Mitotic Clonal Expansion during Preadipocyte Differentiation: Calpain-mediated Turnover of p27*

Yashomati M. Patel§ and M. Daniel Lane

From the Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received for publication, December 31, 1999, and in revised form, March 17, 2000
Published, JBC Papers in Press, April 3, 2000, DOI 10.1074/jbc.M910445199

Evidence is presented that calpain, a calcium-activated protease, degrades the cyclin-dependent kinase inhibitor, p27, during the mitotic clonal expansion phase of 3T3-L1 preadipocyte differentiation. Calpain activity is required during an early stage of the adipocyte differentiation program. Thus, inhibition of calpain with N-acetyl-Leu-Leu-norleucinal (ALLN) blocks clonal expansion and acquisition of the adipocyte phenotype only when added between 12 and 24 h after the induction of differentiation. Likewise, inhibition of calpain by overexpression of calpastatin, the specific endogenous inhibitor of calpain, prevents 2-day post-confluent preadipocytes from reentering the cell cycle triggered by the differentiation inducers. Inhibition of calpain with ALLN causes preadipocytes to arrest just prior to S phase and prevents phosphorylation of the retinoblastoma gene product, DNA replication, clonal expansion, and subsequent adipocyte differentiation but does not affect the expression of immediate early genes (i.e. fos, jun, C/EBPβ, and C/EBPδ). Inhibition of calpain by either ALLN or by overexpression of calpastatin blocks the degradation of p27. p27 is degraded in vitro by cell-free extracts from clonally expanding preadipocytes that contain “active” calpain but not by extracts from pre-mitotic preadipocytes that do not. This action is inhibited by calpastatin or ALLN. Likewise, p27 in preadipocyte extracts is a substrate for purified calpain; this proteolytic action was inhibited by heat inactivation, EGTA, or ALLN. Thus, extracellular signals from the differentiation inducers appear to activate calpain, which degrades p27 allowing density-dependent inhibited preadipocytes to reenter the cell cycle and undergo mitotic clonal expansion.

The adipocyte differentiation program involves several distinct phases (1–3). As preadipocytes reach confluence, they enter a temporary quiescent state arresting at the G0/G1 cell cycle boundary (4). Growth arrest at confluence appears to be a prerequisite for subsequent differentiation. This cell cycle arrest, however, is overcome by mitotic and adipogenic inducers. Confluent 3T3-L1 preadipocytes are unique in that serum alone is unable to induce reentry of contact-inhibited preadipocytes into the cell cycle. Specific adipogenic agents are necessary to induce reentry of density-dependent, growth-arrested preadipocytes into the cell cycle. Upon addition of the differentiation inducers (MDI), i.e a combination of isobutylmethylxanthine (M, a cAMP phosphodiesterase inhibitor), dexamethasone (D), a high level of insulin (I, which acts through the insulin-like growth factor-1 receptor), and fetal bovine serum (FBS) (1, 5), the cells reenter the cell cycle and undergo several rounds of mitosis (6) referred to as mitotic clonal expansion (1, 2, 5). The initiation of this clonal expansion phase involves the expression of “immediate early” genes, fos, jun, C/EBPβ, and C/EBPδ, to drive confluent 3T3-L1 preadipocytes from G0 into S. These genes are expressed during the first few hours following induction of differentiation. During mitosis, DNA replication is hypothesized to alter the accessibility of promoter/enhancer elements to factors required for the transcription of genes involved in the initiation of differentiation (1). This is followed by the expression of transcription factors, notably C/EBPα and PPARγ, that terminate mitotic clonal expansion and coordinately activate transcription of adipocyte genes (7–11). Mitotic clonal expansion of growth-arrested preadipocytes appears to be necessary for optimal differentiation. Thus exposure of subconfluent proliferating preadipocytes to differentiation inducers results in poor differentiation (5).

Entry into the cell cycle is known to be regulated by cyclin-dependent kinases (CDKs) (12). The CDK complex consists of a catalytic serine/threonine kinase subunit and a regulatory cyclin subunit. Several factors contribute to the activity of the CDK complex (12, 13). 1) Synthesis and degradation of the cyclins are regulated at specific stages of the cell cycle. 2) Specific kinases and phosphatases regulate the phosphorylation status of the serine/threonine kinase subunit. 3) The CDK complex also binds inhibitory factors at specific points in the cell cycle. The cyclin-CDK complex is regulated by two families of cyclin-dependent kinase inhibitors (CDKIs). The CIP1/KIP1 family, i.e. p21, p27, and p57, inhibits CDKs by forming ternary complexes with various cyclin-CDKs, whereas the INK4 family, i.e. p15, p16, p18 and p19, inhibits CDK activity by forming binary complexes with CDKs (12).

Progression of quiescent cells through G1 and into S phase requires the coordinated activation of CDK4/CDK6 and CDK2 (12). The interaction between cyclin D and CDK4/CDK6 is thought to link extracellular signals to the cell cycle, whereas the onset of DNA replication per se is regulated by cyclin E-CDK2 complexes (14). The CIP1/KIP1 family of proteins form

* This work was supported by an NIDDK research grant from the National Institutes of Health (to M. D. L.) and a National Research Service award (to Y. M. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biological Chemistry, The Johns Hopkins University, School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-955-3975; Fax: 410-955-0903.

