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C/EBPα Regulation of the Growth-Arrest-Associated Gene *gadd*45

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CCAAT/enhancer-binding protein alpha (C/EBPα) is expressed in postmitotic, differentiated adipocytes and is required for adipose conversion of 3T3-L1 cells in culture. Temporal misexpression of C/EBPα in undifferentiated adipoblasts leads to mitotic growth arrest. We report here that growth arrest- and DNA damage-inducible gene 45 (*gadd*45) is preferentially expressed in differentiated 3T3-L1 adipocytes similar to phenotype-associated genes. Furthermore, C/EBPα transactivates a reporter plasmid containing 1.5 kb of the *gadd*45 promoter region. The proto-oncogene *myc*, which inhibits adipocyte differentiation, abrogates C/EBPα activation of *gadd*45. *gadd*45 is known to be a target of the tumor suppressor p53 in a G_1 checkpoint activated by DNA damage. Immunoprecipitation of radiolabeled proteins with conformation-specific antibodies revealed that wild-type p53 is expressed throughout 3T3-L1 adipocyte development, including the postmitotic period characterized by the accumulation of *gadd*45 and C/EBPα. A stable 3T3-L1 subline was engineered to express a dominant negative p53, human $p53^{143ala}$. The $p53^{143ala}$ subline differentiated to adipocytes and showed appropriate developmental expression of *gadd*45. These findings suggest that postmitotic growth arrest is coupled to adipocyte differentiation via C/EBPα stimulation of growth arrest-associated and phenotype-associated genes.

Control of mitotic cell growth is paramount to animal development and the regulation of regenerating cell populations in adults. Mitotic growth control involves the establishment of both reversible and irreversible growth arrest states. Reversible growth arrest in response to such stimuli as serum deprivation, contact inhibition, and DNA damage has been well studied in cultured cells (12, 28). Irreversible growth arrest states can also be studied in culture. Human diploid fibroblasts enter an irreversible growth arrest state, senescence, after limited mitotic growth in culture (34). Irreversible growth arrest is also a hallmark of terminal differentiation. Leukemic cell lines can be induced to differentiate in culture and show a concomitant loss of proliferative capacity (1). Similarly, pluripotent, fibroblast-like cell lines, including myoblast and adipoblast cell lines, that exhibit an irreversible growth arrest state accompanying cellular differentiation have been established (16, 22).

We have been studying growth arrest during adipose conversion of 3T3-L1 cells (33). 3T3-L1 cells can be propagated as adipoblasts and induced to enter reversible growth arrest states in response to serum deprivation and contact inhibition (31). In addition, 3T3-L1 cells can be induced to differentiate to adipocytes through appropriate hormonal stimulation (35). 3T3-L1 cells require the transcription factor CCAAT/enhancer-binding protein alpha (C/EBPα) (25, 30) to differentiate. C/EBPα has been shown to transactivate the promoters of several adipocyte-specific genes (8). Temporal misexpression of C/EBPα in logarithmically growing adipoblasts results in mitotic growth arrest independent of adipogenesis (36). Notably, mitotic growth inhibition by C/EBPα requires the same functional domains as transcriptional activation (36). These observations led us to speculate that C/EBPα may activate growth arrest-associated genes in addition to phenotype-associated genes in postmitotic adipocytes (36).

Freytag observed that overexpression of the proto-oncogene *myc* inhibits 3T3-L1 differentiation (14). Furthermore, *myc* overexpression precludes 3T3-L1 cells from establishing an irreversible growth arrest state (14). *myc* inhibition of differentiation can be overcome by enforced expression of C/EBPα (15). Freytag and Geddes interpreted the latter observation to suggests that C/EBPα-driven adipogenesis is opposed by *myc*-mediated proliferative potential (15). Our discovery of the antimitotic activity of C/EBPα suggest a more direct antagonism between the two transcription factors: C/EBPα stimulates postmitotic growth arrest, while *myc* sustains proliferative potential. We report here that growth arrest- and DNA damage-inducible gene 45 (*gadd*45) was preferentially expressed in differentiated adipocytes. Furthermore, a 1.5-kb *gadd*45 promoter fragment mediated C/EBPα activation of a reporter construct in a transient transfection assay. We also observed that expression of *myc* inhibited C/EBPα activation of *gadd*45. *gadd*45 was originally isolated from transcripts enriched in cells exposed to DNA-damaging agents (12). Subsequently, it was shown that numerous growth arrest stimuli can induce *gadd*45 (13). *gadd*45 is a target of the p53 tumor suppressor protein in a G_1 cell cycle checkpoint (21). We show here that wild-type p53 is expressed throughout adipocyte development, including the postmitotic period characterized by accumulation of *gadd*45 and C/EBPα. However, introduction of a dominant negative p53 does not inhibit adipocyte differentiation or developmental expression of *gadd*45.

MATERIALS AND METHODS

Cell culture and differentiation. 3T3-L1 and NIH 3T3 cells from the American Type Culture Collection were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Differentiation of 3T3-L1 cells was performed by the standard protocol (35). The stable cell line SCX3 was created by transfecting 3T3-L1 cells with plasmid pCMV-SCX3, which expresses both $p53^{143ala}$ and the G418 resistance gene. The plasmid was introduced by the calcium phosphate-BES method (2) and allowed to recover for 24 h; the cells were then split 1:10 and propagated in the presence of G418 until only resistant colonies remained. Thirty-seven colonies were isolated at the 100- to 200-cell stage and propagated as continuous cell lines. The total proteins isolated from these lines were then assayed for expression of the mutant p53 protein by Western blotting (immunoblotting). SCX3 cells were differentiated by the standard protocol. RNA collection and Northern (RNA) blot analysis were conducted as previously described (36).

