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Hormonal Induction of Adipogenesis Induces Skp2 Expression Through PI3K and MAPK Pathways

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Abstract We have previously shown that the F-box protein, S-phase kinase-associated protein (Skp2) plays a mechanistic role in targeting the cell-cycle inhibitor, p27 for degradation by the 26S proteasome during early stages of 3T3-L1 adipocyte differentiation. Here, we demonstrate that protein levels of Skp2 and its accessory protein, Cks1 increased as density-arrested preadipocytes re-entered the cell cycle during clonal expansion, decreased with differentiation-induced growth arrest, and became refractory to hormonal stimulation following the onset of terminal adipocyte differentiation. Component analysis revealed that while maximal Skp2/Cks1 protein accumulation required the complete differentiation cocktail, that insulin was principally involved. Skp2 mRNA accumulation was found to precede the increase in Skp2 protein and succeed the activation of Akt and Erk1/2, mediators of phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signal transduction pathways, respectively. Using specific inhibitors, we found that while activation of both pathways was required for maximal expression, PI3K signaling was primarily responsible for the increase in Skp2/Cks1 accumulation. The increase in Skp2 mRNA was notable 4 h following hormonal stimulation, plateaued by 12 h during mid-G_1 phase progression, and occurred without change to mRNA stability. We further demonstrate that luciferase activity, originating from a pGL3 vector containing 2.4 kb of the Skp2 promoter, increased 2.5-fold with hormonal stimulation. This increase in promoter activity was markedly suppressed following PI3K and MAPK blockade. Deletion studies indicate that responsive elements were located within the proximal Skp2 promoter. These data demonstrate that Skp2 is transcriptionally regulated by PI3K and MAPK pathways as 3T3-L1 preadipocytes transition from quiescence to proliferation during adipocyte hyperplasia. J. Cell. Biochem. 100: 204–216, 2007.

Key words: adipocyte; obesity; cell cycle; proliferation

Obesity is a devastating condition due to its widespread occurrence in all populations regardless of race, age, gender, or socioeconomic status, and its negative effects on life expectancy and quality-of-life due to co-morbidities such as heart disease, stroke, diabetes, and cancer. Early studies demonstrated that adipocyte hyperplasia is largely responsible for childhood [Hager et al., 1978], morbid [Pettersson et al., 1985], and diet-induced obesity [Mandenoff et al., 1982]. A majority of the research-to-date has been focused on adipocyte hypertrophy leaving many unanswered questions regarding adipocyte hyperplasia. There are two phases of hyperplastic obesity; the proliferation of preadipocytes and subsequent differentiation to form mature, lipid-filled adipocytes. While much work has explored the process of adipocyte differentiation, little is known about the mechanisms regulating preadipocyte replication.

The F-box protein, S-phase kinase-associated protein (Skp2) has been shown to promote cell-cycle progression in neoplastic cells, in large part, by targeting the cyclin-dependent kinase inhibitor (CKI) p27(Kip1) for degradation [Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999]. Skp2 functions as a variable receptor component of the SCF^{Skp2} (Skp1/Cul1/F-Box Protein; superscript denotes the F-box protein) E3 ligase that targets specific proteins for polyubiquitylation and subsequent degradation by the 26S proteasome. Skp2 binds

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to the core of the E3 Ligase complex via its F-box motif, a region composed of approximately 40 amino acids common among all F-box proteins. Its peptide structure is further characterized by a region containing leucine-rich repeats that bind Cks1 and p27, bringing p27 in close proximity to the ubiquitin-conjugating enzyme of the SCF complex [Hao et al., 2005].

Morrison and Farmer [1999] first identified a decrease in p27 protein accumulation during early adipocyte differentiation while characterizing the switch between proliferation and differentiation. Recently, Naaz et al. [2004] found that p27 knockout mice had 80% larger fat pads than wild-type controls due to an increase in adipocyte number, not size. Adipose tissue mass of double knockout mice expressing neither p27 nor a related CKI family member, p21(Cip1) increased by more than 500%, disproportionately over generalized organomegly and also due to cell proliferation. In others studies, we first demonstrated that Skp2 protein dramatically accumulates during early phases of adipocyte differentiation and that this increase plays a major role in regulating p27 levels through targeted degradation[1]. While much research has been devoted to the regulation of p27, less is known regarding mechanisms of Skp2 regulation.

Several signal transduction pathways are known to play an important role in initiating cell-cycle progression. Phosphatidylinositol-3 kinases (PI3Ks) exert their effects on proliferation through targets such as E2F [Brennan et al., 2002], NFκB [Shah et al., 2001], Forkhead transcription factors [Brunet et al., 1999; Kops et al., 2002], and mTOR [Gao et al., 2003]. These effects can occur through PI3K directly or indirectly by its downstream targets, PDK1 and Akt/PKB. Activation of Akt also augments this process as it can lead to increased expression of the insulin-like growth factor 1 (IGF-1) receptor [Tanno et al., 2001]. This is of particular interest as the IGF-1 receptor is a predominant cell-surface growth factor receptor expressed during early adipocyte differentiation [Smith et al., 1988]. A second pivotal pathway is regulated by mitogen-activated protein kinases (MAPKs). The activation of this pathway leads to increased Cyclin D1 expression, Rb hyperphosphorylation, and formation of Cyclin E/A–Cdk2 complexes [Lavoie et al., 1996]. The IGF-1 is known to activate both pathways and is required for differentiation of 3T3-L1 preadipocytes [Smith et al., 1988].

Considering the pivotal role p27 plays in adipose tissue development, we sought to elucidate mechanisms regulating Skp2 protein accumulation during differentiation of murine 3T3-L1 preadipocytes as a model of adipocyte hyperplasia. When grown to a state of density arrest, these quiescent preadipocytes synchronously re-enter the cell cycle for one to two rounds of cell proliferation following exposure to a hormonal cocktail of methylisobutylxanthine, dexamethasone, and insulin (MDI). This period of cell-cycle progression, referred to as 'mitotic clonal expansion,' is a prerequisite for differentiation of this cell line and precedes the irreversible growth arrest that characterizes terminal differentiation [Morrison and Farmer, 1999; Tang et al., 2003a,b]. In this report, we demonstrate that Skp2 protein levels increase, at least in part, by transcriptional mechanisms. Skp2 expression was coupled to cell-cycle progression, refractory to the hormonal cocktail following the onset of terminal differentiation, and synergistically dependent on PI3K and MAPK pathways. Furthermore, data are presented demonstrating that effectors of these pathways mediate Skp2 expression through its proximal promoter. These data represent the first study of Skp2 regulation during adipogenesis and provide a link between this cell-cycle protein, early signaling events, and adipocyte hyperplasia.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Cellgro by Mediatech. Bovine Serum, Fetal Bovine Serum (FBS), and Trypsin-EDTA were from Invitrogen Corporation. Propidium Iodide (PI) and RNase A was purchased from Sigma. Chemical inhibitors were purchased from the following: LY294002 and U1026 (Promega); Actinomycin D (Sigma); Rapamycin (Calbiochem). Antibodies used for immunoblotting were as follows: Skp2 (Zymed); Cks1, Skp1, Cyclin A, Cul1, and peroxisome proliferator-activated receptor (PPAR)γ (Santa Cruz Biotechnology). Enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences.

