Posttranscriptional and Posttranslational Regulation of C/EBPδ in G₀ Growth-arrested Mammary Epithelial Cells

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Previous work from our laboratory demonstrated that CCAAT/enhancer-binding protein δ (C/EBPδ) functions in the initiation and maintenance of G₀ growth arrest in mouse mammary epithelial cells (MECs). In this report, we investigated the posttranscriptional and posttranslational regulation of C/EBPδ in G₀ growth-arrested mouse MECs. The results of transcriptional inhibitor studies demonstrated that the C/EBPδ mRNA exhibits a relatively short half-life in G₀ growth-arrested mouse MECs (t₁/₂ ~ 35 min). In contrast, C/EBPδ mRNA has a longer half-life in G₀ growth-arrested mouse fibroblast cells (t₁/₂ >100 min). Oligo/RNase H cleavage analysis and rapid amplification of cDNA ends-poly(A) test both confirmed the short C/EBPδ mRNA half-life observed in MECs and demonstrated that the C/EBPδ mRNA poly(A) tail length was not shortened during C/EBPδ mRNA degradation, which suggested a deadenylation-independent pathway. The C/EBPδ protein also exhibited a relatively short half-life in G₀ growth-arrested mouse MECs (t₁/₂ ~ 120 min). The C/EBPδ protein was degraded in a ubiquitin-dependent manner, primarily in the nucleus, during G₀ growth arrest. In conclusion, these studies indicated that the C/EBPδ mRNA and protein content are under tight regulation in G₀ growth-arrested mouse MECs, despite the general concept that G₀ growth arrest is associated with a decrease in cellular activity.

It is well documented that global gene transcription and translation dramatically decrease as cells exit the cell cycle and enter the quiescent G₀ growth arrest state (1, 2). However, a small subset of genes become activated, and these gene products function in the initiation and maintenance of G₀ growth arrest (3, 4). Currently, little is known about the regulation and function of G₀ growth arrest-specific genes in cell biology. Recently, an increase in expression and activity of the retinoblastoma (Rb) family member p130 was observed in the initiation and maintenance of G₀ growth arrest (5). Formation of the p130-E2F complex sequesters members of the E2F family to repress the expression of genes necessary for cellular proliferation. Alterations in the structure and function of G₀ growth arrest genes are linked to some types of cancers. For example, germ line mutations found within the von Hippel-Lindau tumor suppressor gene have been linked to hemangioblastomas of the retina and central nervous system and renal carcinogenesis (6). The von Hippel-Lindau protein functions in cell cycle control in a variety of ways, including up-regulating the cyclin-dependent kinase inhibitor p27 (7).

Previous reports from our laboratory (8–13) demonstrate that CCAAT/enhancer-binding protein δ (C/EBPδ) functions in the initiation and maintenance of G₀ growth arrest in mouse mammary epithelial cells (MECs). C/EBPδ mRNA, protein, and DNA binding activity increase during mouse MEC G₀ growth arrest (11–13). STAT3 activation/phosphorylation is necessary for C/EBPδ transcription in G₀ growth-arrested and cytokine-treated mouse MECs (10). In addition, the C/EBPδ promoter exhibits autoregulation during G₀ growth arrest (12).

C/EBPs are a widely expressed, highly conserved family of leucine zipper (bZIP)-type transcription factors (14, 15). Most C/EBPs are encoded by intronless genes and exhibit a high degree of homology in the basic and bZIP regions (14, 15). To date, six family members are characterized including C/EBPα, C/EBPβ (also called CRP2, NF-IL6, LAP, AGP/EBP, IL6BP, or NF-M), C/EBPδ (also called CRP3, NF-IL6b, and CELF), C/EBPε, C/EBPγ, and C/EBP-Homologous Protein10 (GADD153) (15). C/EBPδ binds to DNA as homodimers or as heterodimers with other C/EBP family members or other bZIP proteins such as c-Fos and CREB/ATF (14, 15). Functional C/EBP-binding sites are present in the promoters of genes that function in cell growth arrest (gadd45γ), cell growth (c-fos), and differentiation (phosphoenolpyruvate carboxykinase and β-casein) (16–19).

C/EBPs are directly involved in the regulation of cell fate determination (20–26). Early reports (20, 27) demonstrate that the sequential expression of C/EBPδ, C/EBPδ, and C/EBPα is required for optimal adipocyte differentiation. Further studies (28–30) have identified additional roles for C/EBPα in hepatocyte metabolism and granulocyte differentiation. C/EBPδ also plays an essential role in ovarian granulosa cell biology and the development and differentiation of the mammary gland (22–25, 31). Furthermore, C/EBPα functions in the differentiation and development of neutrophils and eosinophils (32, 33).

Control of gene expression can occur at the transcriptional, posttranscriptional, or posttranslational level (34–37). At the posttranscriptional level, mRNA stability is emerging as a key

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The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; MEC, mammary epithelial cell; gas, growth arrest-specific; STAT, signal transducer and activator of transcription; ARE, A/U-rich element; UTR, untranslated region; bZIP, leucine zipper; RACE-PAT, rapid amplification of cDNA ends-poly(A) test; GAM, growth arrest medium; CGM, complete growth medium; LlαL, N-acetyl-Leu-Leu- Norleu-Al; MeSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; DRB, 5,6-dichlorobenzimidazole 1-β-N-ribosaroside; cp, cyclophilin; BGH, bovine growth hormone.

