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Maternally encoded stem-loop-binding protein is degraded in 2-cell mouse embryos by the co-ordinated activity of two separately regulated pathways

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Abstract

Oocytes accumulate mRNAs and proteins that direct early embryonic development. Although subsequent development requires the timely degradation of these maternal products, little is known of the underlying mechanisms. The stem-loop-binding protein (SLBP), which regulates the stability and translation of mRNAs encoding histones and is synthesized during S-phase and degraded during G2 in somatic cells, accumulates during oogenesis. Maternal SLBP is required for mouse embryos to develop beyond the 2-cell stage, but must be degraded to allow the cell-cycle-regulated expression of somatic cells to be established. We report that the quantity of maternal SLBP changes little following fertilization until 44–52 h post-hCG, corresponding to mid-/late G2 of the 2-cell stage, when it decreases by 75%. Efficient degradation requires two pathways. The first requires activity of cyclin-dependent kinases (cdk) and embryonic transcription, preferentially targets nuclear SLBP, and likely corresponds to the pathway that degrades SLBP at G2 in somatic cells. The second does not require cdk activity or transcription and becomes active at 44–52 h post-hCG independently of cell-cycle progression to mid-/late G2, but is not solely regulated by the time elapsed since hCG injection. Thus, the co-ordinated activity of two separately regulated pathways eliminates maternally encoded SLBP from early mouse embryos.

Keywords

Oocyte-to-embryo transition; Maternal factors; Histones; Cell cycle; Oogenesis; Embryogenesis; Protein degradation

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Introduction

Growing oocytes accumulate messenger RNAs (RNAs) and proteins that direct early embryonic development (Bettegowda et al., 2007; Cui and Kim, 2006; Stitzel and Seydoux, 2007). Following fertilization, these maternal gene products are degraded. This degradation, combined with the transcriptional activation of the embryonic genome, brings development under the control of the embryonic genome. Recent studies have begun to reveal the mechanisms underlying the degradation of maternal products. In *Caenorhabditis elegans*, phosphorylation of the OMA-1 protein during meiotic maturation of the oocyte targets it for degradation in the early embryo (Lu and Mains, 2007; Nishi and Lin, 2005; Shirayama et al., 2006; Stitzel et al., 2006). Importantly, embryos that fail to degrade OMA-1 (or anther maternally supplied protein, MEI-1) die during early development (Bowerman and Kurz, 2006; Lin, 2003; Stitzel and Seydoux, 2007), illustrating that correctly regulated degradation of maternal products is essential for normal embryogenesis. Apart from this case, however, this process is poorly understood. In mammals, for example, maternal mRNA begins to be degraded during meiotic maturation and in the mouse is largely complete by the late 2-cell stage when embryonic transcription begins (Alizadeh et al., 2005; Paynton et al., 1988); however, its molecular basis has not been elucidated. Moreover, almost nothing is known of the fate of the encoded proteins.

The stem-loop-binding protein (SLBP) plays a central role in the metabolism and translation of most histone-encoding mRNAs (Martin et al., 1997; Wang et al., 1996). SLBP binds to a stem-loop structure in the 3′-untranslated region of these mRNAs and this event promotes binding of the U7 snRNP to a site 3′ of the SLBP-binding site. The new transcript is then cleaved between the stem-loop and U7-binding site (Dominski et al., 1999, 2001, 2005; Jaeger et al., 2006; Kolev and Steitz, 2005). This processing reaction stabilizes the transcripts, which would otherwise be rapidly degraded. SLBP then accompanies the histone mRNAs to the cytoplasm, where it bridges the 3′- and 5′-ends of the mRNA through an interaction with eIF4G and promotes their translation (Cakmakci et al., 2008; Gorgoni et al., 2005; Ling et al., 2002; Sanchez and Marzluff, 2002; Whitfield et al., 2004); the role of the stem-loop and SLBP in translation of histone mRNAs is analogous to that of the poly(A) tail and poly(A)-binding proteins in translation of polyadenylated mRNAs.

In somatic cells of vertebrates, SLBP is present only during S-phase of the cell cycle, thus co-ordinating histone synthesis with DNA replication (Whitfield et al., 2000). Following the completion of DNA replication, SLBP-bound histone mRNAs become degraded (Mullen and Marzluff, 2008) and the now free SLBP is transported to the nucleus (Erkmann et al., 2005). It becomes phosphorylated on Thr-61 by the cyclin-dependent kinase (cdk) complex, CDC2A (cdk1)-cyclin A (Koseoglu et al., 2008), which is active during G2 and is localized in the nucleus (Pines and Hunter, 1991). This phosphorylation and subsequent phosphorylation of Thr-60 by casein kinase-2 trigger degradation of SLBP (Koseoglu et al., 2008; Zheng et al., 2003). Degradation requires the activity of the skp1-cullin-F-box (SCF) ubiquitin ligase (Yen and Elledge, 2008). Thus, in somatic cells, SLBP expression is cell cycle-regulated, and its degradation at G2 is controlled by a ubiquitin-mediated pathway that requires SLBP phosphorylation by CDC2A-cyclin A and, based on the intracellular location of this kinase, likely nuclear localization.

SLBP is also expressed in oocytes, although these are not undergoing DNA replication (Allard et al., 2002; Wang et al., 1999). It begins to accumulate when G2-arrested oocytes enter the growth phase (Arnold et al., 2008) and a further substantial accumulation occurs during meiotic maturation. Following fertilization, SLBP remains abundant throughout the first cell cycle and following first cleavage. It is then degraded during the latter portion of the second embryonic cell cycle (Allard et al., 2002). Embryos that lack maternally encoded SLBP are unable to progress beyond the 2-cell stage, owing to a failure to accumulate sufficient histones to support chromatin assembly (Arnold et al., 2008). Thus, maternally supplied SLBP is required for early embryonic development; however, it must be degraded to allow the S-phase-restricted pattern of expression that characterizes somatic cells to be established. In the experiments reported here, we have defined that precise timing and underlying mechanisms that regulate the degradation of this maternal gene product.

