CCAAT/enhancer-binding protein α (C/EBPα) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein

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C/EBPα has a role in growth arrest and differentiation of mouse preadipocytes. To study the mechanism of C/EBPα-induced growth arrest, we developed a cell line, HT1, that contained the human C/EBPα gene under Lac repressor control. IPTG-induced C/EBPα caused inhibition of cell proliferation and DNA synthesis as measured by colony growth assays, cell counting, and BrdU uptake. A number of proteins that are known to be involved in the regulation of the cell cycle, such as cyclin-dependent kinase (CDK) 2 and CDK4, proliferating cell nuclear antigen (PCNA), p53, c-*fos*, and the CDK inhibitor p16 and p27 were investigated by Western analysis. No change in their expression was observed. However, the p21 (WAF-1/CIP-1/SDI-1) protein was significantly elevated in growth-arrested HT1 cells. Elevation of p21/SDI-1 mRNA (threefold) and activation of the p21/SDI-1 promoter by C/EBPα did not account for the 12- to 20-fold increase in p21/SDI-1 protein. Protein synthesis inhibition by cycloheximide (CHX) treatment indicated that the half-life of p21/SDI-1 in dividing HT1 cells was ~30 min. However, in C/EBPα growth-arrested cells, the level of the p21/SDI-1 did not change for >80 min after CHX addition. Our studies demonstrate that C/EBPα activates p21/SDI-1 by increasing p21/SDI-1 gene expression and by post-translational stabilization of p21/SDI-1 protein. Furthermore, induction of p21/SDI-1 is responsible for the ability of C/EBPα to inhibit proliferation because transcription of antisense p21/SDI-1 mRNA eliminated growth inhibition by C/EBPα.

[*Key Words*: C/EBPα; growth inhibition; p21 (WAF-1/CIP-1/SDI-1); protein stabilization]

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CCAAT/enhancer-binding protein α (C/EBP α) was first identified in rat liver nuclear extracts as a heat-stable, sequence-specific, DNA-binding protein (Landschulz et al. 1988a). C/EBPα belongs to the bZIP family of proteins that contain a basic DNA-binding region and a leucine zipper domain (Landschulz et al. 1988b). The leucine zipper region is necessary for formation of functionally active homo- and heterodimers (Landschulz et al. 1989). C/EBPα binds specifically to DNA as a homo- or heterodimer and activates transcription of target genes (Friedman et al. 1989; Williams et al. 1991). High levels of C/EBPα expression are restricted to highly differentiated cells such as hepatocytes, adipocytes, and certain cells in the lung (Birkenmeier et al. 1989). C/EBPα has a general role in regulation of energy metabolism (McKnight et al. 1989; Wang et al. 1995), growth arrest (Umek et al. 1991; Freytag and Geddes 1992), and differentiation of mouse preadipocytes in culture (Cao et al. 1991; Lin and Lane 1994; Yeh et al. 1995). Several lines of evidence indicate that C/EBPα has a crucial role in regulating the balance between cell proliferation and differentiation. Umek et al. (1991) have shown that expression of mouse C/EBPα resulted in growth arrest of preadipocytes and promoted differentiation. Other investigators showed that C/EBPα expression promotes the adipogenic program in a variety of mouse fibroblastic cells (Freytag et al. 1994; Lin and Lane 1994). Expression of antisense C/EBPα has been shown to prevent both growth arrest and terminal differentiation of mouse 3T3 L1 adipoblasts (Lin and Lane 1992). However, in spite of its antimitogenic effect in many cell lines, some cells expressing a high level of C/EBPα can grow (Freytag et al. 1994).

In several circumstances, expression of C/EBPα shows an inverse correlation with cell proliferation. C/EBPα was reduced in regenerating liver with a maximal decrease before DNA synthesis (Mischoulon et al. 1992; Diehl and Yang 1993). Proliferating hepatoma lines HepG2 and Hep3B2 contained low levels of C/EBPα compared with normal liver (Friedman et al. 1989). Numerous experiments with a transiently transfected C/EBPα expression vector indicated that human C/EBPα

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inhibited proliferation of different human cells, including HeLa, Saos-2 (osteosarcoma), Hep3B2 (hepatoma), and diploid fibroblasts (Hendricks-Taylor and Darlington 1995). Taken together, these observations suggest that C/EBPα has an important role in regulation of cell growth.

Cell proliferation has been shown to be regulated by a new class of proteins, the cyclin-dependent kinase (CDK) inhibitors. These include p21 (known as WAF-1, CIP-1, SDI-1, and CAP20) (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994), p27 (Polyak et al. 1994), p57 (Matsuoka et al. 1995), and p16 and p15 (Serrano et al. 1995; Sherr and Roberts 1995). p21/SDI-1 was the first of the CDK inhibitors to be discovered (Noda et al. 1994). p21/SDI-1 has been shown to form a quaternary complex with CDKs, cyclins, and proliferating cell nuclear antigen (PCNA) resulting in inhibition of kinase activity and DNA synthesis (Xiong et al. 1993; Sherr and Roberts 1995). Expression of p21/SDI-1 is regulated by several proteins including p53 (El-Deiry et al. 1993), transforming growth factor-β (TGF-β) (Li et al. 1995), and MyoD (Halevy et al. 1995). p53 and MyoD activate p21/SDI-1 mRNA transcription through interaction with the p21/SDI-1 promoter (El-Deiry et al. 1993; Halevy et al. 1995). Induction of p21/SDI-1 during differentiation of muscle cells was accompanied by cessation of growth. Activation of p21/SDI-1 was also observed during differentiation of human HL-60 leukemia cells (Jiang et al. 1994). Macleod et al. (1995) described the elevation of p21/SDI-1 mRNA and protein coincidently with murine erythroleukemia cell differentiation suggesting a universal role for p21/SDI-1 in growth arrest and differentiation. In addition, CDK inhibitors p27 and p57 have sequence similarity to p21/SDI-1 in the region responsible for interaction with the CDKs (Matsuoka et al. 1995). It is possible that they inhibit cell proliferation through the same pathways as p21/SDI-1.

