Coordinate regulation of histone mRNA metabolism and DNA replication

Cyclin A/cdk1 is involved in inactivation of histone mRNA metabolism and DNA replication at the end of S phase

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S phase is characterized by the replication of DNA and assembly of chromatin. This requires the synthesis of large amounts of histone proteins to package the newly replicated DNA. Histone mRNAs are the only mRNAs that do not have polyA tails, ending instead in a conserved stemloop sequence. The stemloop binding protein (SLBP) that binds the 3' end of histone mRNA is cell cycle regulated and SLBP is required in all steps of histone mRNA metabolism. Activation of cyclin E/cdk2 prior to entry into S-phase is critical for initiation of DNA replication and histone mRNA accumulation. At the end of S phase SLBP is rapidly degraded as a result of phosphorylation of SLBP by cyclin A/cdk1 and CK2 effectively shutting off histone mRNA biosynthesis. E2F1, which is required for expression of many S-phase genes, is regulated in parallel with SLBP and its degradation also requires a cyclin binding site, suggesting that it may also be regulated by the same pathway. It is likely that activation of cyclin A/cdk1 helps inhibit both DNA replication and histone mRNA accumulation, marking the end of S phase and entry into G_2-phase.

Key words: histone mRNA, cell cycle, E2F, cyclin, protein degradation, DNA regulation

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Progression through the cell cycle is driven by sequential activation of a series of protein kinases, the cyclin/cdks.[1] Activation of the G_1 cyclins, cyclin D/cdk4/6 and then cyclin E/cdk2 result in the activation of cell growth pathways and then initiation of DNA replication. During S phase, cyclin A/cdk2 is essential for ongoing DNA replication. Prior to entry into mitosis, activation of cyclin B/cdk1 results in nuclear envelope breakdown. Completion of mitosis requires the inactivation of both mitotic cyclins, cyclin B/cdk1 and cyclin A/cdk1 by destruction of the cyclin subunits by the anaphase-promoting complex (APC). It is less clear whether there is a specific transition that occurs at the end of S phase and entry into G_2-phase.

Histone protein synthesis is restricted to S phase and regulation of histone protein synthesis is accomplished by regulation of histone mRNA levels. Replication histone mRNAs have a unique structure since they are the only eukaryotic mRNAs that are not polyadenylated, ending instead in a conserved stemloop. A novel RNA-binding protein, stemloop binding protein (SLBP), binds the 3' end of histone mRNA and participates in many aspects of histone mRNA metabolism.[2] SLBP is cell cycle-regulated and the protein is rapidly degraded at the end of S phase.[3,4] In a recent study, we demonstrated that the degradation of SLBP, a protein that is limiting for histone mRNA accumulation, requires phosphorylation by cyclin A/cdk1 which in turn primes the phosphorylation of an adjacent threonine by casein kinase 2 (CK2).[5] SLBP is subsequently degraded by a still-unknown ubiquitin ligase. The degradation of SLBP

effectively stops the accumulation of histone mRNA until the next cell cycle.[3] Here we show that a major S-phase transcription factor, E2F1, is regulated in parallel with SLBP, raising the possibility that the activation of cyclin A/cdk1 at the end of S phase may help stop both histone synthesis and DNA replication, just as the activation of cyclin E/cdk2 in late G_1 provides the signal for both DNA replication and accumulation of histone mRNA.

Regulation of DNA Replication

The initiation of DNA replication in vertebrates has been studied extensively and clearly depends on activation of cyclin E/cdk2, which phosphorylates a number of target proteins leading to initiation of DNA replication. As in many biochemical pathways, there are likely cascades of kinases which ultimately modify many target proteins required for DNA replication. E2F1 is a critical transcription factor that regulates transcription of many genes encoding proteins required for DNA replication, including enzymes of deoxynucleotide metabolism, members of the Mcm and Orc complexes and components of the replication apparatus.[6] One set of cyclin E/cdk2 target proteins are members of the pRb family (pRb, p130 and p107).[7] Phosphorylation of these proteins results in their release from the transcription factor E2F1 and upregulation of transcription of these genes.

