CELL CYCLE REGULATION OF THE STEM LOOP BINDING PROTEIN:

A KEY REGULATOR IN HISTONE mRNA METABOLISM

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ABSTRACT

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(Under the direction of Prof. Dr. William F. Marzluff)

Metazoan replication-dependent histone mRNAs are unique among eukaryotic mRNAs since they lack polyadenylated tails, instead ending in a conserved stem-loop. Expression of these histone mRNAs is tightly cell cycle regulated mostly via posttranscriptional mechanisms which are mediated by the 3' stem-loop. The stem loop binding protein (SLBP), which binds to 3' end of histone mRNAs, plays a major role in histone mRNA metabolism by participating in multiple steps including histone mRNA processing, translation and stability. SLBP expression is limited to S phase, and cell-cycle regulation of SLBP is one of the major mechanisms that restrict histone mRNA biosynthesis and thus histone production to the S phase.

The SLBP level is quite low during G1 and it dramatically increases towards S phase. It has been shown that the low level of SLBP at early G1 is due to the low translation efficiency of SLBP mRNA and the translation of SLBP is activated as cells approach S phase. At the end of S phase, SLBP is rapidly degraded depending on phosphorylations of two threonines in N-terminal SFTTP sequences.

In this dissertation, I have further elucidated the molecular details of the mechanisms involved in cell cycle regulation of SLBP expression. I have determined Cyclin A/Cdk1 as the kinase that triggers degradation of SLBP at the end of S phase by phosphorylating SLBP Thr61. Cyclin A/Cdk1 activity is cell cycle regulated similar to SLBP Thr61 phosphorylation and is the major activity in late S-phase cells that phosphorylates Thr61. We propose that as Cyclin A/Cdk1 activity reaches to certain level near the end of S-phase, and it triggers SLBP degradation as one of the mechanisms to shut down histone mRNA biosynthesis.

Moreover, I have shown that the SLBP coding sequence is sufficient to induce low translational efficiency of SLBP mRNA as a regulation to keep the SLBP protein level low at early G1. I further showed that at some point in early G1, SLBP translation efficiency recovers back to S phase level but protein stability decreases. It is likely that there is then regulated degradation of SLBP limiting SLBP level until next G1/S.

TO MY BELOVED WIFE

BEYZA NUR

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INTRODUCTION

CELL CYCLE and ITS CONTROL

Progression through the eukaryotic cell cycle is highly controlled series of events that lead to eukaryotic cell reproduction. In each cycle, cells must coordinate DNA synthesis and chromosomal segregation to generate an exact replica of themselves. These events are confined to discrete periods called synthesis (S phase) when DNA is replicated once and mitosis (M phase) when resulting duplicated chromosomes are equally segregated into two daughter cells. In most of the cycles, these periods are separated by two gap phases called G1 and G2. The first gap phase, G1, occurs between M and S phase and the second gap phase, G2 occurs between the end of S-phase and M phase.

Eukaryotic cell cycle progression is a tightly regulated process that depends on the orderly activation and inactivation of a series of Ser/Thr kinases called Cyclin-dependent kinases (Cdks) (Hunter and Pines, 1991; Hunter and Pines, 1994; Norbury and Nurse, 1992). Cdks are active only after association with certain Cyclins (Morgan, 1995), whose synthesis and degradation are tightly controlled during cell cycle (King et al., 1996; Hershko, 1996; Elledge and Harper, 1998). Different Cyclin/Cdks have been shown to be involved in regulation of different phases of the cell cycle: Cyclin D/Cdk4/6 controls G1 progression, Cyclin E/Cdk2 mediates S phase entry, CyclinA/Cdk2 is important for S phase progression,

and Cyclin A/B/Cdk1 controls G2/M transition during which active CyclinB/Cdk1 is the actual Mitosis Promoting Factor (MPF) (Sanchez and Dynlacht, 2005).

In addition to binding the Cyclin subunit, Cdk activity is regulated by posttranslational modifications such as phosphorylation/dephosphorylation, and association with group of inhibitory proteins collectively called CKIs (Morgan, 1995; Sherr and Roberts, 1995; Sherr and Roberts, 1999; Xiong, 1996). One class of CKIs, comprised of p21, p27, and p57, binds and inhibits Cyclin/Cdk complexes, whereas the other class of Cdk inhibitors, Ink4, p15, p16 and p18, binds to Cdk4/Cdk6 and prevent their activation by the G1 Cyclin, Cyclin D.

The eukaryotic cell cycle is characterized by a series of transitions from one phase to another. Different Cyclin/Cdk complexes are activated at a certain time window and regulate several critical downstream effectors by specific phosphorylation. This sequential activation results in accomplishment of various biochemical events characteristic of each cell-cycle stage and triggers entry into the next cell cycle phase. The places where cells monitor the completion of previous phase and then start the initiation of next phase are called checkpoints. The first checkpoint is called Start or Restriction point. This is particularly important because at this point cells evaluate external signals such as growth conditions and inhibitory or activating signals and decide to continue dividing or to exit from the cell cycle into a quiescent state which is called G0.

In resting cells (G0), activation of Cyclin D transcription in response to mitogens is considered as the first step to drive a cell towards division. Cyclin D protein then associates with Cdk4/6 and initiates G1 progression (Weinberg, 1995). Cyclin D is very a unstable protein and this in part maintains the requirement for continued synthesis of Cyclin D, and

hence the presence of mitogens at this point. The main targets of Cyclin D/Cdk4/6 are members of the retinoblastoma tumor suppressor protein (RB) family, which occupy a central role in G1 progression. In the hypophosphorylated form, Rb interacts and represses the E2f transcription factor family (Weinberg, 1995). E2fs in association with Dp proteins (Dp1 and Dp2 in mammals) regulate the expression of various critical genes for G1/S progression. In the phosphorylated form, Rb dissociates from the E2f/Dp complex which then can activate numerous genes required for G1/S progression and S phase including Cyclin E and Cyclin A which are called G1/S and S phase cyclins respectively (Duronio et al., 1995; Sherr and Roberts, 1999). Cyclin E/Cdk2 (also Cyclin A/Cdk2) can further phosphorylate Rb keeping it inactive, and producing a positive feed back loop which allows to cell to reach the point where cell cycle progression become independent of mitogen presence and the cell passes through the so called Restriction point.

When G1/S and S phase specific Cyclin/Cdks, Cyclin E/Cdk2 and Cyclin A/Cdk2 are activated, they phosphorylate downstream substrates not only required for triggering next cell cycle events but also remove negative impediments to G1 progression. For example, Cyclin E/Cdk2 phosphorylates p27, a Cyclin/Cdk2 inhibitor that prevents G1/S progression and triggers p27 degradation (Vlach et al., 1997). They also phosphorylate factors involved in DNA replication, including Mcm 2-7, Cdc7, Cdc6, Cdt1 allowing the cells both enter and then complete S-phase. At the end of S-phase cells enter G2, defined as a period without DNA replication prior to nuclear envelope breakdown, chromosome condensation and entry into mitosis. Exit from G2 into M-phase requires passage through the G2/M checkpoint, which then results in activation of M phase Cyclin/Cdk complexes which are Cyclin A/Cdk1 and Cyclin B/Cdk1. These M phase Cyclin/Cdks drives the cell to the next checkpoint called

the metaphase to anaphase checkpoint where cells make sure of proper spindle assembly and chromosome alignment. At metaphase-anaphase transition, the anaphase promoting complex (APC) is activated, result in degradation of the Cyclin A and B as well as other proteins involved in chromosome cohesion. This then results in the separation of sister chromatids, exit from mitosis and cytokinesis.

Regulation of the Cyclin/Cdks:

In yeast (*S.pombe and S.cerevisiae*) all cell cycle events are controlled by a single Cdk called Cdk1 which is also called Cdc2 (*S.pombe*) and Cdc28 (*S.cerevisiae*). The activity and substrate specificity of this kinase is controlled by different cyclins at different cell cycle stages. In multicellular eukaryotes, there are multiple Cdks as well as multiple Cyclins. In mammals, there are four Cdk1 like proteins that have a direct role in regulation of the cell cycle. In mammalian cells, two of these cdks, Cdk1 and Cdk2 control cell cycle transitions, and operate primarily at M and S phase, respectively. In addition to these, two other Cdks called Cdk4 and Cdk6 are involved in control of cell cycle entry in response to extracellular signals. Also another family member, Cdk7, is involved in cell cycle regulation indirectly by acting as Cdk Activating kinase that phophorylates other Cdks.

Activation of all Cdks requires binding of Cyclins and in most cases full activation also requires a phosphorylation of a threonine residue near the kinase active site. During the cell cycle, periodic synthesis and rapid targeted degradation of different cyclins is a major mechanism that irreversibly drives cell cycle progression by changing the activity of different Cyclin/Cdk complexes.

Most of the information about the structure of the Cdks has been obtained from the crystal structures of Cdk2, Cyclin E/Cdk2 and Cyclin A/Cdk2 (De Bondt et al., 1993; Jeffrey et al., 1995; Honda et al., 2005). Cdks, which have a two-lobed structure like other kinases, are inactive in the absence of Cyclins due to two structural modifications. The active site of the Cdks is present in a buried cleft between these two lobes. ATP binds deep in the cleft with its phosphates oriented outwards. The substrate protein normally interacts with the entrance of this active-site cleft. But in the absence of Cyclins, this region is blocked by large flexible loop called T- loop or activation loop. Also, key residues in the ATP binding site are not ideally oriented which further suppresses the Cdk activity. Thus, Cdks require significant changes in the structure of their active site to become active.

Cyclin binding introduces significant conformational changes in the Cdk active site. The major change occurs within the T- loop, in which L12 helix changes to a beta strand, which no longer blocks the substrate binding site and makes the active site available. Also major changes occur at the ATP binding site resulting in the proper positioning of the ATP phosphates for an efficient phosphotransferase reaction. As mentioned above, full activation of a Cdk requires phosphorylation of a threonine residue near to the kinase active site (Thr161 in Cdk1, Thr162 in Cdk2). In mammalian cells, this phosphorylation can occur after Cyclin binding and causes the T-loop to flatten and interact more extensively with the binding Cyclin partner. Further, this phosphorylation enhances the interaction with the substrate with SPXK/R, the canonical Cdk target sequence (Russo et al., 1996). The activating Thr 160 phosphorylation is catalyzed by enzymes called Cdk activating kinases (CAKs) (Harper and Elledge, 1998). These enzymes are constantly active during the cell cycle and are not regulated by any known cell cycle pathway. Therefore, Cyclin binding but

not the activating phosphorylation is considered the highly regulated and rate-limiting step in Cdk activation, in vivo.

Although the activating phosphorylation is not regulated, two inhibitory phosphorylations on Tyr 15 and Thr 14 have a significant role in regulating Cdk activity. These residues are present at the roof of the kinase ATP-binding site and their phosphorylations introduce inhibition by interfering with the orientation of ATP phosphates. The phosphorylation state of these two inhibitory phosphorylations is regulated by opposing effects of kinase and phosphatases. Wee1/Mik1 is the kinase that introduces the Tyr 15 inhibitory phosphorylation. Also in vertebrates another kinase called Myt1 can phosphorylate both Tyr 15 and Thr 14. Dephosphorylation of these inhibitory sites is mediated by a family of phosphatases called Cdc25 (Morgan, 1997).

These inhibitory phosphorylations are particularly important in regulation of M phase Cdk activation at the onset of mitosis and also have been shown to be involved in G1/S and S phase Cdk activation. During late S phase and G2, Cyclin B accumulates and associates with Cdk1 but the Cyclin B/Cdk1 complex stays inactive due to these inhibitory phosphorylations. At G2/M, there is a large increase in Cdc25C activity and a decrease in Wee1 activity that together triggers a rapid increase in Cyclin/Cdk1 activity and cause M phase entry. Also removal of inhibitory phosphorylations is important for activation Cyclin/Cdk2 complexes for G1/S and S phase progression.

In vertebrates, Cdc25 has three members, Cdc25A, Cdc25B and Cdc25C, and their activity level differs throughout the cell cycle (reviewed in (Boutros et al., 2006). Cdc25A expression and activity appears at G1/S and peaks at M phase. It is involved primarily in Cyclin/Cdk2 activation to allow S-phase entry and S phase progression, and also may

participate in Cyclin/Cdk1 activation at G2/M. Cdc25B expression and activity starts to increase at late S phase and peaks at G2 and believed to have a role in G2/M regulation of Cyclin/Cdk1. Finally, although Cdc25C expression doesn't change significantly during cell cycle, its activity peaks at G2/M and triggers M phase by activating Cyclin B/Cdk1. Recent studies suggest that Cdc25B,C may also have some role in S phase progression supporting the recent model that the activities of these three phosphatases are in coordinated collaboration throughout cell cycle (Boutros et al., 2006).

Cyclin/Cdks and substrate recognition:

The optimal target sequence for recognition by Cdk1/2 in vitro has been established as S/TPXK/R with absolute requirement proline at +1 position and a less stringent need for +3 positive charged residue. Although S/TPXK/R sequence appears to be a consensus Cdk target site (Nigg, 1995; Songyang et al., 1994; Srinivasan et al., 1995), it is clear that different Cylin/Cdks show differences in substrate specifity towards several physiological substrates (Dowdy et al., 1993; Dynlacht et al., 1994; Ewen et al., 1993; Krek et al., 1994; Peeper et al., 1993; Xu et al., 1994). Despite the crucial role of Cyclin/Cdk dependent phosphorylations during the cell cycle, there is limited knowledge about how individual Cyclin/Cdks target different substrates specifically throughout the cell cycle.

Cyclin subunits are involved in refining the substrate targeting by the core Cdk subunit. For example, in mammals Cyclin A/Cdks but not Cyclin B/Cdks can phosphorylate pRb related protein p107. Recently, the S-phase Cyclins, Cyclin E and Cyclin A have been shown to be involved in substrate specificity by specifically binding to different substrates at

the so called Cy or RXL motif to bring the bound Cdk to the proximity of the substrate. In addition to the Cdk inhibitors p21 and p27 (Adams et al., 1996; Zhu et al., 1995), several cell cycle key regulators such as E2f1, p107, p130, pRb, p53, Cdc6 have been shown to use certain RXL motifs to recruit Cyclin/Cdks for specific phosphorylation events (Dynlacht et al., 1997; Lees et al., 1992; He et al., 2005; Hannon et al., 1993; Luciani et al., 2000; Takeda et al., 2001). Although the RXL motif appeared to be crucial for several substrates to be recognized by Cyclin E or Cyclin A/Cdks, there is very limited example of RXL dependent recognition by Cyclin B/Cdk1 (Liu et al., 1999; Malathi et al., 2005).

Finally, it is clear that substrate recognition is more complex than just the RXL motif for cyclins A and E, but residues other than RXL are important for Cyclin recruitment to the substrate. For example, although both Cyclin E and Cyclin A can interact with the peptide spanning the RXL region of E2F1, full-length E2F1 can only recruit Cyclin A/Cdk2 but not Cyclin E/Cdk2.

In summary, in metazoans, it appears that in vivo each Cyclin/Cdk heterodimer is likely to have its own specific group of substrates, and specificity can be determined by Cyclins along with the kinase domain.

Ubiquitin mediated degradation and cell cycle:

Along with specific phosphorylation, a major component of cell cycle regulation is selective and time-controlled degradation of the critical players by the ubiquitin pathway. Ubiquitination of cellular proteins is performed by three classes of enzymes: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and ubiquitin-protein ligase

complex (E3). Repeated transfer of additional ubiquitin molecules results in a polyubiquitin chain on the substrate protein which labels it for degradation by the proteosome.

The specificity of ubiquitin mediated degradation is determined by E3 ligases that are generally defined to mediate two distinct functions: catalyzing the isopeptide bond formation and recruiting specific substrates (Hershko and Ciechanover, 1998; Hochstrasser, 1996). Based on their characteristic motifs, there are two major groups of E3 ligases: RING domain and HECT domain E3s. RING domain E3s contain either an intrinsic ring finger or an associated Ring finger protein subunit essential for ubiquitin ligase activity (Deshaies, 1999; Pickart, 2001). Multiple different Ring finger E3 ligases, including SCF and APC, have been shown to have important roles in the regulation of the cell cycle.

The SCF (Skowyra et al., 1997; Bai et al., 1996; Feldman et al., 1997) helps control the G1/S transition by inducing degradation of Cdk inhibitors p27 and sic1(Vlach et al., 1997; Pagano et al., 1995; Verma et al., 1997) and degradation of key players like Cyclin E (Moberg et al., 2001; Koepp et al., 2001). SCF is a protein complex, consists of a Cullin protein, Cul1, a Ring finger protein, Roc1, a F box protein and a linker protein called Skp1 (Seol et al., 1999; Skowyra et al., 1997; Skowyra et al., 1999). In this complex, the F box protein specifically recognizes the phosphorylated SCF target and brings it to Cul1 by binding to a linker protein called Skp1 that can bind both Cul1 and F box protein (51). Roc1 interacts with E2 and binds to the other end of Cul1, which functions as bridge to bring the target and E2 together. Regulation of degradation by SCF occurs by regulation of the phosphorylation of the substrates as well as regulated expression of F box proteins.

The other key E3 ligase involved in the cell cycle is the APC, which is required to induce M phase progression and exit by mediating degradation of cell cycle regulators like

Pds1/securin and Cyclin A/B. APC is a large complex consists of at least 11 subunits including a catalytic core formed by a cullin-like protein, Apc2, and a Ring finger protein called Apc11. The targets of APC have conserved motifs that target them for ubiquitination. The most widespread motif is the so called Destruction box (D-box) with some version of an RXXL sequence. Another important motif is the Ken-Box with KENXXXN sequence.

The activity of APC is controlled by binding of different activator subunits Cdh1 and Cdc20 which triggers APC ubiquitin ligase activity towards appropriate substrates during different cell cycle stages. At M phase, Cyclin/Cdk1 complexes phosphorylate core APC subunits and enhance Cdc20 binding. APC/Cdc20 targets securin for triggering sister chromatid separation and also inactivates Cyclin/Cdk1 by triggering Cyclin A and Cyclin B destruction (Shirayama et al., 1999). As a result of cyclin destruction at the end of mitosis, due to lack of Cyclin/Cdk1 activity, Cdc20 is released and APC/Cdc20 is no longer active (Peters, 2002).