To investigate the molecular mechanism of C/EBPα-mediated growth arrest, we have generated stable cell lines HT1 and HT2 that contain the human C/EBPα gene under Lac repressor control. In this study we describe evidence that human C/EBPα inhibits proliferation of human fibrosarcoma cells by induction of the p21/SDI-1 protein. The elevation of p21/SDI-1 protein is caused, in part, by transcriptional activation of the gene; however, stabilization of the protein accounts for sustained increases in p21/SDI-1 levels. The p21/SDI-1 protein is the final mediator of C/EBPα-induced growth arrest because expression of antisense p21/SDI-1 mRNA released the cells from inhibition of proliferation.

Results

Transiently expressed human C/EBPα inhibits proliferation of human fibrosarcoma cells.

Several transformed human cell lines were growth inhibited following transient expression of C/EBPα. HT1080 human fibrosarcoma cells were chosen for further study. This cell line has a nearly normal karyotype, expresses normal p53, and does not express C/EBPα as measured by Western, bandshift, and "supershift" assays. As an initial step, we studied the effect of C/EBPα expression in transient experiments. Two C/EBPα-expressing vectors were used: cytomegalovirus (CMV)–C/EBPα and pOP13–C/EBPα (see Materials and methods). These two vectors produced a high level of functionally active C/EBPα after transfection into HT1080 cells (data not shown). CMV–C/EBPα and pOP13–C/EBPα were cotransfected with a β-gal-expressing vector by calcium phosphate precipitation into HT1080 cells. One, 3, and 5 days after transfection, the proportion of β-galactosidase-positive cells forming clusters of one, two, and more than two clusters was calculated. Cells transfected with the control CMV–stop plasmid formed cell clusters containing more than two cells at 3 and 5 days indicating that they had undergone division. However, very few cells expressing C/EBPα formed clusters of more than two cells at day 5 (Fig. 1) showing that transient expression of human C/EBPα inhibited proliferation of HT1080 cells.

HT1 and HT2 clones express functionally active 42-kD C/EBPα that is regulated by IPTG

To study the molecular mechanisms of C/EBPα-induced growth inhibition, we developed two clonal derivatives of HT1080 cells, HT1 and HT2, which contain the human C/EBPα gene under the control of the Lac repressor (LacSwitch-inducible mammalian expression system, Stratagene). The coding region of C/EBPα was cloned into the pOP13CAT vector by replacing the chloramphenicol acetyltransferase (CAT) gene with 1.3 kb of the human C/EBPα gene. The pOP13–C/EBPα plasmid and the Lac repressor-expressing vector were transfected into HT1080 cells, and stable clones were selected in hygromycin and G-418. Two clones, HT1 and HT2, were selected for further investigation from among several inducible clones. The kinetic induction of C/EBPα by IPTG was analyzed by Western and bandshift assays at 2, 4, 8, and 24 hr. In the absence of IPTG, C/EBPα protein and its binding activity were not detectable (Fig. 2, lanes 0, 24G). Two hours after addition of IPTG, induction of the 42-kD C/EBPα was observed. The maximum level of C/EBPα induction was at 8 hr in both HT1 and HT2 (Fig. 2A). Bandshift experiments indicated that induction of C/EBPα-binding activity correlated with the induction of protein level. Incorporation of anti-C/EBPα sera in the bandshift analyses verified that the induced binding complex contained C/EBPα (Fig. 2B). Three translation products of C/EBPα mRNA with molecular masses of 42, 40, and 30 kD have been described previously (Lin et al. 1993; Ossipow et al. 1993). All three proteins are alternative translation products initiated from the first, second, and third methionine codons (Lin et al. 1993; Ossipow et al. 1993). The 30-kD C/EBPα isoform lacks activation domain one, as defined by Friedman and McKnight (1990) and seems to have a role as a negative regulator of the full-length 42-kD C/EBPα by competition for binding or by formation of inactive hetero-