[1]C.A. Auld and R.F. Morrison, submitted manuscript.

Cell Culture and Differentiation

Murine 3T3-L1 preadipocytes were propagated in growth medium containing DMEM supplemented with 10% calf bovine serum as described previously [Morrison and Farmer, 1999]. By standard differentiation protocol, preadipocytes were propagated in growth medium until reaching a state of density arrest at 2 days post-confluence. Growth medium was replaced at density arrest with differentiation medium comprised of DMEM supplemented with 10% FBS, 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). The term "post-MDI" refers to the time elapsed since the addition of MDI to the culture medium. At 2 days post-MDI, medium was changed to DMEM supplemented with 10% FBS and 0.4 μM insulin. From 4 days post-MDI until harvest, maintenance medium containing DMEM supplemented only with 10% FBS was changed every 48 h. Throughout the study, "time 0" refers to density-arrested cells immediately before chemical induction of differentiation with the addition of MDI to the culture medium. All experiments were repeated three to five times to validate results and ensure reliability.

Flow Cytometry

Cell-cycle progression was assessed by flow cytometry. Briefly, cell monolayers were washed with phosphate-buffered saline (PBS), trypsinized, and detached cells diluted in ice-cold PBS. Cells were gently pelleted by centrifugation (300*g*, 5 min, 4°C), PBS was decanted and cells were fixed and permeabilized by drop-wise addition of 70% ethanol at −20°C while vortexing. Fixed cells were washed with PBS and incubated in the dark for 30 min with PI staining solution containing 50 μg/ml PI and 100 μg/ml RNase A in PBS. DNA fluorescence was measured with a FACS Calibur Flow Cytometer (Becton Dickinson) equipped with a 488-nm argon laser. Width (FL2W) and area (FL2A) of PI fluorescence was recorded for at least 10,000 counts. DNA histograms were extracted from FL2W-FL2A dot plots. The percentage of cells in each phase of the cell cycle was analyzed using ModFit software (Verity).

Immunoblotting

Cell monolayers were washed with PBS and scraped into ice-cold lysis buffer containing 1.0 M Tris, pH 7.4, 150 mM NaCl, 1% Triton X, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and 10 mM N-ethylmaleimide (NEM). Lysis buffer was freshly supplemented with protease (0.3 μM aprotonin, 21 μM leupeptin, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride) and phosphatase (20 mM β-glycerophosphate, 10 mM NaF, and 2 μM sodium vanadate) inhibitors. Cell lysates were clarified by centrifugation (13,000*g*, 10 min, 4°C) and protein concentration was determined by BCA assay (Pierce). Lysates were resuspended in loading buffer containing 0.25 M Tris, pH 6.8, 4% SDS, 10% glycerol, 10% dithiotreitol, 0.01% bromophenol blue, heated for 5 min at 80°C, and placed on ice. Lysates were resolved on SDS–PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). Following transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4°C and horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoblots were developed with ECL and visualized by autoradiography CL-XPosure film (Pierce).

RNA Isolation and Analysis

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to manufacturer's instructions. Total RNA (1 μg) was subjected to Reverse-Transcriptase Polymerase Chain Reaction using One-Step RT-PCR kit (QIAGEN) according to manufacturer's instruction. Briefly, RT-PCR reactions were carried out in the presence of the supplied buffer, dNTPs (400 μM), enzyme mix (reverse transcriptases and DNA polymerase), and RNase inhibitor (10 U). Gene-specific primers were used at a final concentration of 0.6 μM and designed as follows: Skp2 forward, 5′-GGCAAAGGGAGTGACAAA GA-3′; Skp2 reverse, 5′-TCAAAGCACCAGGAGAGATT-3′ (product size = 590 bp); Cks1 forward, 5′-TGTCTGAATCTGAATGGAGG-3′; Cks1 reverse, 5′-TGCTTCTGCCAAATGACTAA-3′ (product size = 381 bp). QuantamRNA Classic and Classic II 18S primer/competimers (Ambion) were used in the reactions as internal standards at a final concentration of 0.6 μM.

Plasmids

Promega's pGL3-basic vector was used to create mouse Skp2-pGL3 constructs for luciferase

assays. Primers flanking the region of −2,473 to +74 (2,478 bp) were used to amplify the murine Skp2 promoter from 3T3-L1 preadipocyte genomic DNA. Positive DNA fragments were gel purified. Vector and isolated genomic DNA were double digested with KpnI and BglII. The resulting DNA fragment was subcloned into the pGL3-basic vector. Nucleotide sequencing was conducted by SeqWright DNA Technology Services. This process from genomic DNA to sequenced vector was repeated to ensure reliability of results. Progressive 5′ truncations of the 2.4 kb promoter were constructed using existing endonuclease restriction sites as follows: 2.2 kb (−2,248 to +74) using KpnI and BbvCI; 1.5kb (−1,573 to +74) using KpnI and EcoRI; 1.0 kb (−1,073 to +74) using KpnI and NsiI; 0.4 kb (−0.454 to +74) using KpnI and MluI. 5′ and 3′ overhangs were flushed using T4 DNA Polymerase (Promega), resulting DNA fragments gel-purified, and blunt-end ligations were carried out using T4 DNA ligase (Promega). All vectors were transformed into JM109 competent cells (Promega) and screened for positive ligations using ampicillin-containing LB agar plates. DNA was isolated using the endotoxin-free Wizard Pure-Fection Plasmid DNA Purification System (Promega). Restriction digests were performed to confirm positive clones.

Luciferase Reporter Assays

3T3-L1 preadipocytes (2×10^6 cells) were transiently transfected with 5 μg of firefly reporter plasmid and 20 ng *Renilla*-SV40 plasmid using Amaxa's Nucleofector (program T20), then incubated with pre-warmed proliferation media for no more than 10 min at 37°C. Cells were subsequently replated at a confluent density, fresh media replaced 24 h post-transfection, and further cultured as indicated. Firefly and *Renilla* luciferase were measured using the Dual-Glo Luciferase Assay System (Promega) and reported as relative firefly luciferase counts (firefly/*Renilla*).