In contrast to the case with Cdc20, the Cyclin/Cdk activity inhibits binding of the other activating subunit Cdh1 to APC by phosphorylating Cdh1 throughout the S and M phases until the metaphase to anaphase transition where Cyclins are degraded by APC/Cdc20 causing inactivation of Cdks (Kramer et al., 2000; Zachariae et al., 1998a; Blanco et al., 2000). This allows Cdh1 dephosphorylation and interaction with APC, which keeps the APC/Cdh1 activity high during G1 until the next S phase. Several S phase related proteins such as Cyclin A, Cdc6, Skp2 are targeted by APC/Cdh1 during G1(Harper et al., 2002).

In human cells, there are six other cullin family proteins in addition to Cullin1. Also there is at least one more cullin-like protein, the PARkin-like cytoplasmic protein (PARC), in addition to Apc2 (Zachariae et al., 1998b; Yu et al., 1998; Nikolaev et al., 2003). Each of the

cullin family members nucleates a multisubunit E3 ligase complex that probably has a similar modular architecture as the SCF. All cullins associate with a RING protein (which is Roc1 for all cullins except Cullin 5 which prefers Roc2) through their C-terminal domain and the Roc1 protein recruits E2 to form the catalytic core, whereas the N-terminal region interacts with specific adaptor proteins to recruit a wide variety of receptor proteins that confer substrate specificity (Ohta et al., 1999). Each cullin has its own adaptor protein(s). While Cullin1 and 7 interact with Skp1, Cullin 2 and 5 interact with elongin BC, Cullin 3 interacts with BTB domain containing proteins and Cullin 4 interacts with DBB1 (Michel and Xiong, 1998; Dias et al., 2002; Geyer et al., 2003; Furukawa et al., 2003; Wertz et al., 2004). The cullin adaptor complexes then interact with substrate binding proteins: Cullin 1/Skp1 interacts with F box proteins, elongin BC interacts with suppressor of cytokine signalling/elongin-BC (SOCS/BC box) proteins (Bai et al., 1996; Kamura et al., 1998). In the case of Cul3, both adaptor protein and substrate receptor functions merged in the multiple proteins that bind to Cul3 via their N terminal BTB domain and to the substrate through their C-terminal domain (Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003; Furukawa et al., 2003).

The large number of F box, BTB-domain and SOCS/BC box proteins results in a huge number of potential Cullin E3 ligases that can target a wide variety of proteins which are potentially performing various cellular functions, including many related to cell cycle that are yet to be discovered.

Translation control and cell cycle:

Proper cell cycle progression requires the expression and activation of key proteins at certain time periods. As explained above, transcriptional regulation or posttranslational regulations like phosphorylation and regulated degradation are very important for cell cycle progression. Recently, control of gene expression at the translation level has emerged as an essential and relatively poorly studied mechanism in cell cycle regulation. Expression of the some the key players of cell cycle such as Cyclin D, Cyclin E, p27, p58, p53, Cdk4, SLBP have been shown to be controlled at the translation level (Cornelis et al., 2000; Millard et al., 2000; Millard et al., 1997; Polymenis and Schmidt, 1997; Whitfield et al., 2000).

In general, translational regulation may occur at two different levels: "global" control in which activity of translation machinery is regulated resulting in changes in translation efficiency of many cellular mRNAs, and "selective" control where translation of certain mRNAs is regulated specifically without affecting the general translation.

Global translation regulation is mostly regulated at the translation initiation level by affecting the level and activity of initiation factors. As mRNAs are exported from nucleus, the eukaryotic translation initiation multiprotein complex eIF4F assembles on the 5'Cap structure-m7GpppN (N is any nucleotide). The eIF4F complex consists of 3 subunits: eIF4E (the cap-binding protein), eIF4A (an RNA helicase), and eIF4G which functions as scaffold protein that can interact with eIF4E and eIF4A and several other proteins like PABP (poly A binding protein) and Mnk1 (the eIF4E kinase) at the same time. It also interacts with the 40S subunit of the ribosome through eIF3.

The activity and availability of eIF4E is regulated as an important mechanism to introduce global control on Cap-dependent translation which represents the majority of the translation in the cell. Generally the amount of active eIF4E is less than the number of

mRNAs in the cell, resulting in a competition between the mRNAs for the available eIF4E. Increasing the amount of active eIF4E thus results in the translation of several mRNAs which normally may compete poorly for eIF4E. eIF-4E is a phosphoprotein and its phosphorylation by the kinase called Mnk1 enhances the translation rate. Also there is a family of translation inhibitory proteins called eIF4E-binding proteins (4E-BPs), whose hypophosphorylated forms compete with eIF4G to bind same binding site on eIF4E that eIF4G binds. High amounts of hypophosphorylated 4E-BP reduce the rate of Cap-dependent translation. Phosphorylation of the 4E-BPs prevents their binding to eIF4E, effectively increasing the concentration of active eIF4E, thus translation rate (Richter and Sonenberg, 2005).

During the cell cycle, translation is regulated at the global level such that the translation level is robust to allow cells to progress through G1 and S-phase, and translation becomes low at M phase (Hay and Sonenberg, 2004; Pyronnet and Sonenberg, 2001). It has been shown that the MAP kinase and Tor kinase pathways are involved in the G1/S transition by affecting translation efficiency (Fukunaga and Hunter, 1997; Gingras et al., 2001; Burnett et al., 1998). mTor can directly phosphorylate and activate p70 S6 ribosomal kinase, P70S6K, which is particularly important for translation of special class of mRNAs with 5' oligopyrimidine tract such as mRNAs of several ribosomal proteins and translation elongation factors. mTor also phosphorylates 4E-BP providing more active eIF4F. In addition to mTor, MAP kinase mediated phosphorylation and activation of Mnk1 is also important during G1 progression. Mnk1 is eIF4E kinase which also enhances translation. Activation of these pathways allows the cell to enhance the ribosome production that it will need for cell growth prior to cell division (Hay and Sonenberg, 2004).

Although the global regulation of translation effects translation of most of the mRNAs, it can end up with relatively specific effects on translation of certain mRNAs due to some cis-elements such as a secondary structure at their 5'UTR (Gingras et al., 1999) or upstream AUGs. These classes of mRNAs consist mostly of mRNAs encoding proteins related to cell growth and cell-cycle progression (e.g. the growth factor thrombopoetin, FGF2 and Cyclin D1, c-myc)(Muise-Helmericks et al., 1998). Translation of these mRNAs is more sensitive to eIF4F assembly for example because of their higher need for the unwinding activity brought by eIF4A RNA helicase. Thus increasing the concentration of active eIF4F in the cell specifically stimulates the translation of many mRNAs required for cell growth and cell cycle progression.

During M phase general translation level diminishes significantly. At M phase, 4E-BP is hypophosphorylated and this causes the disruption of the eIF4F complex and an overall low translation level. Although Cap-dependent general translation is inhibited during M phase, a subset of mRNAs are translated via cap independent translation where ribosomes are brought to mRNAs by an internal ribosome entry site (IRES) (without need for eIF4G complex), which is considered as mechanism of cell cycle dependent translation control on expression of certain proteins.

In addition to global translation regulation, selective regulation mostly occurs by recognition of specific cis-elements in the target mRNAs by specific proteins or miRNAs. For example, as a critical cell cycle protein, translation of p27 mRNA is regulated by binding of RNA binding proteins HuR and hnRNP C1, C2, which interact specifically with U-rich element in its 5'UTR (Millard et al., 2000). As example for translation inhibition by miRNAs, specific miRNAs has been determined to target 3'UTRs and inhibit translation of

the important cell cycle proteins such as E2F1, c-myc and p27 (O'Donnell et al., 2005). The regulation of translation by miRNAs is a rapidly developing field and the large numbers of miRNAs have the potential to regulate a large number of mRNAs. The selective translation control, as a mechanism of regulation of several critical cell cycle proteins, is becoming an important but yet understudied control mechanism during cell cycle.

S PHASE: REPLICATION OF CHROMOSOMES

In eukaryotic cells, the DNA in the chromosomes is highly packaged into a DNA-protein assembly called chromatin. The chromatin structure is composed primarily of histones and as well as non-histone proteins. The chromatin structure is not only important for DNA packaging which is required for genomic stability but also is very important for regulation of gene expression.

During each S phase, eukaryotic cells must not only replicate their DNA, but must also assemble the chromatin structure in each daughter chromosome properly. The first step in chromosome assembly is to assemble new nucleosomes on the newly synthesized DNA. This requires a rapid production of for raw materials, namely histone proteins to package newly replicated DNA into nucleosomes. In metazoans, a unique set of genes, the replication dependent histone genes, which are primarily expressed in S phase, encode the majority of histone proteins. These mRNAs have several unique properties which distinguish them from the rest of the eukaryotic mRNAs, and they require a distinct set of factors for many aspects of their expression and regulation (Marzluff, 2005). A minority of the histone proteins, the

so-called replacement variant histones, are constitutively expressed at low rate from polyadenylated mRNAs, which are metabolized like rest of the eukaryotic mRNAs.

Replication dependent histone genes and cell cycle regulation:

In all metazoans, replication dependent histone genes have distinct characteristics: first, they lack introns, second they are physically linked in large clusters, and finally they encode unique mRNAs which don't have polyA tails but instead they end in a highly conserved 26 nucleotides sequence with a stem loop structure. Other than 5' capping, the only processing step necessary to produce mature histone mRNA is endonucleolytic cleavage after 3' stem loop structure (Gick et al., 1986; Dominski and Marzluff, 1999). This cleavage reaction requires two cis-elements, the stem loop structure and the purine rich element called the histone downstream element (HDE) which binds to the U7snRNP (Mowry and Steitz, 1987; Mowry et al., 1989; Cotten et al., 1988). Along with the U7snRNP, a 31 kD protein called stem loop binding protein (SLBP) that binds to stem loop structure, is required for histone mRNA processing.

The HDE interacts with U7 snRNP which is a small nuclear ribonucleoprotein (snRNP), by base pairing with 5'end of U7 snRNA. In addition to the HDE complementary site, U7 snRNA has a unique Sm binding site that recruits 7 Sm-proteins, five of which are shared with spliceosomal snRNPs, and two novel proteins, Lsm10 and Lsm11 which are likely to be restricted to U7snRNP. In mammals, U7snRNP contains an additional protein called ZFP100 that interacts both to SLBP and stem loop complex and Lsm11, possibly bridging the Stem loop structure with the U7snRNP. The Cleavage reaction occurs between

the stem loop structure and HDE by a complex that contains CPSF-73 and several other factors that are known to play a role in polyA mRNA maturation (Dominski et al., 2005).

Replication dependent histone mRNAs are tightly cell cycle regulated. As cells progress G1 to S phase the level of histone mRNAs increase 35 fold and decrease back to the same amount at the end of S phase (Harris et al., 1991). Most of this regulation is done by posttranscriptional mechanisms which are mediated by stem loop structure (Pandey and Marzluff, 1987; Marzluff and Pandey, 1988). As cells enter S phase, the transcription of histone genes increases only 3 to 5 fold and as major regulatory step, pre-mRNA processing efficiency increases 10 fold, and together these account for the 35 fold increase in histone mRNA level. At the end of S phase, histone mRNA processing is inhibited and the half life of histone mRNA is reduced to 10 minutes (Harris et al., 1991).

A major component of this regulation is the cell-cycle regulation of the key player SLBP, which is synthesized just before entry into S-phase and rapidly degraded at the end of S-phase, effectively regulating histone pre-mRNA processing, in vivo (Zheng et al., 2003). SLBP appears to be the only factor involved in histone mRNA processing that is cell cycle regulated. It is the sole player needed for processing that is absent in G1 and G2 nuclear extracts.

Although both histone mRNA and SLBP are regulated in parallel during the cell cycle, the signals regulating their expression differ. Although stability of histone mRNAs is directly coupled to DNA replication, SLBP expression is controlled most probably by cell cycle signals and is not affected by the status of DNA replication. When G1 cells are treated with aphidicolin, SLBP accumulates normally after cells commit for S phase. However, histone mRNA levels never increase. In parallel with that observation, when we treated cells

with DNA replication inhibitor, hydroxyurea, histone mRNA level rapidly go down while the SLBP level remains constant. Finally, when SLBP degradation is blocked at the end of S phase, histone mRNA levels go down normally when replication is completed.

SLBP is the key player in histone mRNA metabolism, participating in multiple steps of histone mRNA metabolism. After the processing, SLBP stays on the histone mRNA and is involved in both translation and mRNA stability (Wang et al., 1996; Dominski et al., 1999; Sanchez and Marzluff, 2002). Consistent with its role in histone mRNA synthesis and translation, when SLBP levels are knocked down by RNA interference, the rate of progression through S-phase is greatly slowed (Wagner et al., 2005). In contrast U7 snRNP is present constitutively during the cell cycle, and the limiting factor for histone pre-mRNA processing is the presence of SLBP.

Cell cycle regulation of SLBP- the key player in histone mRNA metabolism:

The SLBP level is quite low at G1 and it dramatically increases as cells enter S phase. It has been shown that the low level of SLBP at early G1 is due to the low translation efficiency of SLBP mRNA and as cells approach S phase the translation of SLBP is activated (Whitfield et al., 2000). At the end of S phase, SLBP levels drop due to rapid degradation by the proteosome. The degradation of SLBP at S/G2 requires two threonines and proline residues in the N-terminal SFTTP motif and a downstream KRKL motif which is a putative Cyclin binding site. Previous work by Lianxing Zheng has shown that both of the threonines get phosphorylated by different kinases and trigger late S phase SLBP degradation.

In this dissertation, I have further elucidated the molecular details of the mechanisms involved in cell cycle regulation of SLBP expression. In the second chapter, I showed that Cyclin A/Cdk1 triggers degradation of SLBP by phosphorylating Thr 61 and this is one of the mechanisms to shut down histone mRNA biosynthesis at the end of S phase. In the third chapter, I have tried to determine the mechanisms involved regulating low level of SLBP at G1. I have shown that SLBP coding sequence is sufficient to introduce low translational efficiency of SLBP mRNA as a regulation to keep the SLBP protein level low at early G1. I also provide evidence that after certain point in G1, SLBP translation efficiency recovers back to S phase level but SLBP protein level kept low until S phase possibly by controlled proteindegradation.

CHAPTER II:

Cyclin A/Cdk1 Triggers Degradation of SLBP At The End of S Phase by Phosphorylating Thr 61.

INTRODUCTION

Progression through the cell cycle is driven by a class of protein kinases, the cyclin-dependent kinases (Cdks), which are sequentially activated and deactivated as cells progress from one cell cycle stage to the next. In particular, progress into S-phase requires the activation of Cyclin E/Cdk2, ultimately resulting in the initiation of DNA replication, and progression through mitosis requires the activation of Cyclin B/Cdk1 resulting in nuclear envelope breakdown and chromosome condensation (Sanchez and Dynlacht, 2005). Cyclin A/Cdk2 activity is required for continued progression through S-phase (Yam et al., 2002). The targets of the cdks are activated or inactivated by phosphorylation. A second critical regulatory mechanism for cell cycle progression is targeted proteolysis of key protein regulators (Hoyt, 1997). These include the cyclin subunits of the Cdks, critical proteins in initiation of DNA replication, and proteins responsible for maintaining chromosome pairing (Clarke, 2002).

While much is known about the events that occur for cells transition from G1 to S-phase and for cells to enter and exit mitosis, much less is known about molecular events at

the end of S-phase, as cells progress from S to G2-phase. S-phase is characterized by replication of the chromosomes, and in addition to DNA replication, there must be extensive histone protein synthesis to provide the histones for assembly of the newly replicated chromatin. Histone mRNAs are cell-cycle regulated and their expression is restricted to S-phase. The metazoan replication-dependent histone mRNAs are the only eukaryotic mRNAs that are not polyadenylated. Instead they end in a conserved stem-loop sequence (Marzluff, 2005). Since the replication-dependent histone genes lack introns, the only processing reaction required for histone mRNA biosynthesis is cleavage of the nascent transcript to form the 3' end of the mRNA (Marzluff, 2005).

The 3' end of histone mRNAs is a major cis element responsible for the coordinate regulation of histone mRNA levels (Lüscher et al., 1995). The 3' end is bound by the stem-loop binding protein (SLBP) which is required for both histone pre-mRNA processing and histone mRNA translation. The processing of histone pre-mRNA is cell-cycle regulated, as is the stability of histone mRNAs (Harris et al., 1991). SLBP is also a cell-cycle regulated protein, accumulating only in S-phase cells, and regulation of SLBP levels is an important component of the cell-cycle regulation of histone mRNA (Whitfield et al., 2000). SLBP is rapidly degraded at the end of S-phase, as a result of phosphorylation of two threonines in an SFTTP sequences (amino acids 58-62). Proline 62 is also required for SLBP degradation as is a KRKL sequence (amino acids 95-98) (Zheng et al., 2003), that is a consensus cyclin binding site (Loog and Morgan, 2005; Archambault et al., 2005; Adams et al., 1996; Adams et al., 1999; Schulman et al., 1998; Brown et al., 1999).

I show here that Cyclin A/Cdk1 triggers SLBP degradation at the end of S phase by phosphorylating Thr61. Cyclin A/Cdk1 activity is cell cycle regulated similar and is the

major activity in late S-phase cells that phosphorylates Thr 61 of SLBP. We propose that Cyclin A/Cdk1 is activated near the end of S-phase resulting in degradation of SLBP and down regulating histone mRNA synthesis. It is likely that Cyclin A/Cdk1 phosphorylates other targets at the same time, helping the cell transition from S-phase to G2-phase.

RESULTS

Cyclin/Cdk is involved in degradation of SLBP at S/G2 transition.

SLBP expression is limited to S phase (Whitfield *et al.*, 2000). At the end of S phase, SLBP is rapidly degraded (Fig. 1A, B). Previously, we have shown that SLBP degradation requires phosphorylation of two threonine residues Thr 60, 61 by two different kinases (Zheng *et al.*, 2003). Further, we have shown that along with Thr60 and Thr61, proline 62 and a downstream KRKL region is required for SLBP degradation at the end of S phase (Zheng *et al.*, 2003). The fact that TP (Thr 61, Pro 62) is a possible Cdk target site and KRKL is a putative cyclin binding site suggests that a Cyclin/Cdk is involved in SLBP degradation by phosphorylating Thr61. To test this possibility, I examined the affects of roscovitine, a known Cdk1/Cdk2 specific inhibitor, on SLBP degradation at S/G2 in Hela cells. At G2, SLBP level remained relatively high in cells treated with roscovitine in comparison to DMSO treated control cells (Fig. 2A). FACS analysis confirmed that both cell populations successfully completed S phase and accumulated at G2. There was not a significant difference in their cell cycle profile at the time of collection (Fig. 2B). Since roscovitine is specific to Cdk1/Cdk2, we hypothesized that one or both of these Cdks is involved in SLBP degradation possibly by phosphorylating Thr 61. Several other kinase

inhibitors were tested at late S phase, however none of these inhibitors except roscovitine and inhibitors of CKII (see Fig. 13) showed inhibitory effects on SLBP degradation (Table 1).

