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CCAAT/Enhancer Binding Protein δ (C/EBPδ) Regulation and Expression in Human Mammary Epithelial Cells: II. Analysis of Activating Signal Transduction Pathways, Transcriptional, Post-Transcriptional, and Post-Translational Control

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Abstract CCAAT/enhancer binding protein δ (C/EBPδ) plays a key role in mammary epithelial cell G_0 growth arrest. C/EBPδ gene expression is down-regulated in rodent mammary tumorigenesis and in human breast cancer, suggesting that ''loss of function'' alterations in C/EBPδ gene expression are common in mammary gland malignancies. The goal of this study was to systematically investigate the mechanisms controlling C/EBPδ gene expression in MCF-10A and MCF-12A human mammary epithelial cell lines. The results demonstrate that G_0 growth arrest conditions (i.e., serum and growth factor withdrawal or Oncostatin M (OSM) treatment) result in the activation of JAK1, JAK2, and Tyk 2, members of the Janus kinase family of non-receptor tyrosine kinases, in MCF-10A and MCF-12A cells. Growth arrest or OSM treatment also specifically increases activated (phosphorylated) signal transduction and activators of transcription 3 (STAT3) levels, demonstrating that STAT3, not STAT1 or STAT5, is the downstream target of the activated Janus kinases in MCF-10A and MCF-12A cells. Whole cell lysates from G_0 growth arrested (GA) and OSM-treated MCF-12A cells exhibit increased acute phase response element (APRE) binding compared to lysates from growing (GR) MCF-12A cells. Transient transfection using C/EBPδ promoter-luciferase constructs demonstrated that the APRE (STAT3) consensus binding site is essential for growth arrest or OSM induction of the C/EBPδ promoter. Mutation of the C/EBPδ promoter STAT3 site or expression of a dominant negative STAT3 construct (STAT3β) reduces C/EBPδ promoter activity in response to growth arrest conditions. The human C/EBPδ promoter also contains an Sp1 site at −61 bp (relative to the transcriptional start site) which is required for basal transcriptional activation. Mutation or deletion of the Sp1 site decreases promoter activity in response to growth arrest conditions. Treatment with the transcriptional inhibitor actinomycin D demonstrated that the C/EBPδ mRNA exhibits a relatively short half-life (~40 min). Similarly, treatment with the translational inhibitor anisomysin demonstrated that the C/EBPδ protein half-life was also relatively short (~160 min). These results indicate that the human *C/EBPδ* gene is controlled at multiple levels, consistent with a role for C/EBPδ in cell cycle control and/or cell fate determination. J. Cell. Biochem. 93: 844–856, 2004. © 2004 Wiley-Liss, Inc.

Key words: CCAAT/enhancer binding protein δ (C/EBPδ); growth arrest; mammary epithelial cells; cytokine; Oncostatin M

Mammalian cells exit the cell cycle and enter G_0 growth arrest in response to environmental conditions, such as the presence of specific growth inhibitors, removal of growth stimuli, or contact inhibition [Pardee, 1989; Ford and Pardee, 1999]. Alterations in G_0-specific signaling pathways, or the genes that function in the initiation and maintenance of G_0 growth arrest, can result in aberrant cell proliferation and progression to neoplasia. The Von Hippel–Lindau (*VHL*) tumor suppressor gene, for example,

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functions in cell cycle exit/G_0 entry in response to serum and growth factor withdrawal [Pause et al., 1998]. Hereditary loss of the VHL tumor suppressor gene predisposes individuals to a rare cancer syndrome characterized by an increased incidence of renal cell carcinomas, pheochromocytomas, and vascular tumors [Latif et al., 1993; Baba et al., 2003].

We previously reported that CCAAT/enhancer binding protein δ (C/EBPδ) functions in cell cycle exit/G_0 entry in mouse and human mammary epithelial cells, and that ectopic C/EBPδ expression inhibits in vitro mammary epithelial cell growth [O'Rourke et al., 1997, 1999b; Sivko and DeWille, 2004]. In addition, in vivo studies further demonstrate the growth suppressive role of C/EBPδ, in that nulliparous C/EBPδ (−/−) knockout female mice exhibit aberrant mammary epithelial cell growth control, resulting in excessive ductal branching [Gigliotti et al., 2003]. C/EBPδ is a member of the highly conserved C/EBP family of leucine zipper proteins which are important in the regulation of growth and differentiation in a variety of tissues. There are six C/EBP family members: α, β, γ, δ, ε, and ζ (also called CHOP (C/EBP homologous protein, or GADD153). C/EBPs function as transcription factors, forming homo or heterodimers with both C/EBPs and other leucine zipper proteins [Cao et al., 1991; Hurst, 1995; Ramji and Foka, 2002]. C/EBPs also influence cell cycle progression by binding to key cell cycle regulatory proteins and protein complexes such as retinoblastoma protein (pRb), E2F, p107, p21, and Cdk2 and Cdk4 [Timchenko et al., 1996, 1999; Park et al., 2000; Charles et al., 2001; Gheorghiu et al., 2001; Harris et al., 2001; Wang et al., 2001].

The growth suppressor/tumor suppressor role of C/EBPδ in the mammary gland is also supported by gene expression profile studies in rodent carcinogen-induced mammary tumors and in human breast cancer. In rodent carcinogenesis, DMBA and PhIP reproducibly induce mammary tumors; however, DMBA and PhIP induce different genetic lesions, and presumably alter the structure and expression of different cellular genes [Kuramoto et al., 2002]. Despite differences in genetic alterations, both DMBA and PhIP-induced rat mammary tumors exhibit down-regulation of C/EBPδ gene expression [Kuramoto et al., 2002]. This suggests that loss of C/EBPδ expression is a common factor in the development or progression of mammary tumorigenesis, irrespective of the primary genetic lesion. The growth suppressor/tumor suppressor role of C/EBPδ is also supported by gene expression profiles in human breast cancer patients. Polyak et al. recently utilized serial analysis of gene expression (SAGE) to investigate the molecular alterations associated with breast cancer progression. Their analysis of approximately 50,000 unique transcripts identified C/EBPδ as one of a small subset of genes (17 genes) that were consistently down-regulated with progression from normal human mammary epithelium to breast carcinoma [Porter et al., 2001]. In a follow-up study, Polyak et al. used SAGE to assess molecular markers of ductal carcinoma in situ (DCIS) [Porter et al., 2003]. Their comparative analysis, which included normal breast epithelium, DCIS, and invasive and metastatic breast cancer isolates also identified C/EBP among a limited subset of genes (34 genes) consistently down-regulated in association with the transition from normal to DCIS [Porter et al., 2003].