1 The abbreviations used are: MDI, methylisobutylxanthine, dexamethasone, insulin; ALLN, N-acetyl-Leu-Leu-norleucinal; FBS, 10% fetal bovine serum; CDK, cyclin-dependent kinase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; CS, calf serum; Rb, retinoblastoma; PAGE, polyacrylamide gel electrophoresis; TET, tetracycline; BrdUrd, bromodeoxyuridine; HA, hemagglutinin; PPAR, peroxisome proliferator-activated receptor.
ternary complexes with cyclins and CDKs, including the cyclin D-CDK4/6 complexes. The primary target of cyclin D-CDK4 complexes is the retinoblastoma susceptibility gene product, Rb (15). Activation of cyclin D-CDK4 leads to phosphorylation of the retinoblastoma protein, Rb (15). This initial phosphorylation event is required for further phosphorylation of Rb by cyclin E-CDK2 complexes at the G$_1$/S boundary, which allows cells to enter S phase (16). The active (hypophosphorylated) form of Rb acts by sequestering transcription factors, such as E2F, that regulate genes required for S phase (17, 18). Phosphorylation of Rb causes its inactivation, thereby releasing these transcription factors from inhibition and allowing cell cycle progression. Although the process by which contact-inhibited 3T3-L1 preadipocytes are induced to reenter the cell cycle upon exposure to adipogenic factors is poorly understood, one (or more) of the signaling events described above is likely to be involved.

p27 has been shown to be regulated via its degradation. Previous studies have demonstrated that p27 is a substrate for the ubiquitin-proteasomal degradation pathway (19). In the present study, we provide evidence that during the mitotic clonal expansion phase of adipocyte differentiation calpain is also able to degrade p27. Calpain is known to degrade several different factors involved in the cell cycle, including cyclin D1 (20). Previous studies in our laboratory have shown that inhibition of calpain, either by addition of the calpain inhibitor, ALLN, or by overexpression of the endogenous calpain inhibitor, calpastatin, blocks the differentiation of 3T3-L1 preadipocytes (21), and this inhibition occurs prior to the expression of C/EBPα (21). Evidence presented in the present study demonstrates that calpain catalyzes the degradation of the CDK inhibitor, p27, thereby allowing cells to make the G$_1$/S transition, reenter the cell cycle, and proceed through the mitotic clonal expansion phase of adipocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Materials—**N-Acetyl-Leu-Leu-norleucinal (ALLN) and purified rabbit skeletal muscle calpain were purchased from Sigma and Oil Red O (Coulter Electronics). Cell Culture—3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% calf serum (CS) until confluent (day –2) and then were maintained in the same medium for an additional 2 days (until day 0). Differentiation was induced on day 0 by addition of 0.5 mM methylisobutylxanthine, 1 μM dexamethasone, 1 μg/ml insulin, and 10% FBS in DMEM. After 48 h (day 2), the medium was replaced with DMEM containing 1 μg/ml insulin and 10% FBS (5). After day 4 the cells were fed every other day with 10% FBS in DMEM without insulin. Between day 0 and day 2 the cells synchronously reenter the cell cycle, undergo 2–3 rounds of mitotic clonal expansion, and then begin to express genes indicative of adipocyte terminal differentiation. Cytoplasmic triglyceride droplets become abundant between days 4 and 5, and by day 7 the cells are fully differentiated (5). ALLN (26 μM) was added at the time of MDI treatment (day 0) unless otherwise indicated.

**Oil Red O Staining of Cytoplasmic Triglyceride—**Day 7 3T3-L1 cell monolayers were washed twice with PBS and fixed with 3.7% formaldehyde for 2 min. A 0.2% Oil Red O-isopropyl alcohol solution was added to the cells for 1 h after which the monolayers were washed several times with distilled water, and stained cytoplasmic triglycerides were visualized (22).

**Determination of Cell Number—**Cells at various stages of differentiation were trypsinized, pelleted, and resuspended in a solution containing 10% CS and DMEM. Cell number was determined using a Coulter counter (Coulter Electronics).

**Stable Transfection of the Calpastatin Vector—**A tetracycline-regulated expression system (TET-OFF) (Life Technologies, Inc.) was employed to inducibly express calpastatin (generously provided by Dr. M. Maki, Nagoya University, Nagoya, Japan), in 3T3-L1 preadipocytes, as described previously (21).

**Analysis of RNA—**Total cellular RNA was isolated at various time points during the first 24 h after the induction of differentiation, by the acid-phenol guanidinium isothiocyanate method (23). Ten micrograms of total RNA were separated by electrophoresis, and Northern blot analysis was performed as described previously (21, 24). cDNA fragments of C/EBPβ, C/EBPδ, c-fos, c-jun, c-myc, and 18 S rRNA (full-length clone) were used to probe for the corresponding mRNAs. Probes were labeled to high specific activity by random priming (25).

**[3H]Thymidine Incorporation—**Two-day post-confluent preadipocytes were induced to differentiate with MDI in the presence or absence of 26 μM ALLN. [3H]Thymidine was added to the medium for 30 min at various times following induction of differentiation after which aliquots were assayed for thymidine incorporation as described previously (26).