In order to determine whether phosphorylation of these two residues is sufficient to trigger SLBP degradation, I designed a GST protein SLBP fragment (aa 51-108) chimera protein in which all of the phosphoacceptor sites except these two threonines are mutated to alanine (Fig 3). I cloned this fusion protein into the myc- tagged pcDNA3 vector and stably expressed this protein in HeLa cells. Next, to analyze the regulation of expression of this chimeric protein, cells were synchronized by double thymidine block and collected cells at S phase (3 hrs after release) and G2/M (9 hrs after release) (Fig. 4). The chimeric protein was degraded at G2 in parallel with the endogenous SLBP (Fig. 4, lane 1and lane 2). To confirm that the GST SLBP fragment was degraded by a similar mechanism as full length SLBP, I mutated Thr61 to alanine in the GST SLBP fragment. The GST SLBP fragment containing this mutation was stable at the end of S-phase (Fig. 4, lanes 3 and 4) and the same mutation of Thr61 to alanine in the full length SLBP also prevented SLBP degradation at the end of S-phase (Zheng *et al.*, 2003).

Next, I expressed the GST-SLBP fusion protein fragment (51-108 aa) in bacteria that is sufficient and required for S/G2 degradation and used the recombinant protein as a substrate for in vitro kinase assays (Fig. 5). I also constructed and expressed the mutant forms TAP, ATP in the TTP (aa 60-62) sequence and a mutant with the KRKL sequence changed to 4 alanines (Fig. 3, 5). I prepared an extract from late S-phase cells (6hrs after release from double thymidine block) and used this extract as a source of kinase activity. The extract phosphorylated the wild-type TTP (Fig. 5, lane 1), and ATP protein but not the TAP mutant or GST protein (Fig. 5, lanes 3 and 4). These results suggest that Thr 61 is the only

site phosphorylated in this lysate. The activity was sensitive to roscovitine, demonstrating that this phosphorylation is mediated by Cdk1 or Cdk2 (Fig. 5, lane 2).

Timing of in vitro phosphorylation of Thr61 corresponds to the time of SLBP degradation.

In order to determine whether the kinase activity responsible for the Thr 61 phosphorylation was regulated during the cell cycle, I performed in vitro kinase assays using the bacterially expressed SLBP fragment as a substrate and lysates from HeLa cells isolated at different time points of cell cycle as the kinase source. I tested extracts prepared 4, 6, 10, 11, 12, and 14 hours after release from thymidine block. These cell cycle points correspond to G1/S (0 hrs), mid-S phase (4 hrs), S/G2 (6 hrs), M/G1 (10, 11 hrs), early and mid G1 (12 and 14 hrs). With TTP SLBP Fragment, I detected peak phosphorylation at late S phase (S/G2) overlapping the point where SLBP degradation started (Fig 6A). I did not detect significant phosphorylation using lysates from G1 or G1/S cells. With TAP SLBP substrate, I could not detect phosphorylation suggesting that the phosphorylation I detected is on Thr 61. Since we have previously shown that in vivo, the KRKL region is required for both Thr61 phosphorylation and SLBP degradation at S/G2, I tested the phosphorylation of KRKL/4A SLBP Fragment and I showed that in the absence of KRKL sequence I detected almost no phosphorylation in S and S/G2 lysates and some phosphorylation using an M-G1 phase lysate (Fig 6B). Addition of roscovitine to the in vitro kinase assays, abolished the phosphorylation of the SLBP Fragment as well as the residual phosphorylation of KRKL/4A, suggesting all Thr 61 phosphorylation including the residual phosphorylation of KRKL/4A mutant in the M-G1 extract, was mediated by a Cyclin/Cdk (Fig 6B).

Next, we have concentrated on S phase only and repeated the same kinase assays with different time points throughout the S phase. We detected phosphorylation peaking at late S phase (S/G2) where SLBP is gone (Fig. 7). We detected phosphorylation on GST SLBP fragments TTP and ATP but not TAP, showing that the phosphorylation we detected occurred on Thr61 (Fig 7). Interestingly, although phosphorylation on Thr 61 has significantly increased at S/G2, there is no significant increase in the phosphorylation of H1, indicating that in this experiment the cells had not reached the point at G2/M where Cyclin B/Cdk1 activity rapidly increases.

Cyclin A/Cdk1 phosphorylates SLBP on Thr 61

In order to determine whether Cdk1 or Cdk2 was the major kinase responsible for late S phase phosphorylation of Thr 61, I performed immunodepletion experiments using late S phase lysates and cdk1 or cdk2 antibodies. In the case of Cdk2 depletion, although most of the Cdk2 was removed (Fig 8, lane 2), there was no significant difference in Thr61 phosphorylation detected. On the other hand, removal of even 30-40% Cdk1 from the lysate, significantly reduced the phosphorylation of Thr 61 suggesting that Cdk1 was the major kinase that is responsible for the phosphorylation detected in vitro (Fig 8). Mock depletion using the protein A beads alone had no effect on the kinase activity, or on the levels of cdk1 or cdk2.

Next, to determine the ability of different Cyclin/Cdks to phosphorylate SLBP Thr 61, I immunoprecipitated Cyclin A and Cyclin B complexes and Cdk2 or Cdk1 from late S phase lysates and tested their ability to phosphorylate GST-SLBP Fragment. With the

substrates that I used, I only detected phosphorylation on the ones with SLBP TTP (wildtype) but not TAP confirming that I am detecting phosphorylation on Thr61 of SLBP (Fig 9).

In order to determine the Cyclin partner, I immunoprecipitated Cyclin A/Cdks and Cyclin B/Cdks with anti cyclin A or anti cyclin B and tested those kinase complexes for the ability to phosphorylate SLBP on Thr 61 using substrates with a wild type KRKL sequence or a 4A mutation (Fig 9A). Cyclin A/Cdk required the KRKL region to phosphorylate Thr61 (Fig. 9A). However, Cyclin B/Cdk immunoprecipitates phosphorylated the substrates with or without the KRKL to a similar extent. Thus the cyclin B kinases are likely not the kinases that are responsible for Thr 61 phosphorylation, in vivo.

I compared the ability of the Cyclin/Cdk2 complexes and the Cyclin/Cdk1 complexes from the same extracts to phosphorylate both SLBP Fragment and histone H1, and normalized the SLBP Thr 61 kinase activity of each immunoprecipitate to the activity seen with histone H1 as a substrate. I have showed that Cyclin/Cdk1 phosphorylates SLBP Thr 61 much more efficiently than Cyclin/Cdk2. The Cdk2 immunoprecipitates phosphorylated the SLBP fragment only 1/3 as efficiently as they phosphorylated histone H1 (Fig. 9B, lanes 1 and 3). The activity on SLBP was completely dependent on the KRKL sequence. In contrast, the Cdk1 immunoprecipiates phosphorylated the SLBP fragment better than they phosphorylated histone H1 (Fig. 9B, lanes 5 and 7). The great majority of this activity by Cdk1 was dependent on the KRKL sequence, too. When I normalized their ability to phosphorylate Thr61 to H1, I found that Cdk1 can phosphorylate the SLBP substrate much more efficiently (3-4 times) than Cdk2. This result is consistent with the interpretation that a Cyclin/Cdk1 is the kinase that phosphorylates SLBP on Thr61 and triggers its degradation at late S phase.

The Cdk1 complexes that could be present in the cell are either Cyclin A/Cdk1 and Cyclin B/Cdk1, and Cyclin B/Cdk1 is likely to be the only Cyclin B /Cdk complex. Since most of the Cdk1 activity that phosphorylates Thr 61 is dependent on the KRKL sequence, this activity must be mostly due to Cyclin A/Cdk1 (Fig, 9A, lanes 5 and 6) but not Cyclin B/Cdk1. When it comes to Cdk2, it can be in a complex with both Cyclin A and Cyclin E. Since Cyclin E level is high at early S and has already dropped at S/G2 (Dulic et al., 1992), cyclin A/Cdk2 is most of the Cdk2 activity we detected with Cdk2 immunoprecipitates (Fig 9B, lane 1).

In conclusion, I conclude that Cyclin A/Cdk1 is the major kinase that phosphorylates Thr 61 depending on KRKL sequence in S/G2 cells.

Knocking down Cdk1 results in stabilization of SLBP

To further examine the role of Cdk1, we knocked down Cdk1 in synchronized HeLa cells using a Cdk1 specific RNAi (Fig. 10). Using the strategy shown in Fig. 10A, where siRNA treatment was combined with double thymidine block, we have been able to knockdown a number of proteins in synchronized cells. Control cells were treated with the same siRNA protocol using a control, C2, siRNA (Wagner and Marzluff, 2006). The levels of Cdk1 protein were reduced about 70% by the RNAi treatment. Six hrs after release from the thymidine block, the cells were in late S-phase. As shown at Fig. 10B, there was less SLBP degradation at late S phase when Cdk1 is knocked down compared with the control cells. FACS analysis of these cells that the cell cycle distribution of the cells was comparable in the treated and control cells (Fig 10C).

Timing of the SLBP degradation does not depend on Thr60 phosphorylation.

Previously we showed that changing either Thr60 or Thr61 to alanine stabilized SLBP. Lianxing Zheng had created some possible phosphorylation mimics, none of which resulted in an SLBP that was properly regulated. I further attempted to mimic phosphorylations by creating SLBPs containing SFDTP and SFETP in place of SFTTP, and then selected populations of cells stably expressing the mutant SLBPs at a similar level to the wild-type SLBP. The SFDTP mutant was stable at the end of S-phase, indicating that the aspartic acid did not mimic the phosphothreonine (Fig. 11A, bottom). However, the SFETP mutant was regulated properly (Fig. 11A top) and was degraded like stably expressed wild type SLBP, a little slower than endogenous SLBP but yet still unstable at G2. Treatment of the cells at late S phase with the proteosome inhibitor MG132 stabilized the endogenous SLBP as well as the SFETP SLBP (Fig. 11A, lane 4). This result demonstrates that the E at position 60 mimics phosphothreonine, and likely targets SLBP to the proteosome when T61 is phosphorylated. Since the SFETP SLBP is expressed at normal levels during S-phase and then degraded, I conclude that phosphorylation of Thr61 is essential for proper timing of the degradation of SLBP. It has been shown that Cyclin A/Cdk1 is getting activated near the end of S-phase (Pagano et al., 1992; Mitra et al., 2006). The mutagenesis data taken together with my biochemical results implicate Cyclin A/Cdk1 as the kinase that phosphorylates Thr61 of SLBP, and that this phosphorylation is necessary to trigger degradation of SLBP at the end of S phase.

CKII may be the kinase that phosphorylates Thr60.

The identity of the kinase that phosphorylates the other threonine, Thr60, also required for S/G2 SLBP degradation still remains as a question. Unfortunately, under my

conditions I could not detect any phosphorylation of Thr 60 with my cell lysates (Fig 4, 6). Therefore, I have taken a bioinformatic approach and searched for kinase(s) that have target consensus sequences with similarity to the sequence flanking Thr 60 in SLBP. As candidate kinase, CkII is known to target a consensus motif with n+1 and/or n+3 negative charges from either E/D or phosphoT/S (Wojda, 2000).This consensus fits quite well SLBP TTPE sequence where n+1 could be a phosphothreonine and n+3 is a negatively charged glutamic acid.

Based on this analysis, I tested two different commercial CKII inhibitors, TBB and DMAT, and added them to mid-S phase cells. Each of these inhibitors inhibited S/G2 degradation of SLBP to a certain extent (Fig. 13). For the next experiment, I reasoned that if SLBP degradation block by CKII inhibitors is a result of preventing Thr60 phosphorylation, the Thr60E phosphomimic should rescue the blocking effect of CKII inhibitors on SLBP degradation. When I treated HeLa cells stably expressing his tagged-T60E SLBP with CKII inhibitor at mid- S phase, degradation of both the endogenous and exogenous SLBP were inhibited (Fig 14A). The finding that Thr60E phosphomimic can not rescue the stabilizing effect of CKII inhibitors, suggests that CKII may be required for SLBP degradation at some other step than phosphorylation of SLBP. Even if CKII is required for an additional step in SLBP degradation, this doesn't exclude the possibility that it is also functioning as Thr60 kinase.

Finally, I have mutated negative charged Glu63 at n+3 position to alanine to check whether it is required for SLBP degradation. When I synchronized HeLa cells that are stably expressing his-tagged E63A SLBP, this mutation didn't stabilized SLBP at G2 but yet degradation rate appears to be quite slower than wild type SLBP (Fig. 14B).

DISCUSSION

Among the cell cycle stages, molecular events at late S phase and S/G2 transition, where major S phase events-DNA replication and rapid production of new histone proteins-are completed, are may be the least understood. Here, I introduce a cell cycle signaling mechanism that occurs at the end of S phase where Cyclin A/Cdk 1 induces an inhibitory signal on histone mRNA biosynthesis by triggering degradation of SLBP which is the key and only cell cycle regulated player in histone mRNA metabolism. I believe that Cyclin A/Cdk1 may be the kinase that also regulates several other events at S/G2 transition.

Thr 60 and Thr 61 phosphorylations mediates SLBP degradation at S/G2.

We have previously shown that phosphorylation of Thr 60 and Thr 61 by different kinases are required for SLBP degradation at S/G2. Along with those thereonines a proline residue just near Thr 61 (proline 62) and a downstream putative Cyclin binding site (KRKL) is required for SLBP degradation. We also have shown that T61 phosphorylation and degradation requires a downstream putative Cyclin binding site, KRKL.

By stable expression of GST fused SLBP fragment containing both thereonines and KRKL region but all other possible phosphoacceptor sites (thr and serines) changed to alanine, I have shown that phosphorylation of these threonines are not only required for SLBP degradation at S/G2 but also constitutes sufficient phosphorylation signal for SLBP degradation at the end of S phase (Fig 4).

Cyclin A/Cdk1 triggers degradation of SLBP at S/G2.

The TP motif and requirement of a downstream KRKL region which is putative Cyclin binding site (RXL) suggested a Cyclin/Cdk involvement in SLBP degradation by phosphorylating Thr 61. In support of this idea, I have shown that treatment of Cdk1/Cdk2 inhibitor at late S phase cells inhibits SLBP degradation at S/G2. Further using my GST SLBP fragment as a substrate in in vitro kinase assays, I have shown significant Thr 61 kinase activity appeared at S/G2 border where SLBP degradation starts. I also confirmed that this Thr 61 phosphorylation is Cdk mediated (roscovitine sensitive) and requires downstream KRKL region just as in the in vivo situation.

Based on pattern of Thr 61 kinase activity, we concluded that Cyclin E/Cdk2 is very unlikely to be the Thr 61 kinase since Cyclin E/Cdk2 activity peaks at early S and goes down at late S phase which is just opposite of the Thr 61 activity pattern that we detect. Among the possible Cyclin/Cdks known to be functioning at these cell cycle stages, I investigated the possible role of cyclin/cdk1 and cyclin/cdk2 kinases.

When I compared the Thr 61 phosphorylation ability of Cyclin A/Cdk and Cyclin B/Cdk, I showed that Cyclin A/Cdk but not Cyclin B/Cdk requires the downstream KRKL region. Since we know that in vivo both Thr 61 phosphorylation and SLBP degradation requires KRKL region, I conclude that a Cyclin A/Cdk but not Cyclin B/Cdk is the kinase that phosphorylates Thr 61 in vivo.

Our results strongly suggest that a Cyclin A/Cdk is triggering SLBP degradation at the end of S phase by Thr 61 phosphorylation, and further experiments support the conclusion that Cyclin A/Cdk1 is the major kinase that phophorylates Thr 61 and triggers SLBP degradation. The Thr 61 phosphorylation profile we detected fits very well with Cyclin A/Cdk1 activity which increases towards late S phase with a gradual increase toward

the point it peaks at G2 (Pagano et al., 1992; Mitra et al., 2006). With immunodepletion experiments, I confirmed that majority of the Thr 61 kinase activity detected in late S phase lysate is performed by Cdk1 instead of Cdk2. Also, I have shown Cyclin/Cdk1 immunoprecipitated from late S phase phosphorylates Thr 61 in the SLBP fragment significantly more efficiently than Cyclin/Cdk2 obtained from same lysate and since the phosphorylation by Cyclin/Cdk1 complex mostly requires KRKL region we conclude that Cyclin A/Cdk1 (but not Cyclin B/Cdk1) is the kinase that is responsible for most of the Thr 61 phosphorylation we detect with Cdk1 immunoprecipitates. Finally, fitting with our conclusion, that Cyclin A/Cdk1 is the kinase that phosphorylates Thr 61 and triggers SLBP degradation, Cdk1 knock down introduces inhibitory effect on SLBP degradation at the end of S phase.

In summary, it seems that SLBP uses RXL motif to recruit Cyclin A but its non-perfect Cdk2 target sequence around Thr 61 requires Cdk1 for full phosphorylation. This may be the case for several other Cyclin A/Cdk mediated events which waits until G2 although there is high Cyclin A/Cdk2 activity throughout S phase.

Cyclin A/Cdk1 activity is likely the determinant of timing of SLBP degradation.

I have tried to mimic the Thr60 and Thr 61 phosphorylation by phosphomimic residues. I successfully mimicked Thr 60 phosphorylation since T60E SLBP is degraded at G2 similar to endogenous SLBP. Since in this mutant SLBP, Thr 60 phosphorylation is not regulated, (since I mimic the phosphorylation constitutively), it seems that Thr61 phosphorylation determines the timing of SLBP degradation at G2. According to my model, as CyclinA/Cdk1 activity reaches to a significant critical level at S/G2 border, it

phosphorylates SLBP at Thr 61 to trigger SLBP degradation (Fig 12). Whether Thr60 is constitutively phosphorylated during S-phase (or whether phosphorylation of Thr 60 is normally induced by phosphorylation of Thr 61) is not clear. Once SLBP is phosphorylated, the adaptor which recognizes doubly phosphorylated SLBP and directs in degradation is not known. It is possible that this factor is also cell-cycle regulated.