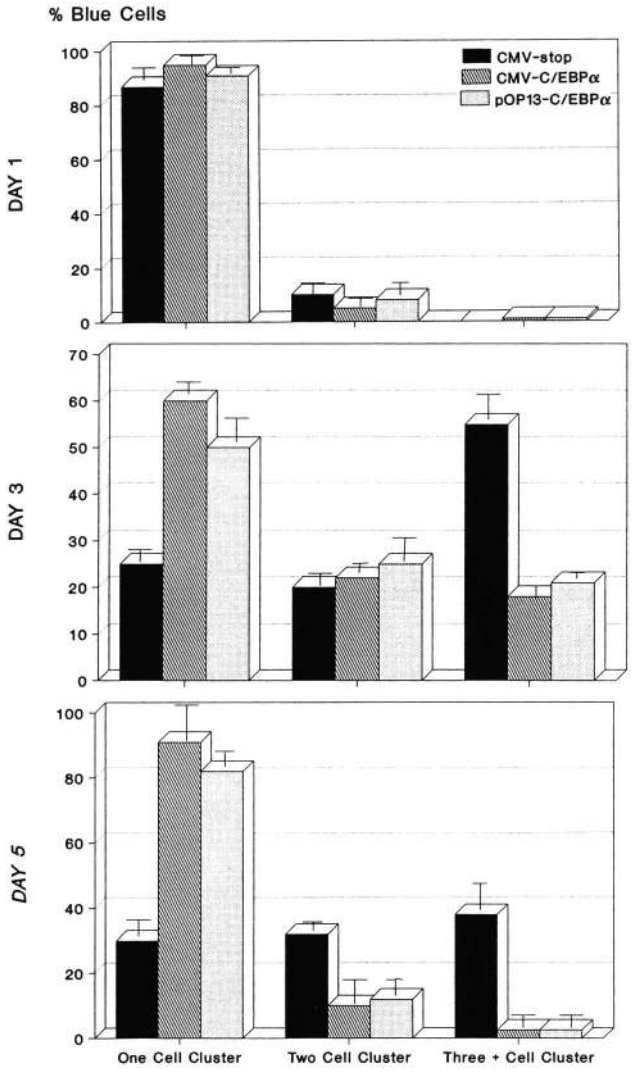

Figure 1. Transient expression of C/EBPα results in growth arrest of HT1080 human fibrosarcoma cells. CMV β-gal was co-electroporated into HT1080 cells with CMV–stop, CMV–CEBPα or pOP13–C/EBPα. The number of cell clusters containing one, two, and more than two cells was scored at day 1, 3, and 5.

dimers. In HT1, the 42- and 40-kD isoforms of C/EBPα were detected. The 30-kD C/EBPα isoform was not observed on Western analysis. The IPTG-induced C/EBPα *trans*-activated a promoter from the human C3 gene (C3luc 200, which contains a high affinity binding site for C/EBP proteins) following its transfection into HT1 cells. In these experiments luciferase activity was measured 18–24 hr after IPTG stimulation. No change in luciferase activity was registered in HT1080 cells. However, a three- to fivefold induction was observed in HT1 cells treated with IPTG, indicating specific activation of the C3 promoter by the C/EBPα protein expressed from the integrated, inducible C/EBPα gene construct (Fig. 2C).

IPTG-induced C/EBPα inhibits cell proliferation

A colony growth assay was used to determine whether

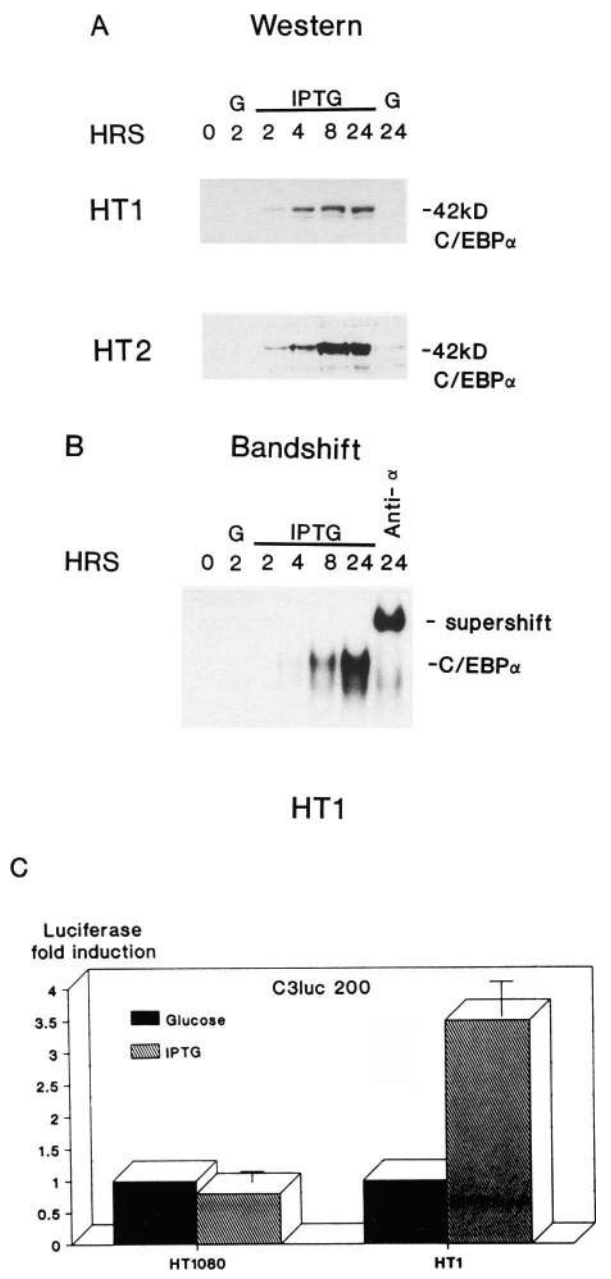

Figure 2. IPTG-mediated C/EBPα induction in HT1 and HT2 stably transformed clones. WCEs were isolated at different times (shown on the *top*) after IPTG or glucose addition and used in bandshift and Western experiments. (*A*) Western: 100 μg of WCEs was loaded on 10% PAAG–0.1% SDS and blotted on a NitroBind membrane. The filter was probed with specific antibodies to human C/EBPα. The position of the full-length 42-kD C/EBPα isoform is indicated. (*B*) Bandshift: WCEs were incubated with the bZIP oligonucleotide containing a high-affinity C/EBPα-binding site. DNA–protein complexes and free probe were separated in a native 5% acrylamide gel. (Anti-α) Antibodies to C/EBPα were added to the binding reaction with WCE isolated from HT1 cells 24 hr after IPTG stimulation. (*C*) IPTG-induced C/EBPα activates the C3luc 200 promoter containing a C/EBPα-binding site after transient transfection of C3luc 200.

the IPTG-induced C/EBPα level was sufficient to cause inhibition of cell proliferation. HT1, HT2, and HT1080 cells were plated in medium containing IPTG or glucose. Seven days after plating, cells were fixed and stained with hematoxylin. The density of the cells is shown in Figure 3A. Control cells (HT1080) formed colonies equally well in IPTG and glucose. However, a dramatic difference was observed between HT1 cells grown in the absence or presence of IPTG. The number of HT1 and HT2 colonies in glucose was identical to that in HT1080 control cells. In the presence of IPTG, colony formation was not observed, indicating strongly that IPTG-induced human C/EBPα caused growth inhibition of HT1 and HT2 cells. The bottom panel of Figure 3A (HT1×40) shows the small size of colonies in IPTG-treated HT1 cells compared with those in glucose.