Materials and methods

Collection of eggs and embryos

All experiments were performed using CD-1 mice (Charles River Canada) in compliance with the regulations and policies of the Canadian Council on Animal Care. Ovulated eggs and fertilized embryos were obtained as described (Allard et al., 2002) For parthenogenetic activation, ovulated eggs collected at 18 h post-hCG were exposed to 10 mM $SrCl^{2+}$ for 2 h in Ca^{2+}-free KSOM. Activated eggs were identified by the presence of two pronuclei. For each experiment, embryos collected from several different females were pooled. Groups of embryos from this pool were randomly collected and allocated to each treatment group.

Immunoblotting

Eggs or embryos at the appropriate stage of development were transferred into a 0.5-ml microtube to which was added 10 μl of Laemmli buffer. Tubes were heated to 95 °C for 5 min, then used immediately or stored at −80 °C until use. All treatment groups from a particular experiment were loaded onto the same gel and processed identically. Proteins were separated by polyacrylamide gel electrophoresis in 12% polyacrylamide gels and transferred to PVDF membrane (GE Healthcare, Montreal, QC) following standard protocols (Harlow and Lane, 1999). Membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 5% non-fat milk and 0.1% Tween-20 (Sigma Chemicals, Windsor, ON), then incubated in primary antibody diluted appropriately in TBS containing 5% bovine serum albumin (Sigma) and 0.1% Tween-20 buffer overnight at 4 °C with gentle agitation. The membranes were then washed in TBS, 0.1% Tween-20, incubated in anti-rabbit-IgG or antimouse-IgG conjugated to horse-radish peroxidase (Promega, Madison, WI) diluted 1:5000 in blocking buffer for 1 h at room temperature, and washed as above. Signals were detected using ECL+ (GE Healthcare) and recorded on film and using a Phosphoimager (GE Healthcare). All data analysis was carried out using the quantitative data obtained using the phosphoimager. For each experiment, values were normalized to a control group on the same blot.

Immunofluorescence

Embryos were exposed briefly (~10 s) to warm acid Tyrode's medium (Sigma) to remove the zona pellucida, washed in HEPES-buffered KSOM medium, then fixed for 15 min in freshly prepared 4% *para*-formaldehyde in phosphate-buffered saline (PBS). Embryos were blocked in PBS containing 3% BSA, 0.1% Tween-20, then incubated in primary antibody diluted in blocking buffer overnight at 4 °C with gentle agitation. They were then washed twice in blocking buffer, incubated in anti-rabbit-IgG conjugated to Alexa 488 (1:200, Molecular Probes, Eugene, OR) or to Cy3 (Jackson Immunoresearch) for 1 h. DNA was stained using DAPI (2.5 μg/ml, Roche) or YOYO-1 (1:20000 from stock; Molecular Probes) added to the solution containing the secondary antibody. Embryos were then washed, mounted in Moviol and covered with glass cover slips. In the experiments employing drug treatments, control and treated embryos fixed at the same time were mounted on the same slide. Images were analyzed using a Zeiss CLSM 510. For each experiment, the conditions for collecting images were established using 44-hour embryos such that the signal remained below saturation. The optical section containing the brightest signal was identified for each embryo and the signal was recorded. Thus, for each experiment, all embryos were sampled using identical parameters. Images were stored as TIFF files and quantified using ImageJ (National Institutes of Health). For each experiment, the mean value at 44 h was set to 1 and the mean and standard error of the mean for each group were calculated and normalized to this value.

Antibodies

Affinity-purified anti-SLBP (Arnold et al., 2008) was diluted 1:4000 for immunoblotting and 1:200 for immunostaining. Anti-tubulin (Cedarlane Laboratories, Burlington, ON) was used at a dilution of 1:5000. Anti-MAPK1/3 (Santa Cruz Biochemicals, Santa Cruz, CA) was used at a dilution of 1:2000.

Drug treatments

Stock concentration, working concentration and supplier for drugs used in this study were as follows. Aphidicolin (1 mg/ml water, 1 μg/ml, Sigma); α-amanitin (1 mg/ml water, 10 μg/ml, Sigma); MG132, roscovitine, 6-dimethylaminopurine (DMAP) (10 mM DMSO, 25 μM, Sigma). The activity of roscovitine, α-amanitin, and 6-DMAP was verified in each experimental replicate by their ability to inhibit cleavage of an aliquot of embryos from the 2- to 4-cell stage.

Real-time reverse transcription and polymerase chain reaction (RT-PCR)

Fifty embryos were deposited into a microfuge tube and 10 pg of EGFP mRNA was added to the tube. RNA was isolated using a Picopure RNA kit (Arcturus, MDS Analytical Technologies, Toronto, ON) following the manufacturer's instructions and resuspended in a final volume of 20 μl. To generate cDNA, 8.5 μl of the RNA solution was combined with 50 ng of random hexamers (Invitrogen, Burlington, ON), dNTPs and 50 U of Superscript II RT (Invitrogen) following the manufacturer's recommendations in a final volume of 20 μl. For each sample, an identical RT reaction was assembled but omitting the reverse transcriptase. PCR was performed using Qiagen Quantitect SYBR-Green (Qiagen, Mississauga, ON) and

a Lightcycler (Roche). Standard curves for SLBP and EGFP were generated to calculate the efficiency of amplification for each primer pair. For analytical reactions, 1 μl of 1:10 diluted reverse-transcription product was used in a final volume of 20 μl. The cycle conditions were as follows: 10 s at 94 °C, 10 s at 50 °C and 30 s at 72 °C for 50 cycles. Melting curve analysis and agarose gel electrophoresis were performed to verify that only the expected product was amplified. The relative expression ratio between samples was calculated from the amplification efficiencies and the crossing points as described (Pfaffl, 2001).