Possible role of CKII in SLBP degradation.

I have identified CKII as a candidate kinase that could target Thr 60 based on the sequence flanking the phosphorylation sites in SLBP. Consensus CKII target site is with a negative charge at n+1 and/or n+3 residues which is consistent with the sequence flanking Thr 60 in SLBP, which has phosphothreonine at n+1 and a glutamic acid at n+3 position (Wojda, 2000). Thus it is possible that phosphorylation of Thr 61 is necessary for efficient phosphorylation of T60 by casein kinase II.

To test for a role of CKII I treated late S phase cells with two different commercial CKII inhibitors. Both of these inhibitors blocked the degradation of SLBP at the S/G2 border (Fig. 13).

On the other hand, since the degradation of SLBP containing the T60E phosphomimic is also sensitive to the CKII inhibitors, CKII must have some other effect on SLBP degradation. As one possible indirect effect, inhibition of CKII may affect the Cyclin A/Cdk1 activity which is the kinase for the other phosphorylation required for SLBP degradation. In parallel with that, it has been shown that CKII is involved in Cyclin/Cdk1 activation as one of the signals that triggers Wee 1 degradation (Watanabe et al., 2005).

In conclusion, at this point it is difficult to assess whether CKII is directly involved in SLBP degradation as a kinase for Thr 60 and/or via an indirect role such as activating Cyclin A/Cdk1 activity.

Cell Cycle regulation of SLBP and histone biosynthesis.

SLBP is the sole factor involved in histone mRNA processing that is not present other than S phase. Along with regulation of histone mRNA transcription and stability, cell cycle regulation of SLBP is one of the mechanisms that control histone mRNA biosynthesis and thus histone production to the S phase. We haven't detected any obvious phenotype due to the presence of stable SLBP at G2 in our experiments. Since we have used HeLa cells, it is very possible these transformed cells can survive excess amount of histones outside the S phase. Moreover, there are other mechanisms to regulate cell cycle dependent SLBP expression and also to limit the histone production to S phase.

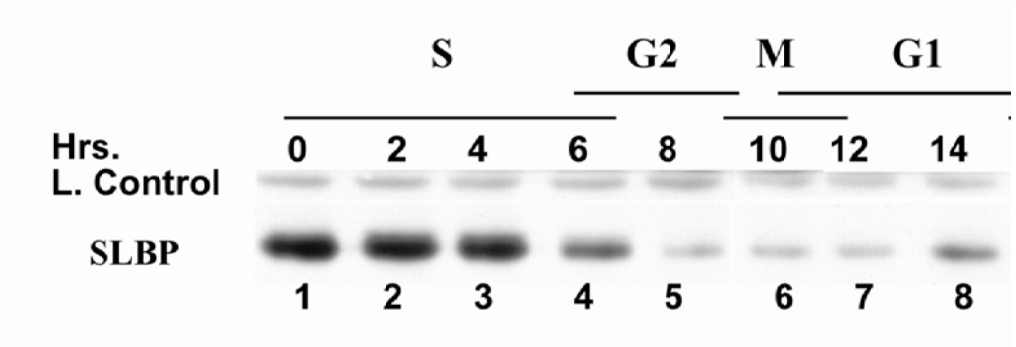
Other than TTP sequence mediated degradation at S/G2, at G1 until the next S phase, SLBP level kept low by different mechanisms ((Whitfield et al., 2000), and Koseoglu and Marzluff , unpublished data) such as low translational efficiency which may decrease the need for SLBP degradation at S/G2 border. Also, more importantly, cells use several different mechanisms to limit excess histone production to the S phase including the low stability of histone mRNAs outside S phase. I believe as well as the redundancy of these different mechanisms, some mechanisms may be more critical at various times in the cell cycle or during development.

Table 1: List of kinase inhibitors analyzed for their effect on SLBP S/G2 degradation. HeLa cells were treated with indicated inhibitors (10μM final) or DMSO at 4 hours after double thymidine block for another 4 hours. SLBP levels were determined by western blot analysis using SLBP antibody.

Inhibitor	Target	Stable SLBP at G2
Roscovitine	Cdk1, Cdk2	+
Wortmanine	PI3 Kinase	-
PD 98059	MEK 1	-
SB 202190	p38 MAPKs	-
SB 203580	p38 MAPKs	-

Figure 1: SLBP expression during cell cycle A) Western blot analysis of SLBP expression in HeLa cells collected at indicated time points after release from double thymidine block. **B)** FACs analysis of the cells collected at indicated times after the release.

A.


S
G2
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Hrs.
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SLBP
1
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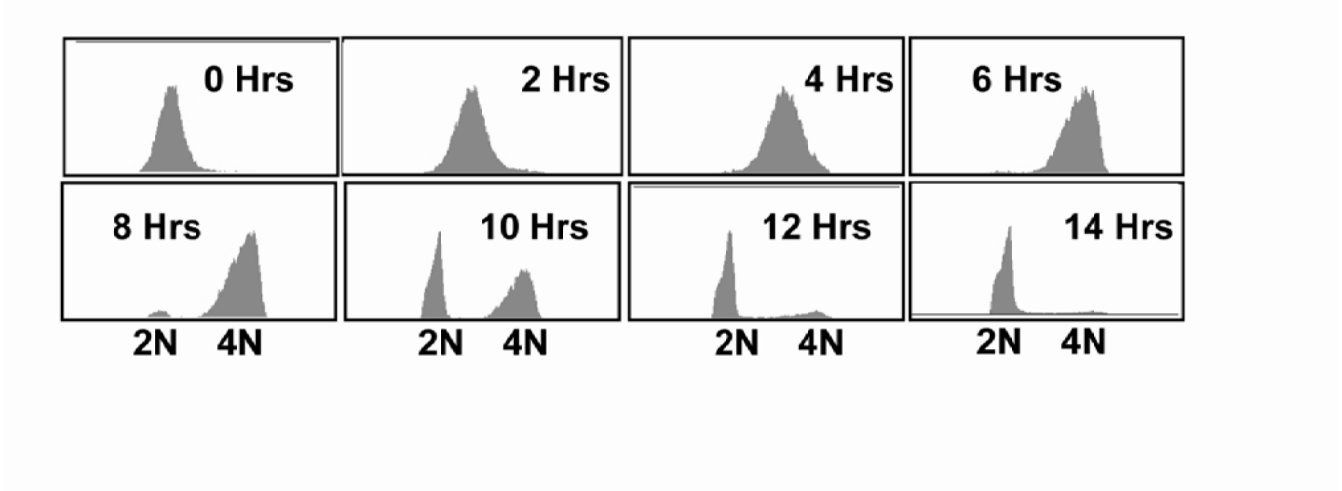
B.


0 Hrs
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6 Hrs
8 Hrs
10 Hrs
12 Hrs
14 Hrs
2N
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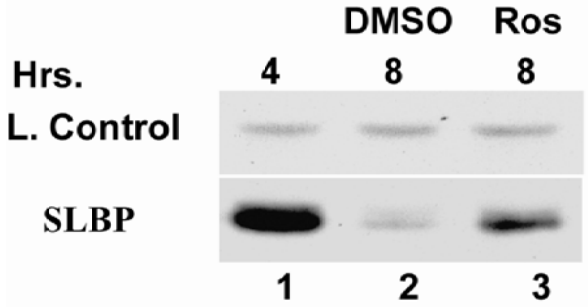
2N
4N

Figure 2: S/G2 degradation of SLBP is Roscovitine sensitive. A) Western blot analysis of SLBP level in HeLa cells which were treated with either dimethylsulfoxide (DMSO) or 20microM Roscovitine (Ros) in DMSO after 4 hours after release from double thymidine block for 4 more hours. **B)** FACs analysis of both Ros and DMSO treated cells at the time of collection at A.

A.



B.

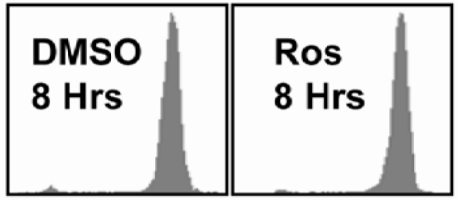


Figure 3: SLBP Fragment (aa.51-108) that is designed as substrate for kinase assays. Regions that are required for late S phase degradation are shown underlined. Possible mutations that have been introduced to those regions were introduced above the wild type. Ser/Thrs have been changed to alanine has been shown by A below the wild type sequence.

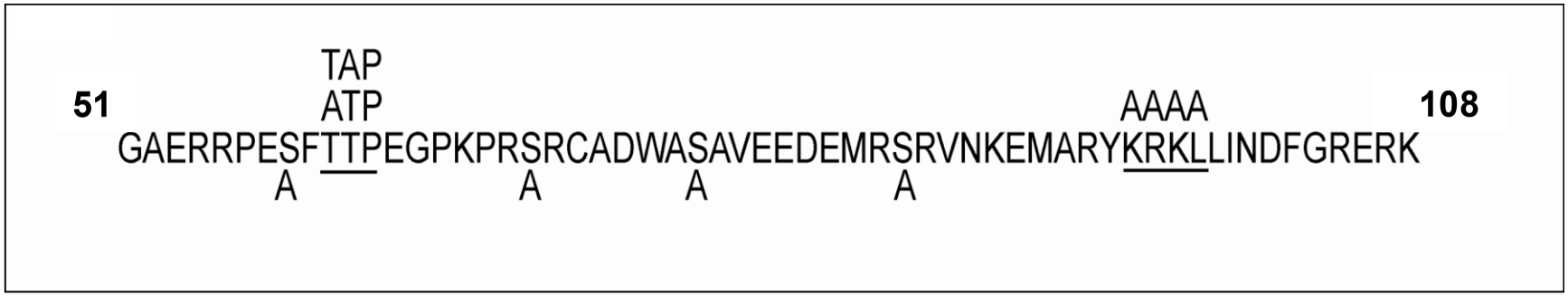

TAP
ATP
51
AAAA
108
GAERRPESFTTPEGPKPRSRCADWASAVEEDEMRSRVNKEMARYKRKLLINDFGRERK
A
A
A
A

Figure 4: SLBP Fragment with only two phosphoacceptor sites (Thr61/Thr62) is sufficient to introduce cell cycle dependent degradation, in vivo. HeLa cells stably expressing mycGST-SLBP fragment with wt TTP or TAP, were synchronized by double thymidine block and collected at S (4hrs after release) or G2 (9 hrs after release) phase. The level of mycGST-SLBP Frag. and endogenous SLBP were detected by western blot analysis using anti-myc and anti-SLBP antibody.


MYC
GST
SLBP F.
Stably Expressed
in He La
S
G2/M
S
G2/M
L. Cont.
SLBP
□SLBP
GST SLBP F.
□Myc
1
2
3
4
TTP
TAP

Figure 5: SLBP Fragment is phosphorylated on Thr 61 by a Cyclin/Cdk. In vitro kinase assays with bacterially expressed GST-SLBP Fragment (TTP, ATP, TAP) or GST alone were performed in the presence of γ32P ATP, using lysate from late S phase cells. The effect of Roscovitine (Cdk1/Cdk2 inhibitor,10microM) on phosphorylation was also determined. Samples were run on SDS PAGE and phosphorylation level of each substrate was determined by autoradiography.

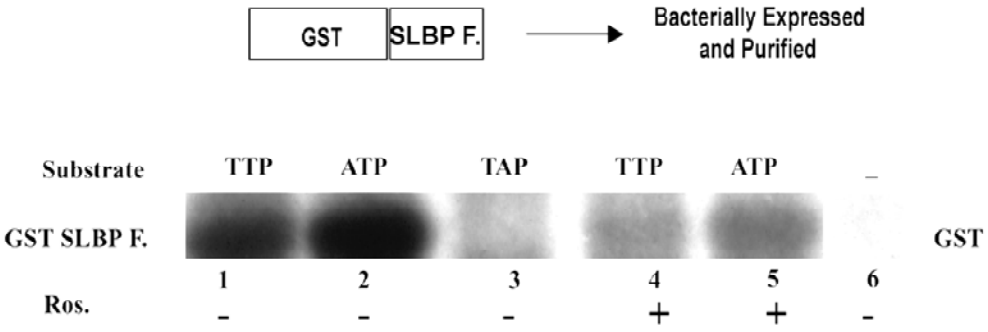

GST
SLBP F.
Bacterially Expressed
and Purified
Substrate
TTP
ATP
TAP
TTP
ATP
GST SLBP F.
GST
1
2
3
4
5
6
Ros.
-
-
-
+
+
-

Figure 6: Peak KRKL dependent and roscovitine-sensitive phosphorylation was detected with late S phase lysate. A) In vitro kinase assays were performed in the presence of γ32P ATP, using lysates from HeLa cells collected at indicated time points after the double thymidine block (panel 1). Samples were run on SDS PAGE and phosphorylation level of each substrate was determined by autoradiography (panel 1). Levels of SLBP and Cyclin A proteins in the lysates were examined by western blot analysis using anti-SLBP and anti-CycA antibody (panel 2,3) **B)** Effect of Roscovitine (10 microM) and KRKL region on phosphorylation of SLBP fragment were analyzed. GST SLBP fragment with wild type KRKL region or 4A mutation were used as indicated below the panel.

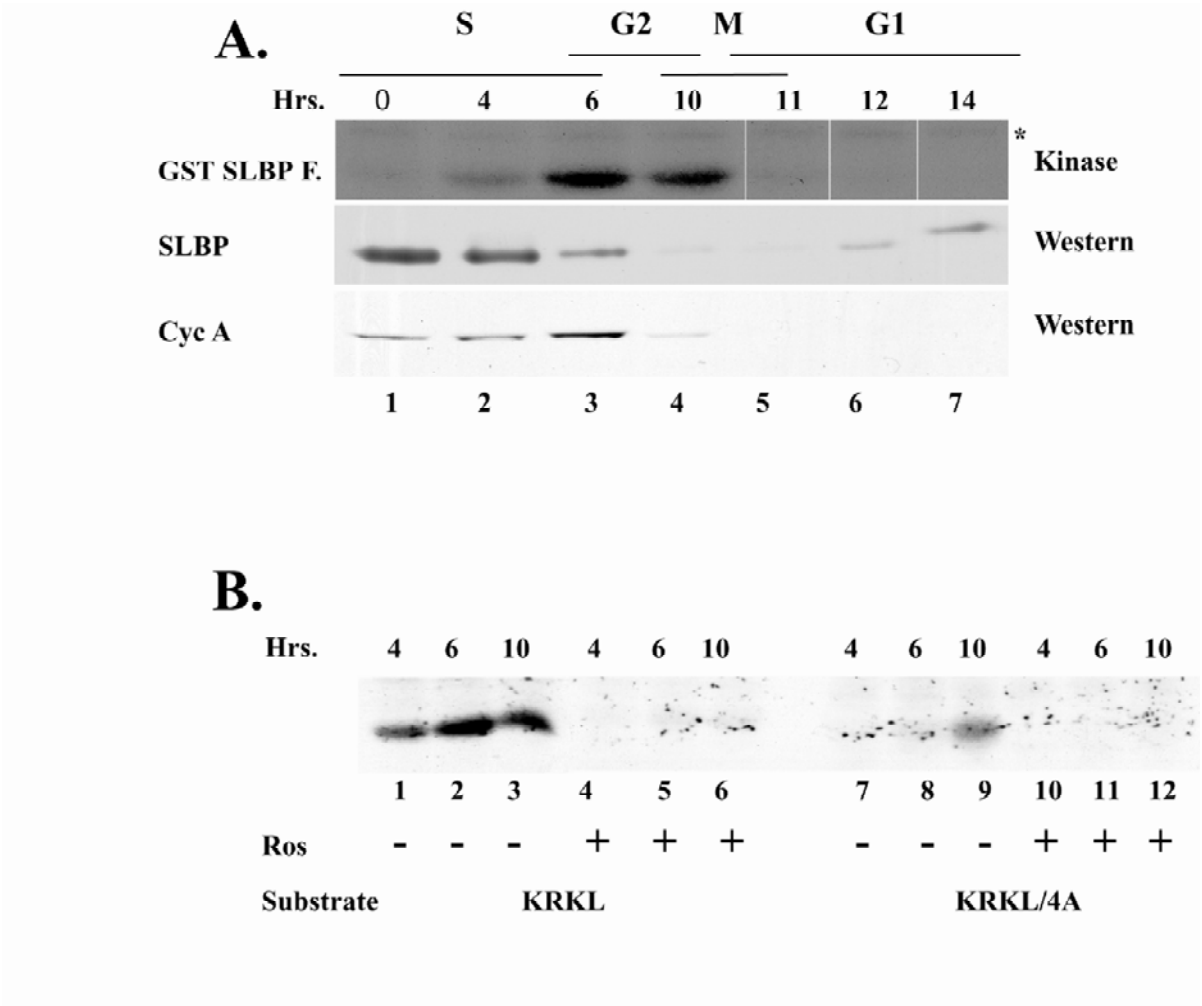
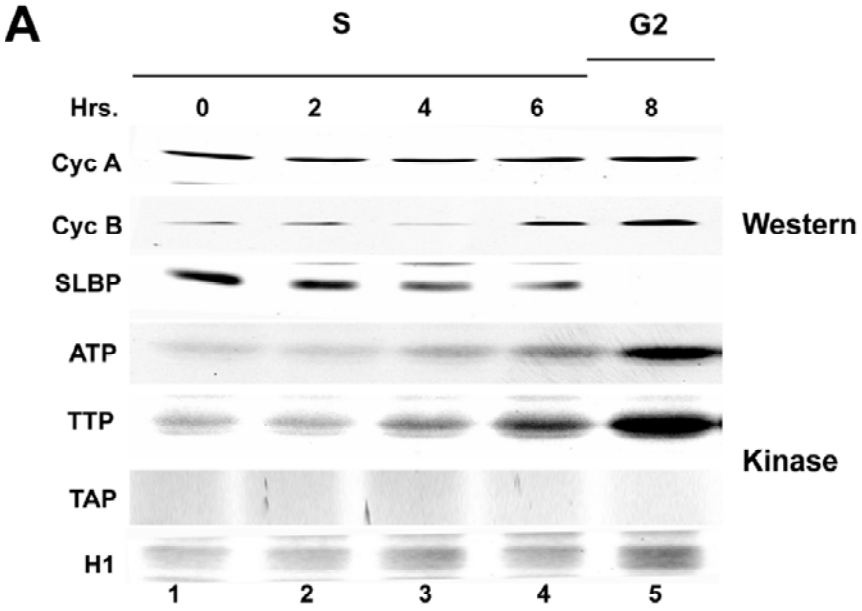

A.
S
G2
M
G1
Hrs.
0
4
6
10
11
12
14
GST SLBP F.
*
Kinase
SLBP
Western
Cyc A
Western
1
2
3
4
5
6
7
B.
Hrs.
4
6
10
4
6
10
4
6
10
4
6
10
1
2
3
4
5
6
7
8
9
10
11
12
Ros
- - - + + + - - - + + +
Substrate
KRKL
KRKL/4A

Figure 7: Phosphorylation detected at late S phase is on Thr61. A) In vitro kinase assays with indicated GST-SLBPFragment (ATP, TTP, TAP) or H1 protein were performed in the presence of γ32P ATP, using lysates from HeLa cells collected at indicated time points after double thymidine block (panel 4,5,6,7). Samples were run on SDS PAGE and phosphorylation level of each substrate was determined by autoradiography (panel 4,5,6,7). Level of Cyclin A, Cyclin B and SLBP protein in the lysates were examined by western blot analysis using corresponding antibodies (panel 1,2,3). **B)** Whole gel appearance of the kinase reactions performed in **A**-panel 5 detected by autoradiography.