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DNA probes. Murine *gadd*45 expression was originally detected by cross-hybridization with a hamster cDNA (gift of A. Fornace). A 619-bp fragment of murine *gadd*45 was amplified by reverse transcriptase-PCR with a sense primer (5′-GGAGCAGCCCTCGAGCCGAGGGAG-3′ [nucleotides 123 to 137]) and an antisense primer (5′-GAGTGACTGCTCGAGTAACTACAA-3′ [nucleotides 719 to 739]). cDNA was synthesized by using a cDNA Cycle kit (Invitrogen) with RNA isolated from 3T3-L1 cells on day 8 of a standard differentiation. cDNA was initially denatured at 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min. pCMV-ORFgadd45 was constructed by subcloning the *gadd*45 fragment into the *Xho*I site of plasmid pCMVneo. The *gadd*45 insert was excised by restriction digestion with *Xho*I, separated by agarose gel electrophoresis in low-melting-temperature agarose (Gibco BRL), and purified by GeneClean (Bio 101). cDNA probes were labeled with [^{32}P]dCTP (3,000 Ci/mmol; NEN) by using a random-primed labeling kit (Oligolabelling Kit) in accordance with the manufacturer's (Pharmacia) directions.

Immunoprecipitation and Western blotting. 3T3-L1 cells were differentiated by the standard protocol (35), and RKO cells were driven into G_0 as previously described (40). Ltk$^-$ cells were transiently transfected with a wild-type murine p53-encoding plasmid (plasmid p441, kindly provided by A. Levine) by the DEAE-dextran method (2). Cells were switched to methionine-free medium, equilibrated for 1 h, and fed [^{35}S]methionine (1,175 Ci/mmol; NEN) for 2 h. For immunoprecipitation, cells were lysed, sonicated, and cleared by centrifugation as previously described (21). Incorporated counts were determined by trichloroacetic acid precipitation, and equivalent trichloroacetic acid-precipitable counts were used in the immunoprecipitation. Samples were sequentially preadsorbed to fixed *Staphylococcus aureus* cells, incubated with either a cocktail of two monoclonal antibodies (PAb421 and PAb1620), PAb1620 alone, or PAb240 alone, in accordance with the supplier's (Oncogene Science) recommendation, and precipitated with protein A-agarose (Gibco BRL). The immunoprecipitates were subjected to polyacrylamide gel electrophoresis, fixed, dried, and visualized with a PhosphorImager (Molecular Dynamics). Ltk$^-$ cells were transfected as described above, with plasmid pCMV-SCX3 (gift of B. Vogelstein), and immunoprecipitation was done as already described, with PAb240, or a total protein extract was prepared from the cells. The samples were resolved by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunodetected with an anti-p53 polyclonal antibody raised in sheep (Ab-7; Oncogene Science). A biotin-conjugated rabbit anti-sheep antibody (Oncogene Science) served as the secondary antibody, and the immunoreactive species were visualized with horseradish peroxidase-conjugated streptavidin in a chemiluminescence reaction (ECL; Amersham) and subsequent autoradiography. p53^{143ala} expression in the stable SCX3 line was detected in the same way.

Transfection and chloramphenicol acetyltransferase (CAT) assays. One-tenth of a 100-cm-diameter plate of nearly confluent NIH 3T3 cells was used to seed a fresh dish and incubated overnight at 37°C. The cells were transfected by the calcium phosphate precipitation method (2). Precipitates were left on the cells for 18 h and included 2 μg of a *gadd*45-CAT plasmid (dc45-40) (40) cotransfected with various amounts of pMSV-C/EBPα, pBS-MSV-C/EBPβ, pBS-MSV-C/EBPδ, pcDNA1-Hu-c-myc-f.s. (nonsense mutation at amino acid 49; kindly provided by L. Penn) or pcDNA-Hu-c-myc (exons 2 and 3 of human c-*myc*, kindly provided by L. Penn). After 18 h, the cells were washed with phosphate-buffered saline and incubated for an additional 24 h in complete medium. Cells were harvested into reporter lysis buffer (Promega), and the CAT assay was performed as recommended by the manufacturer (CAT Enzyme Assay System; Promega).

RESULTS

***gadd*45 is a target of C/EBPα stimulation.** C/EBPα expression during adipose conversion of 3T3-L1 cells is restricted to the postmitotic period coincident with phenotypic differentiation (6). Temporal misexpression of C/EBPα in logarithmically growing adipoblasts results in mitotic growth arrest (36). Combined, these observations suggest that C/EBPα stimulates growth arrest-associated genes in postmitotic adipocytes.

We examined the expression of *gadd*45 as a function of adipose conversion of 3T3-L1 cells (Fig. 1). Adipose conversion includes several separate periods of mitotic growth arrest (reviewed in reference 37). In the standard differentiation protocol, logarithmically growing, subconfluent 3T3-L1 adipoblasts are incubated for 48 h beyond confluence to a postconfluent monolayer (day 0), resulting in growth arrest due to contact inhibition. The cells are then exposed to adipogenic hormones for 48 h, which results in an increase in cell number during a period called clonal expansion (day 1 to day 2) (37). After the differentiation inducers are removed, the cell number again plateaus on day 3 and remains constant throughout phenotypic differentiation (days 3 to 8), which is characterized by accumulation of cytoplasmic fat droplets and expression of adipocyte-specific genes (5, 16). We monitored *gadd*45 mRNA abundance by Northern blot analysis (Fig. 1A). *gadd*45 expression during adipose conversion was biphasic (Fig. 1B). *gadd*45 was undetectable in logarithmically growing adipoblasts and was first detected in the contact-inhibited culture. *gadd*45 levels declined during the postconfluent mitoses of clonal expansion and then reached a maximum in postmitotic, phenotypically differentiated adipocytes.