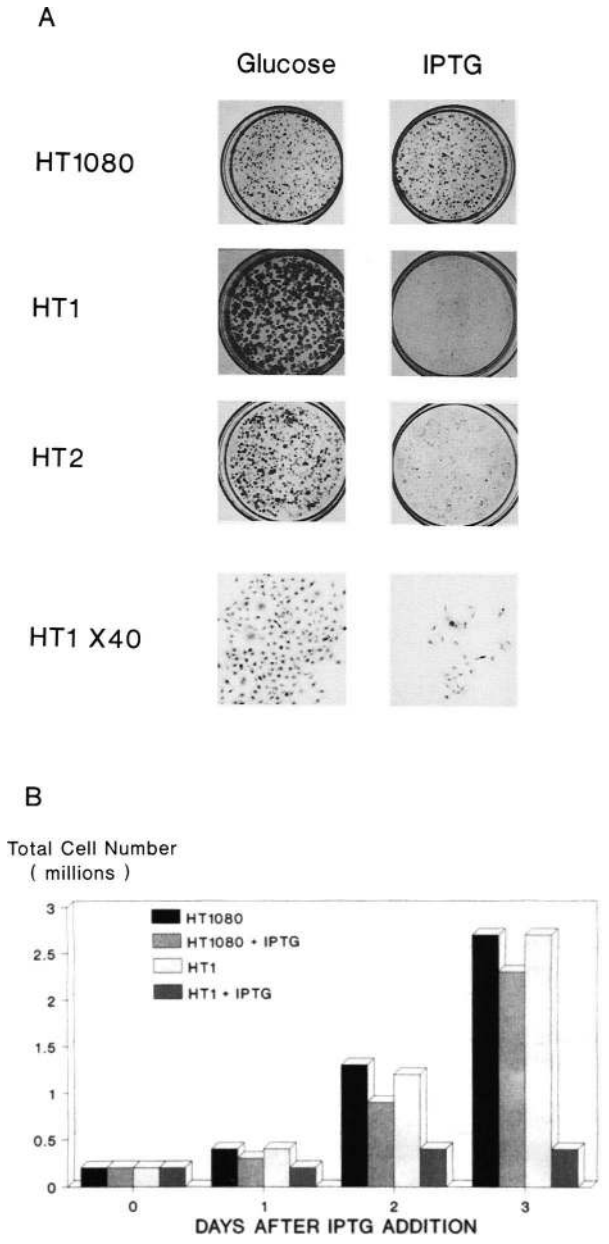

Figure 3. IPTG-induced C/EBPα inhibits proliferation of HT1 and HT2 cells. (*A*) Colony growth assay. HT1, HT2, and HT1080 cells were plated with 10 mM IPTG or with 10 mM glucose. Cells were fixed and stained with hematoxylin at 7 days. HT1X40 – colonies of glucose- and IPTG-treated HT1 cells were at 40× magnification. (*B*) The number of HT1 and HT1080 cells grown in the absence and in the presence of 10 mM IPTG was counted every 24 hr.

Table 1. *Inhibition of DNA synthesis by C/EBP α*

Harvest time (hr)	Percent BrdU-positive cells: 10 mM glucose	Percent BrdU-positive cells: 10 mM IPTG
4	53.9	48.5
12	56.5	50.5
24	52.4	33.0
48	42.5	17.5

DNA synthesis was measured by BrdU uptake. At the time of plating, 10 mM glucose or 10 mM IPTG was added. Between 1500 and 2000 cells were scored for each point. The results are the compilation of three experiments.

Further investigations were carried out with the HT1 clone. To examine the kinetics of IPTG-mediated growth inhibition of HT1 cells, we counted the number of cells 1, 2, and 3 days after plating in IPTG or glucose. As shown in Figure 3B, IPTG addition to HT1 cells caused inhibition of cell proliferation. The inhibition of growth was specific to cells expressing C/EBPα, as IPTG addition did not affect growth of control HT1080 cells. We also analyzed DNA synthesis in C/EBPα growth-arrested HT1 cells by measuring uptake of the thymidine analog BrdU at different times after induction of C/EBPα. Table 1 shows that incorporation of BrdU is reduced at 24 and 48 hr indicating that C/EBPα inhibits DNA synthesis. However, DNA synthesis was detectable in >15% of the HT1 cells even at day 3. In summary, transient expression, colony growth, and BrdU uptake revealed that IPTG-induced C/EBPα caused growth arrest of human fibrosarcoma cells.