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regulatory mechanism in cell cycle control and DNA damage repair (34, 35, 38–40). For example, the stability of growth arrest and DNA damage-inducible mRNA increases after exposure to DNA-damaging agents or other growth arrest treatments (40). In addition, the growth arrest-specific gene 5 (gas-5) exhibits a marked increase in mRNA stability in density-arrested NIH 3T3 cells versus exponentially growing and differentiating cells (41). Alterations in the posttranscriptional regulation of genes that function in cell growth control and cell cycle progression can play a crucial role in tumorigenesis (42, 43). For example, alterations of trans-acting factors that function in c-myc and c-myb mRNA turnover results in increased c-myc and c-myb mRNA stability, which is linked to acute myeloid leukemia (44). Additionally, an increase in the basic fibroblast growth factor mRNA half-life, due to defects in posttranscriptional regulation, has been implicated in a variety of human tumors (45). Although several reports demonstrate that C/EBPα is regulated at the transcriptional level (12, 13), posttranscriptional control of C/EBPα has not been investigated extensively.

In addition to posttranscriptional control, several genes that play critical roles in cell cycle control are regulated posttranscriptionally, at the level of protein degradation (46–49). For example, the rate of p27 protein degradation decreases in response to growth arrest, which results in an accumulation of p27 protein (50, 51). Blocking ubiquitination-dependent protein degradation increases p27 protein half-life and demonstrates that the p27 protein is degraded via the ubiquitin/proteasome pathway (52, 53). In addition, increased p53 protein stability occurs during cellular genotoxic stress (54). This is accomplished by N-terminal phosphorylation of the p53 protein, which decreases the degree of ubiquitination and increases protein stability (54). Accumulating evidence indicates that cellular proteins may be degraded by ubiquitin-mediated mechanisms localized to either the nucleus or cytoplasm (55, 56). Nuclear localized ubiquitin-mediated degradation appears to provide a rapid mechanism for the disposal of nuclear cell cycle regulatory proteins (57). For example, the tumor suppressor protein product, p53, is degraded within the nucleus via a ubiquitin-proteasome pathway during post-stress recovery (58).

The overall goal of this study was to investigate the posttranscriptional and posttranslational regulation of C/EBPα in G0 growth-arrested mouse MECs. Our laboratory has reported previously (12) that C/EBPα exhibits increased transcription and growth suppressor activity in G0 growth-arrested mouse MECs. However, the posttranscriptional and posttranslational regulation of C/EBPα has not been systematically investigated. Previous studies (35) have demonstrated that key cell cycle regulatory proteins are encoded by unstable mRNAs. Because G0 growth arrest is associated with a period of decreased cellular activity, we hypothesized that both the C/EBPα mRNA and protein would exhibit extended half-lives in G0 growth-arrested MECs. Unexpectedly, the results demonstrate that C/EBPα mRNA exhibited a novel short mRNA half-life in G0 growth-arrested mouse MECs (t1/2 ~ 35 min) and contained a relatively short poly(A) tail of ~100 nucleotides. In addition, the C/EBPα protein also exhibited a short half-life in G0 growth-arrested mouse MECs (t1/2 ~ 120 min). Furthermore, ubiquitination inhibitor studies indicated that C/EBPα protein degradation is ubiquitin-dependent and occurs predominantly within the nucleus. The results of these studies demonstrate that the C/EBPα mRNA and protein are under tight regulation in G0 growth-arrested MECs, suggesting that C/EBPα plays a key role in mouse MEC growth control.
cDNA was subsequently used to set up standard PCR (5 min at 93 °C, followed by 30 cycles of 30 s at 93 °C, 30 s at 60 °C, 1 min at 72 °C, and a 7-min extension at 72 °C) using an end-labeled (5'-P)dATP C/EBPβ upstream primer (5'-CCATTGCGGTAAGGCTG-3') and an oligo(dT) anchor downstream primer (5'-GGGATCCGCGGATCCGCTG-3') or the poly(A) tail (poly(dT)12-18) were denatured at 65 °C for 10 min. A digestion mixture (4 μl of 5× buffer (200 mM HEPES (pH 7.9), 50 mM MgCl2, 300 mM KCl, 5 mM dithiothreitol (DTT)), 1 unit of RNase H (Invitrogen) and 1 μl of RNasin (Promega, Madison, WI), in 20-μl reactions) was added to each sample and incubated at 37 °C for 30 min (58). Reactions were stopped with 1 μl of 0.5 M EDTA and precipitated with 0.1 volume of 3 M NaOAc and 2.5 volumes of 100% ethanol. Samples were pelleted by centrifugation, resuspended in Northern blot tracking dye, and subsequently analyzed by Northern blot analysis as described previously (11).

RESULTS

C/EBPβ mRNA Exhibits a Short Half-life in G0 Growth-arrested Mouse HC11 MECs—To investigate the posttranscriptional regulation of the C/EBPβ mRNA in mouse HC11 MECs, we utilized transcriptional inhibitors followed by Northern blot analysis. Briefly, confluent HC11 MECs were G0 growth-arrested by serum and growth factor withdrawal. After 48 h, HC11 MECs were either maintained in growth arrest medium alone (GAM–) or in GAM with the addition of the transcriptional inhibitor, actinomycin D (GAM+) for the indicated times. mRNA half-life was analyzed for a panel of cellular mRNAs including cyclophilin (cp), which was used to confirm equal loading. Consistent with previous reports from our laboratory, C/EBPβ mRNA was detected in G0 growth-arrested HC11 MECs (Fig. 1A, lanes 1–4). C/EBPβ mRNA levels rapidly declined with a half-life of ~35 min (t1/2 ~35 min) following actinomycin D treatment (Fig. 1A, lanes 5–8 and Fig. 1C). After 60 min of actinomycin D treatment, C/EBPβ mRNA was undetectable (Fig. 1A, lane 7). Similar results were observed with a second transcriptional inhibitor, DRB (Fig. 1B).