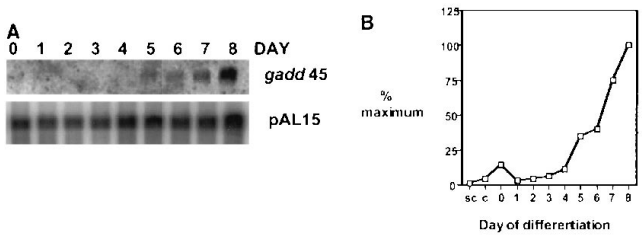

FIG. 1. *gadd*45 is preferentially expressed in postmitotic adipocytes. (A) Total RNA was harvested from 3T3-L1 cells throughout the conversion from adipoblasts to adipocytes. 3T3-L1 cells that were 48 h postconfluency, on day 0 were exposed to differentiation inducers on days 1 and 2 and refed differentiation medium every 48 h thereafter (from day 3 on). RNA was harvested from cells each day, and 10 μg was analyzed by Northern blotting. The blot was probed with *gadd*45 cDNA, stripped, and reprobed with a pAL15 cDNA, a ribosomal protein subunit-encoding transcript whose abundance is constant throughout the period (5). (B) The relative abundance of *gadd*45 was determined by quantification of band intensity (ImageQuant software; Molecular Dynamics); the samples were normalized to the pAL15 signal and graphed by including the samples from the logarithmically growing subconfluent (sc) and newly confluent (c) cells. The sample expressing *gadd*45 at the highest level (days 8) was set at 100%, and all other samples are reported relative to that amount.

The profile of *gadd*45 expression from day 3 to day 8 was similar to that of a number of phenotype-associated genes, including those for adipose fatty acid-binding protein (gene 422) (4), glycerol-3-phosphate dehydrogenase (32), and stearoyl coenzyme A desaturase 1 (20). Adipocyte-specific expression of these phenotype-associated genes is coincident with the accumulation of C/EBPα (6), and each gene promoter is transactivated by C/EBPα in transient transfection assays (8, 29). We wondered whether the promoter region of *gadd*45 would likewise be transactivated by C/EBPα. We obtained a reporter plasmid wherein the 1.5-kb sequence 5′ to the *gadd*45 start site of transcription is appended to the CAT reporter gene (*gadd*45-CAT) (40). The *gadd*45-CAT plasmid was transfected with or without pMSV-C/EBPα, which constitutively expresses C/EBPα, into NIH 3T3 cells. C/EBPα transactivated the *gadd*45 promoter, as evidenced by increased CAT activity in cotransfected cells over cells transfected with *gadd*45-CAT alone (Fig. 2).

Adipocytes express at least three C/EBP isoforms, albeit with different expression profiles, during differentiation (7). C/EBPβ and C/EBPδ are most abundant during days 1 and 2 (7), the period of postconfluent mitoses when *gadd*45 levels decline (Fig. 1B). The abilities of the three isoforms to transactivate *gadd*45 were compared in the transient transfection assay (Fig. 2). Cotransfection with pBS-MSV-C/EBPβ resulted in significantly less transactivation than did cotransfection with pMSV-C/EBPα. At low input levels of the transactivator plasmid (100 ng), pMSV-C/EBPα also transactivated better than pBS-MSV-C/EBPδ. At higher input levels, pBS-MSV-C/EBPδ transactivation was comparable to that of pMSV-C/EBPα. Several mechanisms might account for the observation that C/EBPδ accumulation during adipocyte differentiation did not lead to activation of *gadd*45 (see Discussion).