Despite the growing evidence that C/EBPδ plays a key role in mammary epithelial cell growth control and breast cancer progression, the molecular mechanisms controlling human C/EBPδ gene expression have not been extensively investigated. Transcriptional activation of the mouse C/EBPδ promoter has been investigated in cultured hepatocytes and mammary epithelial cells [Cantwell et al., 1998; Hutt et al., 2000]. The results indicate that a consensus signal transduction and activators of transcription 3 (STAT3) site (located at −106 base pairs (bp) upstream of the transcription start site) and two Sp1 sites (−117 and −53 bp upstream of the transcription start site) play essential roles in basal and regulated expression from the mouse C/EBPδ promoter [Cantwell et al., 1998; Hutt et al., 2000]. The structure of the human C/EBPδ promoter, however, varies from the mouse. For, example, the human C/EBPδ promoter lacks the upstream Sp1 site, which is essential for basal expression in the mouse. In addition, the remaining STAT3 (−282) and Sp1 sites (−61) are not in close linear proximity to each other as they are in the mouse. These structural differences may alter interactions between key transcriptional activators and the human C/EBPδ promoter and these alterations may influence C/EBPδ gene expression.

In addition to transcriptional control, gene expression can also be regulated at the post-

transcriptional and post-translational levels [Ross, 1996; Wilkinson, 2000; Guhaniyogi and Brewer, 2001]. Post-transcriptional regulation is mediated by interactions between transactivating factors and mRNA consensus elements located in the 5′UTR, coding region, 3′UTR, and 3′ poly[A]tail [Guhaniyogi and Brewer, 2001]. The C/EBPδ mRNA exhibits a short half-life ($t_{1/2}$ ~35 min) in G_0 growth arrested (GA) mouse mammary epithelial cells [Dearth and DeWille, 2003b] that parallels other important cell cycle control proteins, growth factors, cytokines, and proto-oncogenes [Guhaniyogi and Brewer, 2001]. Relatively small changes in transcription or half-life of short-lived mRNAs can result in significant (~1,000-fold) differences in mRNA content over a short period of time [Ross, 1996]. Changes in mRNA content due to alterations in gene transcription or mRNA stability have been implicated in a wide range of pathological conditions such as myotonic dystrophy, α-thalassemia, Alzheimer's, and neoplastic transformation [Conne et al., 2000; Guhaniyogi and Brewer, 2001].

Timely destruction of cell regulatory proteins is important for appropriate cell cycle progression. We previously reported that the mouse C/EBPδ protein exhibits a relatively short biological half-life ($t_{1/2} \sim 120$ min) in mouse mammary epithelial cells, paralleling the half-life the mouse p27 ($t_{1/2} > 150$ min) [Dearth and DeWille, 2003b]. The proteolysis of many important positive and negative cell cycle regulatory proteins is mediated by the ubiquitin–proteosome pathway [Bashir and Pagano, 2003]. This degradation pathway targets proteins for proteosome destruction by marking them with polyubiquitin chains [Bashir and Pagano, 2003]. Regulation of the ubiquitin–proteosome pathway is important, as enhanced degradation of negative cell cycle regulators (i.e., p27) or reduced degradation of positive cell cycle regulators (i.e., cyclin E) can influence cell cycle control and oncogenesis [Slingerland and Pagano, 2000; Bashir and Pagano, 2003; Bloom and Pagano, 2003].

The overall goal of this study was to investigate the signal transduction pathway involved in regulating C/EBPδ transcription in G_0 growth-arrested and OSM-treated human mammary epithelial cells, locate specific regions within the C/EBPδ promoter important for transcriptional activation, and determine post-transcriptional and post-translational regulatory mechanisms involved in C/EBPδ regulation. The work described here demonstrates that JAK1, JAK2, and Tyk2 are all activated during G_0 growth arrest and OSM-treatment of immortalized human mammary epithelial cells MCF-10A and MCF-12A. Activation of STAT3 and not STAT1 or STAT5 by these kinases is responsible for C/EBPδ promoter activation in both GA and OSM-treated cells. Transfection and EMSA assays demonstrate that STAT3 binding is increased during growth arrest and is important for C/EBPδ transcription. Co-transfection of a dominant negative STAT3 or mutation of this STAT3 binding site reduces C/EBPδ promoter activity by over 50%. An Sp1 site 61 bases upstream of the transcriptional start site plays a role in C/EBPδ basal promoter activity; as removal or mutation of this site results in diminished promoter activity under growth arrest conditions and limited inducibility of the C/EBPδ promoter by OSM. C/EBPδ mRNA and protein both have short half-lives, ~40 and ~160 min, respectively. This suggests that C/EBPδ expression is tightly regulated during the G_0 growth arrest of human mammary epithelial cells.

MATERIALS AND METHODS

Cell Culture

Cell culture media and components were purchased from Invitrogen/Life Technologies, Inc. (Carlsbad, CA) and Sigma-Aldrich, Inc. (St. Louis, MO). MCF-10A and MCF-12A cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in 1:1 DMEM:F-12 phenol red free media with the addition of 20 ng/ml human recombinant EGF, 100 ng/ml cholera toxin, 10 μg/ml bovine insulin, 500 ng/ml hydrocortisone, 5% horse serum, 100 U/ml penicillin:100 μg/ml streptomycin, and 500 ng/ml Fungizone. MCF-10A and MCF-12A cell lines were GA in 1:1 DMEM/F-12 with 0.1% horse serum.

Growth Arrest, Cytokine Addition, and Drug Treatments

Near confluent (70%) cells were rinsed and then cultured in serum and growth factor deprived media for 24 h. Human Oncostatin M (OSM) was obtained from Peprotech (Rocky Hill, NJ), reconstituted in sterile water and added at a dose of 50 ng/ml for 12 h. Interferon-γ (IFN-γ) was obtained from Peprotech, reconsti-

tuted in 1× PBS pH 8.0 and added at a dose of 10 ng/ml for 12 h. AG490 was purchased from Calbiochem (La Jolla, CA), reconstituted in DMSO and added at doses of 100 and 150 μM. Transcriptional inhibitor actinomycin D (Sigma) was added at 5 μg/ml. Translational inhibitor studies utilized anisomycin (Sigma) at a dose of 10 μg/ml.

Northern Blot Analysis

Total RNA was isolated using RNAzol B (Tel-Test, Inc., Friendswood, TX), run on 1.2% agarose gels and transferred to Duralon UV membrane followed by UV crosslinking. Filters were hybridized for a minimum of 2 h. DNA probes were labeled with [alpha-^{32}P]dCTP using a Random Primers Labeling Kit (Invitrogen), added to the hybridization mixture and allowed to hybridize a minimum of 2 h. C/EBPδ probe was a 700 bp fragment isolated from Research Genetics Clone 82850. Cyclophylin (CP) was used as a loading control.