A
S
G2
Hrs.
0
2
4
6
8
Cyc A
Cyc B
SLBP
ATP
TTP
TAP
H1
Western
Kinase
1
2
3
4
5

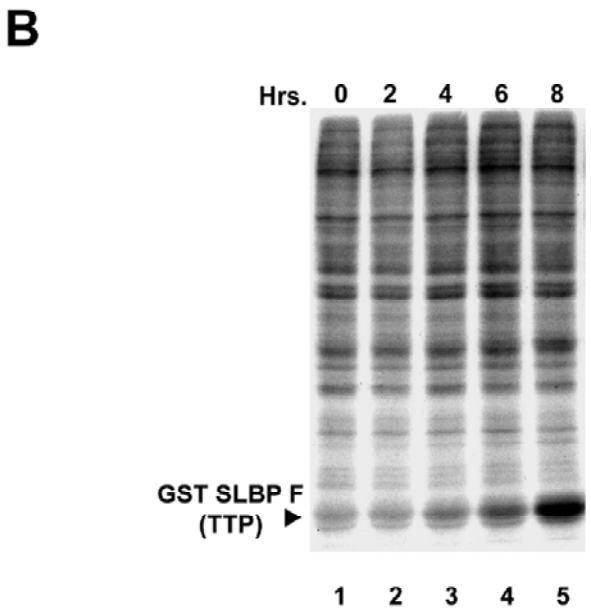

B
Hrs.
0
2
4
6
8
GST SLBP F
(TTP)
1
2
3
4
5

Figure 8: Cdk1 is the major Thr 61 kinase in late S phase lysate. In vitro kinase assay using Cdk1, Cdk2 or mock (just beads) immunodepleted late S phase HeLa cell lysates (panel 3). Both Cdk 2 (panel 1) and Cdk 1 (panel 2) level in each lysate were deteremined by western blot analysis with corresponding antibodies.

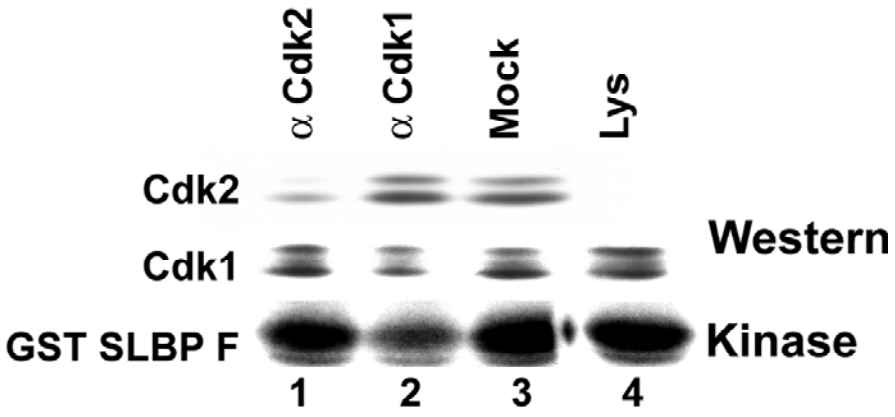

α Cdk2
α Cdk1
Mock
Lys
Cdk2
Cdk1
GST SLBP F
Western
Kinase
1
2
3
4

Figure 9 A) cyclin A/Cdk but not cyclin B/Cdk requires KRKL region for phosphorylating Thr 61. Cyclin A or Cyclin B immunoprecipitates from late S phase HeLa cell lysate were used in in vitro kinase assays in the presence of γ32P ATP. Phosphorylation on H1 and GSTSLBPfragment (TTP, KRKL/4A, TAP) were detected by autoradiography. Phosphorylation level of each substrate were quantified by phosphoimager. Each value is normalized against the level of H1 phosphorylation and indicated below corresponding lane**. B) cyclin/Cdk1 phosphorylates Thr61 much more efficiently than cyclin/Cdk2.** Cdk1 or Cdk2 immunoprecipitates from late S phase HeLa cell lysate were used in in vitro kinase assays in the presence of γ32P ATP. Phosphorylation on H1 and GSTSLBPfragment (TTP, KRKL/4A, TAP) were detected by autoradiography. Phosphorylation level of each substrate were quantified by phosphoimager. Each value is normalized against the level of H1 phosphorylation and indicated below the corresponding lane. In the mock lanes (in A lane 5,10 and in B lane9) IPs were done with beads alone.

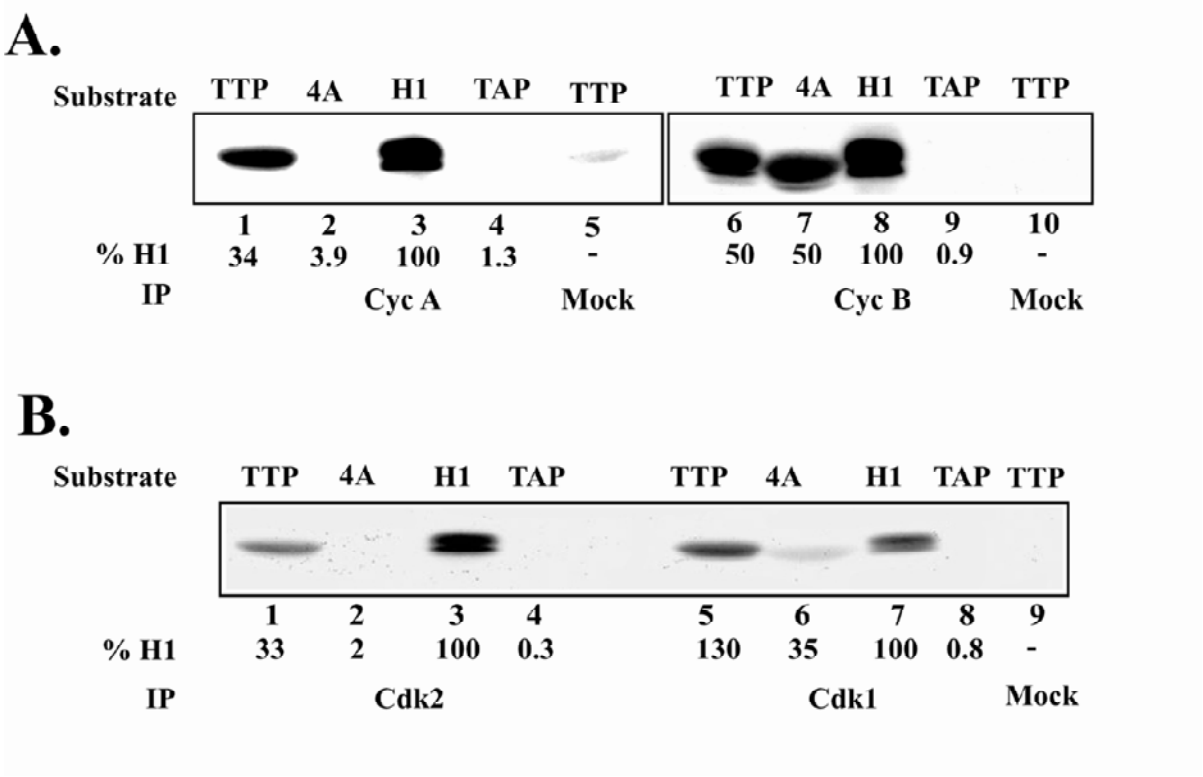
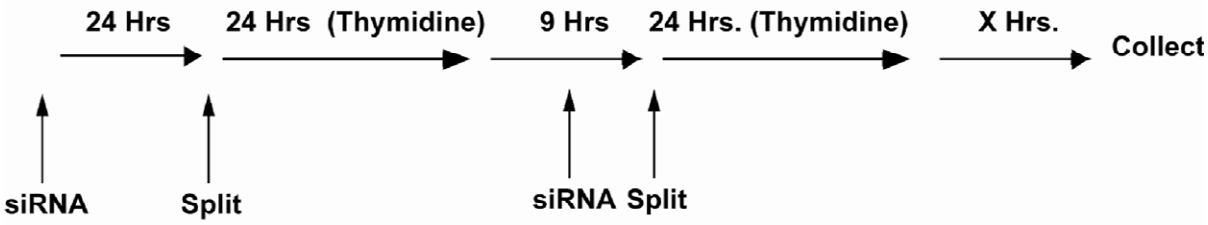
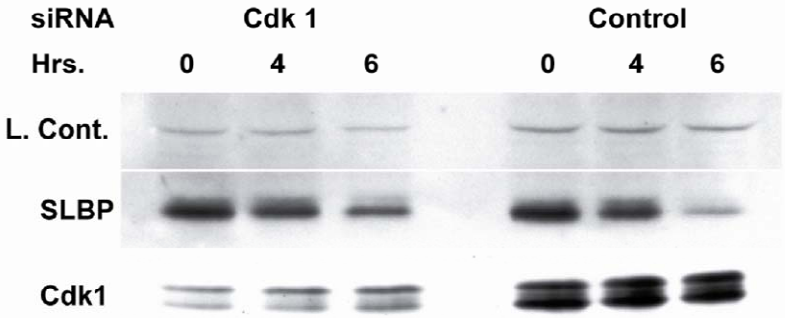

A.
Substrate
TTP 4A H1 TAP TTP
TTP 4A H1 TAP TTP
1 2 3 4 5 6 7 8 9 10
% H1
34 3.9 100 1.3 - 50 50 100 0.9 -
IP
Cyc A
Mock
Cyc B
Mock
B.
Substrate
TTP 4A H1 TAP
TTP 4A H1 TAP TTP
1 2 3 4 5 6 7 8 9
% H1
33 2 100 0.3 130 35 100 0.8 -
IP
Cdk2
Cdk1
Mock

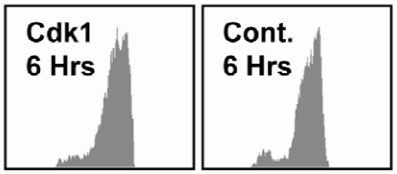
Figure 10: Cdk1 knock down inhibits late S phase SLBP degradation. A) Outline of experimental setup for RNAi/synchronization procedure. **B)** HeLa cells were transfected with Cdk1 or Control siRNA followed by double thymidine block synchronization. Cells were collected at indicated time points after the release from double thymidine block. Levels of Cdk1 and SLBP were determined by western blot analysis with corresponding antibodies. **C)** FACs analysis of the 6 hrs time point cells in B with percentage values of each cell cycle phase.



B.



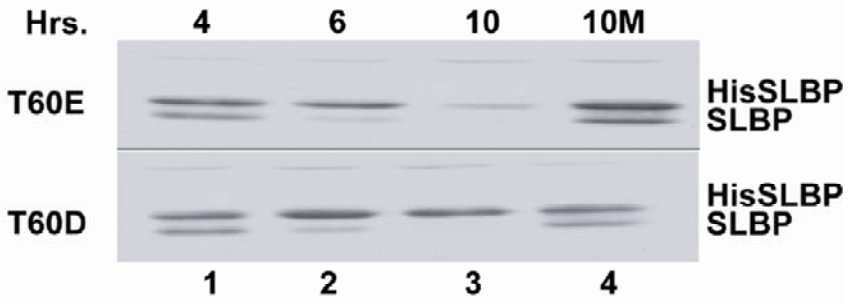
C.



G1%	3	5
S %	46	53
G2%	51	42

Figure 11: Thr 61 to Glu change mimic phosphorylation of Thr 61 for degradation purposes. A) First threonine in the SFTTP motif has been changed to either aspartic acid or glutamic acid and stably expressed in HeLa cells (hisSLBP). Cells were synchronized by double thymidine block and collected at indicated hours after release. Levels of exogenous mutated histagged SLBP and endogenous SLBP were determined by western blot analysis using SLBP antibody. Cells used in lane 10 M were treated with MG132 (proteosome inhibitor) 4 hours after release. **B)** Summary list of the effect of different mutations in TTP motif on G2 SLBP degradation. Regulated represents the ones with similar G2 degradation profile as endogenous SLBP.

A.



B.

SFTTP	→	Regulated
SFATP	→	Stable
SFTAP	→	Stable
SFTEP	→	Stable
SFTDP	→	Stable
SFEEP	→	Stable
SFEDP	→	Stable
SFDTP	→	Stable
SFETP	→	Regulated

Figure 12: Model for triggering SLBP degradation at S/G2. Different than cyclin B/Cdk1 activity which is rapidly boosts at G2/M, gradual increase in cyclin A/Cdk1 activity reaches to sufficient level at S/G2 border to trigger SLBP degradation by phosphorylating Thr 61.

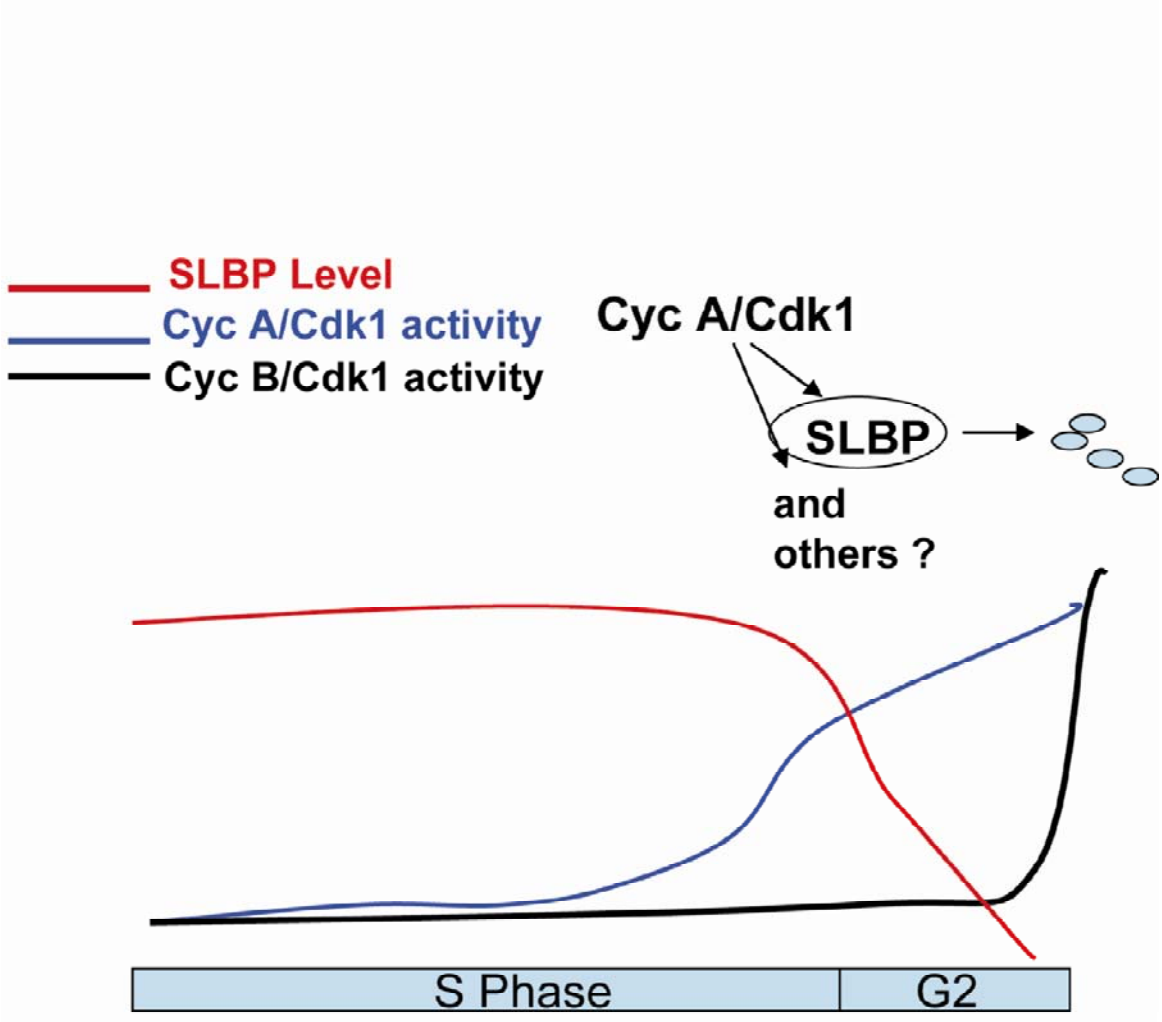

SLBP Level
Cyc A/Cdk1 activity
Cyc B/Cdk1 activity
Cyc A/Cdk1
SLBP
and
others ?
S Phase
G2

Figure 13: CkII inhibitors inhibit SLBP degradation at S/G2. HeLa cells were synchronized by double thymidine block and CKII inhibitors TBB (panel 1 lane3, panel 2 lane 5, 75microM) DMAT (Panel 2 lane2, 10μM, panel 2 lane1, 20 μM), or corresponding amounts of DMSO (Carrier) were introduced at 3.5 hours after release for 4.5 hours. Cells are collected at indicated time points and western blot analysis was performed with SLBP antibody. Panel 1 and Panel 2 are two different experiments done similar way.