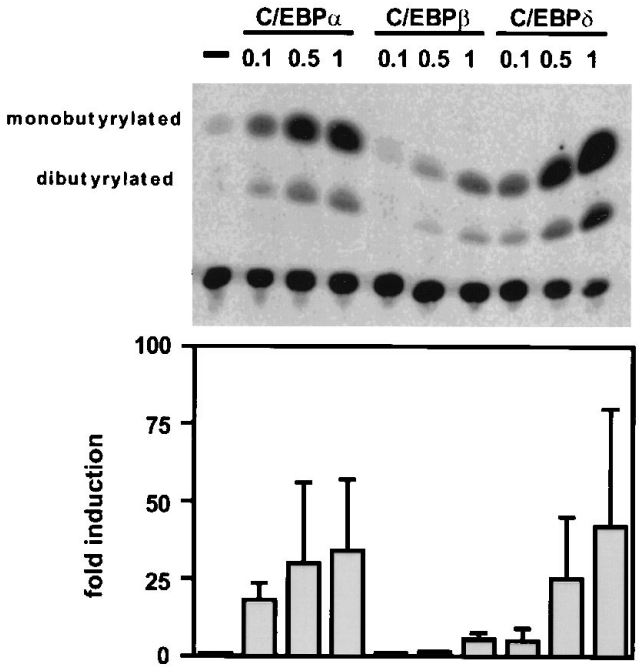

FIG. 2. C/EBP transactivates the *gadd*45 promoter. NIH 3T3 cells were transfected with 2 μg of a *gadd*45-CAT plasmid (40) containing 1.5 kb of the hamster *gadd*45 promoter (−2578 to +149) inserted into *Hin*dIII-*Xba*I sites of pCAT-Basic. This plasmid was transfected alone or cotransfected with 0.1, 0.5, or 1 μg of pMSV-C/EBPα, pBS-MSV-C/EBPβ, or pBS-MSV-C/EBPδ. CAT assays were performed on transfected populations, and the amount of the butyrylated product was quantified after separating the product from the substrate by xylene extraction. In addition, an aliquot of each reaction mixture was subjected to thin-layer chromatography to visualize the products. The fold induction observed in each condition relative to *gadd*45-CAT alone is shown graphically. Each transfection was performed in duplicate, except for those including C/EBPα, which were performed in triplicate. The error bars indicate standard deviations. An exemplary thin-layer chromatograph is shown at the top.

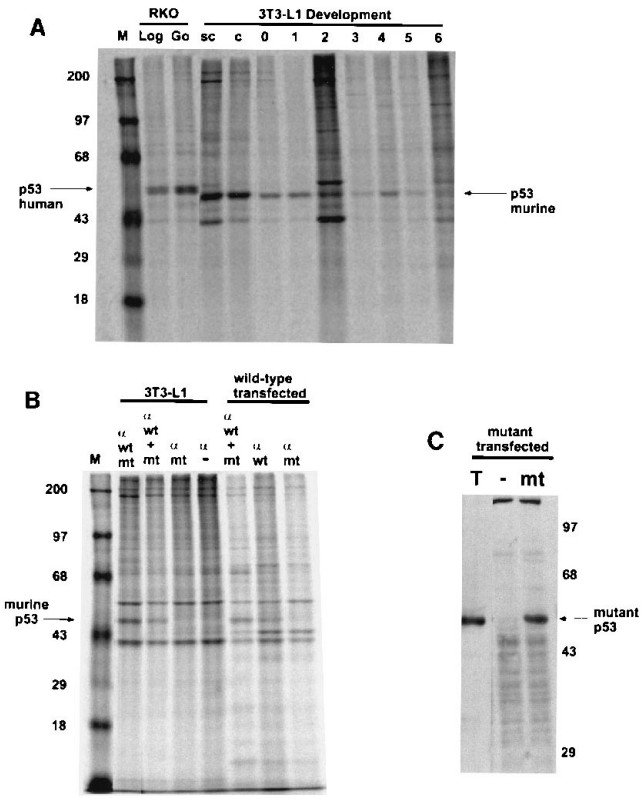

FIG. 3. Immunoprecipitation of wild-type p53 from 3T3-L1 cells throughout adipocyte development. (A) Equivalent trichloroacetic acid-precipitable counts were harvested from [^{35}S]methionine-labeled 3T3-L1 cells throughout adipose conversion or from RKO cells (derived from a human colorectal tumor expressing wild-type p53) in the log phase (Log) or in G_0 growth arrest (Go). A cocktail of two monoclonal antibodies (PAb421 and PAb1620) that recognizes human and murine p53 proteins was used to immunoprecipitate the tumor suppressor from these labeled protein extracts. The samples were analyzed alongside radiolabeled molecular weight markers (lane M). Lane sc, subconfluent cells; lane c, confluent cells. (B) Monoclonal antibodies were used to immunoprecipitate p53 from [^{35}S]methionine-labeled confluent 3T3-L1 cells or Ltk$^-$ cells 48 h after transfection with a wild-type murine p53 expression plasmid. The immunoprecipitations were performed with a cocktail of PAb421, which recognizes wild-type and mutant p53, and PAb1620, which is specific for the wild-type form (α wt + mt); with PAb1620 alone (α wt); with PAb240, which is specific for the mutant form, alone (α mt); or with no primary antibody (α −). (C) Ltk$^-$ cells were transfected with pCMV-SCX3, which expresses mutant p53. A total protein extract was prepared from the cells (lane T) and analyzed by Western blotting alongside immunoprecipitates obtained from the same cells in the presence (lane mt) or absence (lane −) of the primary antibody, PAb240. The samples were immunodetected by using an anti-p53 polyclonal antibody as the primary antibody, a biotin-conjugated secondary antibody, and enhanced chemiluminescence to visualize immunoreactive species. The numbers to the left or right are molecular masses in kilodaltons.

***gadd*45 accumulation in adipocytes is independent of the p53 phenotype.** *gadd*45 is known to be a target of tumor suppressor p53 (21). We wondered what role, if any, p53 plays in the developmental expression of *gadd*45. Wild-type p53 has been detected in 3T3-L1 adipoblasts (18). However, p53 expression levels in differentiated adipocytes have not been examined. We sought to quantify p53 expression throughout adipocyte differentiation. Initial attempts to detect p53 by Western blotting were unsuccessful, consistent with a low level of expression in 3T3-L1 cells (data not shown). Instead, conformation-specific monoclonal antibodies were used to immunoprecipitate p53 from 3T3-L1 cells at various time points throughout the standard differentiation protocol. The samples were compared to p53 immunoprecipitated from RKO cells, which were established from a human colorectal carcinoma and previously shown to express low levels of wild-type p53 (3, 23). Murine p53 was detected throughout adipocyte differentiation (Fig. 3A), although it was most abundant in logarithmically growing, semi-confluent and confluent adipoblasts compared to 48-h postconfluent adipoblasts (day 0) or those examined on any day after the initiation of differentiation (days 1 to 6). To ascertain whether the protein precipitated from 3T3-L1 cells is indeed wild-type p53, we performed immunoprecipitation with antibodies specific for wild-type p53 or mutant conformations of p53. Furthermore, we compared the proteins immunoprecipitated from 3T3-L1 cells with proteins immunoprecipitated from cells that had been transiently transfected with wild-type murine p53 expression plasmid p441. A protein with an apparent molecular mass of 53 kDa was immunoprecipitated (Fig. 3B) from the transfected cells with the cocktail of monoclonal antibodies used for Fig. 3A (αwt + mt), or a single monoclonal antibody specific for the wild-type form of p53 (α wt). The same protein was not immunoprecipitated by an antibody specific for the mutant conformation of p53 (α mt). The protein immunoprecipitated from 3T3-L1 cells precisely comigrated with the material immunoprecipitated from the cells transfected with the wild-type murine p53 expression plasmid and was similarly dependent on the presence of a monoclonal antibody that detects the wild-type protein (Fig. 3B). The monoclonal antibody specific for mutant p53 readily immunoprecipitated human p53^{143ala} (Fig. 3B) from cells tran-