The following primers were used, shown in 5′–3′ orientation. *Slbp* (GenBank NM_009193): TGATGTGGAGACGGATGAAA, AGCAGGAGGATCCCAAAAAT; *Egfp* (GenBank U55762): GAAGTTCATCTGCACCACCG, CTCAGGTAGTGGTTGTCG.

Production of embryos lacking chromosomes

Ovulated eggs were recovered 16 h post-hCG and exposed to $SrCl_2^+$ as above. At 18.5 h, those that had emitted the second polar body, indicating that they had been activated, were selected and the chromosomes were removed. To achieve this, the activated eggs were incubated for 10 min in KSOM–HEPES containing the DNA vital stain, bisbenzimide (Hoechst 33342; 5 mg/ml; Sigma), and 7.5 mg/ml cytochalasin B (CB). Oocytes were then transferred into a 50-μl droplet of KSOM–HEPES + CB covered with mineral oil and the chromosomes were removed using a Nikon inverted microscope equipped with epifluorescence. The second polar body and a small portion of the surrounding cytoplasm were removed and briefly exposed to ultraviolet irradiation to confirm that they contained the chromosomes. The time of chromosome removal ranged from 19 to 23 h post-hCG injection and the nuclear stage of the activated eggs ranged from telophase II to early pronuclear. The egg fragments were then incubated in KSOM under standard conditions and samples were harvested at different times as indicated in the results.

Results

To precisely define the timing of the loss of SLBP in 2-cell embryos, unfertilized eggs were collected at 14–16 h post-hCG and 2-cell embryos at 44, 48 and 52 h post-hCG (hereafter we omit post-hCG except where clarity requires it) and immunoblotted to determine the relative quantity of SLBP, tubulin and MAPK1/3 at these times. Embryos had completed the second round of DNA replication by 40 h and begun to cleave to the 4-cell stage by 52–54 h (data not shown), meaning that they were at mid-/late G2 during the period of study. As compared to the amount present in unfertilized eggs, SLBP had declined slightly by 44 h (Fig. 1A). Over the next 8 h, however, the amount of SLBP decreased substantially, so that 52-h embryos contained only about one-quarter of the amount present in 44-h embryos. In contrast, the quantity of α-tubulin and MAPK1/3 did not significantly change during the same period. The decrease in SLBP was completely suppressed when embryos were incubated in the presence of the proteasomal inhibitor, MG132 (Fig. 1B). These results demonstrated that most of the SLBP present in early embryos became degraded during mid-/late G2 of the 2-cell stage and that degradation occurred through a proteasome-dependent pathway.

To determine the abundance of *Slbp* mRNA during the period when the protein was lost, embryos were collected at 24, 44, and 52 h and the relative amounts of *Slbp* mRNA were measured using real-time RT-PCR. Fig. 1C shows that 90% of the *Slbp* mRNA present in 1-cell embryos at 24 h had been lost by 44 h. Little further decline occurred during the next 8 h. The relatively small amount of encoding mRNA present in 44-h and 52-h embryos suggests that little if any SLBP was synthesized during this period. Therefore, most or all of the SLBP that was degraded during this time was maternally encoded and had been synthesized earlier than 44 h.

SLBP is present in both the nucleus and cytoplasm of oocytes and early embryos (Allard et al., 2002). In view of the observation that about one-quarter of the SLBP was not degraded between 44 h and 52 h, we examined whether this remaining SLBP was localized in either cellular compartment. Embryos were fixed at 44 and 52 h, stained using anti-SLBP, and the fluorescent signals in the nucleus and cytoplasm were recorded and quantified. As shown in Fig. 2, both nuclear and cytoplasmic SLBP declined between 44 h and 52 h. One of the blastomeres of the 52-h embryo shown in Fig. 2A had entered metaphase, confirming that by this time the embryos were near the end of the second cell cycle. Nonetheless, most 52-h embryos retained apparently intact nuclei, indicating that the loss of SLBP preceded entry into metaphase. However, there was little change in the nucleo-cytoplasmic ratio between 44 h and 52 h. This result indicated that both nuclear and cytoplasmic SLBP were degraded during this period.

As discussed in the Introduction, degradation of SLBP in somatic cells requires its phosphorylation during G2 by the cdk complex, CDC2A-cyclin A (Koseoglu et al., 2008; Zheng et al., 2003). MG132-treated 2-cell embryos exhibited a slow-migrating species of SLBP that was eliminated by phosphatase treatment prior to electrophoresis (Fig. 1), suggesting that SLBP in embryos also became phosphorylated prior to its degradation. To test whether cdk activity was required for SLBP degradation in embryos, these were transferred to medium containing the cdk inhibitor, roscovitine, at 44 h and samples were collected at 48 h and 52 h. Roscovitine selectively inhibits CDC2A, CDK2 and CDK5 (Meijer et al., 1997), and its effectiveness was confirmed in each experiment by its ability to prevent the second cleavage division (data not shown). At both 48 h and 52 h, the roscovitine-treated embryos contained significantly more SLBP than control embryos at the same time-point (Fig. 3, indicated by asterisks). SLBP nonetheless became degraded in the roscovitine-treated embryos (Fig. 3, indicated by different letters above the columns), albeit at a slower rate than in controls. Similar results were observed when the drug was added at 48 h (Fig. 3) and at two-fold higher or lower doses (not shown). These results indicated that cdk activity was required to fully activate the mechanism of SLBP degradation in 2-cell embryos.