In addition to C/EBPβ, we also investigated the mRNA level of another C/EBP family member, C/EBPα. Reports from a number of laboratories, including our own, demonstrate that C/EBPα plays a significant role in mammary gland development and differentiation (22, 24, 25, 31). C/EBPα mRNA was detected in G0 growth-arrested HC11 MECs, suggesting that C/EBPβ also plays a role in G0 growth arrest (Fig. 1A, lanes 1–4). C/EBPβ mRNA levels declined with a half-life of ~45 min following the addition of actinomycin D (Fig. 1A, lanes 5–8, and Fig. 1C).

gas-1 mRNA levels are known to be induced during G0 growth arrest of NIH 3T3 fibroblast cells (59–61). In this report, gas-1 mRNA was also detected in G0 growth-arrested HC11 MECs (Fig. 1A, lanes 1–4). Addition of actinomycin D reduced gas-1 mRNA levels (t1/2 ~75 min) (Fig. 1A, lanes 5–8, and Fig. 1C). Compared with C/EBPβ, gas-1 mRNA was relatively stable during G0 growth arrest. In agreement with previous work, c-fos, an immediate early gene that is induced at the G1/S transition, was undetectable at the mRNA level in G0 growth-arrested HC11 MECs (Fig. 1A, lanes 1–4).

In summary, results of transcriptional inhibitor studies indicate that C/EBPβ mRNA is highly unstable during G0 growth arrest in HC11 MECs. C/EBPβ, which has been associated previously with cellular proliferation and differentiation, also exhibits a relatively short mRNA half-life in G0 growth-arrested HC11 MECs. In contrast, gas-1 mRNA is more stable during G0 growth arrest. Overall, the results suggest that the C/EBPβ mRNA is undergoing rapid turnover despite the general decline in global gene expression and biosynthetic activity during G0 growth arrest.

C/EBPβ mRNA Half-life during Cell Cycle Re-entry in HC11 MECs—Cell cycle re-entry requires coordination between the inactivation and/or disposal of G0-specific proteins and the expression of early G1 genes, such as c-fos and c-myc. To investigate the posttranscriptional regulation of C/EBPβ mRNA during early G1, mRNA levels were analyzed from HC11 MECs upon addition of complete growth media alone (CGM–) or CGM plus actinomycin D (CGM+). Labeled C/EBPβ mRNA was delayed compared with the decline observed in G0 growth arrest (Fig. 2, lanes 5–8).

When G1 growth-arrested HC11 MECs were induced to re-enter the cell cycle by refeeding with CGM, C/EBPβ mRNA levels increased ~10-fold within the first 90 min (Fig. 2, lane 1–4). This induction of C/EBPβ mRNA levels during early G1 is consistent with a growth-promoting role for C/EBPβ. The addition of actinomycin D blocked the growth-stimulated induction of C/EBPβ mRNA, which suggests that C/EBPβ transcription plays a major role in the increase in C/EBPβ mRNA levels during early G1 in HC11 MECs (Fig. 2, lanes 5–8). gas-1 mRNA levels also declined after G1 development of C/EBPβ mRNA in HC11 MECs were induced to re-enter the cell cycle by refeeding with CGM (Fig. 2, lanes 1–4). Interestingly, addition of CGM and actinomycin D stabilized the gas-1 mRNA, resulting in high levels of gas-1 mRNA even at 90 min (Fig. 2, lanes 5–8). In agreement with previous reports, c-fos mRNA was transiently induced following the addition of CGM and the initiation of the cell cycle (Fig. 2, lanes 1–4). The induction of c-fos mRNA was blocked by actinomycin D treatment, indicating that c-fos gene transcription is required for the increase in c-fos mRNA during early G1 (Fig. 2, lanes 5–8).

The results of the transcriptional inhibitor studies during cell cycle re-entry demonstrate that the C/EBPβ and gas-1 mRNAs are more stable during the G1/G0 transition compared with G0 growth arrest. This suggests that both C/EBPβ and gas-1 mRNA degradation during cell cycle re-entry is dependent on the transcription of gene product(s) important for mRNA decay during the G0/G1 transition. In addition, the increase in C/EBPβ and C-fos mRNA levels during the G1/G0 transition are inhibited by actinomycin D treatment, indicating that the increase in these immediate early mRNAs is transcription-dependent.

C/EBPβ mRNA Is More Stable in G0 Growth-arrested NIH 3T3 Cells Compared with HC11 MECs—NIH 3T3 cells have been utilized extensively as a model system to investigate mechanisms of cell growth control. We reported previously (11) that C/EBPβ mRNA is present in NIH 3T3 cells regardless of growth status. To investigate the posttranscriptional control of the C/EBPβ mRNA in NIH 3T3 cells, transcriptional inhibitor/Northern blot analysis was performed. In agreement with our previous results, C/EBPβ mRNA was detected in G0 growth-arrested (GAM–) NIH 3T3 cells (Fig. 3A, lanes 1–4). C/EBPβ mRNA levels declined following actinomycin D treatment, although the rate of decline is slower than that observed in G0 growth-arrested HC11 MECs (t1/2 >100 min for NIH 3T3 cells versus t1/2 ~35 min for HC11 MECs) (Fig. 3A, lanes 5–8 and Fig. 3B). Following cell cycle re-entry, C/EBPβ mRNA levels decreased by 90 min (Fig. 3A, lanes 9–12). The addition of actinomycin D stabilized C/EBPβ mRNA (Fig. 3A, lanes 13–16), paralleling the results from experiments in HC11 MECs.
Regulation of C/EBPδ in G₀ Growth Arrest

