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Extra Views

Cdk1

Unsung Hero of S Phase?

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ABSTRACT

Cdk2 has been viewed as a key cell cycle regulator that is essential for S phase progression. The recent discovery that Cdk2 is not required for cell proliferation in mice now shows that other factors must be able to replace Cdk2 in stimulating DNA replication. Experiments performed in *Xenopus* egg extracts identify the mitotic protein kinases Cdk1/Cyclin B and Cdk1/Cyclin A as likely candidates. These observations raise the intriguing possibility that Cdk1 normally participates in genome duplication in wild type cells.

Progression through the cell cycle is driven by cyclin-dependent kinases (CDKs) whose catalytic activity and substrate specificity depend on their association with regulatory subunits called cyclins. According to current dogma, vertebrate cells contain at least three essential classes of CDKs.[1] The first CDK to become active during the cell cycle is Cdk4, which associates with Cyclin D in G_1. Cdk4/Cyclin D phosphorylates and inactivates the retinoblastoma gene product, Rb, leading to induction of E2F-regulated genes whose expression promotes G_1/S progression. The second CDK to become active is Cdk2, which associates with Cyclin E late in G_1 and with Cyclin A at the onset of S phase. Cdk2/Cyclin E phosphorylates Rb and is thought to accelerate its inactivation. In addition, Cdk2/Cyclin E and Cdk2/Cyclin A allow progression through S phase by stimulating origin firing, but the relevant substrates are unknown. Cdk2/Cyclin E is also implicated in centrosome duplication. The last CDK to be activated is Cdk1, which associates with Cyclin A and Cyclin B. Cdk1/Cyclin A is thought to stimulate entry into mitosis whereas Cdk1/Cyclin B leads to nuclear envelope breakdown and the other events that characterize mitosis.

Experiments reported in 2003 call into question this orderly picture of the vertebrate cell cycle, particularly the key role of Cdk2 in G_1/S progression. The first signs of trouble came from the observation that Cyclin E is not required for cell cycle progression.[2] Thus, cells lacking both Cyclin E1 and E2 are able to cycle. Although this result is surprising, Cdk2/Cyclin A could plausibly replace the functions of Cdk2/Cyclin E. Soon afterwards, Tetsu and McCormick reported that select human cancer cell lines proliferate when Cdk2 is inhibited by expression of $p27^{Kip}$ or a dominant negative Cdk2 (DN Cdk2), or when endogenous Cdk2 is silenced using antisense and siRNA strategies.[3] Although these results were intriguing, it could not be ruled out that residual amounts of Cdk2 remained, or that Cdk3 was able to replace the function of Cdk2. The fatal blow came recently. Two groups deleted the Cdk2 gene (in a mouse strain that also lacks Cdk3) and found that the resulting mice are viable. [4,5] The only visible phenotype is sterility, which is caused by defects in meiosis.

Given the essential functions ascribed to Cdk2 in cell cycle progression, how do Cdk2-deficient cells proliferate? Here, we focus on the question of how Cdk2-deficient cells initiate DNA replication. Eukaryotic DNA replication is initiated in two steps.[6] The first step takes place in G_1 and involves the binding of the origin recognition complex (ORC), Cdt1, Cdc6, and the minichromosome maintenance complex (MCM) to origins of DNA replication to form a pre-replication complex (pre-RC). During a mitotic cell cycle, pre-RC formation does not require Cdk2 activity, whereas when cells reenter the cell cycle from quiescence, Cdk2/Cyclin E is essential for MCM binding to chromatin.[2,7] The second step occurs at the G_1/S transition and involves the conversion of pre-RCs into active replication complexes. Until recently, this step was considered to be critically dependent on Cdk2 activity in all metazoans organisms. In mammalian cells, this conclusion was based on expression of dominant negative Cdk2 and microinjection of antibodies to Cyclin A and Cyclin E, all of which arrested cells in G_1.[8-11]