The regulation of initiation DNA replication has been extensively reviewed.[8-10] Activation of cyclin E/cdk2 is required to initiate DNA replication, and a critical target of cyclin E/cdk2 is Cdc6. Phosphorylation of Cdc6 protects it from degradation by the APC allowing licensing of origins in late G_1.[11,12] Other direct targets of cyclin E/cdk2 include p27, an inhibitor of entry into S phase,[13] which is degraded prior to S-phase entry and cyclin E itself,[14] each of which are part of an autoregulatory loop. During S phase cyclin A/cdk2 remains active and is essential for continued S-phase progression.[15] The completion of DNA replication occurs only when every region of DNA has been replicated once and only once. Continued activity of cyclin A/cdk2 and cyclin A/cdk1 prevents origin relicensing and rereplication.[16] In the next G_1 phase, the cell must then license the origins so they can be fired again.[9]

Cyclin/cdks and Histone Protein Synthesis

The replication of DNA is accompanied by the immediate assembly of the newly replicated DNA into chromatin. Thus, at the same time that DNA replication starts, there must be a rapid increase in the rate of histone protein synthesis. When DNA replication is complete, histone-protein accumulation must stop quickly. The regulation of histone protein synthesis is mediated by rapid changes in histone mRNA, as well as degradation of excess histone protein.[17,18] Accumulation of histone mRNA is a result of an increase in the rate of histone gene transcription and the efficiency of histone pre-mRNA processing.[19] The molecular mechanisms that lead to activation of histone mRNA expression are not completely understood. However, the activity of cyclin E/cdk2 is required and an essential cyclin E substrate is the protein NPAT,[20-22] which is found in the Histone Locus bodies. These contain factors required for histone mRNA biosynthesis and are adjacent to the histone genes.[23,24] Phosphorylation of NPAT by cyclin E/cdk2 is necessary for accumulation of histone mRNA.[25] Thus, cyclin E/cdk2 is likely at the top of a cascade that results in both initiation of DNA replication as a result of phosphorylation of cdc6 and accumulation of histone mRNAs as a result of phosphorylation of NPAT.

At the end of S phase, histone mRNA is rapidly degraded and histone mRNA biosynthesis is inhibited. The degradation of histone mRNA is directly regulated by changes in the rate of DNA replication, in response to the demand for histone protein to package newly replicated DNA.[26,27] A critical protein for histone mRNA metabolism is the SLBP, which binds to the 3' end of histone mRNA and participates in histone pre-mRNA processing as well as histone mRNA degradation. SLBP is cell cycle regulated and the protein is present only in S-phase cells.[4] Since histone gene transcription continues outside of S phase at a reduced rate,[28] rapid degradation of SLBP at the end of S phase is necessary to shut off histone mRNA biosynthesis, by preventing histone pre-mRNA processing.[3] Nuclear extracts from G_1- or G_2-phase cells do not process histone mRNA, but the addition of recombinant SLBP restores processing.[3] Accumulation of SLBP just prior to entry into S phase is essential to allow the accumulation of histone mRNA necessary for histone protein synthesis. SLBP degradation is the result of phosphorylation of two threonine residues (T60 and 61) and also requires a putative cyclin binding site.[3] Thus regulation of SLBP levels is the critical component of postranscriptional regulation of histone mRNA levels (**Fig. 1**).

E2F1 is Regulated in Parallel with SLBP

E2F1 has been reported to be a cell cycle regulated protein,[29] although the elements in E2F1 responsible for cell cycle regulation have not been defined. A diagram of E2F1 with the domains that have been previously identified is shown in **Figure 2A**. These include a DNA-binding domain; a heterodimerization domain that interacts with DP, the E2F1 partner;[30] a domain that binds Rb and is required for stabilization of E2F1 when Rb is overexpressed;[31-33] a domain that binds the F-box protein Skp2 that has been proposed to mediate E2f1 degradation as part of the cul1 E3 ligase;[29] an Arf binding site also involved in E2F degradation[34] and a domain that contains a cyclin A binding site.[35,36]