Consistent with results from mouse MECs, the C/EBPβ mRNA was detected in G₀ growth-arrested NIH 3T3 cells (Fig. 3A, lanes 1–4). Upon addition of actinomycin D, a steady decline in the C/EBPβ mRNA content was observed (t₁/₂ > 100 min) (Fig. 3A, lanes 5–8, and Fig. 3B). C/EBPβ mRNA levels increased (15-fold after 90 min) following cell cycle re-entry (Fig. 3A, lanes 9–12). This cell cycle-induced increase in C/EBPβ mRNA levels was blocked by actinomycin D treatment (Fig. 3A, lanes 13–16). In agreement with previous reports (59, 62), elevated levels of gas-1 mRNA was detected in G₀ growth-arrested NIH 3T3 cells (Fig. 3A, lanes 1–4). Addition of actinomycin D resulted in a decline in gas-1 mRNA levels (t₁/₂ ~ 75 min) (Fig. 3A, lanes 5–8, and Fig. 3B). Following the addition of complete growth media and the initiation of the cell cycle, gas-1 mRNA content exhibited a decline by 90 min (Fig. 3A, lanes 9–12). However, the addition of actinomycin D stabilized the gas-1 mRNA (Fig. 3A, lanes 13–16). Finally, c-fos mRNA was undetectable in G₀ growth-arrested NIH 3T3 cells at all time points taken (Fig. 3A, lanes 1–8). c-fos mRNA levels rapidly increased following cell cycle re-entry and peaked 60 min after the addition of complete growth media (Fig. 3A, lanes 9–12). Cell cycle-induced increase in the c-fos mRNA level was blocked by actinomycin D treatment, paralleling the results from experiments with HC11 MECs (Fig. 3A, lanes 13–16).

The extended C/EBPδ mRNA half-life detected in G₀ growth-arrested NIH 3T3 cells suggests that C/EBPδ is under less stringent control in mouse fibroblast-derived cells compared with mouse mammary epithelial-derived cells. In contrast, posttranscriptional regulation of C/EBPδ mRNA during cell cycle re-entry is similar between HC11 MECs and NIH 3T3 cells. The posttranscriptional regulation of C/EBPβ, gas-1, and c-fos in both G₀ growth arrest and cell cycle re-entry is comparable between HC11 MECs and NIH 3T3 cells.

C/EBPδ mRNA Contains a Short Poly(A) Tail—Sequences present within mRNAs influence processing, stability, and transport (34, 35, 39, 63). To investigate the role of the poly(A) tail on C/EBPδ mRNA stability, we utilized an oligo/ RNase H cleavage Northern blot analysis (58). In this analysis, a C/EBPδ-specific oligomer complimentary to the C/EBPδ mRNA within the 3′-untranslated region (UTR) ( oligo 1193) was used to form a DNA/RNA heteroduplex that is cleaved by RNase H, producing C/EBPδ 5′ and 3′ mRNA fragments (Fig. 4A). A [α-[³²P]]dCTP-labeled C/EBPδ 3′-UTR-specific probe that
Fig. 3. C/EBPβ mRNA stability during G0 growth arrest and the G0/G1 transition in NIH 3T3 cells. RNA isolated from untreated and actinomycin D-treated G0 growth-arrested or serum and growth factor-stimulated (G0/G1 transition) NIH 3T3 cells was analyzed by Northern blot as described in Fig. 1. A, lanes 1–4, RNA from G0 growth-arrested NIH 3T3 cells treated with actinomycin D (GAM+); lanes 5–8, RNA from G0 growth-arrested NIH 3T3 cells treated with actinomycin D (GAM+); lanes 9–12, RNA from serum- and growth factor-stimulated (G0/G1 transition) NIH 3T3 cells treated with actinomycin D (GAM+); lanes 13–16, RNA from serum- and growth factor-stimulated (G0/G1 transition) NIH 3T3 cells treated with actinomycin D (GAM+). Results are representative of three independent experiments. B, summary of mRNA half-life data obtained from Northern blot/actinomycin D (Act.D) analysis as determine in Fig. 1. Filled triangles, RNA from actinomycin D-treated NIH 3T3 cells; filled circles, RNA from non-treated NIH 3T3 cells.

spanned the oligo/RNase H digestion site was used to detect both of the oligo/RNase H-generated C/EBPβ fragments: the 5′ C/EBPβ cleavage product (−1.4 kb) composed of the remaining 3′-UTR (−260 bp) plus the length of the poly(A) tail.

Initially, we performed the oligo/RNase H cleavage analysis on RNA from G0 growth-arrested (GAM−) HC11 MECs, and Northern blot analysis detected two cleavage products of −1.4 kb and 370 bp (Fig. 4B, lanes 1–3). The 3′-UTR cleavage product contains 260 bp from the 3′-UTR and reveals a poly(A) tail composed of 100 nucleotides. To investigate the mechanism of C/EBPβ mRNA degradation, mRNA was isolated from actinomycin D-treated G0 growth-arrested (GAM+) HC11 MECs. After 30 min of actinomycin D treatment, both cleavage products were detected (Fig. 4B, lane 4). However, after 60 min of actinomycin D treatment only the 3′ oligo/RNase H C/EBPβ mRNA cleavage product was detected (Fig. 4B, lane 5). These results confirmed the short half-life of the C/EBPβ mRNA in G0 growth-arrested HC11 MECs.