RESULTS

Skp2 Protein Accumulation During Adipocyte Differentiation

We previously demonstrated that targeted p27 degradation is partially dependent on Skp2 protein levels that dramatically increase during S and G_2 phase progression of early 3T3-L1 preadipocyte differentiation[1]. To analyze Skp2 expression throughout the course of differentiation, 3T3-L1 preadipocytes were stimulated with MDI and protein abundance was assessed over time by immunoblotting. As shown in Figure 1, Skp2 protein levels were minimal during density-induced growth arrest (d0), increased transiently with Cyclin A expression during clonal expansion at 1 day post-MDI, and returned to low basal levels as preadipocytes exited the cell cycle prior to the onset adipocyte gene expression marked by the onset of PPARγ accumulation. Protein accumulation of Skp1 and Cul1, other members of the SCF^{Skp2} E3 ligase complex, did not vary throughout the course of differentiation. Interestingly, the protein accumulation profile of Cks1 followed that of Skp2 precisely. This is important as Cks1 plays an obligatory role in Skp2-mediated p27

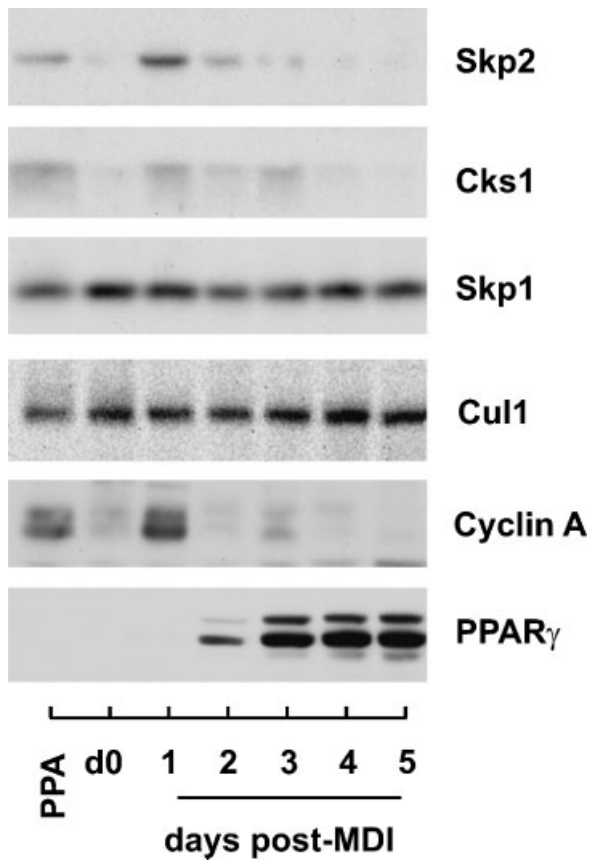

Fig. 1. Expression of SCF-Skp2 E3 Ligase proteins during adipocyte differentiation. Whole cell lysates were harvested from density-arrested 3T3-L1 preadipocytes over time following hormonal induction of differentiation and immunoblotted for SCF complex proteins as well as Cyclin A and PPARγ as indicated. Lysates from untreated proliferating preadipocytes (PPA) at 70% confluency were included for reference of cell-cycle progression.

degradation [Ganoth et al., 2001; Hao et al., 2005]. The synchrony of cell-cycle progression during clonal expansion was demonstrated by elevated Skp2 protein abundance at 1 day post-MDI compared to asynchronously proliferating preadipocytes (PPA) at 70% confluency. Collectively, these data demonstrate that while the SCF complex involving Cul1 and Skp1 may function in protein degradation in the mature adipocyte through utilization of other F-box proteins, the role of Skp2 and Cks1 is confined to preadipocyte replication during early stages of adipocyte differentiation.

Uncoupling Skp2 Regulation Following the Onset of Terminal Adipocyte Differentiation

We next determined if Skp2 accumulation becomes refractory to hormonal stimulation following the onset of differentiation. As shown in Figure 2A, Skp2 and Cks1 protein abundance increased following 22 h of hormonal stimulation as illustrated above. In contrast, however, neither protein accumulated over time following re-stimulation with the same cocktail once the cells had fully differentiated (9 days post-MDI). Accumulation of Cyclin A (Fig. 2A) and S/G_2 phase progression (Fig. 2B), shown at 22 h post-MDI stimulation of density-arrested preadipocytes (d0), were not observed following re-stimulation of mature adipocytes (d9). These data demonstrate that Skp2/Cks1 protein accumulation is coupled to preadipocyte proliferation and not resulting from a non-specific chemical effect of the differentiation cocktail. Furthermore, the refractory nature of Skp2 and Cks1 to growth stimuli following the onset of adipocyte differentiation may contribute to the irreversible state of terminal growth arrest.

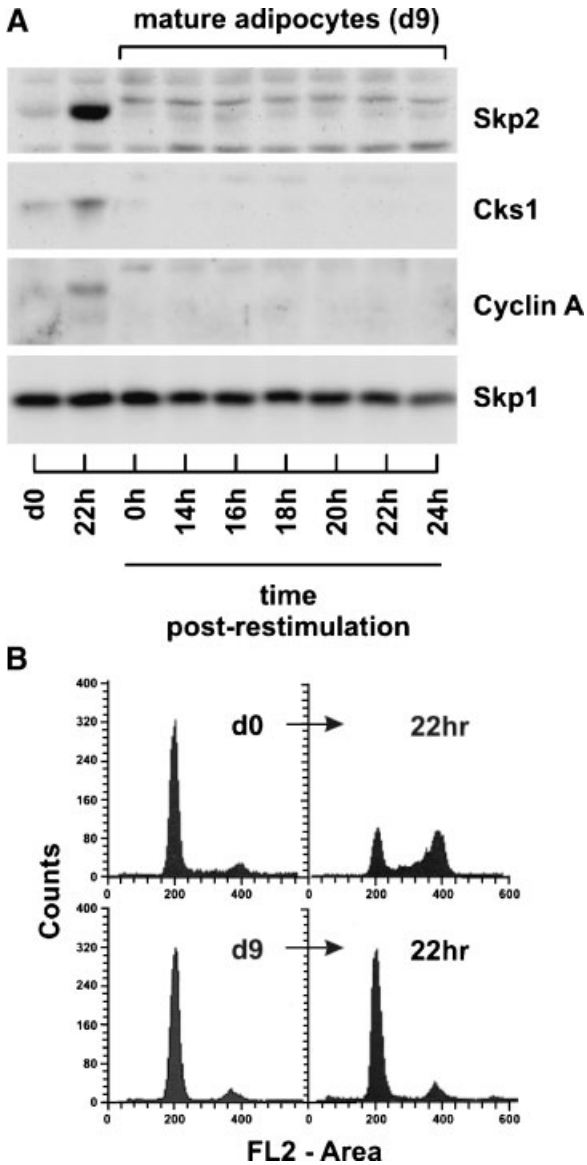

Fig. 2. Skp2/Cks1 protein accumulation confined to clonal expansion of early adipocyte differentiation. **A**: Density-arrested preadipocytes were stimulated with MDI and lysates collected at 22 h during clonal expansion or cultured by standard differentiation protocol for the development of mature adipocytes. At 9 days of differentiation, lysates were harvested over time following re-stimulation with MDI and immunoblotted as indicated. **B**: Cell-cycle progression was assessed with flow cytometric analysis of DNA staining with propidium iodide.