C/EBPα induces p21 (WAF-1/CIP-1/SDI-1) protein in growth-arrested cells

The level of different proteins that are known to be involved in cell cycle control was examined by Western analysis. Whole-cell extracts (WCEs) from glucose (control) and IPTG-treated HT1 cells were prepared at 2, 4, 8, and 24 hr after IPTG addition. The levels of CDK2, CDK4, and PCNA (Fig. 4A,B) and c-Fos, p16, and p27 (data not shown) were identical in both IPTG-induced and uninduced cells. However, the level of p21/SDI-1 protein was significantly increased in HT1 cells expressing C/EBPα and its elevation was coordinate with C/EBPα induction. Note that the results presented in Figure 4 were obtained by reprobing the same filters with different antibodies (see legend to Fig. 4). Little or no decrease in PCNA protein level was observed in growth-

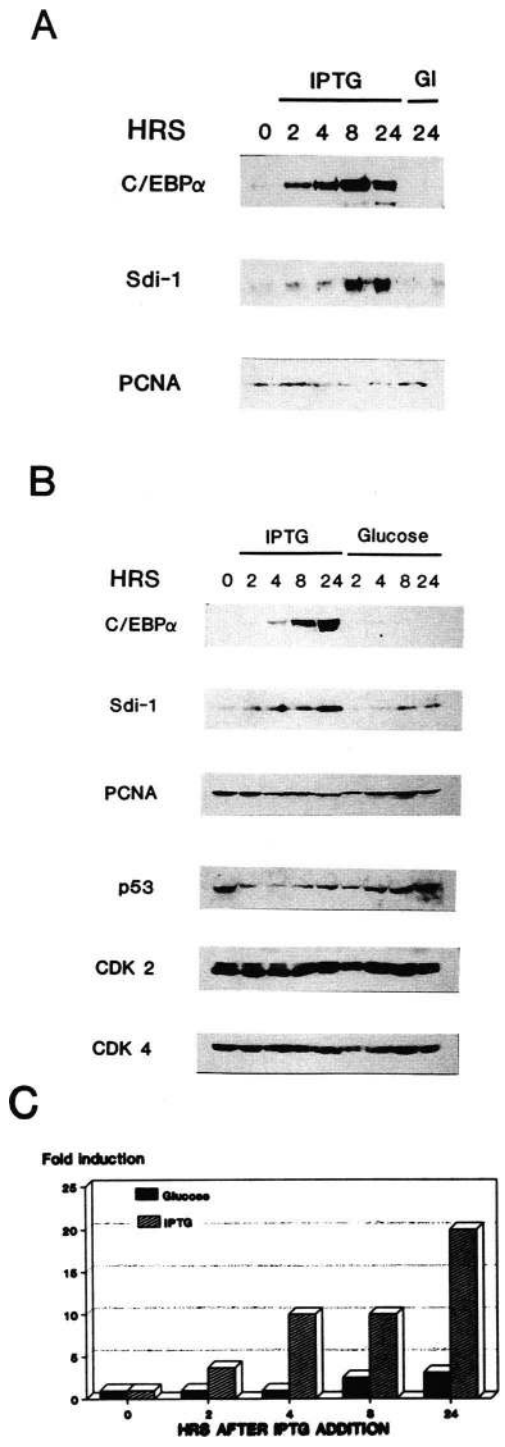

Figure 4. The level of p21/SDI-1 protein is increased in C/EBPα growth-arrested HT1 cells. Western analysis of WCEs from HT1 cells was carried out as described for Fig. 2. Two filters were probed sequentially by antibodies to p21/SDI-1, C/EBPα, PCNA, p53, CDK2, and CDK4 (*B*) and by antibodies to p21/SDI-1, C/EBPα, and PCNA (*A*). (*C*) The density of p21/SDI-1 immunoreactive bands in *B* was determined by laser densitometry, and the histograms were calculated as a ratio to the density of PCNA.

arrested cells. No change or a slight decrease of p53 protein level (Fig. 4B) was observed, suggesting that elevation of p21/SDI-1 is p53-independent. The degree of p21/SDI-1 induction was estimated by laser densitometry of immunoreactive bands comparing each time point with time 0 (Fig. 4C). A 20-fold induction of p21/SDI-1 protein was observed in experiments presented in Figure 4 (A,B). Western blots for p21/SDI-1 were repeated 10 times. The induction of p21/SDI-1 was reproducible with differences in the level varying between 12- and 20-fold. All experiments were carried out with low density cells (<30% confluent) within 24 hr after IPTG addition. We have observed that the level of p21/SDI-1 protein increased in high density cells, both HT1080 and HT1, perhaps as a consequence of cell–cell contact (data not shown).

Transcriptional activation of the p21/SDI-1 gene by C/EBPα is transient and modest

The relative p21/SDI-1 mRNA levels in HT1 in the presence of IPTG or glucose were determined as a ratio of p21/SDI-1 mRNA to 18S rRNA. The expression of p21/SDI-1 mRNA can be regulated by several factors such as fresh serum, cycloheximide (CHX), transforming growth factor-β (TGF-β), p53, and DNA damage (El-Deiry et al. 1993; Li et al. 1995; Macleod et al. 1995). The 1.5-kb C/EBPα mRNA contained within the expression construct was induced within 2 hr after IPTG addition, reached a maximum level at 4 hr and was stable for 24 hr (Fig. 5A). C/EBPα caused induction of p21/SDI-1 mRNA shortly after IPTG addition. The increase was first observed 2 hr after the addition of IPTG, with maximal induction of p21/SDI-1 mRNA occurring at ~4 hr. This increase was transient, however, with return to control levels within 8–12 hr. Activation of p21/SDI-1 mRNA is specific for HT1 cells expressing C/EBPα. Glucose-treated HT1 cells did not show a change in the level of p21/SDI-1 mRNA. Expression of C/EBPα protein resulted in induction of an additional mRNA species (2.7-kb C/EBPα) that hybridized with the C/EBPα probe (Fig. 5A). Although the p21/SDI-1 and C/EBPα probes were hybridized simultaneously in the blot shown in Figure 5A, other blots using only the C/EBPα probe showed that the 2.7-kb band was specific to this gene. The size of human C/EBPα mRNA transcribed from the chrsomosomal gene is identical to that detected in IPTG-induced HT1 cells, suggesting that C/EBPα expression from the pOP13–C/EBPα construct activated the promoter of the endogenous C/EBPα gene. It is known that both mouse and human C/EBPα promoters can be activated by C/EBPα, but the mechanisms of activation are different (Legraverend et al. 1993; Timchenko et al. 1995). Measurements of the Northern blot by phosphorimaging as an p21/SDI-1/18S ratio shows that p21/SDI-1 mRNA is induced 3.5-fold at 4 hr (Fig. 5B). The transient induction of p21/SDI-1 mRNA was reproducible in four experiments with the level of p21/SDI-1 mRNA induction varying between three and fourfold.