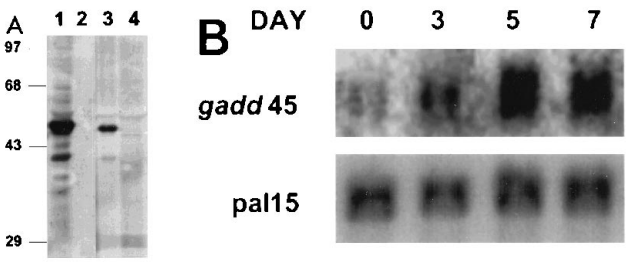

FIG. 4. Expression of *gadd*45 throughout adipocyte development is p53 independent. (A) 3T3-L1 cells were transfected with plasmid pCMV-SCX3, which expresses dominant negative human mutant protein $p53^{143ala}$. G418-resistant clones were isolated and propegated as continuous cell lines. Total protein extracts were prepared from the sublines and analyzed by Western blotting alongside extracts from Ltk^- cells transiently transfected with plasmid pCMV-SCX3 (lane 1). A protein extract of the subline chosen for further study (lane 3) is shown adjacent to an isolated clone that does not express the protein (lane 4). Molecular mass standards are indicated in kilodaltons to the left. (B) Total RNA was harvested throughout a standard differentiation of the subline which stably expresses $p53^{143ala}$. RNA was harvested from cells on the days indicated after initiation of differentiation, and 10 μg was analyzed by Northern blotting as described in the legend to Fig. 1.

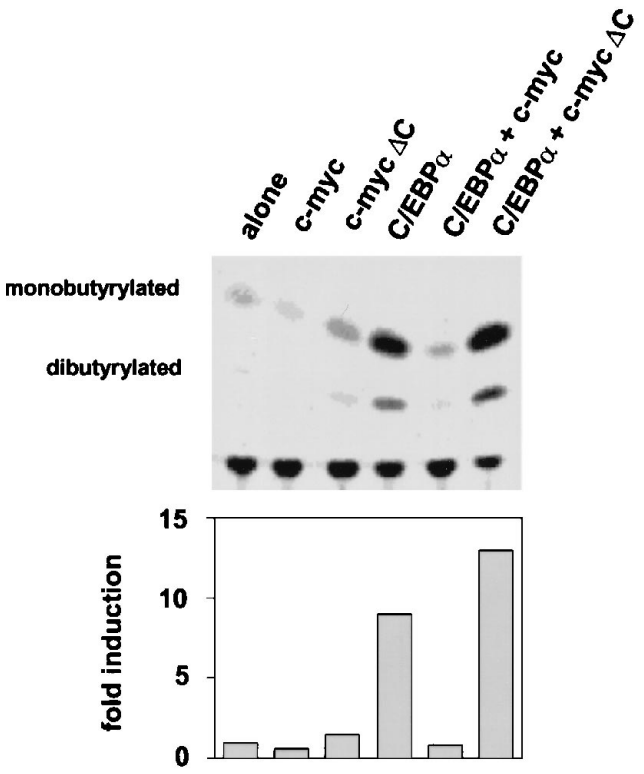

FIG. 5. Myc antagonizes C/EBPα activation of the *gadd*45 promoter. NIH 3T3 cells were transfected with 2 μg of a *gadd*45-CAT plasmid either alone or cotransfected with pMSV-C/EBPα, pcDNA-Hu-c-myc, or pcDNA1-Hu-c-myc-f.s., with a nonsense mutation at codon 49. In addition, the *gadd*45-CAT plasmid was cotransfected with the C/EBPα-encoding plasmid in combination with the plasmids encoding full-length or truncated Myc. After transfection, CAT assays were conducted and quantified as described in the legend to Fig. 2. The experiment was performed twice. The results shown are representative.

siently transfected with plasmid pCMV-SCX3 (provided by B. Vogelstein). The results presented in Fig. 3 reveal that p53 was expressed throughout adipocyte development, including the postmitotic period characterized by the accumulation of C/EBPα (6) and *gadd*45 (Fig. 1).