We confirmed that E2F1 is cell cycle regulated in HeLa cells and that the levels of E2F1 protein parallel the levels of SLBP, increasing as cells enter S phase and decreasing at the end of S phase, suggesting that E2F1, specifically, is degraded at the end of S phase (**Fig. 2B**, lanes 5 and 6). E2F1 accumulates in parallel with SLBP as cells approach the next S phase. Similar results were obtained with E2F2 and E2F3 (Dong J and Marzluff WF, unpublished). Treatment of mid-S-phase cells with MG132 prevented the degradation of both SLBP and E2F1 (**Fig. 2C**, lane 5). To determine which regions of E2F1 were required for degradation of E2F1 at the end of S phase we made a number of

deletions in E2F1 and created cell lines expressing the mutant E2F1 containing an N-terminal HA tag. We deleted a variety of regions previously reported to be involved in degradation of E2F1 under a variety of conditions. These include the SKP2 binding site (amino acids 1–42),[29] the pRB binding site (amino acids 409–426)[37,38] and the ARF binding site (amino acids 426–437).[34] The full-length HA-tagged protein was regulated appropriately (**Fig. 2D**, top) and a mutant (aa 42–379) that removed the Skp2 binding site, the pRb binding site and the Arf binding site was also degraded at the same time as SLBP (**Fig. 2D**, middle). This result indicated that the previously characterized regions that affected E2F1 stability in different conditions were not required for degradation of E2F1 at the end of S phase. Further deletion of the amino-terminus that removed amino acids 1–107 (108–437) and removed the cyclin A binding site resulted in stabilization of the E2F1 protein at the end of S phase (**Fig. 2D**, bottom).

Additional deletions of either amino acids 109–190 or 191–299 from the center of the E2F1 protein had no effect on E2F1 degradation at the end of S phase (**Fig. 2E**, top and middle). However, a mutant E2F1 with amino acids 300–379 deleted was not degraded at the end of S phase (**Fig. 2E,** bottom), demonstrating that there were two regions of E2F1 required for degradation at the end of S phase; a sequence between amino acids 43 and 108 and the region between amino acids 300 and 379 (**Fig. 2F**).

To demonstrate that these two regions were sufficient for degradation of E2F1 we created a miniE2F1 gene with GST substituted for the region between amino acids 49–107 and 300–380 (**Fig. 3A**). The region from 67–108 contains the previously described cyclin binding site,[35] with the core of the cyclin binding site[36,39] at amino acids 89–92 of E2F1. We mutated the core region KRRL to AAAA in the E2F minigene. As a control we also used the SLBP mini gene,[5] which contained aa 56–125 fused to GST (**Fig. 3A**). This region contains both the cyclin binding site and the SFTTP phosphorylation site. We mutated the two threonines in the phosphorylation site to alanines (SLBP-AA). Stable cell lines were created expressing each of these minigenes. Each of the minigenes contained a myc-tag at the N terminus and a FLAG-tag at the C terminus. The wild-type E2F minigene was degraded at the end of S phase (**Fig. 3B**, top), while the mutant E2F minigene was stable throughout S phase and G_2 phase (**Fig. 3B**, bottom). Similar results were obtained for the SLBP minigenes (**Fig. 3C**) as previously reported.[5] Thus both SLBP and E2F1 contain a cyclin binding site that is required for their degradation at the end of S phase.

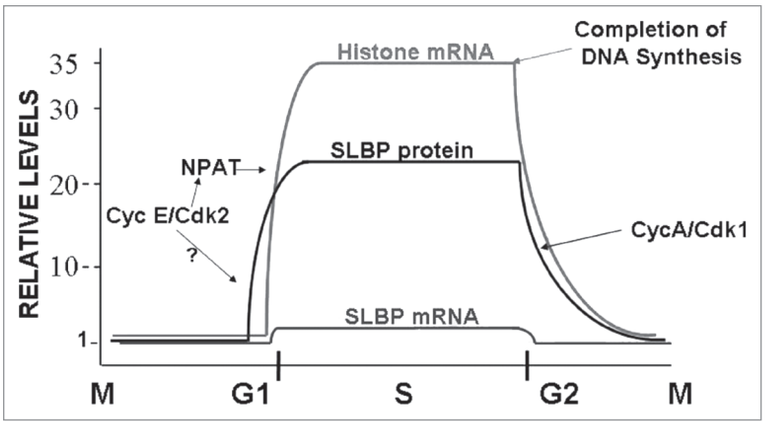

Figure 1. Regulation of histone mRNA during the mammalian cell cycle. The changes in levels of histone mRNA, SLBP and SLBP mRNA during the cell cycle are shown. Cyclin E/cdk2 activation leads to phosphorylation of NPAT and likely translation of SLBP mRNA resulting in activation of histone mRNA accumulation. At the end of S phase, cessation of DNA replication results in degradation of histone mRNA, while activation of cyclin A/cdk1 is required for SLBP degradation.