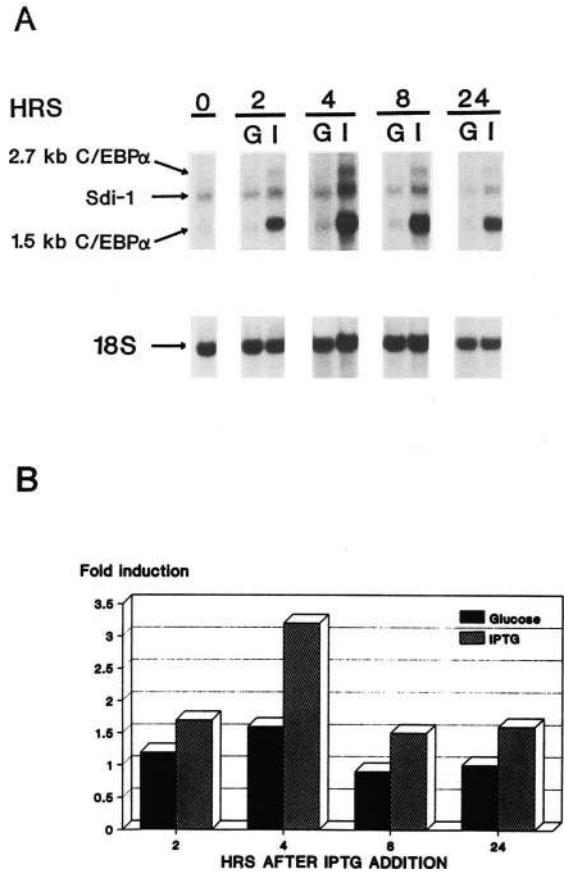

Figure 5. Induction of p21/SDI-1 mRNA by C/EBPα is transient. HT1 cells were incubated in the presence of IPTG (I) or glucose (G). RNA was isolated at different times after IPTG addition (indicated on the *top*) and used for Northern blots as described in Materials and methods. (*A*) *Top*: The filter was hybridized with probes for p21/SDI-1 and C/EBPα in the same hybridization mixture. *Bottom*: the same filter was reprobed with an oligonucleotide complementary to 18S rRNA. (*B*) The level of p21/SDI-1 mRNA was normalized to the level of 18S rRNA. Signal density was quantitated by phosphorimaging.

Human C/EBPα activates the p21/SDI-1 promoter

p21/SDI-1 mRNA can be regulated by several factors including DNA damage. Transient transfection of plasmid DNA (vector only) elevated p21/SDI-1 mRNA (data not shown); therefore, we used stable clones with the p21/SDI-1 promoter coupled to CAT (−550–p21/SDI-1–CAT) integrated into genomic DNA to avoid the high background of p21/SDI-1 expression observed in transient transfection experiments. Several populations of HT1 cells containing −550–p21/SDI-1–CAT were selected (see Materials and methods) and tested by Northern analysis with a specific CAT probe. Figure 6A shows that IPTG-induced C/EBPα stimulates transcription of CAT mRNA through the p21/SDI-1 − 550 promoter. The level of activation varied from two- to fourfold above the baseline in different populations. CAT activity of population 1 after IPTG addition showed a maximal induction of three- to fourfold at 8–14 hr. No change in CAT activity was observed in glucose-treated cells (Fig. 6B), indicating that activation of the p21/SDI-1 promoter was specific to cells expressing a high level of C/EBPα. We conclude that IPTG-induced C/EBPα activates the p21/SDI-1 promoter, but the level and duration of transcriptional activation are not great enough to explain the increased amount of p21/SDI-1 protein present in HT1 cells treated with IPTG.

p21/SDI-1 protein stability is increased in C/EBPα growth-arrested cells

The induction of p21/SDI-1 mRNA is moderate and transient (see Fig. 5). However the p21/SDI-1 protein increased by 12- to 20-fold and was stable for 24 hr, suggesting that p21/SDI-1 expression in C/EBPα growth-arrested cells is regulated at the post-translational level.