Western Blot Analysis

Whole cell protein was isolated using a whole cell lysis buffer containing 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% IGEPAL, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, 1× complete protease (Boehringer Mannheim), 1 mM NaF, 1 mM $NaVO_3$, 1 mM Na_2MoO_4, 100 nM okadaic acid. Proteins were run on precast BioRad 7.5–10% Tris-HCl gels and were transferred to PVDF membrane. Rabbit polyclonal C/EBPδ and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal STAT1, phospho-STAT1(Tyr701), STAT3, phospho-STAT3(Tyr-705), phospho-STAT5(Tyr694), and phospho-Tyk2(Tyr1054/1055) were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit polyclonal phospho-JAK1(Tyr1022/1023) and phospho-JAK2(Tyr1007/1008) were obtained from Calbiochem. Mouse monoclonal STAT5 and p27 were obtained from BD Transduction Laboratories (BD Pharmingen, San Diego, CA). Rabbit polyclonal JAK1, JAK2, and Tyk2 were obtained from Upstate Cell Signaling Solutions (Charlottesville, MD). Westerns were blocked in 10% milk with primary and secondary antibody addition in 5% milk. Signal was developed using ECL and ECL Plus detection systems (Amersham Biosciences, Piscataway, NJ). STAT3, phospho-STAT3-(Tyr705), and β-actin signals were quantified with AlphaImager 2000 Documentation and Analysis System software (Alpha Innotech, San Leandro, CA).

C/EBPδ Promoter Constructs

A 1.7 kb fragment of the human C/EBPδ promoter (generous gift of Dr. Trapman, (Erasmus University, Rotterdam, The Netherlands)) was cloned into the pGL3-basic luciferase vector. Deletion constructs were created by PCR with end points at −1,700, −800, −393, −75, and −43 bp relative to the transcription start site. Site-directed mutagenesis was used to create mutations in the STAT3 and Sp1 sites within the −393 bp C/EBPδ promoter/luciferase construct individually (STAT3 or Sp1) and in combination (STAT3 and Sp1-double mutant).

Transient Transfection Assays

Transient transfections were performed in 60–80% confluent cells in 60 mm culture dishes using the Lipofectamine Plus transfection system (Invitrogen). Cells were co-transfected with 1 μg of each promoter and 50 ng of CMV-Renilla or SV40-β-galactosidase (transfection efficiency controls) for 3 h, rinsed with PBS, and the media changed to either growth arrest media or growth arrest media containing 50 ng/ml OSM. Cells were harvested after 24 h and assayed using the Dual-Luciferase® Reporter Assay System (Promega) and Luminescent β-galactosidase Detection Kit II (Clontech). Luminescence was measured using a Hewlett-Packard Lumicount microplate luminometer. C/EBPδ promoter activities were normalized to renilla or β-galactosidase activities to control for transfection efficiency and the fold induction of OSM over growth arrest media alone was calculated. Results shown are the average fold increase of 3–4 independent experiments with 3 replicates per fragment and treatment per experiment.

Dominant-Negative Stat3 Transfection Assays

An optimized amount (6 μg) of STAT3β (generous gift of Dr. Richard Jove) or pSG5 control plasmid, 1 μg of −393 bp C/EBPδ promoter/luciferase construct, and 50 ng CMV-renilla were co-transfected into 80% confluent cells. Cells were harvested and assayed as described above.

Electrophoretic Mobility Shift Assays

Nuclear extracts were harvested from growing (GR), 48 h GA, and 48 h GA cells treated with

50 ng/ml OSM. Binding assays were carried out in 20 μl of cocktail containing 20 mM HEPES (pH 7.9), 50 mM NaCL, 1 mM DTT, 1 mM PMSF, 10% glycerol, 2 μg of poly (dI-dC), 7 μg of nuclear extracts, and 100,000 cpm of the double-stranded oligonucleotides. Sequence of the upper strand oligonucleotide used with the STAT3 consensus binding site (acute phase response element (APRE)) underlined is as follows: 5′-GAT CC<u>T TCT GGG AAT T</u>CC TAG ATC-3′. The binding reaction mixtures were incubated at room temperature for 20 min. Samples were electrophoresed on 5% native acrylamide gels in 0.5× Tris-borate–EDTA at 200 V at 4°C for 2 h, transferred to 3 mm paper (Whatman), dried under vacuum and exposed to a PhosphorImager cassette.

RESULTS

Activation of Signal Transduction and Activators of Transcription (STAT) Family Members in MCF-10A and MCF-12A, Immortalized, Non-Tumorigenic Human Mammary Epithelial Cells During G_0 Growth Arrest Induction by Serum and Growth Factor Withdrawal, OSM and IFN-γ Addition

We have previously demonstrated the activation of STAT3, induction of C/EBPδ mRNA and protein, and the initiation of G_0 growth arrest in mouse and human mammary epithelial cells following serum and growth factor withdrawal and OSM addition [Hutt et al., 2000; Hutt and DeWille, 2002; Sivko, manuscript submitted]. Three STAT family members, STAT1, STAT3, and STAT5 play essential regulatory roles in mammary epithelial cells [Watson, 2001]. To determine specific STAT activation during serum and growth factor withdrawal and OSM addition, whole cell protein lysates were harvested from GR, growth-arrested, and OSM-treated MCF-10A and MCF-12A human mammary epithelial cells and analyzed by Western blotting. Phosphorylated STAT3 (Tyr705) was detected in MCF-10A and MCF-12A human mammary epithelial cells cultured under growth arrest conditions (serum and growth factor withdrawal) (Fig. 1A,B, GA, lane 2). Phosphorylated STAT3 (Tyr705) levels further increased in both MCF-10A and MCF-12A human mammary epithelial cell lines with OSM treatment (Fig. 1A,B, OSM, lane 3). In contrast, phosphorylated forms of STAT1 and STAT5 were not detected with GA or OSM

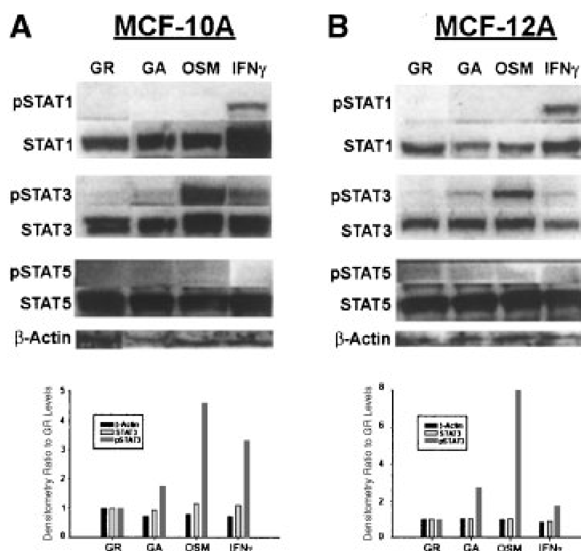

Fig. 1. Signal transduction and activators of transcription 3 (STAT3), but not STAT1 or STAT5, is phosphorylated (Tyr705) during growth arrest and Oncostatin M (OSM) treatment of MCF-10A and MCF-12A mammary epithelial cell lines. Whole cell proteins were harvested from growing (GR, **lane 1**), growth arrested (GA, **lane 2**), and OSM treated (**lane 3**) MCF-10A and MCF-12A cells. Western blots were probed with phospho-specific and phospho-independent STAT1, STAT3, and STAT5 antibodies. Western blot demonstrating phosphorylation of STAT3 (Tyr705), but not STAT1 or STAT5 during growth arrest and OSM treatment in (**A**) MCF-10A and (**B**) MCF-12A cell lines. STAT3, phosho-STAT3, and β-actin levels were quantified by spot densitometry and related to GR levels to calculate induction of phospho-STAT3 with growth arrest and OSM treatment. Interferon-γ (IFN-γ) (**lane 4**) was used as a positive control for STAT1 and STAT3 activation.