We next investigated the C/EBPβ mRNA poly(A) tail length during the G0/G1 transition. G0 growth-arrested HC11 MECs were induced to re-enter the cell cycle by the addition of CGM alone (CGM−) or CGM plus actinomycin D (CGM+). Upon addition of CGM, the reduction of C/EBPβ mRNA oligo/RNase H cleavage products was observed, consistent with a decrease in the C/EBPβ mRNA content upon the onset of early G1 (Fig. 4B, lanes 6–8). After addition of actinomycin D, the reduction of C/EBPβ mRNA oligo/RNase H cleavage products was slightly
pressed HC11 MEC line previously developed in our laboratory. RACE-PAT analysis. RACE-PAT was performed on 5 μg of total RNA isolated from untreated and actinomycin D-treated G0 growth-arrested or serum- and growth factor-stimulated (G2/G1 transition) HC11 MECs at the indicated time points. cDNA was synthesized and subjected to PCR, and products were visualized by PAGE and autoradiography. Lanes 1–3, G0 growth-arrested MECs (GAM—); lanes 4–6, G0 growth-arrested MECs treated with actinomycin D (GAM+); lanes 7–9, serum and growth factor-stimulated (G2/G1 transition) MECs (CGM—); lanes 10–12, serum and growth factor-stimulated (G2/G1 transition) MECs treated with actinomycin D (CGM+). Results are representative of three independent experiments.

delayed compared with GAM+, and a more complex pattern of C/EBPδ mRNA degradation was detected (Fig. 4B, lanes 9–11). The results of the CGM and actinomycin D experiment suggest that transcription of gene products important for mRNA decay is required for efficient C/EBPδ mRNA degradation during cell cycle re-entry. Finally, the estimated size of all the C/EBPδ 3’ oligo/RNase H cleavage products is consistent with a poly(A) tail length of ~100 nucleotides. To confirm the length of the poly(A) tail, we utilized an oligo(dT) in the oligo/RNase H experiment, which generates a C/EBPδ mRNA product lacking a poly(A) tail (Fig. 4B, lane 12). The results reveal that the mobility of this mRNA product compared with full-length C/EBPδ mRNA (Fig. 4B, lane 13) is consistent with a poly(A) tail length of ~100 nucleotides. Overall, the results demonstrate that the C/EBPδ mRNA contains a relatively short poly(A) tail that is not shortened during mRNA degradation in HC11 G0 growth arrest and cell cycle re-entry.

RACE-PAT Analysis Confirms C/EBPδ mRNA Poly(A) Tail Length—To investigate further the C/EBPδ mRNA poly(A) tail length, we utilized a rapid amplification of cDNA ends-poly(A) tail test (RACE-PAT) (58). Initially, mRNA was obtained from G0 growth-arrested HC11 MECs; cDNA was synthesized, and PCR was performed utilizing a radiolabeled C/EBPδ 3’-UTR upstream-specific primer and an oligo(dT) downstream primer. The PCR produced multiple products varying in length from 170 to 270 bp (Fig. 5, lanes 1–3), which is consistent with a C/EBPδ poly(A) tail length of ~100 nucleotides. Following addition of actinomycin D, C/EBPδ mRNA levels declined rapidly as observed previously in the transcriptional inhibitor/Northern blot analysis (Fig. 5, lanes 4–6). This decline is reflected in the decrease in the amount of RACE-PAT product obtained. RACE-PAT analysis was also performed on mRNA obtained from HC11 MECs refed with CGM, which also indicated that the C/EBPδ mRNA poly(A) length is approximately ~100 nucleotides (Fig. 5, lanes 7–9). Additionally, actinomycin D treatment resulted in a more stable C/EBPδ mRNA but had no apparent effect on poly(A) tail length (Fig. 5, lanes 10–12). The results confirm that the C/EBPδ mRNA contains a poly(A) tail of ~100 nucleotides in G0 growth arrest and during the G0/G1 transition and parallel previous mRNA half-life results from experiments in HC11 MECs (Figs. 1A and 2A).

The 3’-Untranslated Region Influences C/EBPδ mRNA Stability—Numerous studies (34, 35, 39) have demonstrated that specific sequences within the 3’-UTR regulate mRNA stability. To investigate the potential role of the C/EBPδ 3’-UTR in mRNA stability, transcriptional inhibitor/Northern blot analysis was repeated utilizing a stably transfected C/EBPδ overexpression HC11 MEC line previously developed in our laboratory (13). This HC11 MEC line expresses an exogenous C/EBPδ mRNA that contains a bovine growth hormone (BGH) 3’-UTR in place of the C/EBPδ 3’-UTR. The cells were grown-arrested for 48 h and maintained in GAM in the presence or absence of actinomycin D. The C/EBPδ/BGH 3’-UTR mRNA levels were compared with the endogenous C/EBPδ mRNA levels. Consistent with previous results, the endogenous C/EBPδ mRNA levels were detected in G0 growth-arrested cells (Fig. 6, lanes 1–4). Similarly, the C/EBPδ/BGH 3’-UTR mRNA was also detected in G0 growth-arrested HC11 MECs at all indicated time points (Fig. 6, lanes 1–4). Consistent with previous results, the addition of actinomycin D resulted in a rapid reduction of endogenous C/EBPδ mRNA levels (t1/2 ~35 min) (Fig. 6, lanes 5–7). Interestingly, the C/EBPδ/BGH 3’-UTR mRNA levels did not decline after actinomycin D treatment (Fig. 6, lanes 5–7). These results indicate that the presence of the BGH 3’-UTR downstream of the C/EBPδ coding region stabilizes the C/EBPδ mRNA compared with the endogenous C/EBPδ mRNA and suggests that the C/EBPδ 3’-UTR plays a role in mRNA stability during G0 growth arrest.