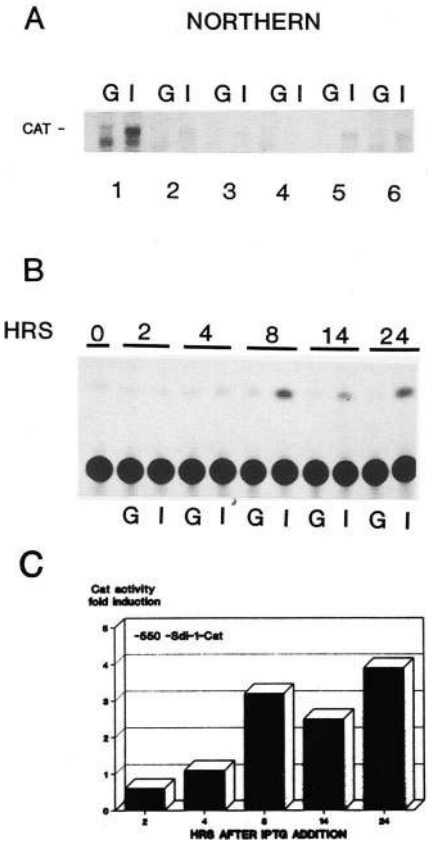

Figure 6. Activation of the p21/SDI-1 promoter by C/EBPα. (*A*) Six populations of HT1 cells (lanes *1–6*) stably transformed with a −550–p21/SDI-1–CAT construct were treated with IPTG (I) or glucose (G). After 12 hr, total RNA was isolated, blotted, and probed for CAT. (*B*) Population 1 (stably transformed with a −550–p21/SDI-1–CAT construct) was treated with IPTG or glucose for 0, 4, 8, 14, and 24 hr. CAT activity (percentage of counts converted to the acetylated form) was determined (Gorman et al. 1982) at these time points. (*C*) Fold induction was estimated as the ratio of CAT activity in IPTG-treated cells to those treated with glucose.

To test this possibility, the half-life of the p21/SDI-1 protein was measured in HT1 cells expressing C/EBPα and in HT1 cells treated with glucose. After overnight incubation with IPTG or glucose, CHX, an inhibitor of protein synthesis, was added (20 μg/ml). Proteins were isolated at 0, 40, and 80 min after CHX addition and analyzed in Western blots. To detect p21/SDI-1 protein in glucose-treated cells, it was necessary to load 300 μg of protein, whereas only 30 μg of protein from IPTG-induced cells was used. In dividing HT1 cells, the half-life of p21/SDI-1 protein is ~30 min. However in HT1 cells expressing C/EBPα, no decrease of p21/SDI-1 was observed >80 min after CHX blockage of protein synthesis (Fig. 7). The same filter was reprobed with antibodies

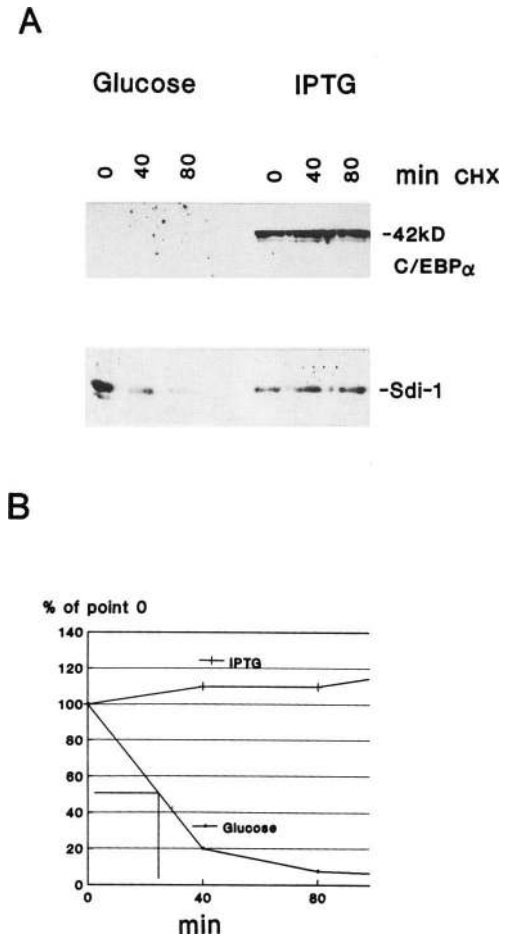

Figure 7. p21/SDI-1 protein is stable in C/EBPα growth-arrested HT1 cells. (*A*) HT1 cells were incubated with 10 mM IPTG or 10 mM glucose overnight. CHX (20 μg/ml) was added, and proteins were isolated at 0, 40, and 80 min after CHX addition. Cell proteins were used for Western analysis as described in Materials and methods. To detect the low level of p21/SDI-1 protein present in dividing cells, 300 μg of total protein from glucose-treated HT1 cells was loaded in each lane. Thirty micrograms of protein from IPTG-induced cells was loaded per lane. The filter was probed sequentially with antibodies to p21/SDI-1 and to C/EBPα. (*B*) Signal intensity was measured by densitometry and calculated as a percentage of point 0.

specific to C/EBPα. The 42-kD C/EBPα is not detectable in control HT1 cells. In IPTG-induced cells, the level of C/EBPα did not decrease within 80 min after CHX addition. This observation indicates that expression of C/EBPα results in stabilization of the p21/SDI-1 protein.