To evaluate the role, if any, of p53 in the developmental expression of *gadd*45 in adipocytes, we sought to inactivate p53 in a clonal subline of 3T3-L1 cells. We followed the strategy employed by Kastan and coworkers, who inactivated p53 in human RKO cells (23). Plasmid pCMV-SCX3 encodes a dominant negative form of p53 that sequesters wild-type p53 into inactive oligomers (19). pCMV-SCX3 (gift of B. Vogelstein) was transfected into 3T3-L1 cells, followed by selection in G418, since the plasmid contains a neomycin resistance cassette (3). Approximately 50 colonies were picked at the 100- to 200-cell stage and propagated as stable cell lines. Protein extracts were analyzed by Western blotting, and several lines expressing mutant p53 were identified upon the basis of (i) the migration of the human mutant protein compared with that of extracts from cells transiently transfected with human or mouse p53 and (ii) the accumulation of high levels of p53 protein in growing cells (a reflection of the relatively long half-life of the mutant protein). A representative Western blot analysis is shown in Fig. 4A. Six of the cell lines identified were subjected to the standard differentiation protocol, since subcloning alone can alter differentiation efficiency (16). Four of the six cell lines derived remained competent to differentiate, as evidenced by the accumulation of cytoplasmic fat droplets (data not shown). Thus, the presence of dominant negative p53 does not inhibit differentiation. Northern blot analysis of the $p53^{143ala}$-producing 3T3-L1 subline throughout adipose conversion revealed that *gadd*45 preferentially accumulated in postmitotic adipocytes (Fig. 4B). Thus, developmental expression of *gadd*45 is independent of the p53 phenotype of 3T3-L1 cells.

C/EBPα activation of *gadd*45 is antagonized by c-*myc*. Constitutive expression of proto-oncogene c-*myc* in 3T3-L1 cells inhibits phenotypic differentiation (14). Moreover, *myc* expression precludes 3T3-L1 cells from establishing a postmitotic growth arrest state (14) that is a prerequisite to phenotypic differentiation (31). Freytag and Geddes demonstrated that the inhibitory effects of Myc on 3T3-L1 differentiation can be titrated by C/EBPα expression (15). They concluded that in 3T3-L1 cells, the two proteins have a reciprocal relationship wherein C/EBPα stimulates differentiation and Myc maintains proliferative potential. Our discovery that constitutive C/EBPα expression leads to mitotic growth arrest (36) suggested a more direct antagonism between the two transcription factors: C/EBPα stimulates the postmitotic growth arrest required for differentiation, while Myc maintains proliferative potential.

We considered that the antagonistic effects of C/EBPα and Myc on 3T3-L1 growth states might be manifest in regulation of *gadd*45. To test this hypothesis, we examined the consequences of simultaneous expression of C/EBPα and Myc on the *gadd*45-CAT reporter plasmid in the transient transfection assay. *gadd*45-CAT was transfected alone or with C/EBPα and Myc expression vectors, individually or in combination (Fig. 5). Expression of *myc* alone did not alter the basal transcription of the *gadd*45 promoter in transfected cells (Fig. 5). However, coexpression of Myc and C/EBPα abrogated the transactivation of *gadd*45 normally afforded by C/EBPα (Fig. 5). A c-*myc* nonsense mutant that expresses only the N-terminal domain failed to antagonize C/EBPα transactivation (Fig. 5). Thus, Myc antagonism of C/EBPα activation of *gadd*45 requires the carboxyl-terminal region of the proto-oncogene that includes the DNA-binding and oligomerization domains (9).

DISCUSSION

The results presented here reveal that *gadd*45 is expressed as a function of adipocyte development in 3T3-L1 cells. The ex-

pression pattern is similar to that of a number of phenotype-associated genes that have proven to be targets of C/EBPα (8, 29), a transcription factor required for adipose conversion of 3T3-L1 cells (26, 30). C/EBPα expression is normally confined to the postmitotic period of adipocyte development (6). Furthermore, C/EBPα induces mitotic growth arrest when prematurely expressed in undifferentiated adipoblasts (36). The latter observation led us to hypothesize that C/EBPα stimulates growth arrest-associated genes in addition to phenotype-associated genes in adipocytes. The expression profile of *gadd*45 during adipocyte development and its transactivation by C/EBPα are consistent with our hypothesis. These findings suggest that the regulation of postmitotic growth arrest and phenotypic differentiation in adipocytes converge at the activity of developmental transcription factor C/EBPα. A similar convergence was recently observed in muscle differentiation. Specifically, MyoD, a myogenic transcription factor, stimulates the *Waf*1/*Cip*1 gene (17), encoding cell cycle-inhibitory protein p21 (38), in addition to muscle-specific genes (24). *Waf*1/*Cip*1, like *gadd*45, is a target of tumor suppressor p53 (11). MyoD stimulation of the *Waf*1/*Cip*1 promoter is p53 independent (17). Furthermore, a comprehensive analysis of p21 mRNA accumulation in p53−/− mice revealed that p21 is expressed in numerous adult tissues at wild-type levels, although p53 is required to elevate p21 in those same tissues in response to DNA damage (27). (We have demonstrated that the presence of dominant negative p53 does not inhibit phenotypic differentiation or adipocyte-specific expression of *gadd*45 [Fig. 4].) While we have not conclusively demonstrated the absence of wild-type p53 activity in these cells, their increased radiation sensitivity is consistent with a reduction in p53 function (Fig. 4). Moreover, the interpretation that 3T3-L1 adipocyte development is p53 independent agrees with the observation that p53−/− mice develop normally (10). Combined with the studies of *Waf*1/*Cip*1 expression, our findings suggest that tumor suppressor p53 and developmental transcription factors share a subset of target genes. Furthermore, the stimulation of growth arrest-associated genes by developmental transcription factors may be a general feature of terminal differentiation.