As the major activation of embryonic gene transcription occurs during the latter half of the 2-cell stage (Bultman et al., 2006; Latham, 1999), we then tested whether SLBP degradation required transcription. Embryos at the late 1-cell stage (32 h post-hCG) were transferred to α-amanitin, an inhibitor of RNA polymerase II, and collected at 52 h. Drug-treated embryos underwent the first cleavage division, but did not undergo second cleavage (data not shown), confirming the effectiveness of the drug. As observed in the roscovitine-treated embryos,

exposure to α-amanitin partially suppressed the degradation of SLBP from 44–52 h (Fig. 4A, left side). Taken together, these results indicated that efficient degradation of SLBP in 2-cell embryos required cdk activity and embryonic transcription.

It was evident, however, that a significant fraction of SLBP was degraded even in the presence of roscovitine or α-amanitin. Although each drug completely prevented the second cleavage division, the incomplete suppression of degradation could indicate that each drug only partially inhibited its cellular target. We addressed this possibility in two ways. First, we reasoned that, if this were the case, incubating embryos in the presence of both drugs would suppress degradation more efficiently. To test this, embryos were incubated in the presence of roscovitine or α-amanitin alone or together and collected at 52 h. As shown in Fig. 4A (right side), both drugs suppressed SLBP degradation, and they did so to the same extent. However, no additional suppression was observed in the presence of both drugs. Second, we tested the effect of a general inhibitor of protein phosphorylation, 6-DMAP. Like roscovitine, 6-DMAP partially suppressed the degradation of SLBP between 44 h and 52 h (Fig. 4B). Moreover, it exerted a quantitatively similar effect – about 50–60% of the SLBP present at 44 h remained at 52 h in the presence of either drug. Thus, two drugs that inhibit cdk-dependent protein phosphorylation by different mechanisms each partially suppressed SLBP degradation. These results argued against the possibility that the partial suppression of SLBP degradation by these drugs was due to incomplete inhibition of their targets.

Cyclin A is localized in the nucleus in somatic cells (Pines and Hunter,1991), and we observed that it is also nuclear in 2-cell embryos as judged by immunofluorescence (data not shown). Yet the cytoplasmic SLBP in these cells was nonetheless degraded between 44 h and 52 h (Fig. 2). This suggested that degradation of cytoplasmic SLBP might not require CDC2A-cyclin A activity. To test this idea, embryos were treated with roscovitine from 44–52 h, then processed for immunofluorescence. As shown in Fig. 2B, the intensity of cytoplasmic fluorescence decreased between 44 h and 52 h, although to a slightly lesser extent than in controls. In contrast, nuclear fluorescence remained unchanged during this period in the presence of the drug. Thus, roscovitine preferentially suppressed the loss of nuclear SLBP. This implies that cytoplasmic SLBP could be degraded by a cdk-independent pathway.

These results described above indicated that SLBP became degraded between 44 h and 52 h post-hCG, corresponding to mid-/late G2 of the 2-cell stage, and that degradation could occur through both roscovitine-sensitive and -insensitive pathways. Although degradation occurred when the embryos were at G2, its timing could in principle be regulated by the time elapsed since hCG injection rather than by cell-cycle progression. Therefore, we sought a means to separate these two parameters. To this end, we activated eggs parthenogenetically at 18 h post-hCG. Since fertilization in vivo occurs at ~12 h post-hCG, cell-cycle progression of the parthenogenetic embryos was retarded by ~6 h as compared to fertilized embryos. We then determined whether SLBP became degraded between 44 h and 52 h in the developmentally delayed parthenogenetic embryos. Fig. 5A shows that SLBP became degraded during this period. However, it was degraded to a lesser extent (~50%) than in fertilized embryos (~75%). We then tested whether this degradation required cdk activity. In contrast to the results observed using fertilized embryos, roscovitine did not detectably

suppress the partial degradation of SLBP in parthenogenetic embryos (Fig. 5B). These results indicated that a pathway of SLBP degradation became active between 44 and 52 h post-hCG independently of cell-cycle progression to mid-/late G2, and that this pathway was not suppressed by roscovitine.

We then tested whether the activity of this pathway of SLBP degradation was regulated solely by the time elapsed since the hCG injection. Eggs were parthenogenetically activated at 16–17 h post-hCG, allowed to complete the second meiotic division so that successfully activated eggs could be selected, and the chromosomes were removed. This produced cytoplasts that would be unable to undergo chromosome-dependent cell cycle and developmental events. Cytoplasts were collected immediately after chromosome removal (19–23 h) and at 38 h, 44 h and 52 h post-hCG. As shown in Fig. 5C, there was no significant change in the quantity of SLBP during this time. This suggests that, although a pathway of SLBP degradation is linked to the time elapsed since hCG injection, its activity requires progression through embryonic development.

Discussion

We have examined the timing and pathways responsible for the degradation of maternally encoded SLBP in mouse embryos. The amount of SLBP changes little after fertilization until the period from 44–52 h post-hCG, corresponding to mid-/late G2 of the 2-cell stage, when it declines by about 75%. Both nuclear and cytoplasmic SLBP are degraded and degradation requires proteasomal activity. Degradation is partially suppressed or delayed by inhibition of cdk activity, and the loss of nuclear SLBP is preferentially suppressed. Degradation is also partially suppressed when embryonic transcription is inhibited; however, no additional suppression occurs when both cdk activity and transcription are inhibited. SLBP is partially degraded between 44 and 52 h post-hCG in parthenogenetic embryos that have not yet reached mid-/late G2, and this degradation is not suppressed by cdk inhibition. Degradation does not occur, however, in embryos from which the chromosomes have been removed. These results identify the pathways that mediate the degradation of maternally encoded SLBP, a prerequisite for the somatic pattern of SLBP expression to be established.