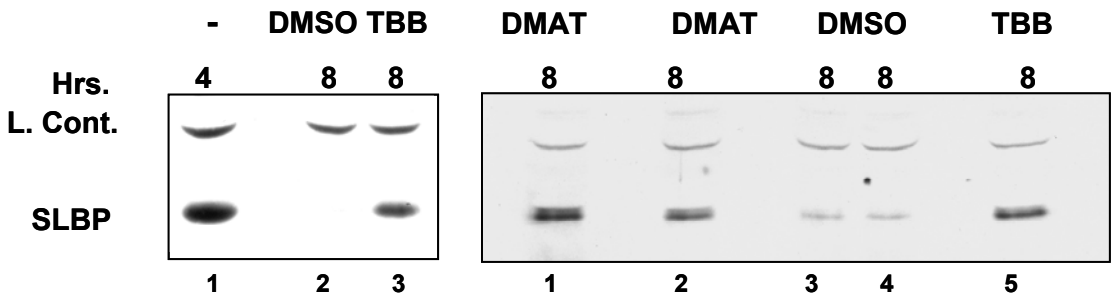
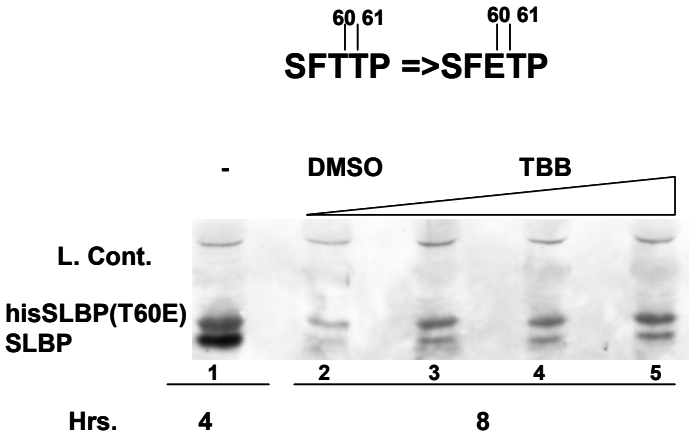
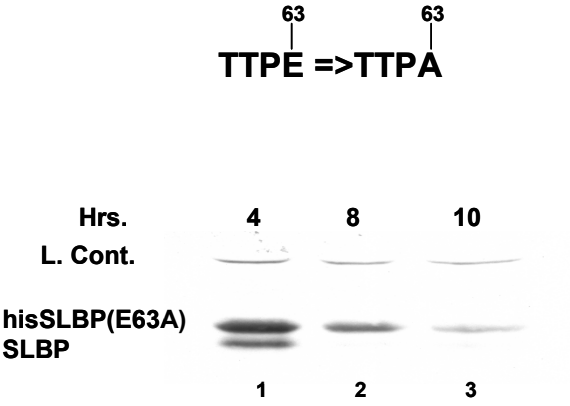

-
DMSO
TBB
DMAT
DMAT
DMSO
TBB
Hrs.
L. Cont.
SLBP
4
8
8
8
8
8
8
8
1
2
3
1
2
3
4
5

Figure 14: Thr60 to Glu change did not rescue CKII inhibitor effect. A) HeLa cells stably expressing exogenous histagged T60E mutated SLBP were synchronized by double thymidine block. 4 hours after release DMSO (Carier, lane 2) or TBB with increasing concentrations (20, 40, 60 microM) were introduced for 4 hours. Cells were collected at indicated time points and western blot analysis was performed using αSLBP. **B)** HeLa cells stably expressing exogenous histagged E63A mutated SLBP were synchronized by double thymidine block and collected at indicated time points after the release. Western blot analysis was performed using αSLBP **C)** Possible effects of CKII in S/G2 SLBP degradation.

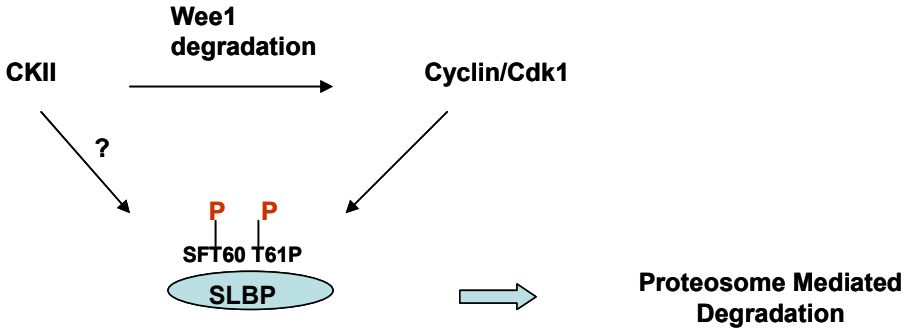
A.


60 61
60 61
SFTTP =>SFETP
-
DMSO
TBB
L. Cont.
hisSLBP(T60E)
SLBP
1
2
3
4
5
Hrs.
4
8

B.


63
63
TTPE =>TTPA
Hrs.
4
8
10
L. Cont.
hisSLBP(E63A)
SLBP
1
2
3

C.


Wee1
degradation
CKII
Cyclin/Cdk1
?
P
P
SFT60 T61P
SLBP
Proteosome Mediated
Degradation

MATERIAL AND METHODS

Construct preparation:

For bacterial expression, the SBLP fragment (51-108) was subcloned into the PGEX2T vector just after the GST tag using PCR-generated insert with EcoRI and BamH1 sites. For expression in HeLa cells, the GST fused SLBP fragment was subcloned into myc-tagged pcDNA3 vector (Invitrogen) by PCR-generated insert with Xho1 and Kpn1 sites. All site-directed mutations were made according to the QuickChange Site Directed Mutagenesis protocols (Stratagene) using appropriate complementary oligonucleotides ranging from 30 to 50 nucleotides in length. All constructs were verified by DNA sequencing.

Transfection and selection of stable cell lines:

HeLa cell plasmid transfections were done with Lipofectamine (GibcoBRL) according to the manufacturer's protocol. 2 micrograms of each plasmid was used with each 6 well-plate. For selection of stably transfected cells, the cells were replated onto a fresh 10-cm plate 24 hrs after transfection with media containing 1 mg/ml G418. The medium containing G418 was changed every 5 days to remove dead cells. Cells were kept under selection until separate colonies could be observed by eye on the plates, and these stably transfected cells (at least 20 colonies per plate) were pooled together. To maintain the stably transfected cells, 200 μg of G418 per ml was kept in the medium and was removed just prior to synchronization.

Cell Culture and Synchronization:

HeLa cells were grown in Dulbecco modified Eagle medium plus 10% fetal bovine serum and penicillin-streptomycin. Cells were synchronized at G1/S border by double-thymidine block. Cells were plated at a low density 24 h before the start of the synchronization protocol when cells were treated with 2mM thymidine for 18 hours for the initial block. After the initial block, cells were washed with PBS and released into fresh medium for 9h, and then blocked again with 2 mM thymidine for 16h to arrest all the cells at the beginning of S phase. The cells were released into fresh media after washing out the thymidine, and lysates were prepared from the cells collected at indicated time points after the release. The cells progressed through G2 and mitosis synchronously. The cell cycle profile of the cells was determined by propidium iodide staining and flow cytometry analysis at UNC Flow Cytometry facility.

Lysate preparation and in vitro kinase assays:

Cells were collected at the indicated time points after release from double thymidine block, resuspended in lysis buffer (50 mM β-glycerophosphate, 20 mM NaF, 1.5 mM EGTA, 0.05% NP-40 with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 500 μM Na_3VO_4) and sonicated 4 times on ice (800 μl per 10 cm dish). Extracts were clarified by centrifugation for 10 minutes at 14000 rpm at 4^oC. In vitro kinase assays were performed in 30 μl reaction mix (supplemented with final 10 mM $MgCl_2$ and 5 μM ATP) with corresponding lysate (1-2 mg/ml protein; 10 μg protein) and 1 μg of substrate (GST-SLBP Fragment or H1) in lysis buffer and 8 μCi of [γ-^{32}P] ATP. In each reaction, immunoprecipitates on beads (from 80-100 μg lysate), the supernatant from the IPs or the lysate directly were used as kinase source as indicated. The reactions were incubated at 30^oC for 30 minutes and stopped by adding 2X SDS loading buffer. Samples were analyzed by

SDS-PAGE and each gel wawas Commassie stained, dried and analyzed by autoradiography and quantified with a PhosphorImager analysis.

RNAi

Down regulation of Cdk1 expression was obtained by transfection of chemically synthesized siRNA using oligofectamine 2000 transfection according to the manufacturer's protocol. Chemically synthesized siRNAs were obtained from Dharmacon (Lafayette, CO.) and had the following sequences of the top strand: 5′AAGGGGUUCCUAGUACUGCAAdTdT3′ and 5′GGUCCGGCUCCCCCAAAUGdTdT3′ (control, C2). A portion of cells were lysed in a buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (Roche), and 0.5% NP-40 and analyzed by Western blotting. The remaining cells were fixed with 70% ethanol and stained with propidium iodide, and were analyzed for their DNA content by flow cytometry with a FACscan and the Summit software (Cytomation, Inc.).

Antibodies and inhibitors:

SLBP was detected with an antibody raised against the C-terminal 13 amino acids of the protein (Wang et al., 1996). Cyclin A and Cyclin B antibodies used in western blots and immunoprecipitation experiments were purchased from Neomarkers (Feremont, CA) and SantaCruz (SantaCruz, CA), respectively. Cdk1 and Cdk2 antibodies were a kind gift from Dr. Yue Xiong (UNC). All the kinase inhibitors tested were purchased from Calbiochem (La Jolla, CA).

CHAPTER III

G1 Phase Regulation of SLBP Expression

INTRODUCTION

Stem loop binding protein is the sole factor involved in histone mRNA processing that is not present other than S phase (Zheng et al., 2003). Along with regulation of histone mRNA transcription and stability, cell cycle regulation of SLBP is one of the major mechanisms that restrict histone mRNA biosynthesis and thus histone production to the S phase. SLBP expression is tightly cell cycle regulated. However, SLBP mRNA level does not change significantly during cell cycle (Whitfield et al., 2000). As described in Chapter 2, at the end of S phase, Cyclin A/Cdk1 triggers rapid degradation of SLBP depending on N-terminal TTP motif. Cells keep SLBP level low during G1 phase until next S phase when it is needed again (Fig 1).

Previously, we have introduced translational regulation of SLBP mRNA as one mechanism that keeps SLBP level low at G1 using CHO cells. In CHO cells, SLBP translation is low at G1 in comparison to S phase although there is not a significant change in SLBP mRNA level (Whitfield et al., 2000). Here, I have further confirmed this regulation in HeLa cells by comparing the amount of newly expressed SLBP by ^{35}S-methionine labeling of both G1 and S phase cells and determining the SLBP mRNA distribution on polysomes in

G1 and S phase HeLa cells by sucrose gradient fractionation. Further, I have shown that coding sequence of SLBP mRNA is sufficient to introduce low translational rate at G1 in comparision to S phase.

As a new insight into SLBP G1 regulation, I have shown that somewhere between early and mid G1, SLBP translational efficiency recovers back to S phase level and I have introduced increased instability of the SLBP protein as a new mechanism that keeps SLBP expression limited during rest of G1. Our findings here suggest regulated degradation as a possible new mechanism that keeps SLBP level controlled during G1.

RESULTS

SLBP mRNA translation rate is low at early G1.

After degradation of SLBP at S/G2, the SLBP level remains low with only limited gradual increase until prior to the next S phase (Fig 1 and Fig 6A). As cells approach S phase, SLBP level increases significantly, reaching the level present in cells arrested at the G1/S border by double thymidine block (Fig 1), without a significant change in SLBP mRNA level (Whitfield et al., 2000). The G1 stage of the cell cycle is often the longest period of mammalian somatic cell cycle, and varies greatly between different cell types and in different growth conditions. In rapidly cycling HeLa cells, it lasts up to 6-8 hours. The SLBP level recovers back to S phase level at late G1 or G1/S border which is about 16-18 hours after release. A problem with studying G1 is that this is also the phase of the cell cycle that varies most within cells in a culture. Cells progress relatively stochastically through G1, resulting in a distribution of times that cells enter S-phase, while cells released from the

double thymidine block progress very synchronously through S-phase, G2 and mitosis. In this study, I have defined early G1 as between 11-12.5 hours after release from double thymidine block and after that point to late G1, I have considered as mid-G1.

In parallel with previous findings from the Marzluff lab using CHO cells, I have confirmed that also in HeLa cells SLBP translation rate is low at early G1. To compare production rate of new SLBP protein between early G1 and S phases, I labeled newly produced proteins, in vivo by treating cells with media containing ^{35}S labeled methionine for 15 minutes after 30 minutes of incubation in media lacking methionine. For these experiments, I synchronized cells at G1/S with double thymidine block and for early G1 cells, I released them for 11.5 hrs where cells have divided and have entered the next G1-phase. For S-phase cells, I have used cells at 2 hours after release from double thymidine block.

After labeling, first, I analyzed an equal amount of total protein of labeled extracts by running on SDS PAGE, I have shown that the general level and pattern of newly produced proteins was comparable demonstrating that the synthesis of the most abundant cellular proteins is very similar in G1 and S phases (Fig 2A). From these lysates, I specifically immunoprecipitated SLBP. The amount of newly produced SLBP in early G1 was 3-4 fold lower than the amount of newly synthesized SLBP precipitated from the S-phase lysates (Fig. 2A).

One reason for keeping protein levels low is to rapidly degrade newly synthesized proteins. This mechanism is used to regulate the level of Cyclin A during G1 in continuously cycling cells (Brandeis and Hunt, 1996). To check whether there is rapid degradation of SLBP at this point of cell cycle, I treated those early G1 cells with the proteosome inhibitor

MG132 for one hour. The level of SLBP protein remained low, indicating rapid degradation of newly synthesized protein is not the case for SLBP. On the other hand, the levels of Cyclin A protein, which is continuously degraded during G1 starting from the late mitosis, increased in response to treatment with proteosome inhibitor in the same early G1 cells (Fig 2B).

A second approach to determine the stability of a protein is to treat cells with an inhibitor of translation, cycloheximide and measure the levels of the protein. Using this approach, I have further shown that there is no rapid degradation of SLBP in early G1. When I blocked new protein synthesis by treating cells with translation inhibitor Cycloheximide for one hour at early G1 the SLBP level did not change, although the low Cyclin A levels did decrease (Fig 2C). Thus two independent approaches confirm that rapid degradation of newly synthesized protein is not the reason for the low level of SLBP we detected with early G1 cells (Fig 2B, C), and is consistent with a lower SLBP translation efficiency in early G1 compared to S-phase cells.

To further confirm the possible translational regulation, I compared the polysome distribution of SLBP mRNA at early G1 and S phase cells. I have performed sucrose gradient fractionation with the cytoplasmic lysate from early G1 and S phase cells. Total RNA from each fraction was isolated and run on agarose gel and ETBR stained. Based on the appearance of 18 S and 28 S ribosomal RNAs, I have detected monosomes around fraction 3, 4 where I have detected both ribosomal RNAs together first time and as fractions get with higher density presumably contains growing chain of polysomes. In order to detect distribution of SLBP mRNA on the polysomes, I synthesized cDNA with oligo dT primed reverse transcription, followed by PCR with specific primers against SLBP mRNA. In the cytoplasmic extract from the early G1 cells, I have determined peak level of SLBP mRNA

around fractions 12 and 13 corresponding with smaller polysomes (Fig 3A). In the case of S phase extracts SLBP mRNA peak shifted to the fractions with highest density corresponding to largest polyribosomes (Fig 3B). As control when I compared the mRNA distribution of a housekeeping gene, GAPDH, which is expressed constitutively I did not detect a significant difference in profile of GAPDH mRNA distribution on polyribosomes (Fig 3). Thus, we concluded that SLBP mRNA translation rate is low in early G1 phase in comparison to S phase.

The SLBP coding sequence is sufficient to introduce G1 translational regulation.

I then tried to determine possible sequence element(s) in SLBP mRNA that are responsible for introducing this cell-cycle dependent translational regulation. First, I asked the question whether SLBP coding sequence is sufficient to introduce the difference in SLBP translational efficiency in G1 and S phase. I cloned the SLBP coding sequence into pcDNA3 vector resulting in a his-tagged protein, which can be distinguished from endogenous SLBP and stably expressed this construct in Hela cells (Fig 4A). Next, I synchronized those cells with double thymidine block and determine the expression of the endogenous and exogenous SLBP protein in G1. I detected similar low expression level of exogenous his-tagged SLBP during G1 as I detected with the endogenous SLBP (Fig 4B).

Next, using immunoprecipitation of SLBP from ^{35}S methionine labeled cells, I have shown that newly produced exogenous SLBP is low in early G1 in comparison to S phase cells just like endogenous SLBP (Fig. 5A). Note that, when analyzing the total extracts, there is no significant difference in labeling of the abundant cellular proteins between G1 and S phases, suggesting that this regulation is specific for SLBP (Fig. 5A). I have further shown

that like endogenous SLBP, exogenous SLBP level does not change in response to cycloheximide or MG132 (Fig 5B, C), showing that like endogenous SLBP, exogenous his-tagged SLBP is not rapidly degraded at that time point. Based on these findings, I conclude that the SLBP coding sequence is sufficient to introduce cell cycle dependent specific translational regulation of SLBP.

Regulated degradation in mid-G1 as a new mechanism to regulate SLBP expression later in G1.

Next, in order to check whether the low level of SLBP translation efficiency is the case for the entire G1 or only limited to very early G1, I compared the rate of newly produced SLBP in later points in G1 (mid G1, 13 hrs after release) with the rate in S phase cells (2 hrs after release) by performing ^{35}S methionine labeling followed by SLBP immunoprecipitation experiments with corresponding cell cycle stage lysates. I have shown that in contrast to early G1, the level of newly produced SLBP later in G1 is similar to S phase level suggesting that translation efficiency recovers to the S phase level somewhere in between early and mid G1 (Fig 6).

Although it seems that the level of SLBP translation increases to S phase level around mid G1, the amount of SLBP protein expression remains relatively low for another 3 hours until late G1 or G1/S before recovering back to S phase level suggesting that there may be additional mechanisms that can keep SLBP expression limited in this period of G1 until next S phase.

A possible mechanism to limit SLBP expression could be regulated degradation. This is the case in G1 for several other S phase related proteins like Cyclin A, Skp2, Cdc6

(Petersen et al., 2000; Bashir et al., 2004; Wei et al., 2004a). To check this possibility, I have repeated cycloheximide experiments to determine the stability of SLBP this time around 1 hour later in G1. In contrast to SLBP in early G1 cells, SLBP level decreased as did the positive control Cyclin A which is already known to be unstable in G1 (Fig 7A). Moreover, like endogenous SLBP, stably expressed exogenous SLBP was also unstable in mid-G1 cells although the small amount present in earlier point of G1 was stable (Fig. 5). In Figure 7B, I have observed rapid degradation of SLBP at S/G2 as expected.