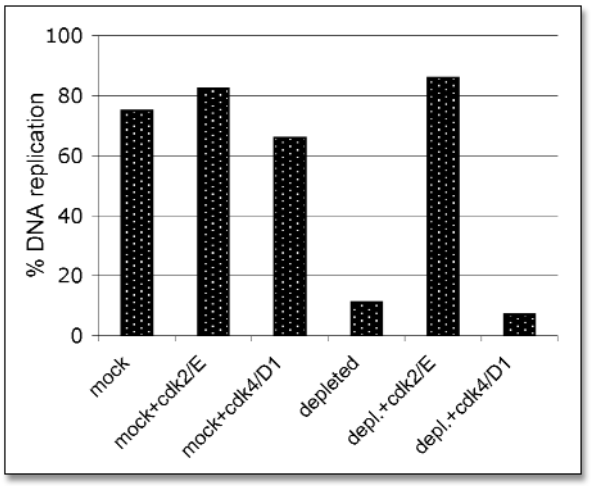

Figure 1: Cdk4/Cyclin D does not support DNA replication in *Xenopus* egg extracts. Egg cytosol was incubated for 30 min with sperm chromatin, followed by addition of either mock or Cdk2 depleted NPE. Recombinant Cdk2/Cyclin E complex (0.4 µM) or recombinant Cdk4/D1 complex (1 µM) was added to depleted and mock depleted NPE, respectively, and the percentage of input DNA replicated after 60 minutes was plotted. For details, see reference 18.

In thinking about which protein kinases might be able to stimulate DNA replication in Cdk2-deficient cells, it is useful to consider experiments performed in *Xenopus* egg extracts. Unfertilized *Xenopus* eggs are arrested in metaphase of meiosis II. When eggs are crushed and thereby activated, Cyclins A and B are degraded, and the resulting egg cytoplasm, which contains Cdk2/Cyclin E, is arrested in interphase. Sperm chromatin added to egg cytoplasm supports assembly of nuclei which undergo DNA replication in the absence of transcription or protein synthesis. This cell-free system has been used to determine which CDKs are able to support the initiation step of DNA replication. Immunodepletion of Cdk2 or Cyclin E from egg extracts abolished DNA replication, providing the clearest evidence that Cdk2/Cyclin E is required for initiation of DNA replication independent of any effects on transcription.[12] Importantly, it was found that Cdk2/Cyclin E could be replaced by Cdk2/Cyclin A, but also by Cdk1/Cyclin A.[13,14] Based on these experiments, the functions of Cdk2/Cyclin E and Cdk2/Cyclin A in stimulating DNA replication could be taken over by Cdk1/Cyclin A. Consistent with this model, in Cdk2$^{-/-}$ mice, Cyclin A associated H1 kinase activity (which almost certainly depends on Cdk1), is detected in early embryo extracts and in spleens from young animals.[4] Moreover, primary Cdk2$^{-/-}$ mouse embryo fibroblasts (MEFs) contain Cyclin A-associated kinase activity. Together, these results argue that Cdk1/Cyclin A could take over the role of Cdk2 in many cell types.

Cdk1/Cyclin A-mediated DNA replication in Cdk2-deficient tissue does not, however, appear to be the entire story, as some of these tissues also lack Cyclin A activity. Thus, adult spleen and immortalized MEFs from Cdk2$^{-/-}$ animals have no detectable Cyclin A-associated kinase activity,[4] nor do colon cancer cells in which Cdk2 has been ablated.[3] How does DNA replication occur in the absence of Cdk2 *and* Cyclin A? A possible candidate is Cdk1/Cyclin B, but until recently, it was unknown whether it had S phase promoting (SPF) activity. This gap in our knowledge was due to the fact that Cdk1/Cyclin B leads to disassembly of the nuclear envelope, a structure that is critically important for DNA replication in the conventional *Xenopus* egg extracts described above.[15,16] We have shown that the requirement for a nuclear envelope in DNA replication in egg extracts can be circumvented with a concentrated nucleoplasmic extract called NPE.[17] Using this system, we showed that Cdk1/Cyclin B is just as active as Cdk2/Cyclin E, Cdk2/Cyclin A, and Cdk1/Cyclin A in stimulating DNA replication.[18] Similar results were obtained by Hunt and colleagues, who showed that when the activity of Cdk1/Cyclin B was attenuated to prevent nuclear envelope breakdown, it exhibited SPF activity in conventional *Xenopus* egg extracts.[19] Based on these results, we propose that Cdk1/Cyclin B is an attractive candidate for the protein kinase that supports DNA replication in Cdk2 and Cyclin A deficient cells. In the yeasts *S. cerevisiae* and *S. pombe*, a single mitotic CDK is sufficient to drive all the major events of the chromosome cycle including DNA replication.[20-22]

Another possibility is that Cdk4 substitutes for Cdk2. Consistent with this idea, Tetsu and McCormick showed that in Cdk2-deficient cells, Cdk4 phosphorylates Rb on sites which are normally targets of Cdk2.[3] Therefore, it is possible that these two protein kinases also have overlapping specificity with respect to DNA replication substrates. To test this model, we immunodepleted *Xenopus* egg extracts of Cdk2 and then supplied high concentrations of active Cdk2/Cyclin E[18] or Cdk4/Cyclin D.[23] While Cdk2/Cyclin E caused a full rescue of DNA replication, Cdk4/Cyclin D had no effect (Fig. 1). Together, the data suggest that Cdk1/Cyclin A or Cdk1/Cyclin B are more likely than Cdk4/Cyclin D to drive initiation of DNA replication in Cdk2-deficient cells.