The characteristics of SLBP degradation in 2-cell embryos resemble those observed in somatic cells in several respects. In both cases, degradation occurs during G2 by a proteasome-dependent pathway (Whitfield et al., 2000; Zheng et al., 2003). As well, efficient degradation requires activity of a cdk. In somatic cells has been identified as CDC2A-cyclin A (Koseoglu et al., 2008; Zheng et al., 2003). While we do not yet know whether CDC2A-cyclin A controls SLBP degradation in embryos, expression of a dominant-negative form of CDK2, which is the other principal cdk that is active during G2, in 2-cell embryos did not inhibit SLBP degradation (W.Z. and H.J.C., unpublished). Certain differences between the two systems also may be identified. Degradation of SLBP in embryos is relatively slow. Its quantity fell by about 75% over a period of 8 h in the 2-cell embryos, whereas its half-life in proliferating cells in culture is ~2 h during S-phase and estimated to be up to 10-fold less during G2 (Whitfield et al., 2000). As well, our results suggest that degradation occurs during mid-/late G2 in embryos, whereas it occurs in early G2 in somatic cells (Whitfield et al., 2000). Notwithstanding these differences, our results

suggest that the mechanism that degrades SLBP during G2 in somatic cells becomes active during G2 in 2-cell embryos, leading to extensive degradation of maternally encoded SLBP.

SLBP does not detectably decline in 1-cell embryos, however, and it accumulates in growing oocytes, which are at G2 (Arnold et al., 2008). Although we cannot exclude that some SLBP is degraded during G2 of 1-cell embryos, which is much shorter than G2 of 2-cell embryos, this suggests that the mechanism of SLBP degradation in 2-cell embryos is not active in oocytes or 1-cell embryos. This cannot be attributed to the absence of CDC2A or cyclin A, as these are present in growing oocytes and 1-cell embryos (Choi et al., 1991; Fuchimoto et al., 2001; Persson et al., 2005). Recent work has revealed that the degradation of SLBP during G2 in somatic cells requires the SCF ubiquitin ligase (Yen and Elledge, 2008). However, the F-box protein that recognizes SLBP has not yet been identified. In view of the observation that embryonic transcription is required for efficient SLBP degradation, it may be speculated that this unidentified F-box protein is not present in oocytes and that it is synthesized upon full activation of the embryonic genome at the mid-2-cell stage, thus enabling phosphorylated SLBP to be efficiently degraded.

Although cdk activity is required for efficient degradation of SLBP, we consistently observed that a portion of SLBP was degraded in the presence of its inhibitor, roscovitine. While we cannot exclude that this is due to incomplete inhibition of cdk activity by the drug, several observations argue against this interpretation. First, treatment with roscovitine completely inhibited the second cleavage division, which requires cdk activity. Second, a similar partial suppression of SLBP degradation was observed when embryos were treated with 6-DMAP or with α-amanitin. Third, no additional suppression of degradation occurred when embryos were treated with both roscovitine and α-amanitin, as might have been expected if each were partially effective. Taken together, these considerations raise the possibility that SLBP in embryos can also be degraded by a cdk-independent pathway. Interestingly, it was recently reported that SLBP in somatic cells is degraded during G1 by a mechanism that requires neither SCF activity nor SLBP phosphorylation (Yen and Elledge, 2008), consistent with the notion of a cdk-independent pathway.

The existence of a cdk-independent pathway of SLBP degradation may account for the degradation of the cytoplasmic SLBP in 2-cell embryos. In somatic cells, SLBP is transported from the nucleus to cytoplasm when bound to histone mRNA and returns to the nucleus when dissociated from the mRNA (Erkmann et al., 2005). At G2, almost all SLBP has returned to the nucleus where it is accessible to CDC2A-cyclin A. In embryos, however, a significant fraction of SLBP remains in the cytoplasm at G2. As cyclin A is nuclear in 2-cell embryos, it seems that this SLBP would not be accessible to CDC2A-cyclin A. Thus, it may be questioned how it becomes degraded. It may be speculated that the cytoplasmic SLBP in 2-cell embryos is degraded by a cdk-independent pathway, whereas the nuclear SLBP is degraded by the cdk-dependent pathway. Consistent with this, roscovitine suppressed the loss of nuclear, but not cytoplasmic, SLBP in treated embryos. A limitation of this interpretation is that we do not know to what extent SLBP can move between the nucleus and cytoplasm in embryos. Although there appears to be little nuclear-cytoplasmic shuttling of free SLBP in somatic cells (Erkmann et al., 2005), we do not know whether the same rules govern its behavior in embryonic cells. Nonetheless, this model offers a potential

rationale for the existence of cdk-dependent and -independent pathways of SLBP degradation and for understanding how SLBP that is not co-localized with cyclin A becomes degraded.

The notion that a cdk-independent pathway of SLBP degradation may exist is further supported by the results observed using parthenogenetic embryos. As these embryos were activated at 18 h post-hCG, they were developmentally retarded by ~6 h as compared to fertilized embryos, which were activated ~12 h post-hCG. Therefore, it may be inferred that between 44 and 52 h post-hCG the parthenotes were not progressing through the period of G2 when SLBP degradation normally occurs. Nonetheless, SLBP became partially degraded during this time, although to a lesser extent than in fertilized embryos. This implies that a pathway of degradation that is not strictly cell cycle-regulated became active in these embryos. Moreover, this partial degradation was not inhibited by roscovitine. Thus, it may be that a cdk-independent pathway of SLBP degradation becomes active at 44–52 h post-hCG independently of cell cycle progression to mid-/late G2.