The region between aa 300 and 379 of E2F1 contains numerous SP and TP sites, suggesting that one or more of these sites is phosphorylated to trigger degradation of E2F1. To demonstrate the phosphorylation of E2F1, we treated lysat es with lambda protein phosphatase and resolved the lysates on a long polyacrylamide gel, using anti-E2F1 to detect the endogenous protein and anti-HA to detect the exogeous protein. Endogenous E2F1 was quantitatively phosphorylated in S-phase cells, and was detected as a broad band (**Fig. 3D**, lane 1). Treatment with phosphatase converted E2F1 to single species (**Fig. 3D**, lane 2). We analyzed HA-tagged full-length E2F1, the mutation of the core cyclin binding site and the deletion of amino acids 370–379. Similar results to the endogenous E2F1 were obtained with the HA-tagged full-length protein (**Fig. 3D**, lanes 3 and 4). Most of the phosphorylation was dependent on the cyclin binding site (**Fig. 3D**, lanes 5 and 6). Mutation of the cyclin binding site resulted in converting E2F1 to two components, one with identical migration to the phosphatase-treated protein and one migrating slightly slower, suggesting there is a single phosphorylation site that did not depend on the cyclin binding site. The deletion of aa 300–379 gave a similar result to mutating the cyclin binding site. E2F migrated as a doublet, the lower band of which migrated identically with phosphatase treated E2F1 (**Fig. 3D**, lanes 7 and 8). We conclude that the cyclin binding site is required for bulk of the phosphorylation of E2F1, which is found on amino acids 300–379. The bulk phosphorylation pattern of E2F1 is similar in early and late S phase, suggesting that there are multiple cyclin-dependent phosphorylations on E2F1. Due to the multiple SP and TP sequences in this region, we have not been able to identify the specific phosphorylation sites on E2F1.

Cyclin A/cdk1 is Required for the Degradation of SLBP at the End of S phase

Our recent study[5] demonstrates that the critical phosphorylation event for SLBP degradation is carried out by cyclin A/cdk1, a kinase which is activated at late S phase.[40] Recombinant cyclin A/cdk1

