Polyamine dependence of normal cell-cycle progression

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Abstract
The driving force of the cell cycle is the activities of cyclin-dependent kinases (CDKs). Key steps in the regulation of the cell cycle therefore must impinge upon the activities of the CDKs. CDKs exert their functions when bound to cyclins that are expressed cyclically during the cell cycle. Polyamine biosynthesis varies bicyclically during the cell cycle with peaks in enzyme activities at the G1/S and S/G2 transitions. The enzyme activities are regulated at transcriptional, translational and post-translational levels. When cells are seeded in the presence of drugs that interfere with polyamine biosynthesis, cell cycle progression is affected within one cell cycle after seeding. The cell cycle phase that is most sensitive to polyamine biosynthesis inhibition is the S phase, while effects on the G1 and G2/M phases occur at later time points. The elongation step of DNA replication is negatively affected when polyamine pools are not allowed to increase normally during cell proliferation. Cyclin A is expressed during the S phase and cyclin A/CDK2 is important for a normal rate of DNA elongation. Cyclin A expression is lowered in cells treated with polyamine biosynthesis inhibitors. Thus, polyamines may affect S phase progression by participating in the regulation of cyclin A expression.

Introduction
In eukaryotic organisms, the size of tissues and organs depends on a balance between cell proliferation, cell differentiation and cell death. These three processes play an active role throughout the life of an individual, as they are necessary for embryogenesis as well as for the functional integrity of the adult organism. All three processes are strictly regulated and are highly dependent on a multitude of intracellular and extracellular factors to progress normally. This paper deals with cell proliferation and concentrates on the role of the polyamines putrescine, spermidine and spermine, putting them into the wider context of various proteins involved in cell cycle regulation. The basis for cell proliferation is the orderly progression of the cell through the cell cycle at the end of which the cell has doubled its content of molecules vital for life. This ensures the production of two new equal-sized healthy daughter cells at the end of a cell cycle.

The cell cycle driving force
Unidirectional progression through the cell cycle is achieved by an underlying biochemical cycle in which cyclin-dependent kinases (CDKs) are activated sequentially by different cyclins [1–4]. The CDKs are constitutively expressed and are found in excess of their cyclin partners in the cell [5]. The cyclins on the other hand are expressed cyclically and the expression of each consecutive cyclin is dependent on the action of a previously expressed cyclin in a complex with a CDK. The activity of the cyclin–CDK complexes is regulated by several mechanisms, including phosphorylation, dephosphorylation, interaction with CDK inhibitors and degradation. Although the driving force of the cell cycle is the phosphorylating activities of cyclin–CDK complexes, there are intercepting overlying levels of control called checkpoints [6].

The synthesis of the D-type cyclins begins in response to mitogenic stimulation in the G1 phase (Figure 1) [7,8]. Cyclin D1 bound to CDK4 mediates the phosphorylation of a number of proteins of which one is the retinoblastoma protein (pRB). pRB has a central role in cell cycle progression from G1 into S phase [9]. In its unphosphorylated form, pRB sequesters the E2F family of transcription factors that are required for transcription of genes involved in the G1/S transition and S phase progression [10,11]. As pRB is partially phosphorylated by cyclin D1–CDK4, the transcription factor E2F-1 is released and stimulates the transcription of genes involved in the G1/S transition and S phase progression [10,11]. As pRB is partially phosphorylated by cyclin D1–CDK4, the transcription factor E2F-1 is released and stimulates the transcription of genes involved in the G1/S transition and S phase progression [10,11]. As pRB is partially phosphorylated by cyclin D1–CDK4, the transcription factor E2F-1 is released and stimulates the transcription of genes involved in the G1/S transition and S phase progression [10,11]. As pRB is partially phosphorylated by cyclin D1–CDK4, the transcription factor E2F-1 is released and stimulates the transcription of genes involved in the G1/S transition and S phase progression [10,11]. As pRB is partially phosphorylated by cyclin D1–CDK4, the transcription factor E2F-1 is released and stimulates the transcription of genes involved in the G1/S transition and S phase progression [10,11]. As pRB is partially phosphorylated by cyclin D1–CDK4, the transcription factor E2F-1 is released and stimulates the transcription of genes involved in the G1/S transition and S phase progression [10,11].
Figure 1 | Schematic presentation of various factors involved in cell cycle regulation

Unidirectional progression through the cell cycle is driven by an underlying biochemical cycle in which CDKs are activated sequentially by different cyclins. The activities of the polyamine biosynthetic enzymes ODC and AdoMetDC increase in conjunction with the G1/S and S/G2 transitions. The enzyme activities are regulated at the transcriptional (mRNA) and post-translational (half-lives of activities) levels.