treatment. IFN-γ treatment was used as a positive control for STAT1 activation, and resulted in the activation of STAT1 and also STAT3, but not STAT5 in both MCF-10A and MCF-12A cell lines (Fig. 1A,B, IFN-γ, lane 4). Phospho-STAT3 quantification revealed a 2–3 fold induction in phospho-STAT3 following GA treatment and a 5–8 fold induction with OSM treatment over levels in GR cells. Nonphosphorylated STAT1, STAT3, STAT5, and β-actin levels do not vary across treatment groups.

Activation of Janus Kinase (JAK) Family Members in MCF-10A and MCF-12A Human Mammary Epithelial Cells and HC11 Mouse Mammary Epithelial Cells During G_0 Growth Arrest Induction by Serum and Growth Factor Withdrawal and OSM Addition

OSM activates receptor-associated tyrosine kinases JAK1, JAK2, and Tyk2 following binding to either the Type I (LIF/gp130) or Type II (OSMβ) receptors [Grant and Begley, 1999;

Hermanns et al., 1999]. To determine which upstream regulators of STAT3 are activated during G_0 growth arrest, whole cell protein lysates were harvested from GR, growth-arrested, and OSM-treated MCF-10A and MCF-12A mammary epithelial cells and analyzed by Western blotting using phospho-specific antibodies. MCF-10A and MCF-12A human mammary epithelial cell lines exhibit approximately similar degrees of increased phosphorylation (activation) of JAK1, JAK2, and Tyk2 in response to growth arrest induced by serum and growth factor withdrawal (Fig. 2A,B, GA, lane 2). In addition, phosphorylated JAK1, JAK2, and Tyk2 levels are further increased in both MCF-10A and MCF-12A human mammary epithelial cell lines in response to OSM treatment (Fig. 2A,B, OSM, lane 3). Both MCF-10A and MCF-12A cell lines demonstrate ~2-fold increases in phospho-JAK1 with growth factor withdrawal and ~4-fold increases with OSM addition over levels in GR cells. MCF-10A cells also increased phospho-JAK2 levels 2-fold with growth factor withdrawal and 4-fold with OSM addition. The MCF-12A cells, however, only minimally activate JAK2 with growth factor withdrawal, but demonstrate a 4-fold increase with OSM addition. Phospho-Tyk2 levels increase 2-fold in both cell lines with growth factor withdrawal and 2–3 fold with OSM addition. The Tyk2 response appears to be greater in the MCF-10A compared to the MCF-12A cells; however, the "growing" MCF-12A cells contain a minimal amount of phospho-Tyk2. This is most likely the result of a small population of GA MCF-12A cells exhibiting contact inhibition due to cell crowding in the GR cell culture. IFN-γ treatment (IFN-γ, lane 4) was used as a positive control for JAK1 and 2 activation in both cell lines. Nonphosphorylated JAK1, JAK2, and Tyk2 and β-actin levels do not vary across treatment groups.

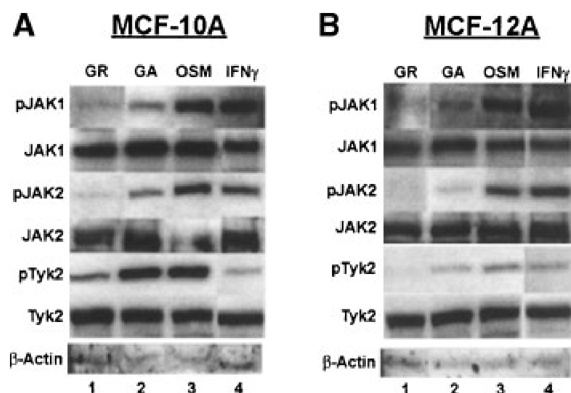

Fig. 2. JAK1, JAK2, and Tyk2 are phosphorylated during G_0 growth arrest and OSM treatment of MCF-10A and MCF-12A mammary epithelial cell lines. Whole cell proteins were harvested from GR (**lane 1**), GA (**lane 2**), and OSM treated (**lane 3**) MCF-10A and MCF-12A cells. Western blots were probed with antibodies to phospho-specific and phospho-independent JAK1, JAK2, and Tyk2. Western blot demonstrating phosphorylation of JAK1, JAK2, and Tyk2 during growth arrest and OSM treatment of (**A**) MCF-10A and (**B**) MCF-12A cell lines. IFN-γ (**lane 4**) was used as a positive control for JAK activation.

Identification of C/EBPδ Promoter Regions That Function in the Transcriptional Activation of the *C/EBPδ* Gene During G_0 Growth Arrest of MCF-12A Human Mammary Epithelial Cells

To determine the growth arrest response region of the C/EBPδ promoter, 5′ promoter deletion constructs were generated from a 1.7 kb C/EBPδ promoter-luciferase construct in pGL3, with end points at −43, −75, −233, −393, −800, and −1,700 bases relative to the transcription start site. The expression level of each C/EBPδ promoter-luciferase construct during G_0 growth arrest is presented in Figure 3A. Expression under GR conditions was minimal for all C/EBPδ promoter-luciferase constructs. Expression under growth arrest conditions was similar for the −1,700 and −800 C/EBPδ promoter-luciferase constructs. The −393 bp C/EBPδ promoter-luciferase construct exhibited greater activity than both the −1,700 and −800 constructs. Deletion to position −233 bp decreased C/EBPδ promoter activity by approximately 50%. Deletion to −75 bp did not further diminish activity, however, deletion to −43 bp eliminated expression altogether. These results indicate that a region or specific site between −233 and −393 is responsible for activation of the human C/EBPδ promoter under growth arrest conditions. OSM addition increased activity of the −1,700, −800, −393, −233, and −75 bp C/EBPδ promoter-luciferase constructs. The −393 C/EBPδ promoter-luciferase construct activity increased approximately 2-fold with OSM addition. A significant decrease in OSM inducibility was observed between the −393 and −233 constructs. These data indicate that the region between −233 and −393 contains a region or specific site that is responsive to OSM. Sequence analysis indicates a STAT3 binding site 282 bp upstream of the transcriptional start site of the human C/EBPδ promoter similar to that previously identified in the

mouse C/EBPδ promoter [Hutt et al., 2000]. These results suggest that STAT3 is important in C/EBPδ promoter activation under growth arrest conditions with enhanced activation following the addition of OSM. Analysis of the −43 to −75 bp region indicates an Sp1 site 61 bases upstream of the transcriptional start site. Since removal of this site decreases promoter activity to basal levels under both growth arrest and OSM-treated conditions, Sp1 appears to play a role in basal C/EBPδ promoter activity during G_0 growth arrest and OSM treatment.