Regulation of Skp2 mRNA Precedes Changes in Protein Accumulation

To determine whether changes in Skp2 protein abundance potentially involved transcriptional mechanisms, RNA and protein were harvested from density-arrested preadipocytes over time following MDI stimulation and relative accumulation assessed by RT-PCR and immunoblotting, respectively. As illustrated in Figure 3, Skp2 protein increased modestly by 12 h post-MDI and abundantly by 20 h, previously determined to coincide with mid-G_1 and S/G_2 phase progression, respectively. These data also show that the increase in Skp2 protein was preceded by a dramatic increase in Skp2 mRNA maximally accumulating by 12 h post-MDI. Accumulation of both Skp2 protein and mRNA decreased as preadipocytes entered terminal stages of differentiation. These data clearly demonstrate that Skp2 is regulated, at

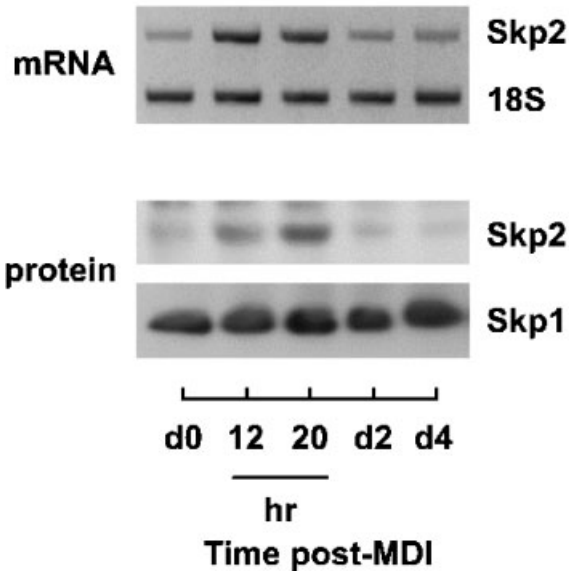

Fig. 3. Accumulation of Skp2 mRNA precedes elevation of Skp2 protein. Whole cell lysates and total RNA were harvested from density-arrested preadipocytes at times indicated and analyzed for relative protein and mRNA accumulation through immunoblotting and RT-PCR, respectively. Abundance of 18S was concomitantly determined for each sample as a reference for loading and consistency.

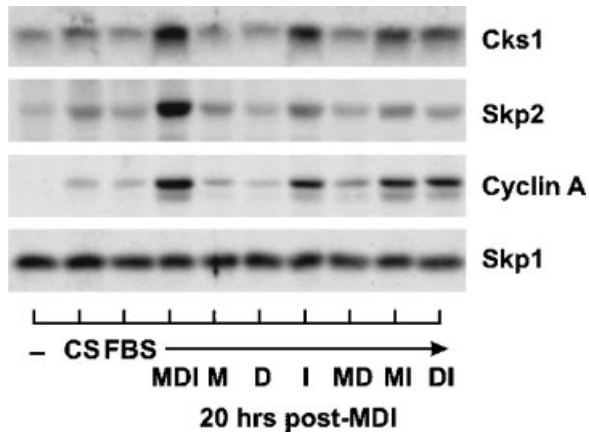

Fig. 4. MDI component analysis for Skp2/Cks1 protein accumulation. Density-arrested preadipocytes were stimulated with MDI, individual components of the cocktail, or a combination of components. In addition, preadipocytes received no medium change (–) or changed to DMEM supplemented with fresh 10% calf serum (CS), or fetal bovine serum (FBS). In all conditions, whole cell lysates were harvested at 20 h post-stimulation and immunoblotted as indicated.

least in part, through transcriptional and/or mRNA stability mechanisms.

MDI Component Analysis and Skp2/Cks1 Protein Accumulation

To identify the pathways leading to Skp2 expression, the effects of individual components of the differentiation cocktail were evaluated. As illustrated in Figure 4, all components of MDI were needed for maximal accumulation of Skp2/Cks1 at 20 h post-stimulation relative to unstimulated, density-arrested cells. These data also revealed that any combination of inducers that included insulin, at a concentration sufficient for IGF-1 receptor activation, resulted in marked accumulation of both Skp2 and Cks1. Moreover, combinations including insulin also resulted in marked accumulation of Cyclin A which is expressed during S/G_2 phases of clonal expansion. No increase in Skp2 or Cks1 was observed following exchange of media containing 10% bovine serum (calf serum; CS) or 10% FBS without MDI supplementation. Moreover, the observation that Skp2 and Cks1 accumulated in parallel with Cyclin A lends further support to the notion that these variable components of the SCF E3 ligase are regulated by pathways ultimately leading to preadipocyte replication.

Role for PI3K and MAPK Pathways in Skp2/Cks1 Regulation

Insulin is known to play a role in proliferation by activating different signaling cascades. Two such pathways are the PI3K and MAPK pathways which have been shown to activate downstream targets (e.g., c-myc, E2F, Cyclin D1) important for cell-cycle progression. These kinase-driven pathways can work independently and/or synergistically through crosstalk between cascades. To determine whether either pathway plays a role in regulating Skp2 expression during preadipocyte proliferation, cells were treated with LY294002 and/or U0126, specific inhibitors of PI3K and MAPK pathways, respectively, 30 min prior and during hormonal induction of differentiation. As shown in Figure 5A, inhibition of either pathway prevented maximal mRNA accumulation of Skp2 and Cks1. Moreover, pharmacological inhibition of both pathways synergistically and completely suppressed mRNA accumulation at 20 h post-MDI to basal, unstimulated levels. Of the two pathways, blocking PI3K had the greatest effect. We determined that Skp2 and Cks1 protein accumulation was similarly affected in a pattern consistent with changes in mRNA levels. To determine whether blocking these pathways also had an effect on