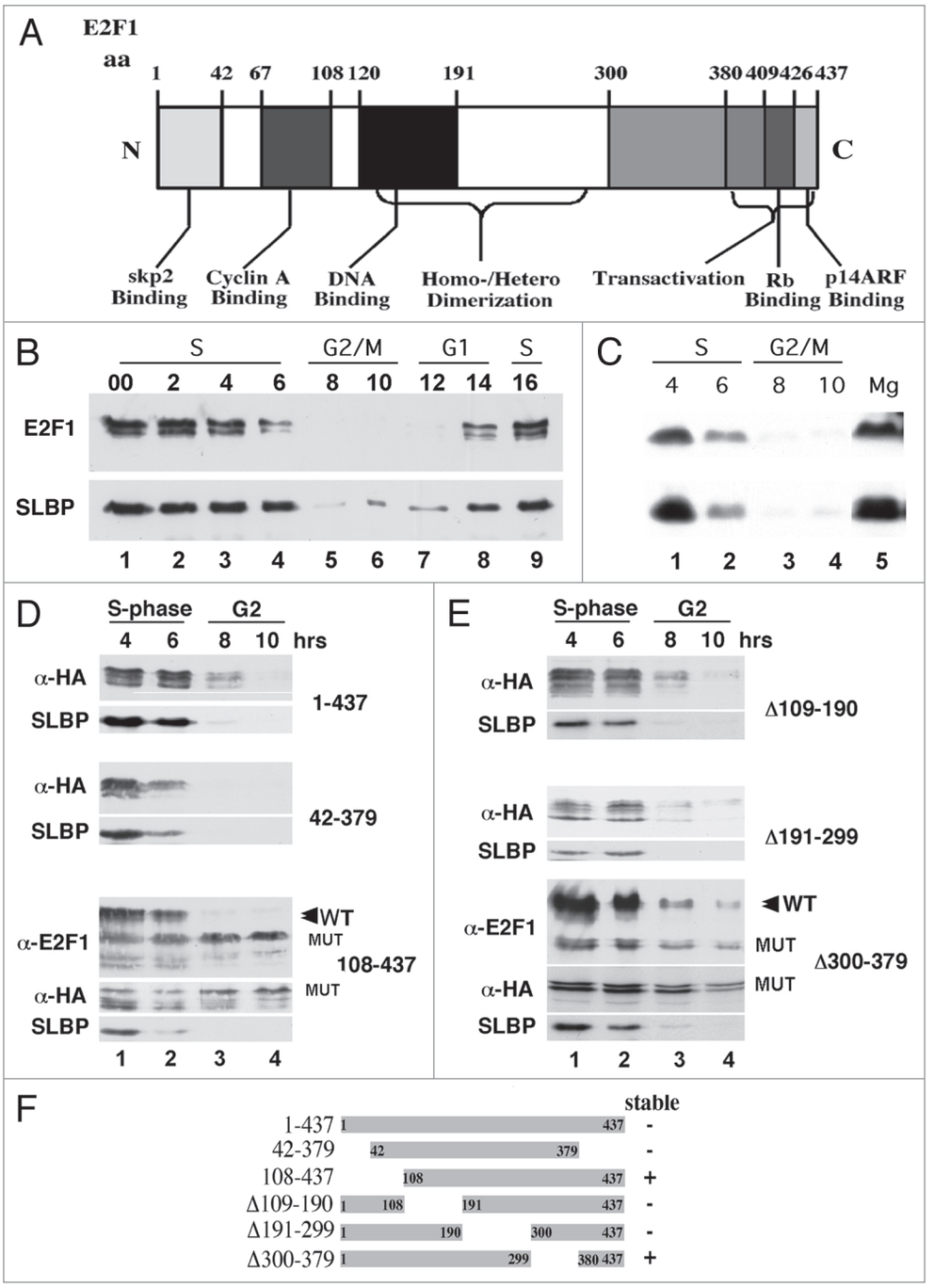

Figure 2. Definition of a region required for E2F1 degradation at the end of S phase. (A) A schematic of the domains of the E2F1 protein is shown. (B and C) HeLa cells were synchronized by double-thymidine block and released into S phase. At the indicated times, lysates were prepared and analyzed for SLBP and E2F1 levels by western blotting. In (C) MG132 was added 4 hrs after release into S phase and lysates prepared 6 hrs later (lane 5). (D and E) HeLa cells stably expressing the E2F1 constructs tagged with HA (shown in F) on the amino terminus were synchronized by double-thymidine block; lysates prepared and analyzed by western blotting for the HA-tagged protein,SLBP and where the tagged protein was resolved from the wild-type protein, for endogenous E2F1. In (D), the top part is full-length E2F1, the middle part deletion of amino acids 1–42 and the bottom part, deletion of amino acids 1–107. The bottom part was probed with anti-E2F1 and the endogenous and mutant protein were detected. In (E), the top part is deletion of amino acids 109–190, the middle part deletion of amino acids 191–299 and the bottom part deletion of amino acids 300–379. This blot was probed with both anti-E2F1 and anti-HA. (F) A summary of the effect of the deletions on degradation of E2F1 at the end of S phase is shown.

phosphorylates T61 of SLBP but recombinant cyclin A/cdk2 does not. Degradation of SLBP requires a KRKL sequence about 40 amino acids after the phosphorylation sites and phosphorylation of T61 of SLBP by cyclin A/cdk1 depends on the KRKL sequence. Given the requirement of the cyclin A binding site for E2F1 degradation (**Fig. 3B**) and the timing of the