To verify the importance of STAT3 in C/EBPδ promoter activity during growth arrest, MCF-12A cells were co-transfected with the −393 bp C/EBPδ promoter-luciferase construct, cytomegalovirus-renilla construct, and increasing amounts of either dominant negative STAT3β construct or pSG5 control plasmid. C/EBPδ promoter-luciferase activities after 24 h of growth arrest are shown in Figure 3B. Compared with the pSG5 construct, the STAT3δ construct decreased C/EBPδ promoter activity by 50%. Similarly, the addition of JAK inhibitor AG490 decreased STAT3 phosphorylation and C/EBPδ protein expression when compared to a DMSO-treated control (Fig. 3C). These results are consistent with a role for STAT3 in the induction of C/EBPδ gene expression in response to growth arrest and OSM treatment.

Mutations in the STAT3 and Sp1 Binding Sites Decrease Inducible and Basal Activity of the C/EBPδ Promoter, Respectively

To further investigate the functional roles of the STAT3 and Sp1 consensus binding sites in

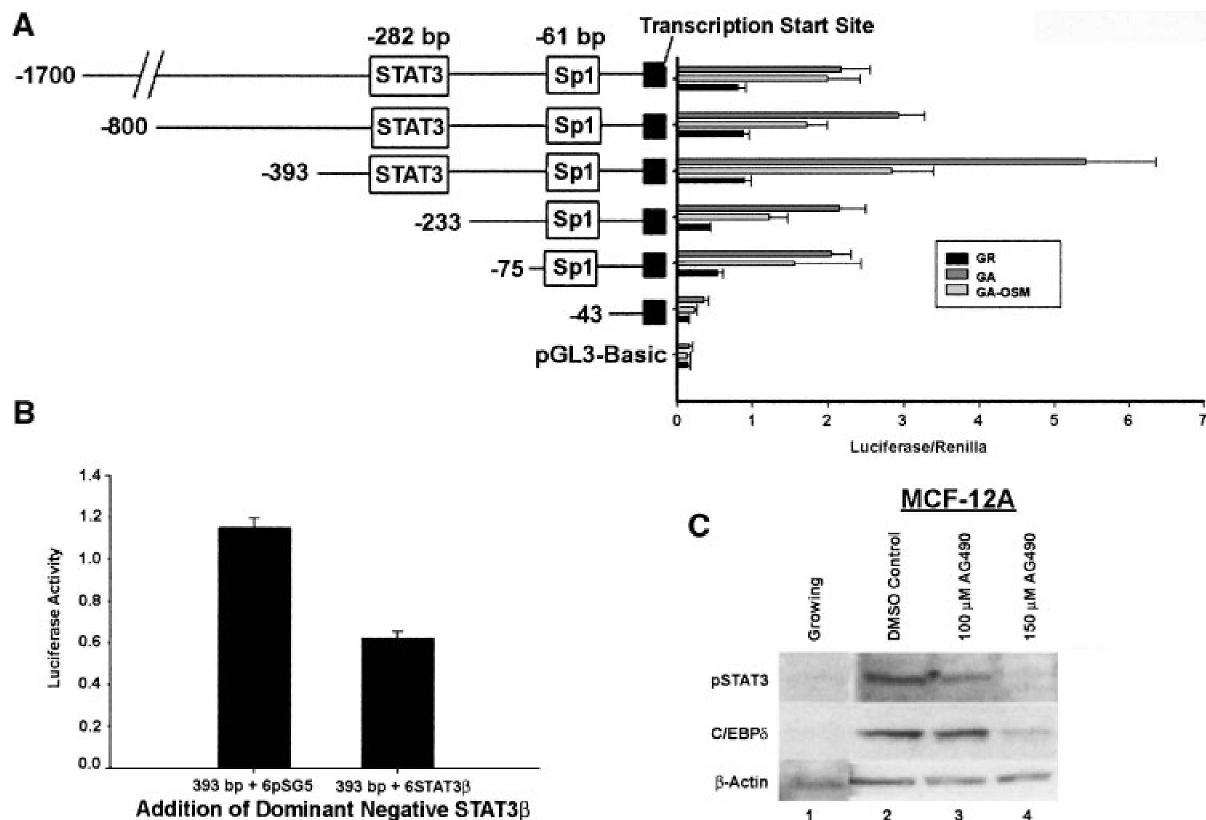

Fig. 3. Identification of an OSM and growth arrest responsive region in the human CCAAT/enhancer binding protein δ (C/EBPδ) promoter. **A**: Luciferase promoter constructs containing 5′ promoter deletions were co-transfected along with CMV-Renilla into MCF-12A cells. Transfected cells were subsequently GA or OSM treated for 24 h. Luciferase data was normalized to renilla values to control for differences in transfection efficiency. Values are representative of 10 transfected plates (four independent experiments) ± standard error. **B**: MCF-12A cells transfected with 1 μg of −393 bp C/EBPδ promoter construct, 50 ng CMV-Renilla, and either 6 μg STAT3β or 6 μg pSG5 (−control) were maintained in growth arrest media for 24 h. Luciferase activity was normalized to renilla values to control for transfection efficiency differences. **C**: Western blot demonstrating the increase in phospho-STAT3 and C/EBPδ protein levels with growth arrest and the decrease in phospho-STAT3 and C/EBPδ protein levels with JAK inhibitor AG490 treatment. Whole cell proteins were harvested from GR MCF-12A cells (**lane 1**) and GA MCF-12A cells treated for 24 h with DMSO (**lane 2**), 100 μM AG490 (**lane 3**), or 150 μM AG490 (**lane 4**). Western blots were probed with antibodies to phospho-STAT3, C/EBPδ, and β-actin.