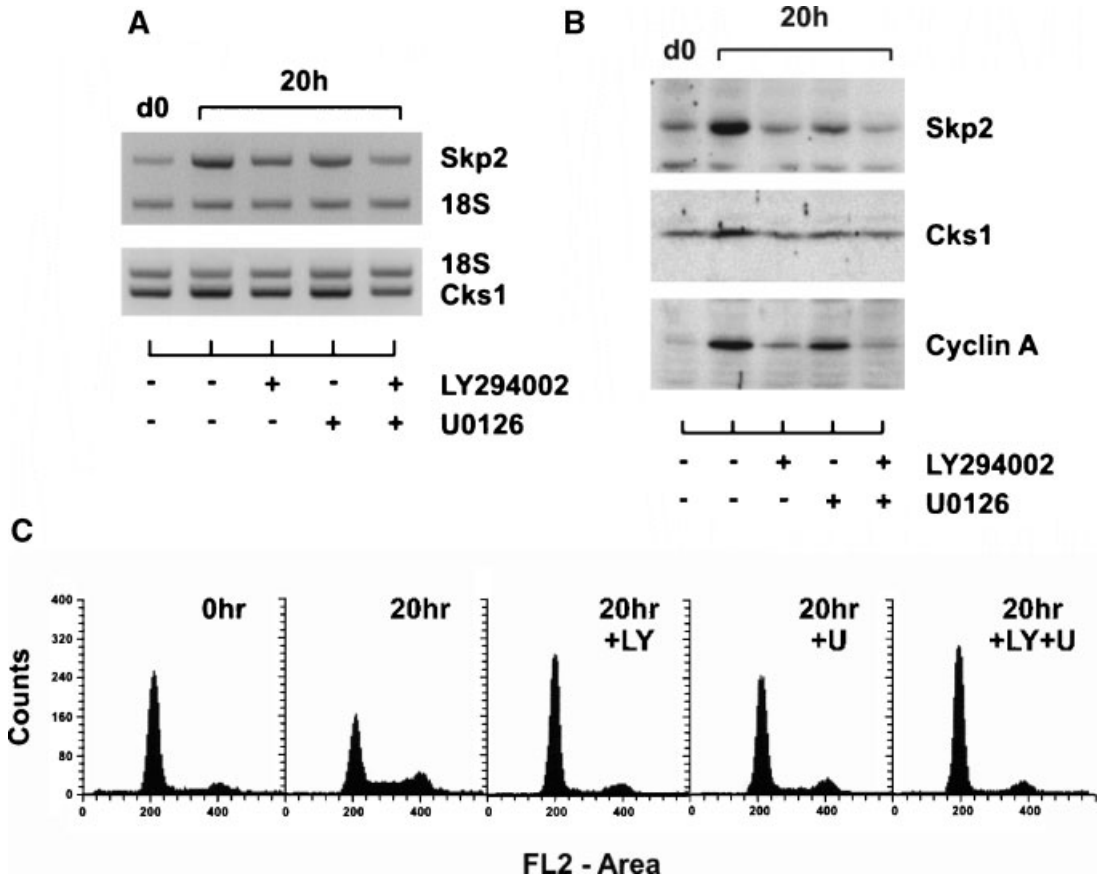

Fig. 5. PI3K and MAPK pathways mediate Skp2/Cks1 expression and cell-cycle progression. Preadipocytes were stimulated with MDI supplement with 10 μM LY294002, 10 μM U0126, specific inhibitors of PI3K and MAPK signaling pathways, respectively. Relative mRNA (**A**) and protein (**B**) accumulation was assessed through RT-PCR and immunoblotting, respectively. Cell-cycle progression was assessed with flow cytometric analysis of DNA staining with propidium iodide (**C**).

preadipocyte proliferation, we examined Cyclin A levels (Fig. 5B) and cell-cycle phase distribution with flow cytometric analysis of PI DNA staining (Fig. 5C). Consistent with data described above, both inhibitors synergistically inhibited S phase progression resulting in G_1 arrest with PI3K blockade producing the greatest effect when considering either inhibitor alone. Collectively, these data demonstrate that PI3K and, to a lesser extent, MAPK signaling is required for the increase in Skp2 mRNA and protein levels during clonal expansion. These data also provide further support to the link between preadipocyte replication and Skp2/Cks1 accumulation.

Kinetic Analysis of PI3K and MAPK Pathway Activity

We next determined the kinetics of pathway activation by examining the phosphorylation state of critical kinases of each pathway in reference to the timing of Skp2 accumulation. Cell lysates were harvested over time following MDI stimulation and immunoblotted with phospho-specific protein antibodies as illustrated in Figure 6. We observed an early, transient activation of the MEK/ERK activation of the MAPK pathway with maximal accumulation of phosphorylated protein within 15 min post-MDI stimulation. We also show that the primary downstream target of the PI3K pathway, Akt, is transiently phosphorylated at residues Ser473 and Thr308, beginning at 30 min post-stimulation and continuing through at least 8 h with maximal phosphorylation occurring between 1 and 3 h post-MDI. Phosphorylation of the indicated residues is a strong indicator of pathway activation. Collectively, these data demonstrate that both pathways were activated during clonal expansion

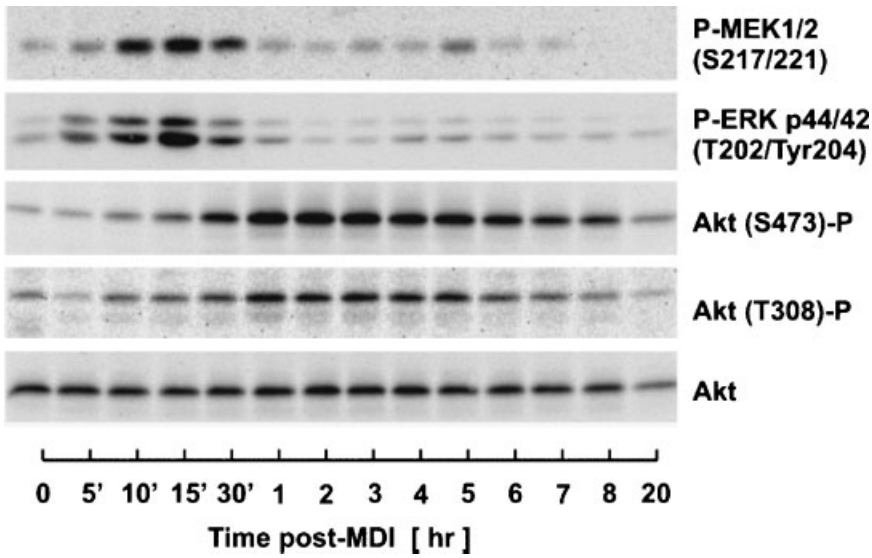

Fig. 6. Kinetic analysis of PI3K and MAPK pathway phosphorylation and activation. Whole cell lysates from density-arrested preadipocytes were harvested over time following MDI stimulation and immunoblotted with phosphospecific antibodies as indicated.

and that the kinetics of activation was consistent with a role of these pathways in coupling hormonal stimulation with Skp2/Cks1 accumulation.