Regulation of polyamine biosynthesis during the cell cycle

The activities of ODC and AdoMetDC have been investigated during the cell cycle in cell populations synchronized by various synchronization methods [22–25]. The general conclusion of all these studies is that ODC and AdoMetDC are activated in a biphasic manner during the cell cycle with a first activation phase in late G1 close to the G1/S transition and a second activation phase in conjunction with the S/G2 transition (Figure 1). From these results it may be concluded that ODC and AdoMetDC are not required for the initial progression through the G1 phase but presumably for processes during the S phase and thereafter.

It is known that ODC is a target gene for c-Myc [26,27] and c-myc in turn is a target gene for E2F-1 [10,12]. Thus, the activation of ODC in conjunction with the G1/S transition may be a consequence of the initial phosphorylating activity of cyclin D1/CDK4 on pRB. Pyronnet et al. [28] found the second activation peak of ODC at the G2/M transition, i.e. somewhat later than other observations [22–25]. They attributed the second increase in ODC activity to the presence of a cap-independent internal ribosome entry site in the ODC mRNA that presumably functions exclusively at the G2/M transition.

To investigate the role of transcriptional and post-translational mechanisms in the regulation of ODC and AdoMetDC activities during the cell cycle of Chinese hamster ovary (CHO) cells, we investigated the mRNA levels of the enzymes and the half-lives of the enzyme activities, respectively [25,29].

Both ODC and AdoMetDC mRNA levels doubled during the cell cycle [25]. The doubling of the ODC mRNA content was found at the end of the S phase in conjunction with a doubling of the enzyme activity (Figure 1). At the same time there was a slight increase in the half-life of the enzyme activity (Figure 1). Thus both transcriptional and post-translational mechanisms were responsible for the increase in ODC activity in the late S phase. However, the increase in ODC activity in conjunction with the G1/S transition took place without a change in the ODC mRNA content while there was an increase in the half-life of the enzyme activity, implying the involvement of post-translational regulation (Figure 1).

The doubling of the AdoMetDC mRNA content took place when there was a doubling of the AdoMetDC activity at the G1/S transition (Figure 1). At the same time, there was also an increase in the half-life of the AdoMetDC
activity (Figure 1). Thus, the initial increase in AdoMetDC activity in conjunction with the G1/S transition was regulated both transcriptionally and post-translationally. The second increase in AdoMetDC activity at the S/G2 transition was presumably regulated at the translational level, as there was no change in the mRNA content or the half-life of the enzyme activity (Figure 1). However, this notion has to be investigated as well as the contribution of translation in the regulation of enzyme activities of both enzymes during the entire cell cycle.

Polyamine levels during the cell cycle

Regarding polyamine levels, Heby et al. [30] found clear biphasic increases in putrescine, spermidine and spermine levels in CHO cells coinciding with the biphasic ODC activity during the cell cycle [23]. Other studies have shown a doubling of the polyamine contents during the cell cycle without marked fluctuations [25,31–33]. In our study using CHO cells, the doubling of the putrescine content mainly took part during late S phase and S/G2 transition, while the doubling of the spermine content mainly took place during G1 and S phases [25]. The spermidine level showed a constant increase during the entire cell cycle. In general, it seems that the rather large fluctuations in ODC and AdoMetDC activities results in more subtle changes in the total cellular polyamine pools. However, there may be pronounced changes in different intracellular polyamine pools that presently cannot be distinguished [34,35].