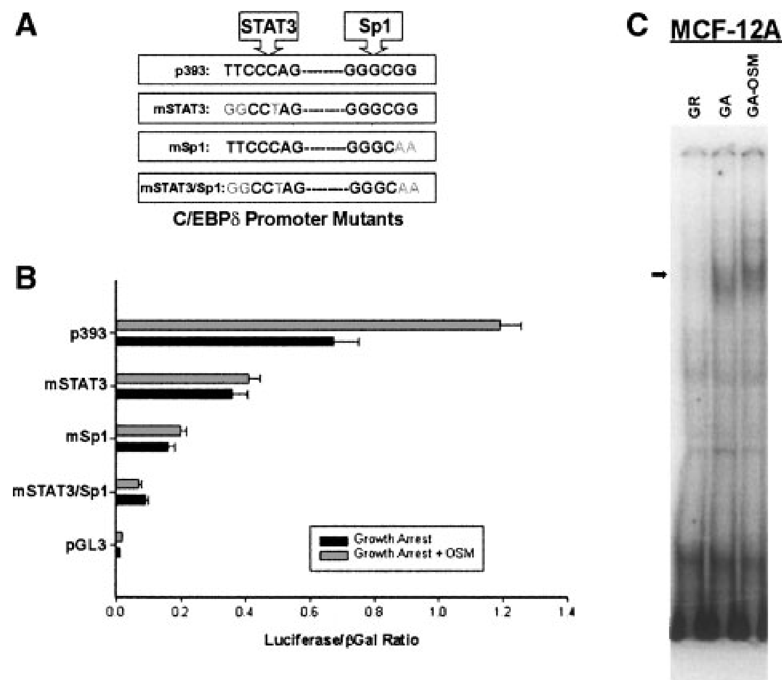

Fig. 4. Mutation of STAT3 and/or Sp1 sites decreases C/EBPδ promoter activity. **A**: Diagram of mutations. Mutations were introduced into the −393 bp C/EBPδ promoter construct at either the STAT3 or Sp1 site or both sites. **B**: Luciferase expression under growth arrest or growth arrest plus OSM addition normalized to β-galactosidase. **C**: EMSA assay confirms increased binding of activated STAT3 to its acute phase response element (APRE) under growth arrest conditions.

the C/EBPδ promoter, single and double STAT3 and Sp1 mutant constructs were produced in the 393 bp C/EBPδ promoter construct (Fig. 4A). Mutation of the STAT3 binding site reduces growth arrest and OSM-inducible C/EBPδ promoter activity by ~50%, consistent with the dominant negative STAT3β results (Fig. 4B). C/EBPδ promoter-luciferase activity decreases by 80% with the Sp1 mutation alone and by 87% with both sites mutated, similar to the deletion construct data (Fig. 4B). These results suggest that STAT3 is the key regulator of C/EBPδ promoter inducibility and Sp1 plays an important role in both basal and regulated promoter activity.

To verify that there is increased binding of activated STAT3 to its APRE during growth arrest conditions, electrophoretic mobility shift assays were performed to demonstrate binding of nuclear proteins to a STAT3 consensus binding site (APRE). The results demonstrate the absence of protein binding to the STAT3 APRE in lysates from exponentially GR MCF-12A human mammary epithelial cells (Fig. 4C). In contrast, there is a significant increase in binding to the STAT3 APRE in lysates from MCF-12A human mammary epithelial cells following serum and growth factor withdrawal (GA) and OSM addition (GA-OSM) (Fig. 4C).

C/EBPδ mRNA Exhibits a Short Half-Life in MCF-12A Human Mammary Epithelial Cells

Because of the significant role of post-transcriptional regulation in cell cycle control, we next investigated C/EBPδ mRNA stability in MCF-12A growth-arrested human mammary epithelial cells using the transcriptional inhibitor actinomycin D. C/EBPδ mRNA levels were detected in G_0 GA human MCF-12A (Fig. 5A, lanes 1–4), consistent with previous work from our lab in mouse and human mammary epithelial cells [Dearth and DeWille, 2003b; Sivko, manuscript submitted]. Addition of actinomycin D resulted in a rapid decline in C/EBPδ mRNA levels ($t_{1/2} \sim 40$ min) (Fig. 5A, lanes 5–7 and Fig. 5B). Comparable results were also obtained in the MCF-10A and in OSM-treated cells (data not shown). These results demonstrate that the C/EBPδ mRNA half-life is short in G_0 GA human mammary epithelial cells, similar to previous results in G_0 GA mouse mammary epithelial cells [Dearth and DeWille, 2003b].

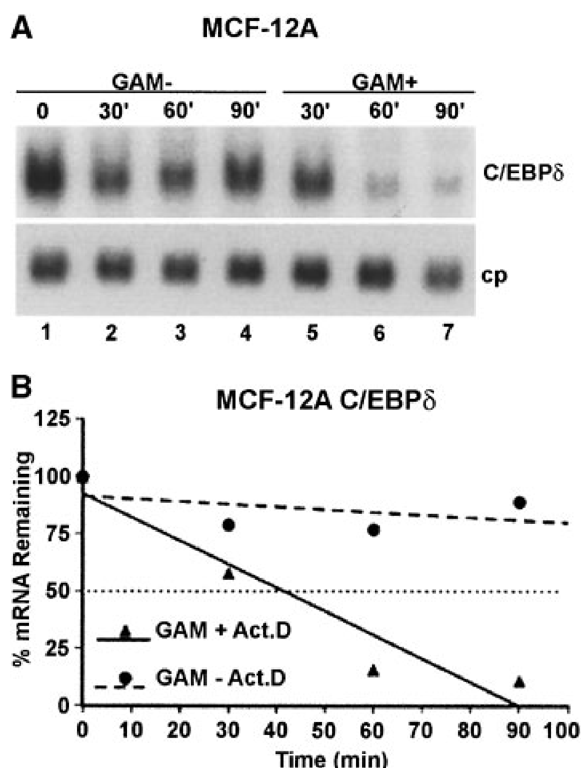

Fig. 5. C/EBPδ mRNA exhibits a short half-life in MCF-12A cells. RNA isolated from untreated and actinomycin D treated G_0 GA MCF-12A was analyzed by Northern blot analysis using C/EBPδ and cp probes as described in Figure 1. **A**: **Lanes 1–4**, RNA from G_0 growth arrested cells (GAM−); **lanes 5–7**, RNA from G_0 growth arrested 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 analysis as determine in Figure 1. Filled triangles (▲), RNA from actinomycin D treated mammary epithelial cells; filled circles (●), RNA from non-treated mammary epithelial cells.