Skp2 mRNA Accumulation Independent of mRNA Stability

We have shown elevated Skp2 mRNA levels in preadipocytes at 12 h post-MDI (Fig. 3). We next determined the kinetics of Skp2 mRNA accumulation with respect to PI3K and MAPK activation as discussed above. As illustrated in Figure 7A, Skp2 mRNA levels progressively increased over time following MDI stimulation with measurable differences noted as early as 4 h post-MDI. Maximal mRNA accumulation plateaued by 12 h corresponding to mid-G_1 phase progression and returned to near basal levels by 32 h post-MDI as the cells synchronously completed the cell cycle to re-enter G_0/G_1. These data demonstrate that first evidence of increased Skp2 mRNA accumulation (4 h) succeeds Akt phosphorylation which peaks at 1 h post-MDI as shown above (Fig. 6). Furthermore, the periodic increase in Skp2 mRNA suggests that mRNA fluctuations are linked to cell-cycle phase transitions resulting from the induction cocktail. These data support the premise that Skp2 is regulated as a function of cell-cycle progression set in motion by these signaling pathways.

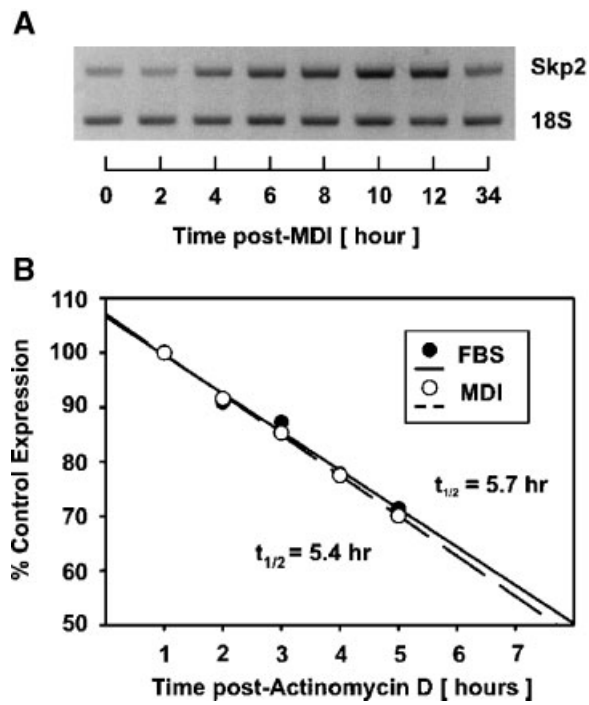

Fig. 7. Skp2 mRNA accumulation is not dependent on changes in mRNA stability. **A**: Total RNA was harvested over time following MDI stimulation for kinetic assessment of Skp2 mRNA accumulation by RT-PCR. **B**: Density-arrested preadipocytes were stimulated with MDI. After 12 h, cell medium was supplemented with Actinomycin D (5 μg/ml). Total RNA was subsequently harvested over time and assessed for Skp2 mRNA stability by RT-PCR.

We have demonstrated the importance of the PI3K and MAPK pathways in the increase in Skp2 mRNA and protein. To determine how these signaling cascades regulate Skp2 in preadipocytes, we next examined mRNA stability and promoter activity. To ascertain potential changes in Skp2 mRNA half-life, density-arrested preadipocytes were stimulated with and without MDI for 12 h. At that point, actinomycin D was added to both groups and mRNA levels determined over time following inhibition of RNA synthesis as a measure of RNA turnover. As illustrated in Figure 7B, no difference in Skp2 mRNA half-life between density-arrested cells (5.7 h) and those progressing through G_1 phase (5.4 h) was observed, ruling out regulation at the level of mRNA stability.

Adipogenic Stimuli Regulates Skp2 Transcription

To determine if Skp2 was regulated at the level of transcription, we amplified and sequenced a 2.4 kb fragment of the 5′ flanking region of Skp2 genomic DNA. This promoter region was subcloned into a pGL3-basic luciferase reporter vector (Promega) and transferred into a single pool of preadipocytes with electroporation (Amaxa). Cells were plated at saturation density and stimulated 2 days post-confluence with MDI supplemented with and without LY294002 and U0126. As illustrated in Figure 8, we observed a 2.5-fold increase in Skp2 promoter activity at 12 h post-MDI relative to unstimulated, density-arrested preadipocytes (d0). This increase in promoter activity was inhibited by 35% and 20% with LY294002 and U0126, respectively. These inhibitors in combination resulted in an additive effect reducing Skp2 promoter activity by approximately 50%. These changes in promoter activity are consistent with Skp2 mRNA and protein accumulation suggesting that Skp2 was regulated, at least in part, through transcriptional mechanisms involving effectors of the PI3K and MAPK pathways.

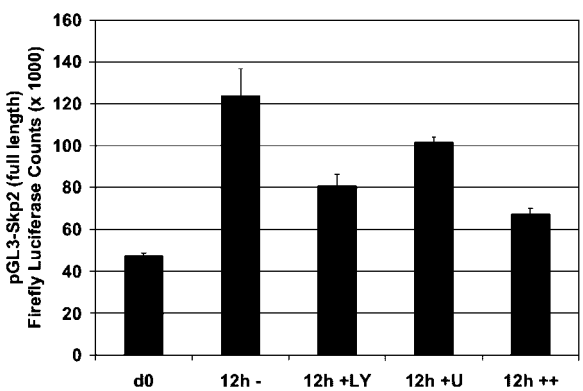

Fig. 8. Induced Skp2 promoter activity following hormonal induction of adipogenesis. Subconfluent 3T3-L1 preadipocytes were transfected with 5 μg pGL3 firefly luciferase vector subcloned with ~2.4 kb of the Skp2 promoter. Cells were grown to 2 days post-confluence and stimulated with MDI, MDI + LY294002 (10 μM), MDI + U0126 (10 μM), or MDI + both inhibitors. Luciferase activity was measured 12 h post-stimulation.

