calcium microdomains in the spindle during mitosis and should stimulate efforts to detect calcium microdomains in other cells, though this will be technically very challenging.

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Figure 1.

Events during mitosis. At metaphase the chromatin is condensed into chromosomes and chromosome pairs are joined at the kinetochores. The kinetochores are attached to the spindle poles (centrosomes). At anaphase, the cohesin complex that joins the two chromosome pairs is destroyed and the pole-chromosome microtubules shorten to separate the chromosomes. For simplicity, only one chromosome pair is shown.

Figure 2. Comparison of the distribution of ER and spindle microtubules during mitosis. ER enters the mitotic spindle as the microtubules polymerize to form the metaphase spindle.

Figure 3.
Schematic of nuclear division, migration and cellularization in the early *Drosophila* embryo

Figure 4.
Schematic of mitosis in the cortex of the syncytial embryo

A

B

Figure 5.

ER and spindle in syncytial *Drosophila* embryos. A. Simultaneous imaging of spindle microtubules and ER: note that the ER remains outside the spindle (from ref. 56), in marked contrast to sea urchin. B. Detail of ER distribution in the spindle, showing accumulation of ER at the spindle poles and in the mid body at telophase.

Figure 6.

Microinjection of the InsP_3 binding protein p130 blocks nuclear division. The protein was a GFP chimera, microinjected beyond the bottom left of the image. It forms a gradient within the embryo. At highest concentrations, it blocks nuclear division immediately, at intermediate concentrations, nuclei progress into mitosis and arrest there. At the lowest concentrations, nuclei proceed into the following interphase, but do not undergo a further mitosis entry.