**In vivo fluorescence—**BrUrd incorporation was performed as described in the protocol provided by Becton Dickinson. Briefly, 2-day post-confluent preadipocytes grown on coverslips were treated with MDI or MDI and 26 μM ALLN for 16 h. The cell monolayers were labeled with 10 μM BrUrd for 30 min, fixed in 70% ethanol for 30 min, air-dried, and then immersed in 0.07 N NaOH for 2 min and neutralized with PBS, pH 8.5. Coverslips were incubated in a solution containing 50 μl of 0.5% Tween 20PBS and 20 μl of anti-BrdUrd (fluorescein isothiocyanate-conjugated) for 30 min, washed with PBS, and dried.

**Analysis of Protein and Immunoblotting—**Cell lysates were prepared from dividing preadipocytes, 2-day post-confluent preadipocytes (day 0), and cells at various time points during the course of differentiation. Each cell monolayer (6 cm) was washed once with 5 ml of phosphate-buffered saline and lysed in 0.5 ml of a solution containing 1% SDS, 60 mM Tris-HCl, pH 8. The lysate was incubated at 100 °C for 10 min and stored at –35 °C. Samples were subjected to 12% acrylamide, SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were stained with Ponceau S to assess total protein loading. Membranes were incubated with various antisera as indicated or HA mouse antiserum to detect HA-tagged human calpastatin followed by a horseradish peroxidase-conjugated secondary antibody (Sigma). Proteins were visualized by enhanced chemiluminescence (ECL) (Amer sham Pharmacia Biotech).

**Degradation of p27 in Vitro—**Two-day post-confluent cells were lysed in a solution containing 40 mM Tris-HCl, pH 7.5, 120 mM NaCl. Lysates (30 μg of protein) were incubated in the presence of 1 unit of purified rabbit skeletal muscle calpain that had or had not been heated to 100 °C for 10 min. Incubations were at 30 °C for 1 h in the presence of 6 mM CaCl$_2$ alone or in the presence of 10 mM EGTA or 26 μM ALLN (20). p27 degradation was assessed by Western blot analysis.

In mixing experiments, confluent (day 0) preadipocytes that were either maintained in CS or induced to differentiate with MDI or ALLN for 24 h after the start of induction were lysed as described above. Lysates (alone or in combination) were incubated at 25 °C for 30 min in the presence of 6 mM CaCl$_2$. To verify the involvement of calpain, cell lysates from preadipocytes, induced to differentiate with MDI, were first incubated for 30 min in the presence or absence of antibody against calpain or calpastatin or in the presence of a calpastatin peptide and then for 30 min with lysates from uninduced preadipocytes (CS). Samples were then analyzed for p27 by Western blot analysis as described previously.

**RESULTS**

**Inhibition of Calpain Activity Blocks the Adipocyte Differentiation Program at an Early Stage—**Induction of differentiation of 3T3-L1 preadipocytes leads to the expression of genes. The coordinate expression of these genes gives rise to the adipocyte phenotype (1–3). Thus, as illustrated in Fig. 1A when preadipocytes are induced to differentiate with MDI, massive amounts of triglyceride accumulate in the cytoplasm. Previous studies (21) showed that by inhibiting calpain, preadipocytes that had been induced to differentiate became growth-arrested. This growth arrest occurred at a stage prior to the expression of the C/EBPα and thus prevented the expression of adipocyte marker genes. To locate the time window in the differentiation program at which calpain acts, confluent, growth-arrested 3T3-L1 preadipocytes were treated with a calpain inhibitor, i.e., ALLN, for a 24-h period at the beginning of day 1, 2, or 4 following induction of differentiation with MDI. After each 24-h treatment, ALLN was “washed out” with a medium change. The extent of differentiation was assessed on day 7 by staining cytoplasmic triglyceride with Oil Red O. As shown in Fig. 1B, differentiation was unaffected by exposure to ALLN beginning
on days 2 or 4; however, differentiation of preadipocytes treated with ALLN on day 1 (concomitant with MDI treatment) was almost completely (>95%) inhibited as evidenced by a lack of Oil Red O staining. It is evident, therefore, that calpain is required at an early stage(s) of the differentiation program. To define the exact time frame of ALLN action, preadipocytes were treated with ALLN for 6–12-h periods within the first 24 h of differentiation. Preadipocytes treated with ALLN from 0 to 6 or 6 to 12 h, following the addition of the differentiation inducers, differentiated normally (Fig. 1B). However, differentiation was markedly inhibited in preadipocytes treated with ALLN between 12 and 24 h. Thus, it appears that the action of calpain is required for only a limited period early (12–24 h) in the differentiation program.

**ALLN Blocks the Mitotic Clonal Expansion Phase of Adipocyte Differentiation**—Since the time window during which differentiation of 3T3-L1 preadipocytes is susceptible to inhibition by ALLN coincides with that of mitotic clonal expansion, it was of interest to determine whether the inhibitor affects clonal expansion. It should be noted that mitotic clonal expansion is a prerequisite for differentiation (2). Clonal expansion occurs during the first 3 days after induction with MDI (6). To address this question, 2-day postconfluent 3T3-L1 preadipocytes were either maintained without differentiation inducers or were treated with MDI or MDI plus ALLN for 48 h. Cell number was then determined on day 7, and the fold increase in cell number was normalized to that of non-induced controls. Preadipocytes treated with MDI exhibit a 4–5-fold increase in cell number relative to non-induced controls (Fig. 1C). In contrast, preadipocytes treated with MDI and ALLN fail to proliferate to a significant extent (Fig. 1C). These findings show that ALLN treatment of preadipocytes blocks the mitotic clonal expansion of the differentiation program.