The results of the transient transactivation assay (Fig. 2) suggest that *gadd*45 is a direct target of C/EBPα. However, *gadd*45 accumulation lags behind the accumulation of certain phenotype-associated genes that are well established as C/EBPα targets (e.g., gene 422) (4). Perhaps *gadd*45 requires higher levels of C/EBPα to be transactivated. In fact, C/EBPα levels increase throughout the period of *gadd*45 accumulation (7). Alternatively, the earlier onset of expression of phenotype-associated genes might be C/EBPβ driven. C/EBPβ accumulates earlier in 3T3-L1 cells, on days 1 and 2 (7). Furthermore, retroviral expression of C/EBPβ converts pluripotent NIH 3T3 cells to adipocytes in a C/EBPα-independent manner (39). The latter observation was interpreted to suggest that C/EBPβ directly activates phenotype-associated genes (39). In contrast, C/EBPβ failed to transactivate *gadd*45 in the transfection assay (Fig. 2). Meanwhile, the DNA-binding properties of C/EBPα and C/EBPβ appear to be very similar (7). Thus, the isoforms must be discriminated via their distinct transactivation domains (7) in the direct activation of phenotype-associated versus growth arrest-associated gene promoters. Alternatively, *gadd*45 may be an indirect target of C/EBPα. C/EBPα may regulate growth arrest through another transcription factor(s) that discriminates and directly regulates growth arrest-associated genes. We are continuing to investigate the transcriptional regulation of *gadd*45 in the hope of understanding the coupling of phenotypic differentiation and postmitotic growth arrest.

Like C/EBPα, C/EBPδ activated *gadd*45 in a transient transfection assay (Fig. 2). However, *gadd*45 levels decline when C/EBPδ levels are highest, on days 1 and 2 of differentiation (7). Low levels of *gadd*45 at this time are consistent with postconfluent mitotic growth of the cells. Several observations might account for the fact that *gadd*45 is not induced by C/EBPδ during its accumulation on days 1 and 2. First, the antimitotic activity of C/EBPα requires a transactivation domain not present in C/EBPδ (7, 26). This protein domain may be required to stimulate *gadd*45 expression in 3T3-L1 cells. Second, C/EBPβ, which failed to stimulate *gadd*45 (Fig. 2), is also abundant at this time (7). Furthermore, C/EBPβ and C/EBPδ form heterodimers (7). Perhaps a C/EBPδ-C/EBPβ heterodimer cannot activate *gadd*45. Finally, Myc also accumulates during this period of postconfluent mitoses (14). Myc antagonizes C/EBPα stimulation of *gadd*45 and likely antagonizes C/EBPδ stimulation during days 1 and 2. Myc declined by day 3 (14) prior to C/EBPα and *gadd*45 accumulation in postmitotic adipocytes (Fig. 1).

Constitutive expression of Myc precludes 3T3-L1 cells from entering a unique growth arrest state that precedes phenotypic differentiation (14). Forced expression of C/EBPα overcomes this Myc-mediated block of differentiation (15). Freytag and Geddes concluded that C/EBPα and Myc have a reciprocal relationship in 3T3-L1 cells wherein C/EBPα promotes phenotypic differentiation and Myc affords proliferative potential (15). Our findings suggest a more direct antagonism between the two transcription factors: C/EBPα stimulates postmitotic growth arrest, while Myc sustains proliferative potential. The antagonistic relationship between these transcription factors is manifest in their regulation of *gadd*45. Specifically, C/EBPα stimulates *gadd*45 while Myc inhibits C/EBP-mediated transactivation. We anticipate that C/EBPα activation of growth arrest-associated genes accounts, in part, for its restricted expression in differentiated cells (6). We speculate that this regulation must be overcome for cells to reenter the mitotic cell cycle. Neoplastic transformation of differentiated cells provides an example in which we anticipate down-regulation of growth arrest-associated genes. We are currently examining the significance of C/EBPα and *gadd*45 for the maintenance of postmitotic growth arrest in adipocytes.

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REFERENCES

1. **Antoun, G. R., G. G. Re, N. H. Terry, and T. F. Zipf.** 1991. Molecular genetic evidence for a differentiation-proliferation coupling during DMSO-induced myeloid maturation of HL-60 cells: role of the transcription elongation block in the c-myc gene. Leuk. Res. **15:**1029–1036.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1995. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Baker, S. J., S. Markowitz, E. R. Fearon, J. K. U. Wilson, and B. Vogelstein.** 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science **249:**912–915.
4. **Bernlohr, D. A., C. W. Angus, M. D. Lane, M. A. Bolanowski, and T. J. Kelly.** 1984. Expression of specific mRNA during adipose differentiation: identification of an mRNA encoding a homologue of myelin P2 protein. Proc. Natl. Acad. Sci. USA **81:**5468–5472.
5. **Bernlohr, D. A., M. A. Bolanowski, T. J. Kelly, Jr., and M. D. Lane.** 1985. Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. **9:**5563–5567.
6. **Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight.** 1989. Tissue-specific expression, develop-