These findings suggest possible change in the rate of degradation of SLBP as a second mechanism to control the level of SLBP expression during G1 prior to the point that SLBP level reaches to S phase level somewhere at late G1. This increased degradation of SLBP occurs somewhere between early and mid-G1 cells, but not in early G1 cells.

DISCUSSION

As a key player in histone mRNA metabolism, SLBP expression is tightly regulated during the cell cycle. Previously, I have shown that Cyclin A/Cdk1 triggers rapid degradation of SLBP at S/G2 border. Here, I have shown that during G1, SLBP level kept low until the next S phase via at least two mechanisms. First, I have confirmed that low translation efficiency as one of the mechanisms to keep SLBP level low at early G1 and have shown that SLBP coding sequence is sufficient to mediate this regulation. Further, I have shown that the low translation efficiency recovers to the S phase level somewhere between early and mid-G1 phase and I have proposed regulated degradation as an additional mechanism that may be keeping SLBP levels low until S phase.

Low translation rate of SLBP in G1 is mediated by its coding sequence.

During G1, SLBP expression is limited until G1/S border (or late G1). After SLBP is degraded rapidly at S/G2 and cells complete M phase, it takes around 8 hrs for HeLa cells to progress to the point at G1/S border where SLBP expression level come back to S phase level.

Previously, Mike Whitfield in the Marzluff lab, showed a low production rate of SLBP in G1 cells in comparison to S phase cells in CHO cells. Further, he had shown that SLBP mRNA level is not regulated during the cell cycle in either CHO or HeLa cells. Here, I have confirmed that translation rate of SLBP mRNA is lower at early G1 point in comparison to S phase rate also in HeLa cells, by comparing new SLBP synthesis rate and SLBP mRNA polysome distribution in G1 and S phase HeLa cells and showing that SLBP is not rapidly degraded at this point of cell cycle (Fig 2).

Although we have determined that SLBP translation rate is low in early G1, the nature of this regulation is still unknown. It is still not clear whether SLBP translation is kept low by active inhibition in early G1 by a binding of specific protein or miRNA, which is simply removed as cells approach S-phase, or whether there is active triggering by activation or expression of certain factors that facilitates SLBP translation. It is also possible that SLBP mRNA translation is activated as a result of an increase in a limiting general translation factor.

In order to elucidate the mechanism, I have tried to find a cis-element(s) in SLBP mRNA that is responsible for the translational regulation. For this purpose, I have first checked whether SLBP coding sequence is sufficient to introduce low translation efficiency

in early G1 cells as endogenous SLBP. In HeLa cells, I have stably expressed vector where I have cloned in only SLBP coding sequence but not the UTRs. When I repeated rapid degradation and ^{35}S experiments with those HeLa cells, the his-tagged exogenous SLBP showed similar profile as endogenous SLBP.

Based on these findings I concluded that SLBP coding sequence is sufficient to introduce the difference between SLBP mRNA translation efficiency in early G1 and S phase cells. Further mapping of possible cis-element in coding sequence and determination of whether there is any contribution from UTRs is yet to be done.

Since in somatic cells there is only a couple of examples of coding sequence dependent translation regulation (Stajduhar et al., 2000; Lin et al., 2000; Tai et al., 2004a), and all miRNAs published so far are targeting 3'UTR, this may be interesting and novel regulation. miRNAs that are targeting coding sequences have been recently discovered and are yet to be published (personal communication with R. Agami).

Regulated degradation is a possible second mechanism to limit SLBP expression level in G1.

In this chapter, I have introduced a second possible mechanism to limit the level of SLBP expression in G1 phase. I have shown that new SLBP production rate increases to the S phase rate somewhere between early and mid-G1 (Fig 6), although it takes another 3 hours for SLBP expression to reach the S phase level (Fig 1). This observation suggested the presence of another mechanism to control SLBP levels until G1/S border. Since the SLBP production rate is the same in mid-G1 as in S-phase, the other possibility to restrict SLBP expression may be the instability of the protein. When I checked the stability of SLBP by

blocking new protein production with cycloheximide, I found that, unlike the earlier point in G1, the SLBP level goes down around 50 % in one hour (Fig. 7), suggesting involvement of regulated degradation as a second mechanism to coordinate SLBP expression and S phase entry.

Thus according to my current model (Fig. 8), at least two mechanisms are involved in keeping SLBP levels low in G1-phase (Fig. 8). At early G1, SLBP translation is low in comparison to S phase, but the small amounts of SLBP protein are stable. At sometime later in G1, the SLBP production rate increases to the S phase rate but regulated degradation keeps SLBP level limited until next S phase. This allows significant increase in SLBP levels just before S phase when this degradation stops.

Possible mechanism for regulation of SLBP degradation in G1.

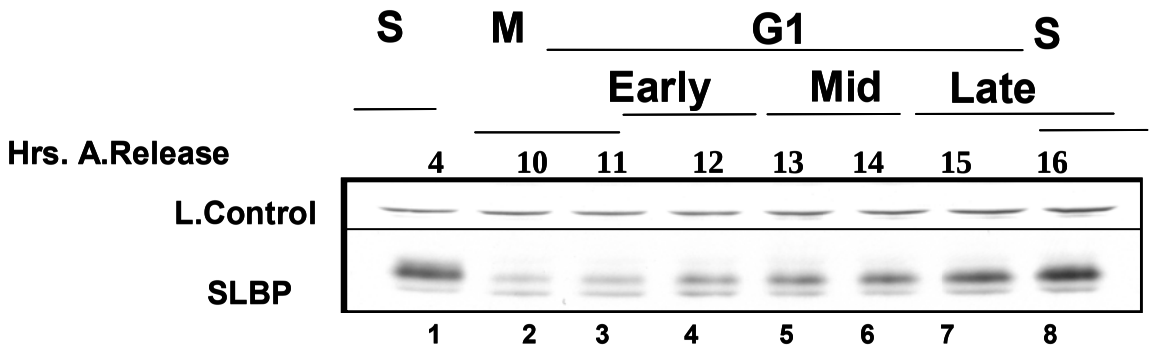
A possible candidate for the factor that targets SLBP for degradation in mid-G1 is the APC. There are at least two forms of the APC: APCCdh1 and APCCdc20. The initial active form is APCCdc20 which is responsible for degradation of Cyclin A and B at the end of mitosis. APCCdh1 becomes active after M phase Cyclins degradation at late M phase and remains active during G1 until it gets inactivated as cells approach S-phase. Cdk activity promotes APCCdc20 activity and prevents APCCdh1 activity. The kinetics of switch from APCCdc20 activity to APCCdh1 activity at M/G1 is not very well determined. It is also possible that there are additional activating factors that have not been described for the APC.

It is possible due to the fact that Cyclin A is strong target for both forms of APC, at immediately after M/G1, we can detect Cyclin A degradation, on the other hand SLBP

degradation can be detected after somewhere in early G1 where APCCdh1 activity and possibly the SLBP level reaches to certain level.

Figure 1) SLBP level stays low until the next S phase in HeLa cells. A) Western blot analysis of SLBP expression in HeLa cells collected at indicated time points after release from double thymidine block. **B)** FACs analysis of the cells collected at indicated times after the release.

A.


S
M
G1
S
Early
Mid
Late
Hrs. A.Release
4
10
11
12
13
14
15
16
L.Control
SLBP
1
2
3
4
5
6
7
8

B.

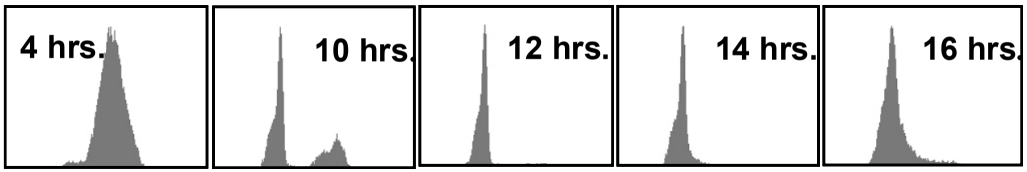
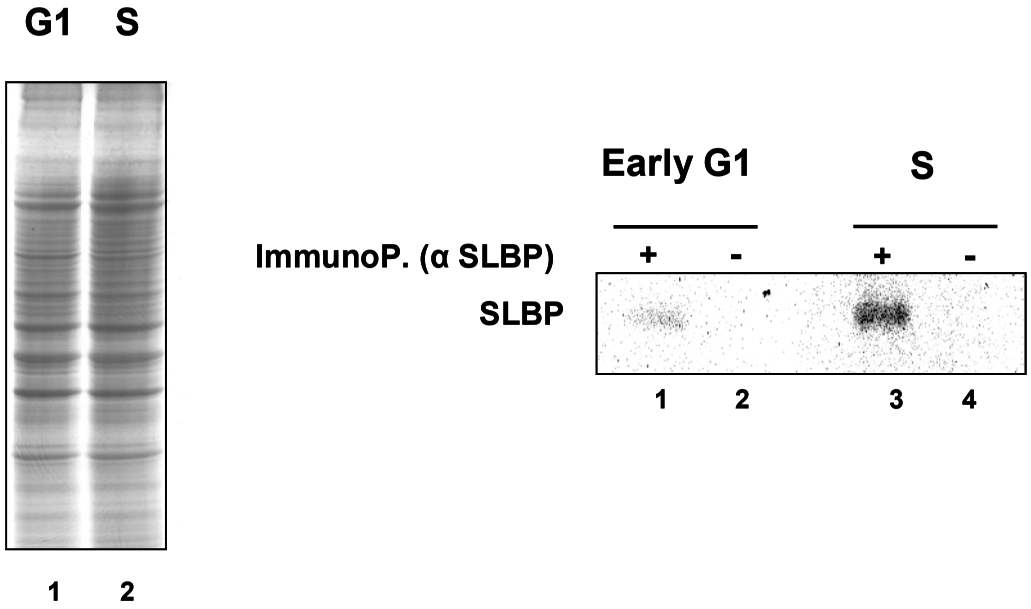
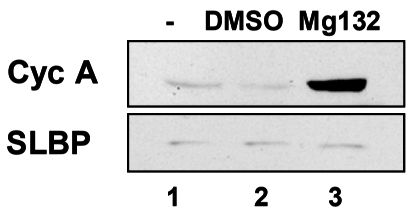

4 hrs.
10 hrs.
12 hrs.
14 hrs.
16 hrs.

Figure 2: SLBP translation efficiency is low in early G1 cells. A) HeLa cells are labeled with ^{35}S-methionine for 15 minutes at early G1 or S phase (12 and 2.5 hrs after double thymidine release, respectively) after half hour incubation in methionine lacking media. Equal protein amounts of total lysates (Panel 1) and immunoprecipitates with SLBP antibody or beads alone (panel 2) were run on SDS PAGE gel and detected with autoradiography. **B)** Western blot analysis of the cells collected after one hour MG132, DMSO (carrier) or just media treatment at early G1 (between 11.30 and 12.30 hrs after release from double thymidine block) using Cyclin A or SLBP antibody as indicated. **C)** Western blot analysis of early G1 (11.30 hrs after release) cells before and after 1hour 0.1 M Cycloheximide treatment, using Cyclin A or SLBP antibodies as indicated.

A.


G1
S
1
2
Early G1
S
ImmunoP. (α SLBP)
+
-
+
-
SLBP
1
2
3
4

B.


-
DMSO
Mg132
Cyc A
SLBP
1
2
3

C.

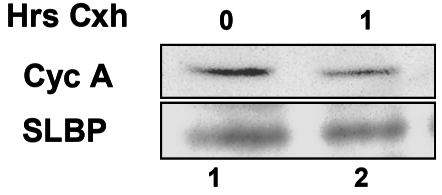
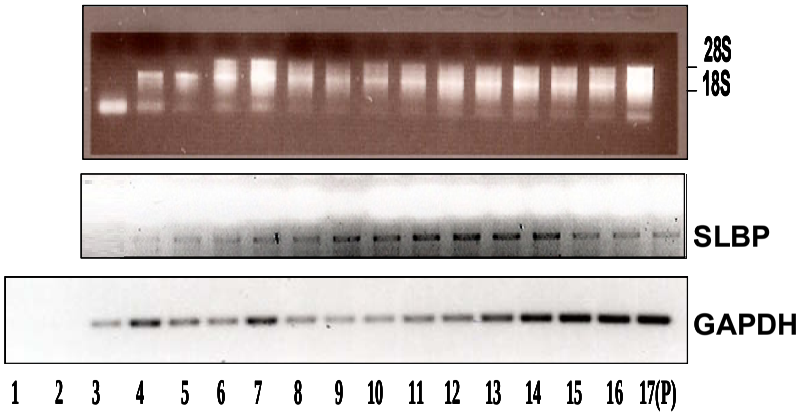

Hrs Cxh
0
1
Cyc A
SLBP
1
2

Figure 3: SLBP mRNA shifts from small to large polysomes between early G1 and S phase. Hela cells were synchronized by double thymidine block and collected at either 2 hours after release **(B)** or 11.30 hours after release **(A).** Cytoplasmic lysates were prepared and fractionated on 15 to 40 % sucrose gradient, and RNA was prepared from each fraction. The RNA was resolved by agarose gel electrophoresis and stained with EtBr (top panels). Using RNA from each fragment, RT PCR targeting either SLBP or GAPDH mRNA were performed.

A.


Early G1
28S
18S
SLBP
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17(P)

B.

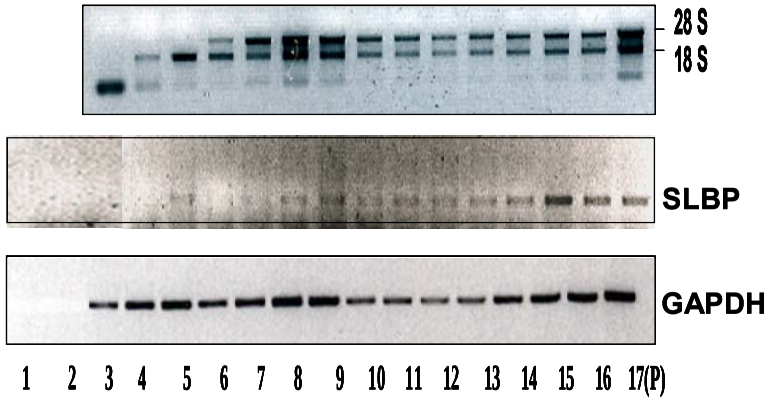

S phase
28 S
18 S
SLBP
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17(P)

Figure 4: SLBP coding sequence clone (his-SLBP) expression is low at G1. A) SLBP coding sequence is cloned in to pcDNA3 vector and stably expressed in HeLa cells. **B)** Western blot analysis of HeLa cells stably expressing exogenous histagged-SLBP collected at indicated times after double thymidine block with SLBP antibody.

A.


5'UTR pcDNA3
Coding Seq.
3'UTR pcDNA3
Stably expressed in He La cells

B.

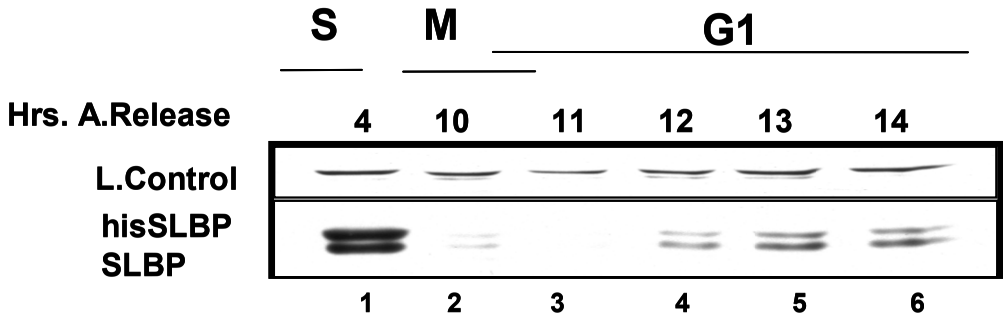
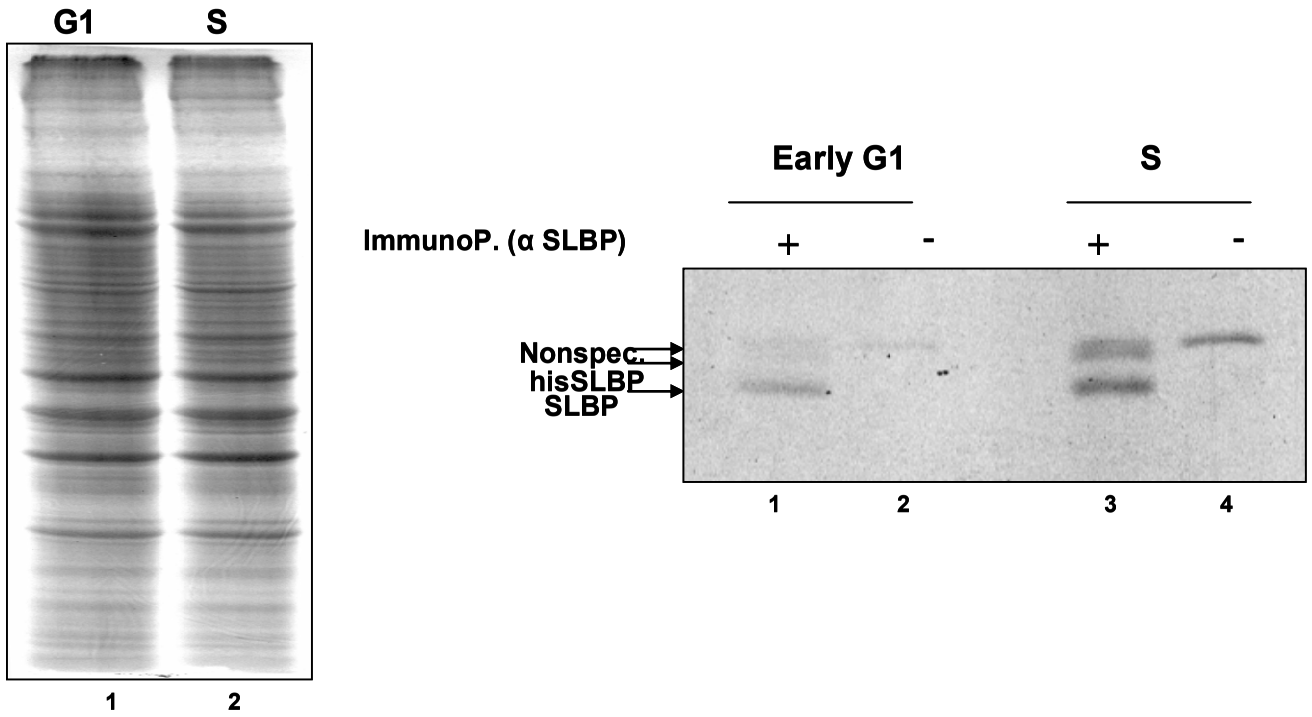
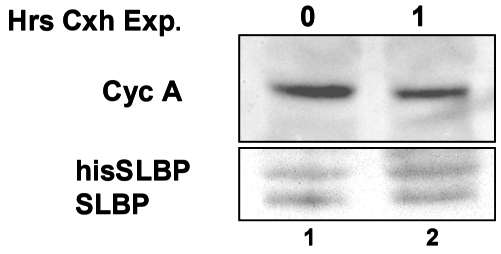

S
M
G1
Hrs. A.Release
4
10
11
12
13
14
L.Control
hisSLBP
SLBP
1
2
3
4
5
6

Figure 5: SLBP coding sequence is sufficient to introduce low translation efficiency in early G1 cells. A) HeLa cells expressing exogenous histagged SLBP are labeled with ^{35}S-methionine for 15 minutes at early G1 or S phase (12 and 2 hrs after double thymidine release, respectively) after incubating half hour in media without methionine. Equal protein amounts of total lysates (Panel 1) and immunoprecipitates with SLBP antibody or beads alone (panel 2) were run on SDS PAGE gel and detected with autoradiography. **B)** Western blot analysis of early G1 (11.30 hrs after release) cells before and after 1 hour 0.1M Cycloheximide treatment using Cyclin A or SLBP antibodies. **C)** Western blot analysis of the cells collected after one hour MG132, DMSO (carrier) or just media treatment between 11.30 and 12.30 hrs after release from double thymidine block using Cyclin A or SLBP antibody as indicated.