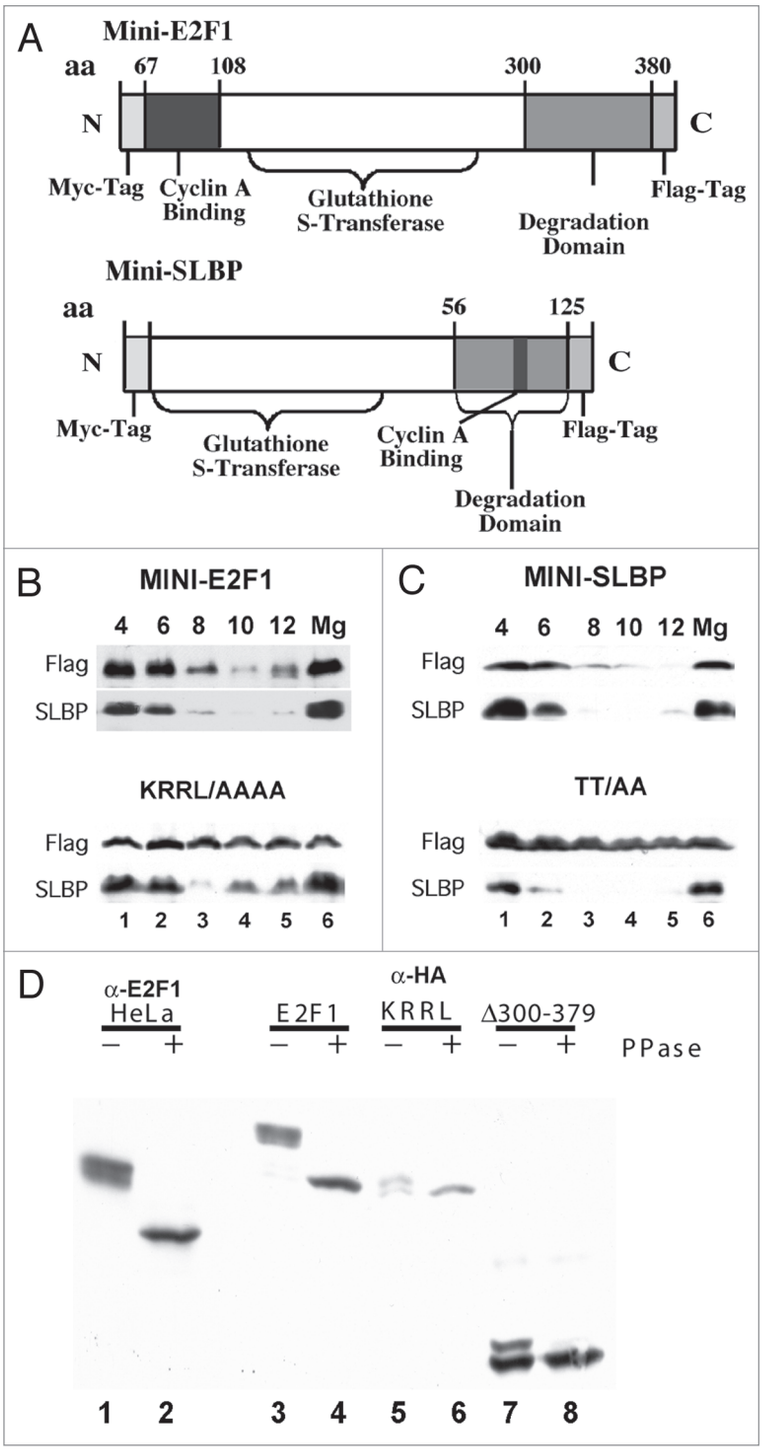

Figure 3. Phosphorylation of E2F1 requires a cyclin binding site for E2F1 degradation. (A) The indicated minigenes for SLBP[5] and E2F1 are shown. Amino acids 56–125 of SLBP are sufficient to direct degradation of the protein. The E2F minigene contains the two fragments of E2F1 identified in **Figure 2**, 67–108 and 300–380 separated by GST. Point mutants of the core of the cyclin binding site of E2F1 and in the TT which is the target of phosphorylation of SLBP were also constructed in the context of the minigene. (B and C) Cells stably expressing the E2F1 minigene and mutant minigene (B) or the SLBP minigene or mutant minigene (C) were synchronized by double-thymidine block, released into S phase. Parallel cultures expressing the SLBP and E2F minigenes were synchronized and lysates were prepared at the indicated times after release and analyzed by western blotting for the FLAG-tag on the minigene or SLBP antibody to detect endogenous SLBP. (D) Cells expressing HA-tagged wild-type E2F1, the cyclin binding site mutant in the wild-type protein, of the 300–379 deletion (lanes 3–8) were synchronized by double-thymidine block and lysate prepared 4 hrs after release into S phase. A portion of the lysate was treated with lambda protein phosphatase. Equal amounts of the treated and untreated lysates were analyzed by western blotting using the anti-HA antibody after resolution of long polyacrylamide gels for an extended amount of time to resolve phosphorylated forms. The endogenous E2F1 was analyzed in lanes 1 and 2 using the E2F1 antibody. Note the difference in mobility of the endogenous E2F1 and the tagged-E2F1 is due to the HA-tag.

degradation, it is likely that cyclin A/cdk1 phosphorylates a critical site on E2F1 leading to its degradation.