mental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev. **3:**1146–1156.
7. **Cao, Z., R. M. Umek, and S. L. McKnight.** 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. **5:**1538–1552.
8. **Christy, R. J., V. W. Yang, J. M. Ntambi, D. E. Geiman, W. H. Landschulz, A. D. Friedman, Y. Nakabeppu, T. J. Kelly, and M. D. Lane.** 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. Genes Dev. **3:**1323–1331.
9. **Dang, C. V., H. van Dam, M. Buckmire, and W. M. F. Lee.** 1989. DNA-binding domain of human c-Myc produced in *Escherichia coli*. Mol. Cell. Biol. **9:**2477–2486.
10. **Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, J. S. Butel, and A. Bradley.** 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature (London) **356:**215–221.
11. **el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
12. **Fornace, A. J., Jr., I. Alamo, Jr., and M. C. Hollander.** 1988. DNA damage-inducible transcripts in mammalian cells. Proc. Natl. Acad. Sci. USA **85:** 8800–8804.
13. **Fornace, A. J., Jr., D. W. Nebert, M. C. Hollander, J. D. Luethy, M. Papathenasiou, J. Fargnoli, and N. J. Holbrook.** 1989. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol. Cell. Biol. **9:**4196–4203.
14. **Freytag, S. O.** 1988. Enforced expression of the c-*myc* oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G_0/G_1. Mol. Cell. Biol. **8:**1614–1624.
15. **Freytag, S. O., and T. J. Geddes.** 1992. Reciprocal regulation of adipogenesis by Myc and C/EBP alpha. Science **256:**379–382.
16. **Green, H., and O. Kehinde.** 1975. An established preadipose cell line and its differentiation in culture. II. factors affecting the adipose conversion. Cell **5:**19–27.
17. **Halevy, B., G. Novitch, D. B. Spicer, S. X. Skapek, J. Rhee, G. J. Hannon, D. Beach, and A. B. Lassar.** 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science **267:**1018–1021.
18. **Hermeking, H., D. A. Wolf, F. Kohlhuber, A. Dickmanns, M. Billaud, E. Fanning, and D. Eick.** 1994. Role of c-myc in simian virus 40 large tumor antigen-induced DNA synthesis in quiescent 3T3-L1 mouse fibroblasts. Proc. Natl. Acad. Sci. USA **91:**10412–10416.
19. **Hinds, P. W., C. A. Finlay, A. B. Frey, and A. J. Levine.** 1987. Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-*ras*-transformed cell lines. Mol. Cell. Biol. **7:**2863–2869.
20. **Kaestner, K. H., J. M. Ntambi, T. J. Kelly, and M. D. Lane.** 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes. A second differentially expressed gene encoding stearoyl-CoA desaturase. J. Biol. Chem. **264:**14755–14761.
21. **Kastan, M. B., Q. Zhan, W. S. el-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein, and A. J. Fornace.** 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell **71:**587–597.
22. **Konigsberg, I. R.** 1979. Skeletal myoblasts in culture. Methods Enzymol. **58:**511–527.
23. **Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh, and M. B. Kastan.** 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA **89:**7491–7495.
24. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell **58:**823–831.
25. **Lin, F.-T., and M. D. Lane.** 1992. Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. Genes Dev. **6:**533–544.
26. **Lin, F. T., O. A. MacDougald, A. M. Diehl, and M. D. Lane.** 1993. A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. Proc. Natl. Acad. Sci. USA **90:**9606–9610.
27. **Macleod, K. F., N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein, and T. Jacks.** 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev. **9:**935–944.
28. **Nathans, D., L. F. Lau, B. Christy, S. Hartzell, Y. Nakabeppu, and K. Ryder.** 1988. Genomic response to growth factors. Cold Spring Harbor Symp. Quant. Biol. **53:**893–900.
29. **Rolland, V., X. Le Liepvre, M. L. Houbiguian, M. Lavau, and I. Dugail.** 1995. C/EBP alpha expression in adipose tissue of genetically obese Zucker rats. Biochem. Biophys. Res. Commun. **207:**761–767.
30. **Samuelsson, L., K. Stromberg, K. Vikman, G. Bjursell, and S. Enerback.** 1991. The CCAAT/enhancer binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte development. EMBO J. **10:**3787–3793.
31. **Scott, R. E., D. L. Florine, J. J. Wille, and K. Yun.** 1982. Coupling of growth arrest and differentiation at a distinct state in the G1 phase of the cell cycle: GD. Proc. Natl. Acad. Sci. USA **79:**845–849.
32. **Spiegelman, B. M., M. Frank, and H. Green.** 1983. Molecular cloning of mRNA from 3T3 adipocytes: regulation of mRNA content for glycerophosphate dehydrogenase and other differentiation-dependent proteins during adipocyte development. J. Biol. Chem. **258:**10083.
33. **Shugart, E. C., A. S. Levenson, C. M. Constance, and R. M. Umek.** 1995. Differential expression of *gas* and *gadd* genes at distinct growth arrest points during adipocyte development. Cell Growth Differ. **6:**1541–1547.
34. **Stein, G. H., and V. Dulic.** 1995. Origins of G1 arrest in senescent human fibroblasts. Bioessays **17:**537–543.
35. **Student, A. K., R. Y. Hsu, and M. D. Lane.** 1980. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. J. Biol. Chem. **255:**4745–4750.
36. **Umek, R. M., A. D. Friedman, and S. L. McKnight.** 1991. CCAAT/enhancer binding protein: a component of a differentiation switch. Science **251:**288–292.
37. **Vasseur-Cognet, M., and M. D. Lane.** 1993. *Trans*-acting factors involved in adipogenic differentiation. Curr. Opin. Genet. Dev. **3:**238–245.
38. **Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach.** 1993. p21 is a universal inhibitor of cyclin kinases. Nature (London) **366:** 701–704.
39. **Yeh, W. C., Z. Cao, M. Classon, and S. L. McKnight.** 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. **9:**168–181.
40. **Zhan, Q., F. Carrier, and A. J. Fornace.** 1993. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. Mol. Cell. Biol. **13:** 4242–4250.