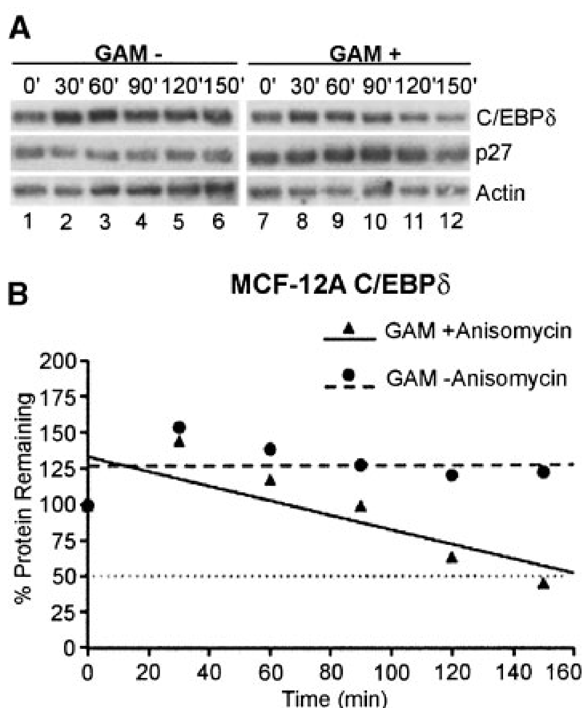

Fig. 6. C/EBPδ protein exhibits a short half-life in G_0 GA MCF-12A cells. Western blot analysis was performed on 50 μg of whole cell protein isolated from untreated and anisomysin treated G_0 GA human MCF-12A cells at the indicated time points as described in the figure. **A**: **Lanes 1–6**, protein from GAM−; **lanes 7–12**, protein from growth arrested cells treated with anisomycin (GAM+). Results are representative of three independent experiments. **B**: Summary of protein half-life data as described in the figure. Filled triangles (▲), protein from anisomycin treated mammary epithelial cells; filled circles (●), protein from non-treated mammary epithelial cells.

C/EBPδ Protein Exhibits a Short Half-Life in G_0 GA MCF-12A Human Mammary Epithelial Cells

Post-translational control is also a major mechanism by which cells regulate the level of cell cycle control proteins [Hochstrasser, 1995; Pagano, 1997; Nakayama et al., 2001]. To investigate the post-translational regulation of the C/EBPδ protein, MCF-12As were G_0 GA for 48 h in the presence or absence of the translational inhibitor anisomycin. C/EBPδ protein was detected in G_0 GA MCF-12A (Fig. 6A, lanes 1–6) and C/EBPδ protein levels declined in GA MCF-12A cells following anisomycin treatment with a half-life of approximately 160 min (Fig. 6A, lanes 7–12 and Fig. 6B). In addition, p27 protein was detected during MCF-12A G_0 growth arrest (Fig. 6A, lanes 1–6). p27 protein levels decreased following anisomycin treatment at a rate paralleling that of C/EBPδ (Fig. 6A, lanes 7–12). These results demonstrate that the C/EBPδ protein exhibits a short half-life, similar to p27.

DISCUSSION

The content and function of key proteins that mediate cell cycle progression, such as cyclins, cdks, cdk inhibitors, p53, growth factor receptors, and cytokines are tightly regulated. Control of these key cell cycle regulatory proteins has been demonstrated at transcriptional, post-transcriptional, and post-translational levels, indicating that no single regulatory mechanism is responsible for cell cycle checkpoint control [Bashir and Pagano, 2003]. In this report, we demonstrate the strict regulation of C/EBPδ expression transcriptionally by JAK/STAT3

activation of the C/EBPδ promoter, post-transcriptionally by rapid degradation of C/EBPδ mRNA and post-translationally by the short half-life of the C/EBPδ protein, suggesting that C/EBPδ plays a key role in the growth control of human mammary epithelial cells.

STATs 1, 3, and 5 all play specific roles in normal mammary gland development and function. STAT5 mediates prolactin signaling, regulates milk protein gene expression and is a survival factor for differentiated mammary epithelium. STAT1 is up-regulated during early pregnancy and late involution and is important for IFN-γ signaling. STAT3 is activated by the mechanical stretching of epithelial cells due to milk stasis and plays an important role in the initial "reversible" phase of post-lactational mammary gland of involution [Chapman et al., 1999; Watson, 2001]. We have previously demonstrated that STAT3 is activated (phosphorylated), translocates to the nucleus, binds to the C/EBPδ promoter, and activates C/EBPδ transcription during mouse mammary gland involution in vivo and during G_0 growth arrest in HC11 mammary epithelial cells in vitro [Hutt et al., 2000]. The mechanism of STAT3 activation in mammary epithelial cells at the onset of involution is thought to be mediated by cytokines signaling through the gp130 receptor [Watson, 2001]. IL-6 family cytokines bind to and signal through individual receptors which complex with gp130 and activate the JAK/STAT signal transduction pathway [Heinrich et al., 1998; Gomez-Lechon, 1999]. We have demonstrated that IL-6 cytokine addition activates STAT3 and induces C/EBPδ in HC11 mouse mammary epithelial cells and in finite-lifespan, immortalized and tumorigenic human mammary epithelial cell lines [Hutt and DeWille, 2002; Sivko and DeWille, 2004]. Here, we establish that STAT3 alone, not STAT1 or STAT5, is activated during serum and growth factor withdrawal or OSM induced growth arrest of MCF-10A and MCF-12A human mammary epithelial cells (Fig. 1).

We and others have demonstrated that IL-6 family cytokine addition, primarily IL-6, OSM and LIF, inhibits growth in both mouse and human cell types including mammary epithelial cells [Liu et al., 1998; Halfter et al., 1999; Badache and Hynes, 2001; Grant et al., 2001; Hutt and DeWille, 2002; Park et al., 2003]. The upstream signaling pathways of STAT3 during the G_0 growth arrest of human mammary epithelial cells, however, had not yet been confirmed. The Janus Kinase family of non-receptor tyrosine kinases (JAKs) are bound to cell surface cytokine receptors and are known to activate STATs during cytokine signaling [Darnell, 1997], making them likely candidates for upstream signaling. Here, we demonstrate that JAK1, JAK2, and Tyk2 are activated during serum and growth factor withdrawal induced G_0 growth arrest and following OSM addition in MCF-10A and MCF-12A human mammary epithelial cells (Fig. 2). It is not surprising to find the activation of more than one JAK, as different JAK family members have been shown to associate with the same cell surface receptor and hence are able to activate the same STAT molecule [Darnell, 1997]. It is also believed that at least two receptor-associated JAKs are required to initiate downstream signaling events following cytokine-induced receptor dimerization [Hermanns et al., 1999]. Blocking JAK2 signaling with AG490 reduces the levels of activated STAT3 and the levels of C/EBPδ protein (Fig. 3C), confirming the role of JAK signaling in the activation of STAT3 and the induction of C/EBPδ and G_0 growth arrest in MCF-10A and MCF-12A cell lines.