Regulatory Regions of the Skp2 Promoter

To identify regulatory *cis* elements mediating Skp2 transcription, we constructed serial deletions (Fig. 9A) of the 2.4 kb promoter vector using available restriction sites. Each vector was introduced into preadipocytes as described above along with a *Renilla* luciferase vector to control for transfection efficiency. As shown in Figure 9B, progressive deletions of the 5′ flanking region from −2,473 to −1,074 had no effect on basal promoter activity in unstimulated, density-arrested cells (d0) or in the approximate twofold increase in activity observed at 12 h post-MDI. While deleting nucleotides −1,073 to −455 modestly decreased promoter activity, the magnitude of difference between basal and MDI-induced activity was similar to larger promoter fragments suggesting the presence of regulatory elements mediating the effects of hormonal stimulation at points downstream of −454 of the Skp2 promoter. We also observed that luciferase activity from all constructs, stimulated with MDI in the presence of PI3K and MAPK inhibitors, was consistent with Skp2 mRNA accumulation with LY294002 resulting in a greater suppression of induced luciferase activity than U0126. When normalizing MDI-treated cells with and without each inhibitor to the values obtained from unstimulated cells, we observed no difference between constructs (Fig. 9C). Taken together, these results demonstrate the presence of a PI3K and MAPK responsive *cis* element(s) within the region of −454 to +74 base pairs of the Skp2 proximal promoter.

DISCUSSION

Preadipocytes, known to exist throughout life, undergo transition from an extended state of quiescence to proliferation under conditions

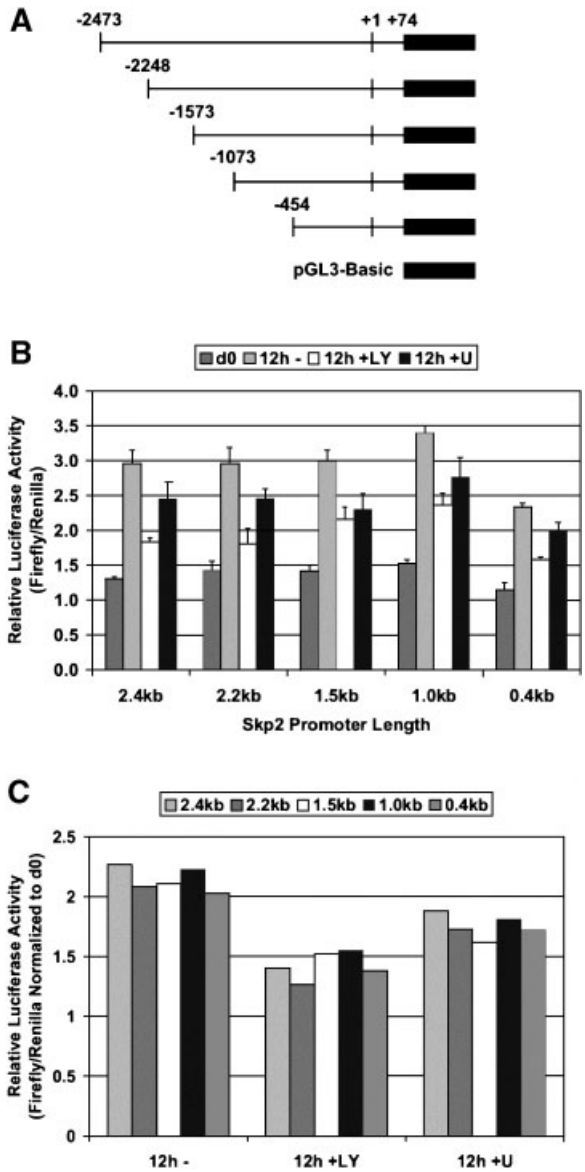

Fig. 9. PI3K and MAPK responsive elements in the proximal Skp2 promoter. **A**: Schematic depiction of pGL3-Skp2 promoter deletion constructs. **B**: Subconfluent preadipocytes were cotransfected with 5 μg of pGL3-Skp2 promoter deletion constructs as indicated and 20 ng *Renilla*-SV40 luciferase vector as a control for transfection efficiency. Cells were grown to 2 days post-confluence and stimulated with MDI, MDI + LY294002 (10 μM), or MDI + U0126 (10 μM). Normalized firefly/*Renilla* luciferase activity was measured 12 h post-stimulation. **C**: Luciferase activity from each vector treated with MDI and/or inhibitors in **panel B** normalized to basal, unstimulated (d0) activity.

of positive energy balance, leading to hyperplastic obesity [Prins and O'Rahilly, 1997; Hausman et al., 2001]. This transition is largely governed near the G_1/S phase border by the CKIs, p27, and p21. The importance of CKI regulation in adipose tissue development was underscored by recent studies demonstrating the marked expansion of adipose tissue mass resulting from adipocyte hyperplasia in mice defective in p27/p21 gene expression [Naaz et al., 2004]. Using 3T3-L1 preadipocyte differentiation as a model of adipocyte hyperplasia, we have previously shown that p27 protein levels decrease during S phase progression, in part, by targeted proteolysis involving Skp2-mediated p27 ubiquitylation and degradation by the 26S proteasome[1]. This process is triggered by phosphorylation of p27 by Cdk2 as well as by the transient accumulation of Skp2 and Cks1 proteins near the G_1/S phase transition. Mechanisms coupling signaling pathways originating from hormonal stimulation to Skp2 accumulation and, therefore, p27 degradation during preadipocyte replication are completely unknown.

Recent evidence has demonstrated Skp2 expression to be upregulated in many cancer cells and to exhibit periodic regulation during normal cell-cycle progression through transcriptional [Imaki et al., 2003; Sarmento et al., 2005; Zhang and Wang, 2005] as well as protein degradation mechanisms [Wirbelauer et al., 2000; Zhang et al., 2003; Bashir et al., 2004; Wei et al., 2004] in a cell type specific manner. In neoplastic cells, Skp2 is upregulated by PI3K [Mamillapalli et al., 2001; Andreu et al., 2005] and negatively regulated by TGFβ [Wang et al., 2004]. Evidence for modulation of Skp2 expression by Notch1 has also been reported [Sarmento et al., 2005]. The data presented here demonstrate that Skp2 is expressed transiently during 3T3-L1 preadipocyte replication, largely through transcriptional mechanisms involving *cis* regulatory elements within the first ~454 base pairs of the proximal Skp2 promoter that are responsive to effectors of PI3K and MAPK signaling pathways. Rapid elevation of Skp2 mRNA levels following hormonal induction of adipocyte differentiation suggests that transcriptional events mediating early-G_1 phase progression are involved.