C/EBPδ Protein Exhibits a Short Half-life in G0 Growth-arrested Mouse MECs—Posttranslational control is a major mechanism by which cells regulate the level of cell cycle control proteins (46–49). To investigate the posttranslational regulation of the C/EBPδ protein, HC11 MECs were G0 growth-arrested for 48 h and maintained in GAM in the presence or absence of the translational inhibitor anisomycin. Protein half-life was analyzed for a panel of cellular proteins by Western blot analysis including actin, which was used to confirm equal loading. Consistent with previous reports from our laboratory (11–13), C/EBPδ protein was detected in G0 growth-arrested HC11 MECs (Fig. 7A, lanes 1–6). Following anisomycin treatment, C/EBPδ protein levels declined with a half-life of ~120 min (Fig. 7A, lanes 7–12, and Fig. 7B). As a control, the stability of p27, a growth arrest-specific protein that is regulated predominantly at the posttranslational level, was assessed (50, 51). As expected, p27 protein was detected during G0 growth arrest of HC11 MECs (Fig. 7A, lanes 1–6). In contrast to the C/EBPδ protein decay kinetics, the p27 protein is relatively stable in G0 growth-arrested HC11 MECs even after treatment with anisomycin (Fig. 7A, lanes 7–12). The results demonstrate that the C/EBPδ protein exhibits a shorter half-life compared with p27, which suggests that C/EBPδ protein is tightly regulated in G0 growth-arrested mouse MECs.

C/EBPδ Protein Is Degraded via a Ubiquitin-dependent Pathway in G0 Growth-arrested Mouse MECs—To investigate the protein degradation pathway utilized by the C/EBPδ protein in G0 growth-arrested mouse MECs, we used the ubiquitination inhibitor, MG-132. HC11 MECs were growth-arrested by addition of GAM for 48 h and subsequently maintained in either growth arrest media alone (control), GAM plus a vehicle control.
**FIG. 7.** C/EBP6 protein exhibits a short half-life in G0 growth-arrested HC11 MECs. Western blot analysis was performed on 50 μg of whole cell protein isolated from untreated and anisomycin-treated G0 growth-arrested HC11 MECs at the indicated time points. Western blots were sequentially probed with C/EBP6, p27, and actin antibodies. Actin was used as a loading control. A, lanes 1–6, protein from growth-arrested MECs (GAM+); lanes 7–12, protein from growth-arrested MECs treated with anisomycin (GAM−). Results are representative of three independent experiments. B, summary of protein half-life data obtained from Western blot/anhysinogen analysis. Signals were quantified, and the relative amount of each protein is expressed as a percentage of the 0-min control time, which was set at 100%. Graphs are plotted as % protein remaining versus time. Filled triangles, protein from anisomycin-treated MECs; filled circles, protein from non-treated MECs.

(-Me2SO), or GAM plus the ubiquitination inhibitor (+MG-132). C/EBP6 protein was detected in both G0 growth-arrested control and Me2SO samples (Fig. 8A, lanes 1–4 and 5–7, respectively). Interestingly, C/EBP6 protein content dramatically increased by 60 min of MG-132 treatment and continued to be elevated up to 180 min (Fig. 8A, lanes 8–10). Similar results were obtained with a second ubiquitination inhibitor, LLnL, in G0 growth-arrested HC11 MECs (Fig. 8B).

As a control, p27 protein levels were monitored in both mouse and human MECs after MG-132 treatment. Previous work (52, 53) in vitro and in vivo demonstrates that the ubiquitin-proteasome pathway regulates the p27 protein level. p27 protein was detected in both G0 growth-arrested control and Me2SO samples (Fig. 8A, A and B). As expected, a modest increase in p27 protein level is detected after MG-132 treatment in HC11 MECs (Fig. 8A, lanes 8–10). Together, these results demonstrate that the C/EBP6 protein is degraded via a ubiquitin-proteasome-dependent pathway that is conserved in both mouse MECs.

**Ubiquitination of the C/EBP6 Protein Is Localized Predominantly to the Nuclear Compartment**—To determine whether C/EBP6 protein degradation occurs in the nucleus and/or cytoplasm, nuclear and cytoplasmic protein was analyzed from G0 growth-arrested HC11 MECs. HC11 MECs were growth-arrested by addition of GAM for 48 h and subsequently maintained in either growth arrest media alone (control), GAM plus a vehicle control (+Me2SO), or GAM plus the ubiquitination inhibitor (+MG-132). Nuclear and cytoplasmic protein fractions were isolated at the indicated times. C/EBP6 protein was detected in the nuclear protein fraction but not the cytoplasmic protein fraction in both the G0 growth-arrested control and Me2SO samples (Fig. 9, lanes 1–6 and lanes 7–10, respectively). In agreement with previous results (Fig. 8), C/EBP6 protein content increased after MG-132 treatment (Fig. 9, lanes 11–14). Importantly, the increase in C/EBP6 protein is restricted to the nuclear compartment (Fig. 9, lanes 11 and 13).

Nuclear and cytoplasmic p27 protein levels were also monitored in MG-132-treated G0 growth-arrested HC11 MECs. Similar to the previous study, p27 protein was detected in G0 growth-arrested control and Me2SO samples (Fig. 9, lanes 1–6 and lanes 7–10, respectively). In contrast to C/EBP6 protein subcellular localization, p27 protein was found in both the nuclear and cytoplasmic compartments. Detection of the p27 protein increased slightly in both compartments after MG-132 treatment (Fig. 9, lanes 11–14).