As ALLN has also been reported to act as a proteosome inhibitor, the effect of a potent irreversible proteosome inhibitor, i.e. lactacystin (27), was investigated. Confluent 3T3-L1 preadipocytes were either induced with MDI or with MDI in the presence of a high concentration (10 μM) of lactacystin for 48 h, after which cell number was assessed. Preadipocytes treated with lactacystin and MDI underwent cellular proliferation (Fig. 1C) and differentiated normally (not shown), albeit at a slightly slower rate than cells treated with MDI alone. Previous studies have shown that a much lower concentration of lactacystin (3 μM) is sufficient to block proteosome action (28). Inhibition of proteosome action by lactacystin has been shown to inhibit progression of the cell cycle (29). To verify that lactacystin, at the concentration used in this study, i.e. 10 μM, is capable of inhibiting proteosome action in 3T3-L1 preadipocytes, we treated dividing preconfluent preadipocytes with lactacystin after which cell number was assessed. Cell cycle progression/proliferation of preconfluent preadipocytes was completely blocked by lactacystin (Fig. 1D). Thus, the proteosomal inhibitor, lactacystin, can inhibit cell proliferation of “preconfluent” 3T3-L1 preadipocytes but does not inhibit cell proliferation (mitotic clonal expansion) of MDI-induced 3T3-L1 preadipocytes. These findings indicate that the MDI-induced mitotic clonal expansion of confluent preadipocytes is distinctly different from cell proliferation of preconfluent preadipocytes. Thus, the inhibitory effect of ALLN on differentiation of 3T3-L1 preadipocytes does not appear to be due to inhibition of proteosome action.

The specificity of calpain action in initiating mitotic clonal expansion was further investigated with 3T3-L1 preadipocytes stably transfected with an inducible expression vector for calpastatin, the specific endogenous inhibitor of calpain (30). This vector contains a TET-promoter/HA-tagged calpastatin transgene that allows conditional expression of calpastatin under the control of the tetracycline promoter. In the presence of tetracycline, calpastatin is not expressed (21), and preadipocytes undergo clonal expansion (Fig. 1C) and differentiation (21). Upon removal of tetracycline from the medium 24 h prior to induction with MDI, calpastatin is expressed (21), and mitotic clonal expansion is almost completely blocked following induction with MDI (Fig. 1C). Therefore, it appears that the inhibition of calpain activity, rather than inhibition of proteo-
some activity, is responsible for the blockade of clonal expansion and differentiation of 3T3-L1 preadipocytes. Nevertheless, it is possible that calpain initiates proteolytic degradation, and proteosomes complete the proteolytic process.

**ALLN Arrests Mitotic Clonal Expansion of 3T3-L1 Preadipocytes Prior to S Phase**—Since ALLN blocks the mitotic clonal expansion phase of the adipocyte differentiation program, it was important to identify the stage of the cell cycle where cells are arrested. To ascertain whether calpain activity is required prior to or during S phase, confluent 3T3-L1 preadipocytes were treated with MDI or MDI and ALLN and then were pulse-labeled with [3H]thymidine at 30-min intervals at various times after induction. It should be noted that induction with MDI induces the synchronous reentry of growth-arrested preadipocytes into the cell cycle. During the first 12 h following induction with MDI, very little [3H]thymidine was incorporated into DNA (Fig. 2A). Initiation of DNA synthesis, as measured by [3H]thymidine incorporation, began about 14 h after induction with MDI (Fig. 2A). In contrast, cells treated with MDI and ALLN failed to incorporate [3H]thymidine. Addition of ALLN prevented the incorporation of [3H]thymidine even after 24 h, indicating that the initiation of DNA synthesis was inhibited, not merely delayed (results not shown).

To verify the timing of DNA synthesis, BrdUrd incorporation was measured 16 h after induction of differentiation. 3T3-L1 preadipocytes were treated with MDI or MDI and ALLN for 16 h and then incubated for 30 min with bromodeoxyuridine (BrdUrd), an S phase-specific marker. The cells were then fixed, and BrdUrd incorporation was assessed by immunofluorescence. As shown in Fig. 2B, preadipocytes induced to differentiate with MDI were heavily stained with fluorescent anti-BrdUrd, indicating entry into S phase by 16 h. In contrast, cells treated with MDI and ALLN for 16 h failed to incorporate BrdUrd into DNA and thus had been arrested prior to S phase, most likely in late G1 or at the G1-S boundary. As a negative control, preadipocytes that had not been induced to differentiate did not incorporate BrdUrd (results not shown). Previous studies (21) have shown that ALLN-induced inhibition of adipocyte differentiation is reversible. Preadipocytes treated with MDI and ALLN for 48 h and then re-exposed to MDI after 7 days underwent mitotic clonal expansion and were then differentiated to the same extent as control cells treated with MDI alone (21). The time when ALLN blocks reentry into the cell cycle, i.e. near the G1-S boundary of MDI-induced preadipocytes, is consistent with the finding that ALLN inhibits differentiation only when added between 12 and 24 h after the induction of differentiation (Fig. 1C).