Several possible objections to a role for Cdk1 in initiating DNA replication come to mind. First, it is commonly assumed that the highly condensed mitotic chromatin generated by this protein kinase would preclude DNA replication. However, our recent data argues that this is not the case. When chromatin containing pre-RCs was driven into mitosis with Cdk1/Cyclin A or Cdk1/Cyclin B it was subsequently still able to initiate and undergo a single complete round of DNA replication.[18] Therefore, mitotic chromatin is not incompatible with the initiation or elongation phases of DNA replication. Second, Cdk1/Cyclin B does not enter the nucleus until midway through mitosis, possibly allowing too little time for DNA replication before anaphase. Interestingly, we found that mitotic chromatin replicated at least 4–5 faster than uncondensed chromatin,[18] suggesting that the kinetics of DNA replication in mitosis might be much faster. Third, Cdk1 might not be able to support DNA replication because it leads to nuclear envelope breakdown. However, while the nuclear envelope is required for DNA replication in conventional *Xenopus* egg extracts, this requirement is not intrinsic, as it can be bypassed via a concentrated nuclear extract.[17] Further, results from Rao and Johnson show that in mammalian cells, DNA replication can take place in S phase nuclei *after* these have been broken down through fusion with a mitotic cell.[24] Fourth, unreplicated DNA is thought to trigger a checkpoint that downregulates Cdk1. However, this checkpoint requires prior initiation of DNA replication, and it may only be active when replication forks are stalled with aphidicolin or hydroxyurea.[25] In summary, we know of no definitive objection to the idea that Cdk1 might be able to execute S phase.

If it is true that Cdk1/Cyclin A or Cdk1/Cyclin B drive S phase in Cdk2-deficient cells, might these protein kinases normally participate in DNA replication in wild type cells? While expression of dominant negative Cdk1 arrests cells in G_2, demonstrating that Cdk1 is not essential for the bulk of genome duplication,[11] Cdk1 may play subtle or redundant roles in DNA replication. Cyclin A becomes complexed with Cdk1 in S phase,[9] so it is conceivable that

Cdk1/Cyclin A might, for example, act on late firing origins. These origins could be equally responsive to Cdk1 and Cdk2, or they might even be critically dependent on Cdk1. Interestingly, work by Laird and colleagues has shown that specific loci comprising ~1% of the genome replicate very late in G_2,[26] at a time when Cdk2 activity has declined. These regions might contain origins that are activated by Cdk1. The concept that some origins can only be activated by a specific CDK has a clear precedent: in *S.cerevisiae*, Cdc28/Clb5, but not Cdc28/Clb6, is able to activate late origins, whereas early origins respond to both protein kinases.[27]

Even if Cdk1 were not essential for origin firing in a wild type cell, might the ability of this kinase to stimulate DNA replication serve some function? We have proposed that Cdk1-mediated origin firing late in the cell cycle contributes to the maintenance of genomic stability by insuring complete DNA replication before mitosis.[18] It is currently unclear whether small amounts of unreplicated DNA are able to trigger the G_2/M checkpoint in otherwise normal cells. If not, cells containing unreplicated DNA might occasionally progress through mitosis, causing chromosome breaks in anaphase. However, if DNA replication and origin firing are possible in mitosis, genome duplication can theoretically continue until the moment cells undergo anaphase. Thus, failure to replicate small amounts of DNA early in the cell cycle is not necessarily a catastrophic event if Cdk1/Cyclin A and Cdk1/Cyclin B can stimulate replication at much later times.

Further experiments will be necessary to determine which protein kinase stimulates DNA replication in Cdk2-deficient cells and whether this kinase participates in replication in wild type cells. If it is Cdk1/Cyclin A or Cdk1/Cyclin B, one might predict that DNA replication in Cdk2-deficient cells takes place immediately before mitosis with a short or non-existent G_2 period. Furthermore, chromatin might adopt a condensed structure during S phase, although it is important to stress that those sections of the genome undergoing active replication are expected to undergo transient decondensation.[18] Ultimately, it will be essential to specifically inactivate Cdk4/Cyclin D, Cdk1/Cyclin A, and Cdk1/Cyclin B in Cdk2-deficient cells to identify which of these protein kinases is the unsung hero of S phase.

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