To date, only two reports have identified *trans*-acting factors that are essential for Skp2 transcription, with some controversy. The first of these studies expressed luciferase vectors subcloned with the mouse Skp2 promoter, containing nucleotides −2,275 to +94 relative to the transcriptional start site, in proliferating human HeLa cells [Imaki et al., 2003]. Serial

deletions between −208 and −103 resulted in an 80% decrease in luciferase activity with greater deletions to +6 (i.e., +6 to +94) having no measurable activity. Critical analysis of this region demonstrated that GA-binding proteins (GABPs) interact with the core binding motif, CACTTCCG, located at −175/−153. While most of these studies were performed with asynchronously proliferating neoplastic HeLa cells, these authors demonstrated that serum-deprived NIH-3T3 fibroblasts expressing a GABP sequence mutation produced less luciferase activity over the course of cell-cycle progression resulting from serum stimulation.

In our study, we examined inducible activity of ~2.5 kb (−2,473 to +74) of the murine Skp2 promoter in density-arrested murine 3T3-L1 preadipocytes synchronously re-entering the cell cycle following hormonal induction of differentiation. Our data demonstrate a ~2.5-fold increase in Skp2 promoter activity during mid-G_1 (12 h post-MDI) when Skp2 mRNA is maximally expressed, nearly identical to that observed with wild-type NIH-3T3 fibroblasts of the GABP report discussed above [Imaki et al., 2003]. Progressive truncations of the Skp2 promoter to −454 had no effect on activity in the absence or presence of PI3K or MAPK inhibitors suggesting that effectors of these pathways impinge upon regulatory elements within this region of proximal Skp2 promoter. While a role for PI3K activity has yet to be determined, GABP-dependent promoter activity as well as GABP phosphorylation has been shown to increase with activation of the MAPK signaling pathway [Flory et al., 1996]. Although GABP has been shown to play a role in mitochondrial thermogenesis in brown adipose tissue [Villena et al., 1998], its role in white adipose tissue proliferation and differentiation has yet to be determined. However, based on the position of the binding motif, GABP represents a likely candidate integrating hormonal signaling events with Skp2 transcription during preadipocyte proliferation.

In direct contrast to the above report, others have demonstrated that serial deletions of the 5′ flanking region (−3,723 to +212) of the human Skp2 promoter down to −59, which included the GABP binding site, had little effect on reporter activity in asynchronously proliferating HeLa cells [Zhang and Wang, 2005]. Surprisingly, however, 3′ deletions resulted in a dramatic fall in activity leading to the identification of a required element between +65 and +149. Critical analysis of this region demonstrated that E2F proteins interact with the core binding motif, TTGCGCGCG, located at +95/+103 of the human Skp2 promoter. Functional studies revealed that this region in the 5′ untranslated region of exon 1 was critical for E2F-1, not E2F-4, directed periodic promoter activity during replication of normal cells and the high level of Skp2 promoter activity in neoplastic cells. Serum stimulation of serum-deprived NIH-3T3 fibroblasts expressing a reporter construct containing a core E2F sequence demonstrated increased promoter activity during cell-cycle progression in parallel with changes in Skp2 mRNA levels. It was postulated that Skp2 transcription increases when 'activation' E2Fs (e.g., E2F1-3), released following phosphorylation of retinoblastoma proteins during G_1/S phase transition, replace 'repressive' E2Fs (e.g., E2F4,5) that function to maintain quiescence during growth arrest. The reason why these deletion studies of the human Skp2 promoter did not identify a required GABP element [Zhang and Wang, 2005] or why the truncated murine Skp2 promoter containing the E2F regulatory element was devoid of reporter activity [Imaki et al., 2003] is unclear.

In our studies, Skp2 mRNA and promoter activity increased during G_1 phase progression of replicating preadipocytes with measurable differences in mRNA accumulation occurring as early as 4 h and maximal levels by 12 h following hormonal stimulation. Nearly identical kinetics were observed in the E2F study discussed above with synchronous cell-cycle progression of NIH-3T3 fibroblasts expressing an E2F binding motif reporter construct [Zhang and Wang, 2005]. In our report, the effect of PI3K and MAPK blockade on promoter activity was consistent with changes in Skp2 mRNA accumulation with effectors of these pathways impinging upon Skp2 promoter activity through regulatory elements located between −454 and +74 base pairs. These data are consistent with a regulatory role for the E2F element, which is located at +40/+48 of the murine promoter and within the fragment used in the studies presented here.

In addition to the putative role of E2Fs in regulating Skp2 expression during preadipocyte replication, this family of transcription factors has recently been shown to function in the transcriptional cascade mediating terminal adipocyte differentiation by regulating the

expression of PPARγ, a principal transcription factor essential for adipocyte gene expression. Recent evidence suggests that E2F4 binds to the PPARγ promoter and suppresses PPARγ expression in density-arrested preadipocytes. Upon hormonal stimulation, E2F1 replaces the repressive E2F4 leading to upregulation of PPARγ during the first day of differentiation [Fajas et al., 2002]. Support for this premise followed with the observation that the loss of E2F4 allows cells to undergo spontaneous differentiation, further illustrating the divergent properties of E2F proteins during adipogenesis [Landsberg et al., 2003]. As clonal expansion is essential for differentiation, it would not be unexpected to find that E2Fs function similarly in regulating the expression of Skp2. As Skp2 has been shown to couple phosphorylated p27 to the SCF E3 ligase, the link between hormonally induced signaling cascades, E2F-1 activation, Skp2 accumulation, p27 degradation, and obligatory cell-cycle progression becomes more evident.

Although clonal expansion is obligatory for 3T3-L1 differentiation, exit from the cell cycle is also essential for adipogenesis as proliferation and differentiation are generally considered mutually exclusive processes. In this report, we further demonstrate that Skp2 expression becomes refractory to hormonal stimulation following the onset of differentiation even though PI3K signaling remains intact (i.e., Glut4 trafficking). While mechanisms uncoupling Skp2 expression from proliferative signaling pathways during terminal adipocyte differentiation are unknown, others have demonstrated that adipocyte differentiation is dependent on E2F repression by C/EBPα, also a principal transcription factor mediating adipocyte gene expression. While a role in Skp2 regulation has yet to be determined, it is plausible that C/EBPα conveys terminal growth arrest, in part, through suppression of E2F activity, Skp2 expression, p27 degradation, and resulting G_1 phase growth arrest. Further studies are needed and underway to determine if either E2F or GABP or both are essential for coupling of PI3K and MAPK signaling pathways to periodic Skp2 expression as preadipocyte undergo the transition from quiescence to proliferation during adipocyte hyperplasia.

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