**ALLN Does Not Block MDI-induced Expression of Genes Expressed within the First 12 h after Induction**—Two-day post-confluent 3T3-L1 preadipocytes become growth-arrested in a distinctive G0 state (4) that is permissive for reentry into the cell cycle upon exposure to the differentiation inducers, i.e. MDI. This induction causes growth-arrested preadipocytes to exit G0 and enter G1 and then progress into S phase. Immediately following induction (within the first 30 min) there is a dramatic increase in the expression of the mRNAs that encode several immediate early genes including c-fos, jun B, and c-myc (Fig. 3). These early events signal the progression from G0 into G1. Inhibition of calpain by ALLN does not, however, affect the induction of gene expression of these immediate early genes, although the pattern of induction was somewhat different (Fig. 3). jun B mRNA is induced within 30 min of MDI induction in both ALLN-treated and untreated preadipocytes, yet the levels of jun B mRNA decline more rapidly in the ALLN-treated cells than the ALLN (−) cells (Fig. 3). In contrast ALLN treatment delayed the onset of expression of c-myc during the 1st...
hour, but the levels were similar to MDI-induced preadipocytes after 2.5 h (Fig. 3). Also, inhibition of calpain did not affect the expression pattern of c-fos (Fig. 3), cyclin D (not shown), or genes (i.e. C/EBPβ and C/EBPδ; Fig. 3) known to be involved in the activation of the terminal adipogenic transcription factors, C/EBPα and PPARγ. As calpain is a protease, the possibility was considered that ALLN might inhibit calpain-catalyzed turnover of the proteins encoded by these mRNAs. Western blot analysis at various times during the first 24 h after induction showed, however, that ALLN had no effect on the expression of C/EBPβ, C/EBPδ, and Fos proteins (results not shown). Thus, although ALLN blocks mitotic clonal expansion and DNA synthesis, it has no effect on factors involved in the G2/G1 transition or the differentiation cascade. Although the pattern of expression of these genes was not altered by ALLN treatment, previous studies have shown that ALLN treatment of MDI-induced preadipocytes did alter the ability of C/EBPδ to bind DNA (21). These findings are consistent with the fact that ALLN exerts its effect 12 h after the induction of differentiation.

**ALLN Prevents the Phosphorylation of Rb**—Since ALLN inhibited DNA replication during mitotic clonal expansion, it was of interest to determine whether this inhibitor prevents preadipocytes from traversing the G1/S boundary. Phosphorylation of the retinoblastoma gene product, Rb, is a recognized indicator of the G1/S transition. Studies by Matsushime et al. (15) demonstrated that Rb is the primary target of phosphorylation by the cyclin D-CDK4 complex. This kinase has been shown to phosphorylate and inactivate Rb (31), thereby allowing cells to pass the restriction point triggering the G1/S phase transition. To determine whether the inhibition of calpain by ALLN treatment affects the phosphorylation state of Rb, confluent preadipocytes were induced to differentiate with MDI in the presence or absence of ALLN. The phosphorylation status of Rb was monitored by the change in its mobility by SDS-PAGE following induction of differentiation. As shown in Fig. 4, Rb is in the unphosphorylated (higher mobility) state at time 0 (prior to induction) and remains unphosphorylated until after 12 h following induction. Phosphorylation of Rb (as indicated by a shift to a lower mobility form) begins between 12 and 16 h and is maximal by 20–24 h. In contrast, Rb remains in the unphosphorylated state, even 24 h after induction in the presence of ALLN (Fig. 4). It should be noted that in other experiments (not shown), it was demonstrated that the slow migrating band in the Rb doublet is converted to the rapidly migrating band by treatment with alkaline phosphatase. Since phosphorylation of Rb was inhibited by ALLN, it appears that the preadipocytes were arrested in G1, and did not enter S phase. This view is consistent with the fact that ALLN blocks differentiation only when added between 12 and 24 h after induction, the period during which preadipocytes progress from G1 to S phase.

**ALLN Prevents Degradation of the CDK Inhibitor, p27**—Since preadipocytes induced to differentiate in the presence of ALLN exit G0, but do not enter S phase, it seemed likely that an event(s) occurring in G1 must be affected by ALLN treatment. The transition from quiescent to proliferating cells is known to be controlled by the interaction of cyclin-dependent kinases (CDKs) with cyclin-dependent kinase inhibitors (12). It has been shown that p27 interacts with the cyclin D1-CDK4/6 complexes in preadipocytes (32). To determine whether expression of p27 is affected by ALLN, the cellular level of p27 was assessed at various times after induction of differentiation. As shown in Fig. 4, B and C, p27 is expressed by quiescent, growth-arrested preadipocytes and begins to decline ~10 h after induction, the decline being complete by 16 h (Fig. 4, B and C). Addition of ALLN at the time of induction prevented the decline of p27 (Fig. 4, B), the initial level being maintained for at least 48 h (results not shown). In contrast, expression of the CDK inhibitor p21, which has been implicated in the withdrawal of cells from the cell cycle signaling the end of clonal expansion (33), was not affected by ALLN treatment (results not shown). The fact that inhibition of calpain action by ALLN prevents the degradation of p27 suggested that p27 is involved in the reentry of quiescent preadipocytes into the cell cycle, signaling the start of clonal expansion.

Phosphorylation of Rb occurred only after the level of p27 had decreased to ~25% that of quiescent preadipocytes prior to DNA replication, as assessed by [3H]thymidine incorporation (see Fig. 4C). It should be recognized that whereas the preadipocytes reenter the cell cycle synchronously, synchrony is not complete. Thus, not all cells enter S phase at precisely the same time. In preadipocytes induced to differentiate in the presence of ALLN, p27 levels did not decline, and Rb remained hypophosphorylated, and DNA replication failed to occur. These findings suggest that calpain may trigger the degradation of p27, thereby releasing CDK from inhibitory constraint leading to the phosphorylation of Rb.