As injection of hCG triggers meiotic maturation of pre-ovulatory oocytes, it may be speculated that activation of the cdk-independent pathway of SLBP degradation is linked to the time elapsed since the initiation of maturation. Events that seem to be regulated by a clock rather than cell-cycle or developmental progression have previously been identified in embryos of various species (Johnson and Day, 2000). For example, timed cortical rearrangements and cyclic changes in the activity of a potassium channel occur in cytoplasts generated by enucleation of 1-cell mouse embryos (Day et al., 1998; Waksmundzka et al., 1984). SLBP degradation, however, does not occur between 44 and 52 h post-hCG in cytoplasts that lack the chromosomes. This suggests that activation of the cdk-independent pathway of SLBP degradation, although potentially regulated by a clock that is set during maturation, requires progression through certain cell-cycle or developmental stages.

In summary, our results suggest that maternally encoded SLBP is degraded in 2-cell embryos by two pathways: a cdk- and transcription-dependent pathway that becomes active during G2, targets nuclear SLBP, and corresponds to the pathway that degrades SLBP at G2 in somatic cells; and a cdk-independent pathway that becomes active at 44–52 h post-hCG independently of cell-cycle progression to mid-/late G2 and targets cytoplasmic SLBP. The implication of two pathways in the degradation of maternally encoded SLBP in the mouse bears a striking similarity to observations made using *D. melanogaster*, where maternal mRNAs are degraded in early embryos by two pathways, of which one is maternally encoded and the other requires embryonic transcription (Tadros and Lipshitz, 2005). Rapid degradation requires the activity of both pathways, but slow degradation nonetheless occurs when only one pathway is active. It may be a common feature of early embryonic development that maternally and embryonically encoded pathways work co-ordinately to eliminate maternal mRNAs and proteins, thus enabling the oocyte-to-embryo transition to be efficiently completed.

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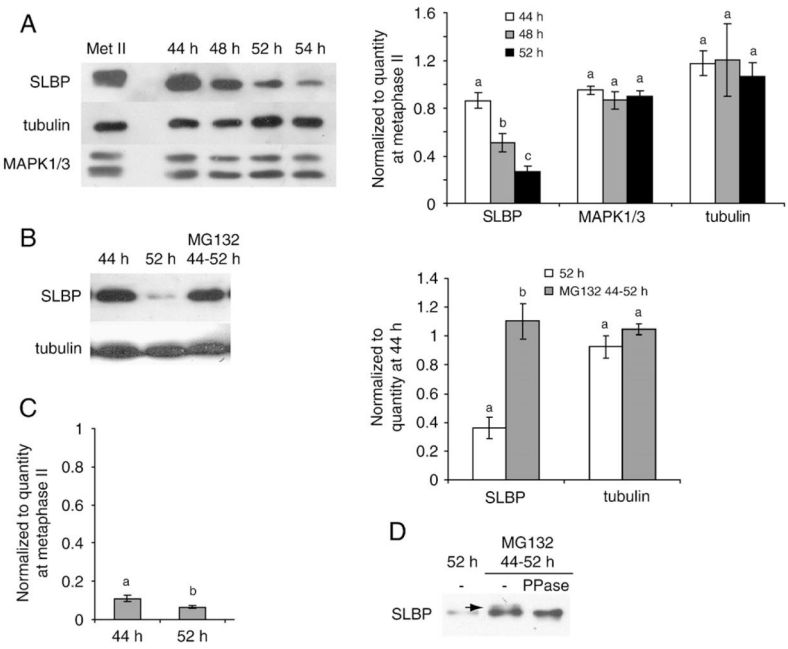

Fig. 1.

SLBP is degraded at the late 2-cell stage. (A) Metaphase II eggs and 2-cell embryos were collected at the indicated times post-hCG injection and immunoblotted using antibodies that recognize SLBP, α-tubulin, and MAPK1/3. Fluorescent signals were quantified using a phosphorimager and normalized to metaphase II eggs on the same blot. For each experiment, all treatment groups contained the same number (25–40) of embryos. Left side shows a representative immunoblot. Right side shows combined results derived from 4–7 independent experiments for each data point, except for 2 values only for tubulin at 48 h. For each protein, different superscripts indicate statistical significance (ANOVA, Tukey, $p<0.05$). (B) 2-cell embryos were collected at 44 h and at 52 h following incubation in the presence or absence of MG132. Graph shows combined results from 5 independent experiments. For each protein, different subscripts indicate statistical significance (t-test, $p<0.05$). (C) Embryos were collected at the indicated times and analyzed using real-time RT-PCR. Results are normalized to quantity of SLBP present in metaphase II eggs. Results of four independent experiments are shown. Different subscripts indicate statistical significance (t-test, $p<0.05$). (D) Some MG132-treated embryos were treated with phosphatase before immunoblotting. Arrow shows the slow-migrating species that disappeared following phosphatase treatment.

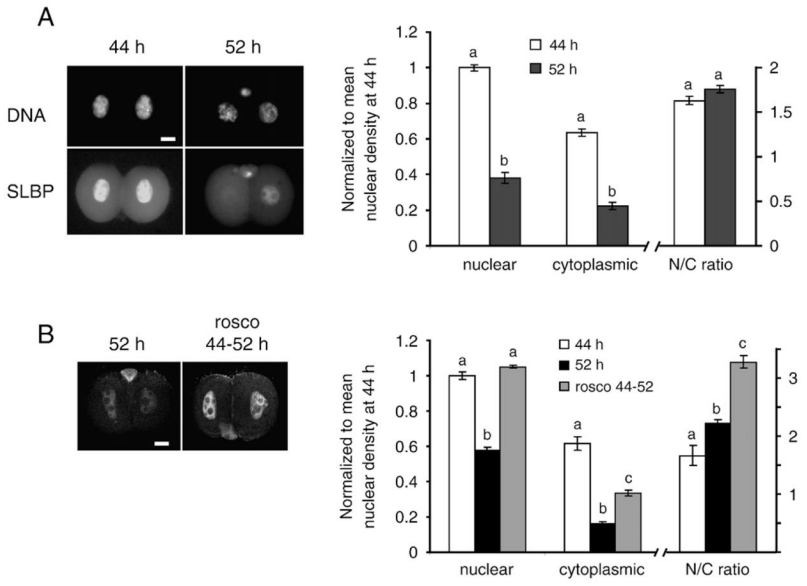

Fig. 2.