A.


G1
S
1
2
Early G1
S
ImmunoP. (α SLBP)
+
-
+
-
Nonspec.
hisSLBP
SLBP
1
2
3
4

B.


Hrs Cxh Exp.
0
1
Cyc A
hisSLBP
SLBP
1
2

C.

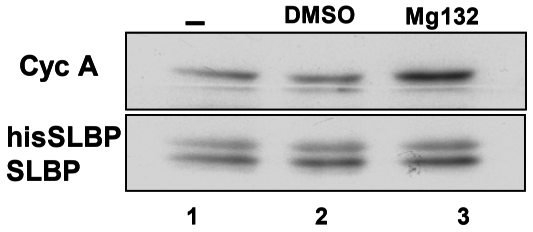
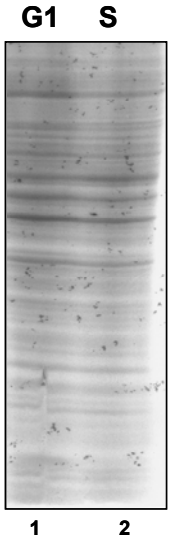

–
DMSO
Mg132
Cyc A
hisSLBP
SLBP
1
2
3

Figure 6: SLBP translation efficiency recovers to S phase level between early and mid-G1. HeLa cells are labeled with ^{35}S-methionine for 15 minutes at early G1 or S phase (13 and 2 hrs after double thymidine release, respectively) after incubation in methionine lacking media for half hour. Equal protein amounts of total lysates (Panel 1) and immunoprecipitates with SLBP antibody or beads alone (panel 2) were run on SDS PAGE gel and detected with autoradiography.


G1
S
1
2

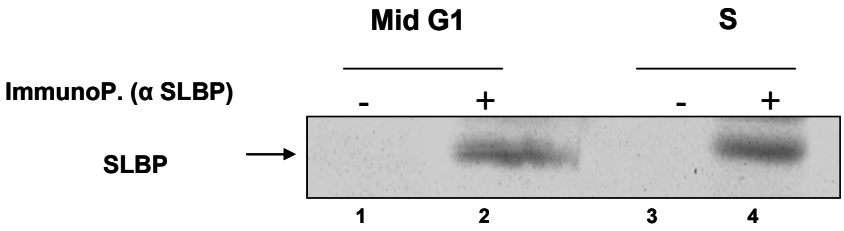
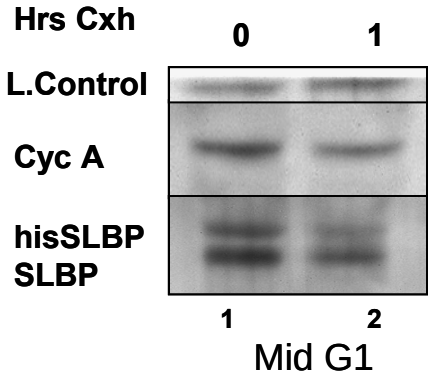

Mid G1
S
ImmunoP. (α SLBP)
-
+
-
+
SLBP
1
2
3
4

Figure 7: SLBP is less stable at mid-G1. A) HeLa cells expressing exogenous histaggedSLBP were treated with 0.1M Cycloheximide for 1 hour at mid-G1 (13 after double thymidine release). Western blot analysis of the cells before and after treatment were performed by using Cyclin A or SLBP antibody. **B)** HeLa cells were treated with 0.1 M Cycloheximide for 1 hour at S phase and S/G2 (2.30 hrs and 6 hrs after double thymidine release). Western blot analysis of the cells before and after treatment were performed by using SLBP antibody.

A.


Hrs Cxh
0
1
L.Control
Cyc A
hisSLBP
SLBP
1
2
Mid G1

B.

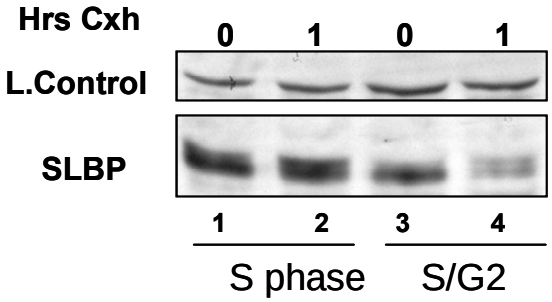
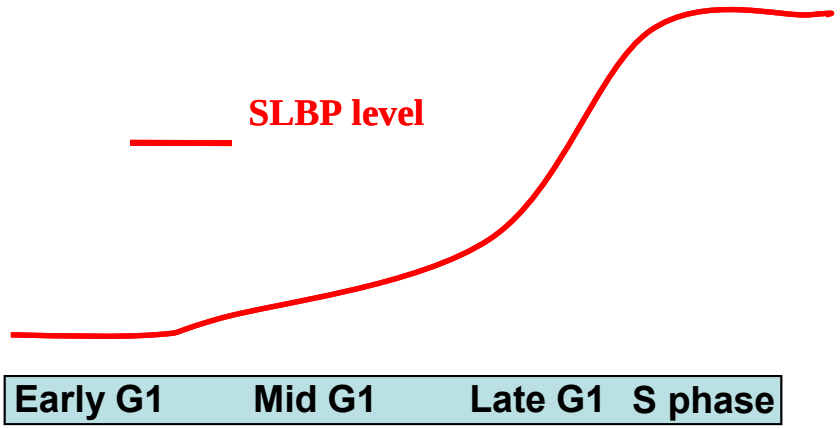

Hrs Cxh
0
1
0
1
L.Control
SLBP
1
2
3
4
S phase
S/G2

Figure 8: Model for regulation of SLBP expression in G1. In early G1 SLBP translation rate is low. Between early and mid G1 SLBP translation efficiency increase to S phase level but yet SLBP protein level kept limited by regulated degradation until next S phase.



Low SLBP Translation

SLBP Translation ↑ Regulated Degradation

MATERIALS AND METHODS:

.

Determination of SLBP synthesis rate:

HeLa cells were synchronized by double tymidine block as explained in Chapter 2. Early G1 cells (11.15 hrs after release) and S phase cells (2 hours after release) were preincubated in DMEM, without methionine supplemented with 10% dialyzed fetal bovine serum, for 45 mins prior to labeling to deplete intracellular stores of methionine. SLBP synthesis rate was measured by pulsing cells with 1 mCi of [^{35}S]methionine for 15 min. Whole-cell extracts were incubated with anti-SLBP and the SLBP–antibody complexes recovered by binding to protein A agarose beads. The bound proteins were eluted in SDS loading buffer and resolved by gel electrophoresis and detected by autoradiography.

Polysome fractionation:

HeLa cells were synchronized as explained as Chapter 2. HeLa cells at early G1 (11.30 hours after release) and S phase (2hrs after release) were lysed in hypotonic buffer and the nuclei and mitochondria removed by centrifugation. The cytoplasm was adjusted to 0.1% NP-40, 0.1 M NaCl, 5 mM $Mg(OAc)_2$ and layered on a 15–40% sucrose gradient containing 0.1 M NaCl, 5 mM $Mg(OAc)_2$, 10 mM Tris, pH 7.5. Polyribosomes were fractionated by centrifugation at 39 000 r.p.m. for 2.5 h in an SW841 rotor. Aliquots of 0.5 ml were collected and RNA was prepared from 0.3 ml of each aliquot using Trizol LS according to manufacturer's direction. RNA prepared from each fraction was analyzed by agarose gel electrophoresis to identify the monoribosome peak. In order to detect SLBP and GAPDH mRNA I have designed primers that are targeting 200 nucleotide region in their coding

sequences with comparable GC content. After RNA isolation from each fraction, RNA was subject to reverse transcription (RT) using the manufacturer's protocols (New England Biolabs, Natick, MA) with random hexamer primers. Approximately 1/10 of the RT reaction was used as a template for 20 cycles PCR to detect GAPDH and SLBP mRNAs.

CHAPTER 4

CONCLUDING DISCUSSION

SLBP expression is limited to S phase where it functions. SLBP expression as major player in histone biosynthesis is tightly regulated during cell cycle. I have determined that cells restrict SLBP expression to S phase via several different mechanisms. At S/G2, SLBP is rapidly degraded depending on specific phosphorylations at Thr 60 and Thr 61, with the phosphorylation on Thr 61 by cyclinA/cdk1 as the likely event that triggers SLBP degradation. At M to early G1, SLBP translation is low due to mechanism mediated by its coding sequence. And finally, as a possible new mechanism to regulate SLBP expression in G1, SLBP expression is controlled by regulated degradation until next S phase.

SLBP Expression and the S/G2 Transition.

Here, I have determined that SLBP degradation at S/G2 is triggered by Cyclin A/Cdk1 mediate Thr 61 phosphorylation. Cyclin A/Cdk1, different than Cyclin B/Cdk1 which shows rapid activation at G2/M, shows gradual activity increase starting in late S phase increasing towards G2 phase where it peaks (Pagano et al., 1992; Mitra et al., 2006). According to our model, at S/G2, Cyclin A/Cdk1 activity reaches sufficient level and triggers SLBP degradation by phosphorylating Thr 61.

The S/G2 transition is one of the least understood stages of cell cycle. Here, we introduce Cyclin A/Cdk1 as player in this stage where it triggers degradation of SLBP as a mechanism to end histone biosynthesis which is one of the major events that is performed in S phase. It is an appealing idea that Cyclin A/Cdk1 plays several other roles including triggering degradation of several other proteins at S-G2 transition. One of the candidates for this kind of regulation is degradation of E2F1 which is also degraded at almost same cell cycle point as SLBP. Dr. Jian Dong in the Marzluff lab has shown that S/G2 degradation of E2F1 is mediated by phosphorylation of a central region of E2F1 which is different than previously determined Skp2 targeting site. Further, this degradation requires a RXL motif and is sensitive to roscovitine just like SLBP. We are currently investigating whether Cyclin A/Cdk1 is the kinase that triggers E2F1 degradation at S/G2 similar to SLBP.

Finally, the fact that SLBP can distinguish between Cyclin A/Cdk2 and Cyclin A/Cdk1 as kinase for the phosphorylation of Thr 61 can provide explanation for cell cycle events that are believed to be mediated by Cyclin A/Cdks but yet don't happen during S phase where there is high Cyclin A/Cdk2 activity, but instead occur in G2 phase. For example, Cyclin A/Cdk is believed to have role in Cyclin B/Cdk1 activation. On the other hand although there is high Cyclin A/Cdk2 activity throughout S phase this event occurs at G2. It is possible that Cyclin A/Cdk1 activity may be needed a possible initial, activating phosphorylation of Cdc25C and/or inhibiting phosphorylation of Wee1 to trigger Cyclin B/Cdk1 activation loop for robust Cyclin B/Cdk1 activation and M phase entry. It is likely that there will be other specific substrates of Cyclin A/Cdk1. Already a few examples have been identified (Henneke et al., 2003; Li et al., 2004; Baldin et al., 1997).

In conclusion, I believe that the role I propose here for Cyclin A/Cdk1 at the time of S phase completion may be an example of several other biochemical events that Cyclin A/Cdk1 is regulating at S/G2 transition.

A remaining unknown question is what ubiquitin ligase is necessary for SLBP degradation at the end of S-phase. Dr. Dong and I knocked down a number of the cullin proteins, but none of these had convincing effects on SLBP levels in exponentially growing cultures. Since knocking down a cullin generally affects the cell cycle enough that we could not subsequently synchronize the cells, it was not possible to definitively ask if any of these ligases had a specific effect at G2. Since the form of SLBP that is recognized for degradation is the doubly phosphorylated form, and it has not been possible to create phosphomimics that are degraded appropriately, it has not been possible to develop biochemical approaches to find binding partners for the SLBP to allow its degradation. The recent finding that the E change at Thr 60 mimics phosphorylation at this site, together with the ability to phosphorylate Thr 61 in vitro, makes potential biochemical approaches possible to identify the ligase.

G1 phase regulation of Stem loop binding protein.

Low translation rate at early G1.

I have further confirmed that SLBP translation is low at early G1 in comparison to S phase in HeLa cells. In contrast to our previous model, I have shown that SLBP translation rate comes back to S phase level not at G1/S (16/18hrs after release) somewhere between early and mid G1. Further, I have shown that coding sequence of SLBP is sufficient to

introduce this low translation rate. The nature of this regulation is yet to be determined. It is critical to determine whether there the sequences in either the 5' or 3' UTR that are also involved in the translational regulation. In addition it should be possible to narrow down the regions required for translation regulation by deletion analysis, creating proteins that react with our antibody but lack a tag which may affect the half-life of the protein. Translational regulatory regions in the coding region are unusual, and it is not clear how they might function to reduce translation efficiency,

During M phase, general translation (Cap dependent translation) is inhibited majorly due to scarce level of active eiF4F complex. From M phase to G1, translation machinery recovers back to active state and at G1 there is robust translation. In G1, mTOR and MAPK pathways further activate the translation machinery and are important for G1 progression as explained in Chapter 1. This kind of global regulation on translation machinery can introduce relatively specific effect on certain mRNAs due to properties like their high 5' GC rich content which introduces secondary structures. It is possible that at M/G1 transition although translation of the most of the mRNAs recovers, some of the mRNAs require further activation of translation machinery by for example mTOR and/or MAPK pathways in G1. It is possible that the low translation rate that we detect with SLBP in early G1 is an example of a selective effect of changes in global translation regulation due to some intrinsic property of SLBP coding sequence that makes it more vulnerable to the any change in the general translation machinery.

Possible signal that causes transition from low to high SLBP translation remains to be an interesting question. It is possible to block candidate signals known to take role in G1 like mTOR, MAPK and Cdks. On the other hand, since blocking these signals introduces cell

cycle block or delay it is difficult to asses whether the possible effect seen on SLBP is direct or indirect. Another possibility about the nature of SLBP translation regulation can be specific regulation by a protein or miRNA that recognizes certain cis-element within the coding sequence. Since there is limited number of examples of coding sequence dependent translation regulation(Stajduhar et al., 2000; Lin et al., 2000; Tai et al., 2004b) , and all miRNAs published so far are targeting 3'UTR , this may be interesting and novel regulation. As recent developments, miRNAs that are targeting coding sequences have been discovered and are yet to be published (personal communication with R. Agami).

As a future direction, being able to determine a possible cis-element responsible for this regulation will be important to understand the nature of this regulation. If we can determine a cis-element that is required and sufficient to introduce this regulation, this sequence can be used as bait to identify any protein that can specifically bind to it. Also, in order to check possible involvement of a miRNA, to be able to block any possible miRNA targeting this cis-element, 2OH methyl modified version of this sequence can be transfected to see if it can block translational regulation of SLBP.

Regulated degradation of SLBP at G1.

Here, I have introduced regulated degradation as an additional mechanism to control SLBP expression in G1. I have shown that low translation efficiency of SLBP recovers back to S phase level during G1 but yet SLBP expression does not recover back to S phase level until G1/S which is around 3-4 hours later. SLBP stability is low during this period which may be the cause low SLBP expression during G1.

APCCdh1 is known to be regulating the expression of several S phase related proteins like Cyclin A, Skp2 and Cdc6 in G1(Petersen et al., 2000; Bashir et al., 2004; Wei et al., 2004b). It becomes active after degradation of M phase Cyclins at late M phase until next S phase where Cyclin A/Cdk2 activity and expression of APC inhibitor Emi-1 block its activity(Herman-Antosiewicz et al., 2007; Fung and Poon, 2005). One of the known target motif for APC is so called Destruction box (D-box) with some version of RXXL sequence. SLBP has two highly conserved RXXL motif at its N-terminal which fits with the appealing possibility that APCCdh1 regulates expression of another S phase protein-SLBP in G1. It is possible that SLBP translation rate is low at M/G1 and towards mid-G1 it comes to S phase level while APCCdh1 activity (and may be SLBP level, too) reaches to the level to be able to target SLBP as a new mechanism to limit the SLBP expression by targeted degradation until next S phase. In order to determine the possible region(s) of SLBP that targets it to regulated degradation, I have produced several deletions of SLBP (including RXXL regions) and I am in the process of stably expressing them in HeLa cells. In order to differentiate G1 degradation from S/G2 degradation, I have also mutated Thr 61 to prevent S/G2 degradation. Next, using those cells, I will try to map the possible region that is needed for G1 regulated degradation of SLBP.

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