As a control for our nuclear and cytoplasmic protein fractionation, we monitored the subcellular localization of Bcl-x, which is known to be localized to the cytoplasmic compartment (64). As expected, the majority of the Bcl-x protein content was localized within the cytoplasmic compartment in both G0 growth-arrested control and Me2SO samples (Fig. 9, lanes 1–6 and lanes 7–10, respectively). Importantly, upon MG-132 treatment, no change in Bcl-x localization was detected (Fig. 9, lanes 11–14). Taken together, these results demonstrate that the C/EBP6 protein is absent from the cytoplasmic compartment, which suggests a nuclear localized ubiquitin-mediated degradation pathway.

**DISCUSSION**

Although most cells in the adult animal exist in a G0 growth arrest state, little is known about the regulation and function of genes expressed during G0 (1–4). This study investigated the posttranscriptional and posttranslational regulation of C/EBP6 in G0 growth-arrested mouse MECs in vitro. Previous reports...
Fig. 9. C/EBPβ protein ubiquitination is localized to the nuclear compartment. Western blot analysis was performed on 25 μg of nuclear and cytoplasmic protein isolated from untreated or MG-132-treated G0 growth-arrested mouse HC11 MECs at the indicated time points as described in Fig. 7. Lanes 1, 3, and 5, nuclear protein from G0 growth-arrested MECs (control); lanes 2, 4, and 6, cytoplasmic protein from G0 growth-arrested MECs (control); lanes 7 and 9, nuclear protein from G0 growth-arrested MECs treated with vehicle (+DMSO); lanes 8 and 10, cytoplasmic protein from G0 growth-arrested MECs treated with vehicle (+DMSO); lanes 11 and 13, nuclear protein from G0 growth-arrested MECs treated with ubiquitination inhibitor (+MG-132); lanes 12 and 14, cytoplasmic protein from G0 growth-arrested MECs treated with ubiquitination inhibitor (+MG-132). Results are representative of three independent experiments.

from our laboratory (11–13) have shown that C/EBPβ gene expression and DNA binding activity increase in G0 growth-arrested MECs. The G0-specific increase in C/EBPβ gene expression is STAT3-dependent (10). In addition, overexpression of C/EBPβ in MECs accelerated G0 growth arrest and apoptosis in response to serum and growth factor withdrawal (13). In contrast, reducing C/EBPβ levels by antisense RNA delayed MEC G0 growth arrest and apoptosis after serum and growth factor withdrawal (13). In this report, we demonstrated that C/EBPβ mRNA exhibits a novel short half-life during G0 growth arrest in mouse MECs (t1/2, 35 min). Interestingly, mRNAs encoding several important cell cycle control proteins, growth factors, lymphokines, cytokines, and proto-oncogenes also exhibit short half-lives (35). For example, the cytokine interleukin 6, which is important in the inflammatory response, has an mRNA half-life of ~20 min (65). We suggest that the short C/EBPβ mRNA half-life in G0 growth-arrested MECs allows the cells to respond rapidly to potential growth stimuli. Interestingly, the short half-life of C/EBPβ mRNA observed in mouse MECs appears to be a property of mammary epithelial derived cell lines. For example, in G0 growth-arrested NIH 3T3 cells, the C/EBPβ mRNA half-life is ~2–3-fold longer. This suggests that tight regulation of the C/EBPβ mRNA is important in the initiation and maintenance of G0 growth arrest in mouse MECs. Similar to our studies in mouse HC11 MECs, the C/EBPβ mRNA exhibited a relatively short half-life of ~40 min in G0 growth-arrested human MCF-12A MECs (data not shown). This suggests a conservation of mRNA decay kinetics for C/EBPβ in both mouse and human MEC systems.

Cell cycle re-entry (G0/G1 transition) is associated with dramatic changes in gene expression. Transcription of growth arrest genes is known to decrease with cell cycle re-entry, although mRNAs encoding growth arrest-specific proteins could persist and may delay or interfere with cell cycle re-entry. The disposal of G0-specific mRNAs and proteins during MEC cell cycle re-entry is not well characterized. This study sought to determine whether or not the decay kinetics of C/EBPβ mRNA were similar between G0 growth arrest and the G0/G1 transition. Results of transcriptional inhibitor/Northern blot studies upon cycle re-entry demonstrate that C/EBPβ mRNA has a longer half-life during the G0/G1 transition compared with G0 growth-arrested HC11 MECs. In fact, both C/EBPβ and gas-1 mRNAs exhibited stabilization during cell cycle re-entry in response to transcriptional inhibitors in two mouse MEC lines, HC11 and COMMA D (later data not shown). Post-transcriptional control is known to play a major role in the regulation of gas family members in many cell types (61, 66, 67). Our results parallel previous work that shows an increase in gas-1 and gas-6 mRNA stability after cell cycle re-entry and treatment with actinomycin D of fibroblastic cell lines (61, 66, 67). Furthermore, actinomycin D treatment of Schwann cells during cell cycle re-entry stabilized the gas-3 mRNA (68). The difference in C/EBPβ mRNA half-life in G0 growth arrest and the G0/G1 transition suggests that there is a specific mRNA degradation pathway for C/EBPβ during the G0/G1 transition that differs from G0 growth arrest. In addition, increased stabilization of the C/EBPβ mRNA suggests that the synthesis of a trans-acting factor(s) or RNA is required to degrade the C/EBPβ mRNA upon cell cycle re-entry.