The second kinase required for degradation of SLBP is casein kinase II (CK2). CK2 phosphorylates T60 of SLBP, but only after T61 has been phosphorylated by cyclin A/cdk1.[5] CK2 has also been shown to be important for proper cell cycle progression. CK2 activity is required for the G_1/S and G_2/M transition in yeast[41] and mammalian cells.[42,43] The consensus CK2 target site requires nearby negative charges and often requires priming phosphorylations by other kinases. Another known example of cooperation of CK2 and cyclin/cdk1 is in G_2, when phosphorylation of Wee1 by a cyclin/Cdk1 primes phosphorylation by CK2 resulting in production of a phosphodegron that is necessary for proper Wee1 degradation and proper G_2/M transition.[43]

Cyclin A/cdk1 and DNA Replication

A number of substrates for cyclin A/cdk1 have been identified recently and many of these share a property that phosphorylation contributes to the inactivation of proteins required for DNA replication. These include Orc1[44] and Fen1,[45] each of which is required for DNA replication, and the CDP/Cux transcription factor,[46,47] which is necessary for transcription of the DNA polymerase α gene. These results are consistent with the possibility that activation of cyclin A/cdk1 at the end of S phase is an event that marks the end of S phase. Cyclin A/cdk1 may also help prevent premature activation of the APC during G_2

by phosphorylating cdh1 on the APC.[40] The other known substrate of cyclin A/cdk1 is cdc25B,[48] which is degraded as a result of phosphorylation by cyclin A/cdk1, possibly as part of an autoregulatory loop. Thus, one role of cyclin A/cdk1 may be to irreversibly inactivate DNA replication to prevent re-replication of DNA, as well as inactivating histone mRNA biosynthesis at the end of S phase.

There are likely other examples of phosphorylations that might be carried out by cyclin A/cdk1 but have only been identified as cyclin A substrates. For example, a cyclin A/cdk is believed to have role in cyclin B/cdk1 activation,[49] another event that occurs at G_2. It is possible that cyclin A/cdk1 activity may be needed for the initial, activating phosphorylation of Cdc25 and/or inhibitory phosphorylation of Wee1 to trigger the cyclin B/cdk1 autoactivation loop for robust cyclin B/cdk1 activation and M-phase entry.

The mechanism by which the cell modulates cyclin A/cdk1 and cyclin A/cdk2 activity remains elusive since all three polypeptides are present throughout S and G_2-phase, until cyclin A is degraded at mitosis. Recent studies by Merrick and colleagues show that cyclin A binds cdk2 early in S phase and only binds cdk1 near the end of S phase. They also point out that the different activation pathways for cdk1 and cdk2 contribute to the initation of cyclin A/cdk1 activity at the end of S phase.[52,53] In addition chk1 kinase acts to prevent premature activation of cyclin A/cdk1, by destabilizing Cdc25A.[52,53] It is possible that cyclin A-bound cdk1 is either more readily dephosphorylated by Cdc25 and/or resistant to inhibitory phosphorylations by kinases such as Wee1. This may be the reason for detection of cyclin A/cdk1 activity starting from late S phase toward G_2, where it peaks, while cyclin B/cdk1 activity stays low until G_2/M when a rapid activity increase causes M phase entry.

Conclusions

The major cell cycle transitions are driven by changes in activities of specific cyclin/cdks. Our results together with those of others, suggest that the transition from S phase to G_2 phase may be mediated by the increase in the activity of cyclin A/cdk1 at S/G_2. This occurs prior to the robust activation of cyclin B/cdk1, which results in nuclear envelope breakdown and entry into mitosis. Activation of cyclin A/cdk1 results in inhibition of histone mRNA synthesis due to degradation of SLBP, as well as degradation of E2F1-3 and inhibition of several components involved in DNA replication. Thus activation of cyclin A/cdk1 helps shut down the chromosome replication machinery and plays a critical role as the cell transitions from S phase into G_2 phase when it prepares to enter mitosis.

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