SLBP is lost from both nuclear and cytoplasmic compartments. (A) Two-cell embryos were collected at the indicated times post-hCG, stained using anti-SLBP and a DNA-binding dye and examined using confocal microscopy. The fluorescent signal was quantified using NIH Image J. Left side shows a representative image. The left-hand blastomere in the 52-h embryo has entered metaphase. Right side shows the density of nuclear and cytoplasmic staining, normalized to nuclear density at 44 h, and the nuclear:cytoplasmic ratio (right-most bars). 38 cells analyzed at 44 h, 50 at 52 h. Different subscripts within each pair of bars indicate statistical significance (*t*-test, $p<0.05$). (B) As in (A) except that some embryos were treated with roscovitine from 44–52 h. 58 cells analyzed at 44 h, 72 at 52 h, and 76 following roscovitine treatment. Different subscripts within each group of bars indicate statistical significance (ANOVA, Tukey, $p<0.05$). Scale bar=20 μm.

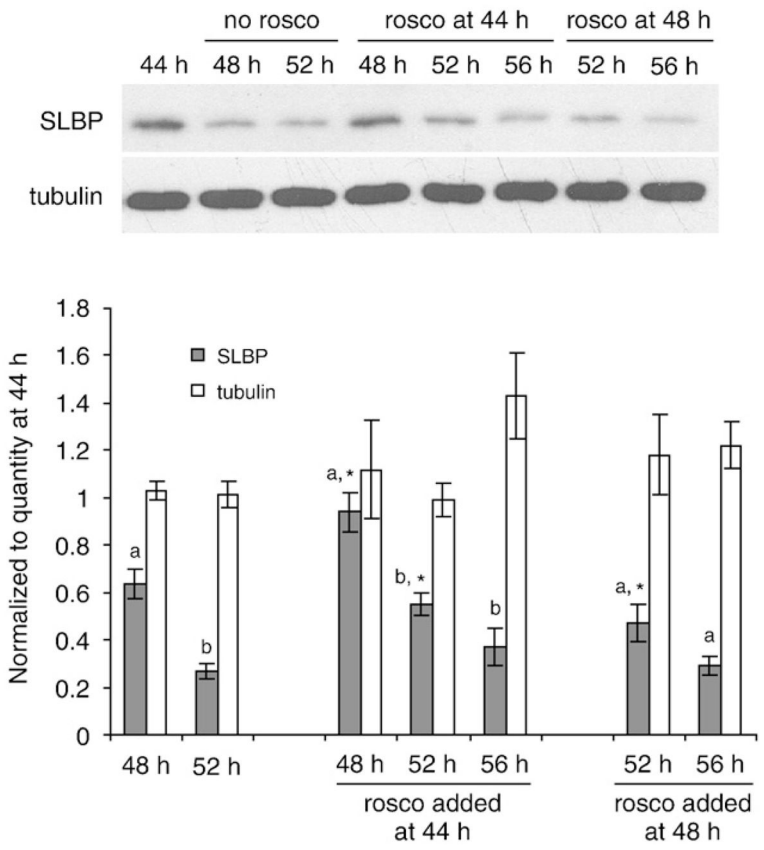

Fig. 3.

Degradation of SLBP is partially suppressed by an inhibitor of cdk activity. Two-cell embryos were incubated in the presence of DMSO or roscovitine added at 44 h or 48 h post-hCG, collected at the indicated times, and immunoblotted using antibodies that recognize SLBP and α-tubulin. Representative immunoblot (upper) and combined results (lower) of five independent experiments. In each experiment, values were normalized to the quantity present at 44 h. All groups contained significantly less SLBP than the 44-h embryos, except for the roscovitine-treated group at 48 h (*t*-test, $p<0.05$). Different superscripted letters indicate significant differences within a group, and an asterisk indicates a significant difference as compared to DMSO-treated embryos at the same time-point (ANOVA with Tukey for group with 3 time-points, *t*-test for groups with 2 time-points, $p<0.05$). No significant changes in the quantity of tubulin were detected.