Previous results from our laboratory have demonstrated STAT3 activation of the C/EBPδ promoter during G_0 growth arrest of HC11 mouse mammary epithelial cells [Hutt et al., 2000]. Johnson and co-workers, found that IL-6 induced STAT3 to bind the C/EBPδ promoter and increased C/EBPδ transcription in cultured hepatocytes [Cantwell et al., 1998]. C/EBPδ promoter analysis by transient transfection assays in MCF-12A cells demonstrated that both STAT3 (−282 bp) and Sp1 (−61 bp) binding sites play important roles in transcriptional activation of the *C/EBPδ* gene. STAT3 binding is important for promoter activity following serum and growth factor withdrawal and is responsible for the enhanced promoter activity following OSM addition (Fig. 3A). Mutation of this STAT3 site or addition of a dominant negative, STAT3β, reduces C/EBPδ promoter activity and prevents the enhanced promoter activity previously demonstrated following OSM addition (Figs. 3B and 4). Sp1 is one of a family of 20 transcription factors which activate transcription by binding GC-rich regions in gene promoters and is important in growth control, apoptosis, and angiogenesis [Suske, 1999; Black et al., 2001]. Sp1 sites are involved

in both the inhibition of growth-promoting genes and the up-regulation of growth-inhibitory genes such as *p21* by *p53* [Black et al., 2001]. Mutation or deletion of the −61 bp Sp1 site in the C/EBPδ promoter dramatically reduces C/EBPδ promoter activity following serum and growth factor withdrawal and also prevents enhanced promoter inducibility by OSM (Figs. 3B and 4B). Mutation of both STAT3 and Sp1 sites reduces C/EBPδ promoter activity to basal levels (Fig. 4B), suggesting that STAT3 and Sp1 play a cooperative role in C/EBPδ promoter activation. STAT/Sp synergy and direct protein/protein interaction has been reported for Sp1 with both STAT1 and STAT3 [Look et al., 1995; Cantwell et al., 1998]. Interestingly, these results are similar to those of the mouse C/EBPδ promoter [Cantwell et al., 1998; O'Rourke et al., 1999a; Hutt et al., 2000] despite differences in promoter structure between the two species. The mouse promoter contains similar STAT3 and downstream Sp1 sites, but has an additional Sp1 (−120 to −115) site immediately upstream of the STAT3 (−110 to −102) site [Cantwell et al., 1998]. In a previous report, Johnson et al. demonstrated that STAT3 and Sp1 interact at these close proximity binding sites to provide the IL-6 inducibility of the mouse C/EBPδ promoter [Cantwell et al., 1998]. Our studies demonstrate that intact STAT3 and downstream Sp1 sites are necessary for both growth arrest and OSM-inducible C/EBPδ promoter activity. Preliminary results from our laboratory indicate that Sp1 is constitutively bound to the C/EBPδ promoter (Y. Zhang, manuscript in preparation). This suggests that STAT3 activation and translocation to the nucleus plays a crucial role in the regulated expression of the C/EBPδ promoter. Current studies are investigating the chromatin structure and assembly of transcriptional co-activators and chromatin remodeling complexes on the C/EBPδ gene promoter.

Having investigated control of C/EBPδ gene expression at the transcriptional level, we next investigated post-transcriptional control of C/EBPδ gene expression by assessing C/EBPδ mRNA stability. Regulation of mRNA stability plays a major role in controlling expression of "immediate early" response genes such as c-fos [Chen and Shyu, 1995]. Control of mRNA stability is mediated primarily by binding of transactivating factors to AU-rich elements (AREs) located in mRNA 3′UTRs [Chen and Shyu, 1995]. The presence of U-rich 3′ domains and AUUUA motifs promotes deadenylation and accelerates mRNA degradation [Chen and Shyu, 1995]. Interestingly, analysis of a subset of human and mouse genes revealed increased nucleotide substitution and greater divergence between species in the UTRs than in the corresponding coding regions [Larizza et al., 2002]. The mouse C/EBPδ 3′UTR contains two U-rich regions, both of which contain extended AUUUA sequences (U1 and U2) [Dearth and DeWille, 2003a]. Analysis of the human C/EBPδ 3′UTR revealed two U-rich regions, the first of which (U1) contains an interrupted AUUUA sequence and folds into a different mRNA secondary structure compared to the mouse C/EBPδ 3′UTR. The second U-rich region (U2) in the human C/EBPδ 3′UTR is similar in sequence and structure to the mouse C/EBPδ 3′UTR and contains an extended AUUUA region (data not shown). Here, we demonstrate that human C/EBPδ mRNA has a relatively short half-life ($t_{1/2} \sim 40$ min) in G_0 GA MCF-12A cells (Fig. 5). These results are similar to the relatively short half-life ($t_{1/2} \sim 35$ min) reported in GA mouse HC11 cells [Dearth and DeWille, 2003b].

Since both human and mouse C/EBPδ mRNA and mouse C/EBPδ protein have short half-lives, we expected C/EBPδ protein to also exhibit a short half-life. The results here demonstrate that human C/EBPδ protein has a similar half-life ($t_{1/2} \sim 160$ min) to both mouse C/EBPδ protein ($t_{1/2} \sim 120$ min) and that of p27 ($t_{1/2} >$ 150 min) (Fig. 6). While p27 mRNA levels do not change significantly during the cell cycle, p27 protein levels oscillate, with maximal protein levels being present during G_0 and G_1 [Polyak et al., 1994; Bashir and Pagano, 2003]. Similar to p27, C/EBPδ protein levels in human mammary epithelial cells also vary with the cell cycle, with maximal levels being present during G_0. In contrast to p27, C/EBPδ is also transcriptionally regulated in human mammary epithelial cells, with C/EBPδ mRNA levels increasing during G_0 and decreasing during other phases of the cell cycle [Sivko, manuscript submitted]. Blocking translation by the addition of anisomycin resulted in a similar decrease in C/EBPδ and p27 protein levels suggesting that C/EBPδ protein degradation occurs rapidly and by a mechanism similar to that of p27. The most common degradation method of short-lived proteins including p27 is the ubiquitin–

proteasome pathway [Conaway et al., 2002]. Our lab has previously demonstrated that C/EBPδ protein undergoes ubiquitination and nuclear degradation in G_0 growth-arrested mouse mammary epithelial cells [Dearth and DeWille, 2003b]. These results suggest that human C/EBPδ protein is most likely degraded via the ubiquitin–proteasome pathway similar to mouse C/EBPδ and other tumor suppressor such as p53, p21, and p27 [Dearth and DeWille, 2003b].

In summary, the data presented here establishes that JAK1, JAK2, and Tyk2 activation is responsible for STAT3 activation during the G_0 growth arrest of human mammary epithelial cells induced by serum and growth factor withdrawal and OSM treatment. Activated STAT3 translocates to the nucleus and binds to its consensus sequence on the C/EBPδ promoter; and in conjunction with Sp1, activates C/EBPδ transcription. Removal or mutation of either STAT3 or Sp1 sites results in decreased C/EBPδ promoter activation. C/EBPδ mRNA and protein both have short half-lives during G_0 growth arrest. These results suggest that C/EBPδ is tightly regulated transcriptionally, post-transcriptionally, and post-translationally during the G_0 growth arrest of human mammary epithelial cells. This maintains cells in a quiescent G_0 state, yet allows cells to quickly re-enter the cell cycle and proliferate upon growth factor stimulation.

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