**Degradation of p27 by Calpain In Vitro**—Since ALLN blocks the degradation of p27, it was important to verify that p27 is, in fact, a substrate for calpain. To determine whether p27 is degraded by calpain in vitro, cell lysates from 2-day post-confluent 3T3-L1 preadipocytes that contain p27 were incubated with purified calpain. At this point in the differentiation program, the cellular level of p27 is high (Fig. 4, B and C; day 0); the level of p27 falls later in the differentiation program as preadipocytes progress from the quiescent into the proliferative.
Calpain Degradation of p27

For that, 30°C with either purified calpain (2-day post-confluent preadipocytes (which contain p27) were incubated in the presence or absence of 10 mM EGTA (Fig. 5A, lane 2) or 26 μM ALLN (lane 5) or calpain that had been heated to 100°C for 10 min (lane 3). Aliquots of the reaction mixture were subjected to SDS-PAGE and Western blot analysis using an antisera to p27. B, cell lysates from 2-day post-confluent 3T3-L1 preadipocytes maintained in calf serum (CS) or induced to differentiate with MDI (MDI) or MDI and ALLN (MDI + ALLN) for 24 h were incubated alone (lanes 1–3) or in combination (lanes 4 and 5) or in the presence of calpain antiserum (lane 6), a calpastatin peptide (lane 7), calpastatin antiserum (lane 8), or preimmune serum (lane 9) for 30 min. Samples were subjected to SDS-PAGE and Western blot analysis using an antisera to p27.

state. Western blot analysis was used to follow the change in immunoreactive p27 in cell lysates incubated with purified calpain (Fig. 5A). Purified calpain rapidly degraded p27 in cell lysates (Fig. 5A, lane 2). To verify that degradation was due to the exogenously added calpain, the effect of several conditions/agents known to block calpain activity were tested and found to prevent the degradation of p27. Calpain, after heat treatment at 100°C, failed to degrade the p27 present in preadipocyte lysates (Fig. 5A, lane 3). Since calpain is activated by calcium, the effect of EGTA was tested. In the presence of EGTA, calpain failed to degrade p27 (Fig. 5A, lane 4). Finally, to verify that ALLN inhibits calpain activity in vitro, as it does in intact 3T3-L1 preadipocytes (see below), cell lysates were incubated with calpain and ALLN. As shown in Fig. 5A (lane 5), ALLN totally blocked the degradation of p27. These findings verify that purified calpain can degrade p27 in vitro.

To verify that the p27-degrading activity in 3T3-L1 preadipocytes is due to calpain, cell-free extracts were examined for this activity. When cell lysates from quiescent preadipocytes, which express p27, were incubated with cell-free extracts from cells induced to differentiate for 24 h, p27 was rapidly degraded (Fig. 5B, compare lanes 1 and 4). However, cell extracts from preadipocytes induced to differentiate in the presence of ALLN failed to degrade p27 (Fig. 5B, lane 5). To verify that the p27-degrading factor is calpain, cell extracts from MDI-induced preadipocytes were preincubated either with antibody against calpain or an inhibitory calpastatin peptide (a peptide corresponding to the domain through which calpain interacts with calpastatin) and then incubated with lysates from uninduced preadipocytes were added. Both anti-calpain antibody and the calpastatin peptide blocked degradation of the p27 present in the uninduced preadipocytes as assessed by Western blot analysis (Fig. 5B, lanes 6 and 7, respectively). In contrast, neutralizing calpastatin with calpastatin antibody or preincubation with preimmune serum had no effect on p27 degradation (Fig. 5B, lanes 8 and 9, respectively). Taken together these results provide compelling evidence that the p27-degrading activity in cell-free extracts from differentiating preadipocytes is calpain.

FIG. 5. Degradation of p27 by calpain in vitro. A, cell lysates from 2-day post-confluent preadipocytes (which contain p27) were incubated for 1 h at 30°C with either purified calpain (lanes 2, 4, and 5) in the presence of absence of 10 mM EGTA (lane 4) or 26 μM ALLN (lane 5) or calpain that had been heated to 100°C for 10 min (lane 3). Aliquots of the reaction mixture were subjected to SDS-PAGE and Western blot analysis using an antisera to p27. B, cell lysates from 2-day post-confluent 3T3-L1 preadipocytes maintained in calf serum (CS) or induced to differentiate with MDI (MDI) or MDI and ALLN (MDI + ALLN) for 24 h were incubated alone (lanes 1–3) or in combination (lanes 4 and 5) or in the presence of calpain antiserum (lane 6), a calpastatin peptide (lane 7), calpastatin antiserum (lane 8), or preimmune serum (lane 9) for 30 min. Samples were subjected to SDS-PAGE and Western blot analysis using an antisera to p27.