Polyamine dependence of cells progressing synchronously through the cell cycle

Cells may be depleted of their polyamines by treatment with drugs that inhibit ODC or AdoMetDC or stimulate catabolism of polyamines [36,37]. Although there are numerous publications on the effects of polyamine biosynthesis inhibitors on asynchronously proliferating cells, results from experiments with inhibitors on synchronously proliferating cells have not been published. We have seeded CHO cells synchronized by the mitotic shake-off technique in the absence or presence of the ODC inhibitor α-difluoromethylornithine and followed the progression through the cell cycle using flow cytometry (Figure 2). At 7 h after seeding, an effect on cell cycle progression was apparent, as there were fewer cells in the S phase in α-difluoromethylornithine-treated cultures than in control cultures. Similar results were found when seeding mitotic CHO cells in the presence of the spermine analogue N1,N11-diethylnorspermine (P.S.H. Berntsson and S.M. Oredsson, unpublished work). N1,N11-Diethylnorspermine depletes the polyamine pools mainly by stimulating polyamine catabolism but also by inhibiting ODC and AdoMetDC [37]. Thus, unperturbed polyamine biosynthesis is needed for unperturbed cell cycle progression.

Because of the overlap in DNA distributions between G1 phase cells and early S phase cells, from these experiments one can only draw the conclusion that there was a cell cycle effect but not if the G1 phase was prolonged, if the G1/S phase transition was affected or if there was a delay in S phase progression. Such overlaps in DNA distribution have a greater impact on the analysis of cell cycle phase distributions of synchronously proliferating cells than on the analysis of asynchronously proliferating cells.

Polyamine dependence of cells progressing asynchronously through the cell cycle

Numerous studies have been performed with polyamine biosynthesis inhibitors and polyamine analogues on asynchronously proliferating cells. The result of polyamine depletion is always growth inhibition, which usually is reversible when the drug is removed or the cells are supplemented with polyamines. Polyamine depletion may also result in apoptosis or senescence [38–40]. When cells stop proliferating because of polyamine deficiency, different effects are found on the cell cycle phase distributions. Cells may accumulate in the G1 phase of the cell cycle [38,41] but also in S and G2 phases [42–44]. The molecular basis for the differences in cell cycle accumulation is not clear, but it is most
probably related to the combination of genetic aberrations in the cell lines studied. So far there are very few publications reporting the effects on different cell cycle regulatory proteins in cells growth-arrested by polyamine depletion. The N,N,N\textsuperscript{11}-diethylnorspermine-induced G\textsubscript{1} arrest found in MALME-3M human melanoma cells was correlated to a hypophosphorylation of pRB and increased levels of the tumour suppressor protein p53 and the two CDK inhibitors p21 and p27 [38]. In CHO cells lacking functional p53, polyamine depletion resulted in an S phase accumulation [45]. However, to draw any general conclusions about the role of p53 in polyamine depletion-induced growth arrest, more studies have to be performed.

It has to be emphasized that effects on specific cell cycle phases are observed before the onset of growth arrest. Using a bromodeoxyuridine–DNA flow cytometry method, we have shown that cell cycle progression is affected within one cell cycle after seeding cells in the presence of drugs that deplete the cellular polyamine pools [46–48]. The S phase was prolonged within the first cell cycle after seeding the cells, while effects on the G\textsubscript{1}/S transition and the G\textsubscript{2}/M phase were found in the following cell cycles. Altogether our results imply that the delay in S phase progression in polyamine-depleted cells is due to a decreased rate of DNA elongation rather than to effects on the initiation of DNA replication [49]. In MCF-7 human breast adenocarcinoma cells treated with N,N,N\textsuperscript{11}-diethylnorspermine, cyclin A expression was decreased on both the mRNA and protein levels before the onset of the delay in S phase progression (P.S.H. Berntsson and S.M. Oredsson, unpublished work). At the same time point, there were no effects of N,N,N\textsuperscript{11}-diethylnorspermine treatment on the expression of cyclin D1, CDK2, cyclin E, E2F-1 and the CDK inhibitor p27 or on the expression or phosphorylation of pRB. Thus, polyamines may be important in a step between the initial release of E2F-1 due to the early phosphorylation of pRB by cyclin D1–CDK2 and the full release of E2F-1 due to hypophosphorylation of pRB, which results in the transcription of cyclin A. However, this notion has to be investigated and the effect of polyamine biosynthesis inhibition on cyclin A expression has to be studied in other cell lines in order to draw any general conclusions from this observation in MCF-7 cells.

**Concluding remarks**

It is clear that normal cell cycle progression requires unperturbed polyamine metabolism. With the current knowledge of factors involved in cell cycle regulation and sensitive methods to study molecular interactions, it should now be possible to pinpoint steps where polyamines are required.

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**References**


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