The mechanism underlying C/EBPβ mRNA degradation is currently not known, although numerous studies have demonstrated that the length of the poly(A) tail is a major factor in the stability of eukaryotic mRNAs (i.e., a decrease in poly(A) tail length results in an decrease in mRNA stability) (34, 35, 39, 63). In this report, analysis of poly(A) tail length by oligo/RNase H cleavage and RACE-PAT demonstrated that the C/EBPβ mRNA has a short poly(A) tail of ~100 nucleotides. This is somewhat shorter than the average eukaryotic mRNA that contains a poly(A) tail of ~200 nucleotides (69).

Structural elements found within the 5′-UTR, coding region, and the 3′-UTR are known to be involved in regulating mRNA stability (34, 35, 39). For example, the 3′-UTR of many labile mRNAs, such as cytokine and oncoprotein mRNAs, contain multiple copies of AU-rich elements (AREs) (34, 35, 39). These cis-acting elements interact with trans-acting factors to destabilize the mRNA. Analysis of ARE sequences from 12 transcription factor-encoding mRNAs that exhibit early G1 instability classified two distinct groups of mRNAs: 1) mRNAs with 3′-UTRs that contain one or more copies of the well recognized “AUUUA” sequence and 2) mRNAs with 3′-UTRs that contain one or more copies of a “non-AUUUA” sequence (70). An example of a non-AUUUA mRNA is c-jun, which contains “U”-rich regions that confer G1 instability (70). Interestingly, analysis of the C/EBPβ 3′-UTR revealed a single AUUUA element and two U-rich regions (region 1, 18 uracils/32 nucleotides; region 2, 17 uracils/26 nucleotides). This indicates that the C/EBPβ mRNA has characteristics of both AUUUA and non-AUUUA AREs. Mutational analysis is ongoing to characterize further the role of these instability elements in C/EBPβ mRNA decay.

Analysis of another C/EBP family member, C/EBPβ, demonstrated similar mRNA decay kinetics as C/EBPβ during mouse MEC G0 growth arrest. Like C/EBPβ, C/EBPβ mRNA displayed a short half-life of ~45 min. The results suggest a conserved mRNA decay pathway shared between C/EBPβ and C/EBPβ in G0 growth-arrested mouse MECs. Although the homology between the C/EBPβ and C/EBPβ 3′-UTRs is ~30%, both 3′-UTR sequences contain multiple U-rich elements that may regulate mRNA degradation.

Because the C/EBPβ mRNA was shown to have a short half-life in G0 growth-arrested MECs, we hypothesized that the C/EBPβ protein would exhibit a similar short biological half-life (39). A yeast genome-wide analysis has demonstrated that unstable mRNAs encode for unstable proteins (71). Examples include translation initiation factors, termination factors, and proteins of the mating pheromone signal transduction pathway (71). The results in this report established that the half-life of the C/EBPβ protein is shorter (t1/2 ~120 min) than the tumor suppressor, p27 (t1/2 >150 min). The short half-life of the C/EBPβ protein in G0 growth-arrested MECs suggests that C/EBPβ function is tightly reg-
ulated during MEC quiescence, which may allow MECs to respond rapidly to growth signals and re-enter the cell cycle when necessary.

The ubiquitin-proteasome pathway is a major selective decay mechanism of short-lived regulatory proteins (49). Cell cycle regulatory proteins that are degraded by the ubiquitin-proteasome pathway include the tumor suppressors, p21 (17 min), p53 (75–150 min) (50). The results in this report established that the C/EBPβ protein is also degraded via the ubiquitin-proteasome pathway in growth-arrested MECs (tG0 – 120 min). It has yet to be determined whether phosphorylation of the C/EBPβ protein precedes ubiquitination, which has been observed in the regulation of p27 protein decay.

It has been known for some time that mammalian proteasome complexes are localized throughout the cell including the nucleus, cytoplasm, and within the endoplasmic reticulum membrane network (55, 56). Proteasomes localized within the nucleus have been shown to be responsible for the turnover of short lived proteins important for many critical cellular processes. Some proteins that undergo ubiquitination within the nuclear compartment include the large subunit of RNA polymerase II (74), the progesterone receptor (75), the nuclear compartment include the large subunit of RNA polymerase II (74), the progesterone receptor (75), the large subunit of RNA polymerase II (74), the progesterone receptor (75), the large subunit of RNA polymerase II (74), the progesterone receptor (75). It is speculated that cells are able to rid themselves of nuclear proteins that are no longer necessary by ubiquitination within the nucleus (57). Results of this study demonstrate that C/EBPβ protein ubiquitination is localized to the nucleus. We speculate that nuclear protein degradation provides a mechanistic explanation for the relatively short half-life of the C/EBPβ protein during MEC G0 growth arrest and allows for proper cell cycle progression during the G0/G1 transition.

In summary, the data presented establish that the C/EBPβ mRNA has a short half-life in G0 growth-arrested MECs. The C/EBPβ mRNA has a relatively short poly(A) tail (~100 nucleotides) that does not vary in length during decay in G0 growth arrest or the G0/G1 transition. It is proposed that the C/EBPβ mRNA is degraded by a mechanism involving endonucleolytic cleavage during G0 growth arrest. Additionally, the C/EBPβ protein has a relatively short half-life in G0 growth-arrested MECs and is degraded by the ubiquitin-proteasome pathway within the nuclear compartment. This study suggests that despite the decrease in cellular activity during G0 growth arrest, C/EBPβ mRNA and protein are tightly regulated in MECs. We predict that this tight regulation allows G0 growth-arrested MECs to proliferate in response to growth stimuli. Studies investigating possible instability elements in the C/EBPβ mRNA 3'–UTR and characterization of trans-acting factors important in C/EBPβ mRNA degradation are currently underway.

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