Overexpression of Calpastatin Inhibits p27 Degradation Ex Vivo—To verify that p27 is a substrate for calpain during mitotic clonal expansion, preadipocytes harboring an inducible expression vector for calpastatin, the specific calpain inhibitor, were employed. The expression vector contains a TET-promoter/HA-tagged calpastatin transgene that allows conditional expression of calpastatin under the control of the tetracycline promoter. Thus, in the presence of tetracycline, calpastatin is not expressed (Fig. 6, lanes 1, 2, 5, and 6) and in its absence expression occurs (Fig. 6, lanes 3, 4, 7, and 8). Tetracycline was removed from the medium 24 h prior to induction of differentiation with MDI to ensure accumulation of calpastatin. Six hours after induction, p27 could be detected whether or not calpastatin was expressed (Fig. 6, lanes 1, 2, 5, and 6). This was expected since degradation of p27 normally occurs later (between 10 and 14 h) in the differentiation program (Fig. 4, B and C). Thus, by 16 h into the program in the absence of calpastatin, p27 was not detected (Fig. 6, lanes 5 and 6). However, when calpain was inhibited by the expression of calpastatin, p27 was detected at 16 h indicating that degradation had been blocked (Fig. 6, lanes 7 and 8). These results are consistent with the time frame when the calpain inhibitor, ALLN, blocks differentiation (Fig. 1), mitotic clonal expansion (Figs. 1C and 2), and the degradation of p27 (Fig. 4, B and C). These findings support the view that calpain is responsible for the degradation of p27 during the mitotic clonal expansion phase of adipocyte differentiation.

Discussion

To initiate the adipocyte differentiation program, confluent growth-arrested 3T3-L1 preadipocytes in G0 are exposed to differentiation inducers (i.e. MDI). The cells synchronously re-enter the cell cycle, undergo several rounds of mitotic clonal expansion, then exit the cell cycle, and terminally differentiate into cells possessing the adipocyte phenotype (1, 2). Previous studies (21) showed that calpain activity is required during the first 48 h following induction of differentiation. The calpain inhibitor, ALLN, blocks differentiation during this period but has no effect when added 48 h after induction. It is during this time window (0–48 h) that preadipocytes undergo mitotic clonal expansion. The inhibitory effect of continuous exposure to ALLN is reversible. Thus, ALLN-arrested preadipocytes re-
tained their undifferentiated characteristics for at least 7 days after an initial 24-h exposure to ALLN (21). If the inhibitor is then removed and the cells are again exposed to the differentiation inducers, differentiation into adipocytes occurs (21). It can be concluded, therefore, that ALLN arrests differentiation, rather than merely delaying its onset, and this arrest is reversible. That calpain is targeted by ALLN is supported by the finding that overexpression of calpastatin, the endogenous specific calpain inhibitor, also blocks differentiation (21).

As shown in this paper (Fig. 1B) inhibition of differentiation by the calpain inhibitor, ALLN, can occur only during a brief time window in the differentiation program, i.e. between 12 and 24 h. Importantly, it is in this time window that mitotic clonal expansion and that the differentiation program are both initiated. Indeed, ALLN blocks the mitotic clonal expansion phase of the program (Fig. 1C) and, as a consequence, prevents differentiation. It should be noted, however, that ALLN has virtually no effect on mitosis of logarithmically dividing preconfluent 3T3-L1 preadipocytes (prior to the induction of differentiation) and therefore is not a general cell cycle inhibitor. The anti-mitotic effect of the calpain inhibitor, ALLN, is apparently limited to differentiation-linked mitotic clonal expansion. Consistent with this site of action of ALLN, both mitotic clonal expansion (Fig. 1C) and differentiation (21) are blocked by overexpression of calpastatin, the specific endogenous calpain inhibitor, in 3T3-L1 preadipocytes.

While inhibition of calpain arrests preadipocytes prior to the G1/S boundary (Fig. 4), this inhibition does not block expression of the immediate early genes, which begins within 0.5 h after induction (Fig. 3) and precedes mitotic clonal expansion. Expression of these genes allows cells to exit G1 and enter G2. As ALLN exerts its effects only between 12 and 24 h, events prior to this point in the program do not appear to be affected. It should be noted that whereas mitotic clonal expansion is a prerequisite for adipocyte differentiation, mitotic clonal expansion alone is insufficient to induce differentiation (34, 35). Although the reason mitotic clonal expansion is necessary for differentiation is unknown, it is possible that during DNA replication chromatin becomes accessible to transcription factors, e.g. C/EBPβ and C/EBPδ, that are expressed during this period. These transcription factors function in an activation cascade that leads to the expression of PPARγ and C/EBPα which, in turn, coordinately activate the adipocyte genes that produce the adipocyte phenotype (36). It appears that the pathways regulating cell cycle progression and differentiation “priming” (i.e. activation of C/EBPβ and C/EBPδ) are interrelated and that calpain may be involved in both proliferation and differentiation. Inhibition of calpain by ALLN blocked both the degradation of p27 and thus the reentry of confluent preadipocytes into the cell cycle and the ability of C/EBPβ to bind DNA and activate the transcription of the adipogenic transcription factors, PPARγ and C/EBPα.