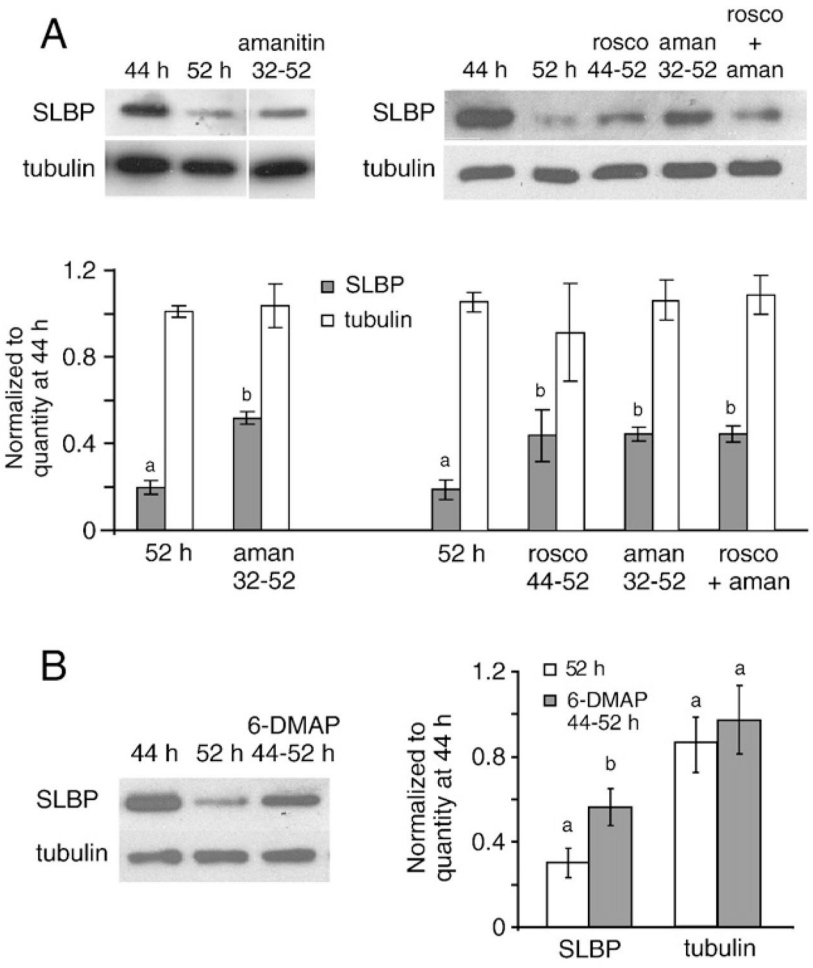

Fig. 4.

Degradation of SLBP is partially suppressed by inhibitors of transcription and protein phosphorylation. (A) Two-cell embryos were incubated in the presence of vehicle or of α-amanitin added at 32 h post-hCG (left blot and group of bars) or of α-amanitin added at 32 h, roscovitine added at 44 h, or both (right blot and group of bars). They were collected at the indicated times and immunoblotted using antibodies that recognize SLBP and α-tubulin. Upper panel shows a representative immunoblot. Lower panel shows combined results of six (left) or three (right) independent experiments. In each experiment, values were normalized to the quantity present at 44 h. All groups contained significantly less SLBP than the 44-h embryos. Different superscripted letters indicate significant differences within a group (ANOVA, Tukey, $p<0.05$). No significant changes in the quantity of tubulin were detected. (B) As in (A) except that embryos were exposed to 6-DMAP from 44–52 h. Representative immunoblot (left) and combined results (right) of three independent experiments. For SLBP, but not tubulin, the 6-DMAP-treated group differed significantly from the 44-h and 52-h groups (*t*-test, $p<0.05$).

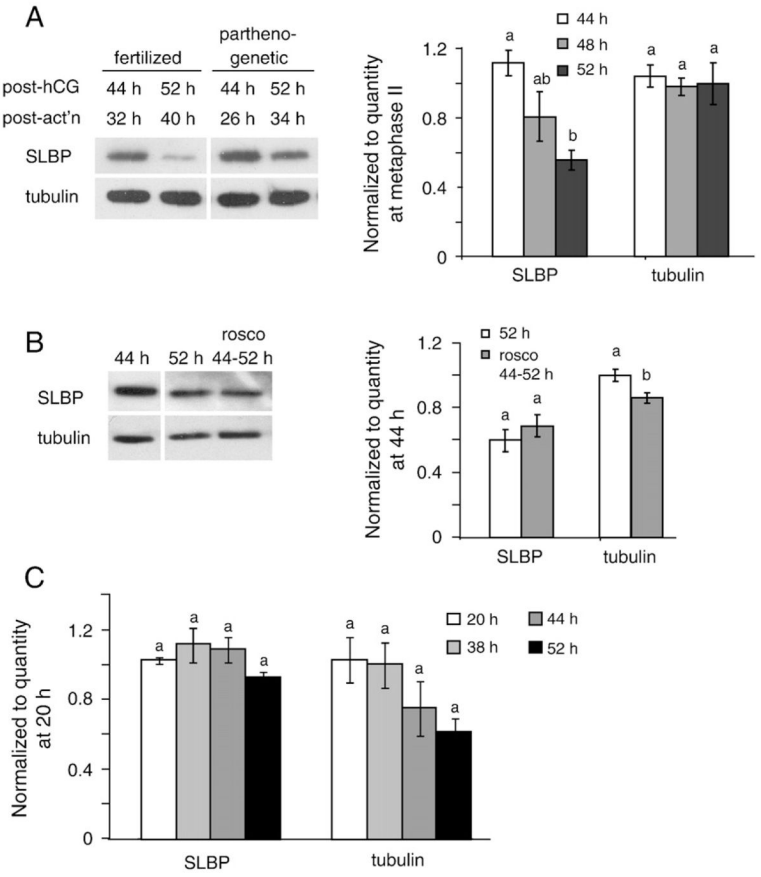

Fig. 5.

A pathway that becomes active independently of progression to mid-/late G2 triggers degradation of SLBP. (A) Unfertilized eggs were parthenogenetically activated at 18 h post-hCG, then collected at the indicated times and immunoblotted using antibodies that recognize SLBP and α-tubulin. Representative immunoblot, including fertilized embryos collected during the same experiment (left) and combined results of five independent experiments (right). In the immunoblot, the time of collection is indicated both in h post-hCG and in h post-activation, assuming that fertilization occurred 12 h post-hCG. (B) As in (A) except that a group of embryos was treated with roscovitine from 44–52 h. Five independent experiments. (C) Unfertilized eggs were parthenogenetically activated at 16.5–17 h post-hCG and the chromosomes were removed at between 19 and 23 h from those that had emitted the second polar body. Samples were collected at the indicated times post-hCG. Three independent experiments. Different superscripts indicate statistical significance (ANOVA with Tukey or *t*-test, $p<0.05$).