As indicated above, exposure of growth-arrested 3T3-L1 preadipocytes to differentiation inducers triggers reentry into the cell cycle and clonal expansion. A large body of evidence (12) has shown that the CDKs and CDK inhibitors, e.g. p27, control the G1 to S transition. Several lines of evidence obtained in the present study indicate that calpain mediates the turnover of p27 that is required for growth-arrested 3T3-L1 preadipocytes to pass through the G1 to S checkpoint to progress through the cell cycle. 1) p27 begins its decline 10–12 h after confluent preadipocytes are induced to differentiate and disappears completely by 16 h (Fig. 4, B and C). This decrease in p27 is blocked by the calpain inhibitor, ALLN, when added just before and during the time window, i.e. between 12 and 24 h (Fig. 1B), when p27 normally disappears (Fig. 4, B and C). 2) In vitro studies show that purified calpain can degrade p27 and that this degradation is blocked by the calcium chelator, EGTA, or by the calpain inhibitor, ALLN (Fig. 5A). 3) Cell lysates from MDI-induced preadipocytes that possess catalytically active calpain degrade p27 present in growth-arrested preadipocytes (Fig. 5B). Moreover, this calpain degradation is prevented by either anti-calpain antibody or a calpastatin peptide (known to selectively inhibit calpain). 4) Overexpression of calpastatin, the specific endogenous calpain inhibitor, prevents the turnover of p27 and subsequent clonal expansion ex vivo (Fig. 6). 5) By blocking degradation of p27 with

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**Image Description:**

![Diagram](image.png)  
**FIG. 7.** Proposed model for the events involving calpain during mitotic clonal expansion of 3T3-L1 adipocytes following induction of differentiation. **CALPAIN** refers to “activated” calpain; **TF** indicates transcription factors involved in the G1-S phase transition; and **Day 1 and 2–7** refers to days during the adipocyte differentiation program.
a calpain inhibitor all subsequent events of the differentiation program are derailed including phosphorylation of Rb (Fig. 4, A and C), induction of expression of the S phase cyclins (results not shown), DNA replication (Fig. 2), cell proliferation (Fig. 1C), and acquisition of the adipocyte phenotype (Fig. 1 and Ref. 21). Although there is evidence that p27 is able to inhibit cell cycle progression, it is possible that calpain-mediated turnover of p27 may not be the critical event resulting in initiating the clonal expansion phase of 3T3-L1 adipocyte differentiation. Calpain is known to cleave a variety of substrates i.e. cytoskeletal proteins as well as phosphatases, kinases, and transcription factors.

Our findings suggest that reentry of confluent growth-arrested preadipocytes into the cell cycle involves the degradation of p27. Other studies have implicated p21 (33), C/EBPα (35), and PPARY (37) as antimitotic factors later in the differentiation program, i.e. in the termination of mitotic clonal expansion. Since the turnover of p27 is necessary for adipocyte differentiation, a lack of p27 would not be expected to have an impact on adipogenesis. Consistent with our findings, p27-deficient mice possess adipose tissue (38–40). Although p27-deficient mice are somewhat larger than normal, they are not obese; rather, organ size/weight increases in proportion to whole body weight. These findings confirm that p27 deficiency in preadipocytes is permissive for adipocyte differentiation.

The model shown in Fig. 7 incorporates our findings on the role of calpain in mitotic clonal expansion into the context of the established framework of the cell cycle. In this model we visualize the sequence of events to include the following: 1) activation of calpain* initiated by the differentiation inducers (MDI); 2) degradation of p27 by calpain* releasing the cyclin D-CDK4 complex from inhibitory constraint; 3) phosphorylation of Rb by the “activated” cyclin D-CDK4 complex; 4) release of sequestered transcription factors, e.g. E2F, from inhibitory constraint by Rb; 5) transcriptional activation by these factors of the genes required for S phase progression and DNA replication; and 6) entry into the mitotic clonal expansion phase and subsequent events of the adipocyte differentiation program.

It has been reported that p27 is degraded via the ubiquitin-proteosome pathway (19), and a recent finding (41) suggests that p27 may also be degraded via a ubiquitin-independent pathway. The findings in this study are consistent with Shirane et al. (41), in that p27 can be degraded in a ubiquitin-independent manner. A possible mechanism by which p27 may be identified for degradation by calpain is through the PEST sequence in p27. Like many proteins that contain PEST sequences, p27 turns over rapidly (Fig. 4, B and C). Although a PEST consensus sequence is not an absolute requirement for proteolysis by calpain, many proteins that are cleaved by calpain contain PEST sequences. By using the PEST-FIND program, a PEST sequence was identified near the carboxyl terminus of p27 (position 169–189) (16). In contrast, other findings (41) suggest that p27 is cleaved at the amino terminus in a ubiquitin-independent manner. We were unable to locate a consensus PEST sequence in the amino terminus of p27. Since the PEST site is not an absolute requirement for cleavage by calpain, it is possible that the calpain proteolytic site on p27 may coincide within the amino terminus as suggested by Shirane et al. (41). Studies are underway to determine the site of calpain-mediated proteolysis in p27. To reconcile the findings that p27 is degraded by both calpain and a ubiquitin-proteosome pathway, it is possible that calpain catalyzes an initial cleavage event at the PEST sequence in p27, thereby targeting p27 for further degradation by proteasomes. Alternatively, both pathways may operate independently. Other recent studies have shown that cyclin D1 is degraded by calpain (20), whereas other cyclins (A and E) have been shown to be degraded via the proteosome system. Conceivably, a coordinated mechanism involving both calpain and proteasomes regulate the activity of factors during the cell cycle. We suggest that calpain may initiate the degradation of p27 and that the proteosomal degradation system may complete the process. Investigations are underway to determine whether the turnover of p27 during the mitotic clonal expansion phase of the adipocyte differentiation program involves both systems.

Acknowledgments—We thank Dr. Ernesto Carafoli (ETH, Zurich) for helpful discussions and Dr. M. Maki, Nagoya University, Nagoya, Japan, for supplying the tetracycline-regulated expression vector.