Expression of antisense p21/SDI-1 mRNA eliminates C/EBPα-mediated growth inhibition

To demonstrate that p21/SDI-1 is responsible for C/EBPα-mediated growth arrest, we determined the effect of antisense p21/SDI-1 mRNA expression on the ability of C/EBPα to inhibit proliferation of HT1 cells. Expression of antisense p21/SDI-1 mRNA has been shown to decrease the p21/SDI-1 protein level and to abolish p21/SDI-1-mediated growth arrest in cotransfection assays (Nakanishi et al. 1995). Cotransfection of CMV–stop (control) or CMV–As–p21/SDI-1 (expressing antisense p21/SDI-1 mRNA, +1 to +165) with CMV–β-Gal into HT1 cells was followed by addition of IPTG or glucose 18 hr after plasmid delivery. Cells receiving control DNA (CMV–stop) remained as single cells in the presence of IPTG-induced C/EBPα (Fig. 8A). In contrast, the expression of antisense p21/SDI-1 mRNA abolished C/EBPα-mediated growth arrest, indicating that C/EBPα inhibits cell proliferation through the p21/SDI-1 protein. HT1080 cells are responsive to overexpression of p21/SDI-1 following transient transfection of a p21/SDI-1 expression construct and exhibit significant growth arrest (data not shown). An additional association between C/EBPα and p21/SDI-1 expression was observed in two clones that expressed a relatively high level of C/EBPα in response to IPTG but did not show growth arrest. These two clones (HT2C4 and HT1b9) were among the clones isolated simultaneously with HT1 and HT2. Western blotting of cell extracts showed that no elevation of the p21/SDI-1 protein was detected in these clones following IPTG induction of C/EBPα (Fig. 8B; data not shown for HT1b9). These results demonstrated that induction of p21/SDI-1 by C/EBPα is important for C/EBPα-mediated growth arrest. Taken together, the observations strongly indicate that C/EBPα inhibits cell proliferation through the elevation of p21/SDI-1 protein.

Discussion

An antiproliferative effect of C/EBPα has been described by several investigators (Umek et al. 1991; Freytag and Geddes 1992; Freytag et al. 1994; Lin and Lane 1994); however, the molecular mechanisms that are involved in C/EBPα-mediated growth arrest are unknown. C/EBPα-mediated growth arrest is likely to involve coordinate expression of several genes. Here, we examined gene products known to be important in cell cycling and found a dramatic increase of p21/SDI-1 protein in C/EBPα-arrested HT1 cells. The maximum level of p21/SDI-1 induction (12- to 20-fold) was observed at 8–24 hr after IPTG addition, immediately after the maximal C/EBPα induction. The levels of other proteins, including two other CDK inhibitors, p16 and p27, were unaf-

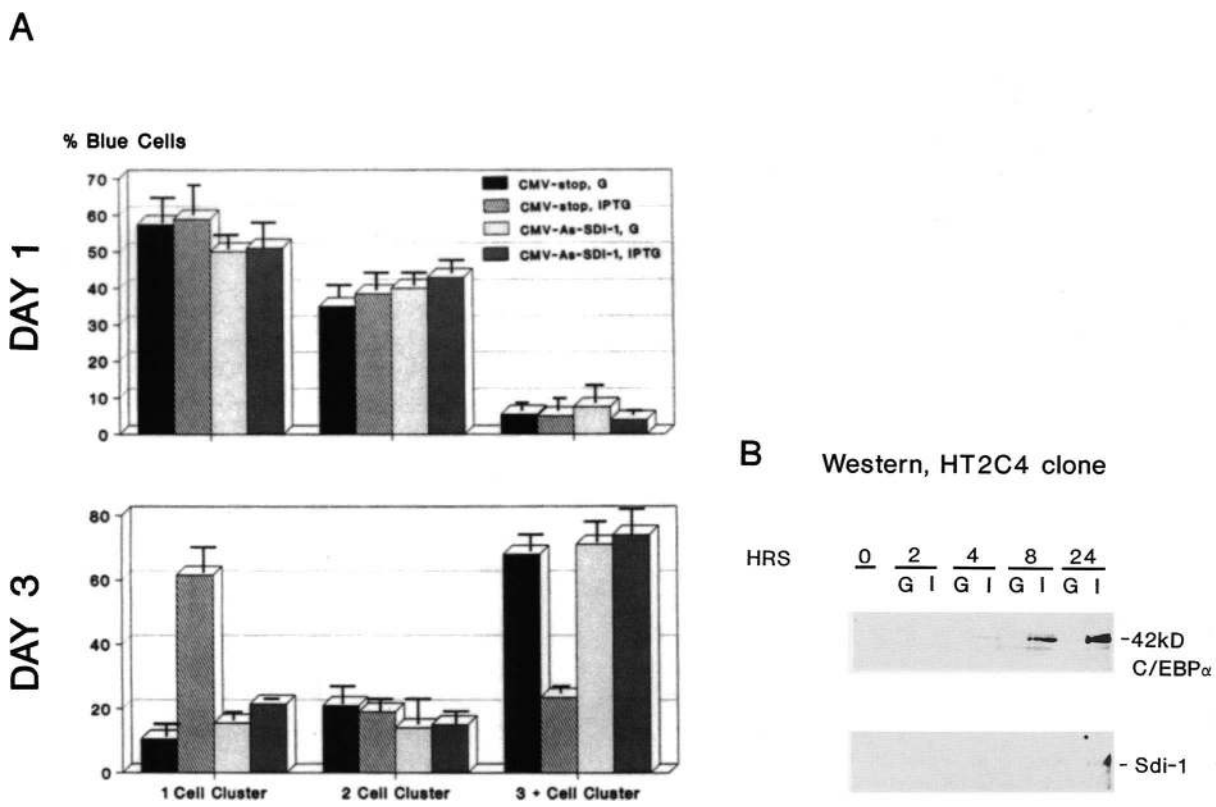

Figure 8. p21/SDI-1 protein is responsible for C/EBPα inhibition of cell proliferation. (*A*) Expression of antisense p21/SDI-1 mRNA blocks C/EBPα growth inhibition. The CMV–As–p21/SDI-1 plasmid (expressing antisense p21/SDI-1 mRNA) was cotransfected with CMV–β-Gal into HT1 cells, and growth inhibition was measured as described in Materials and methods. CMV–stop was used as the control. (*B*) C/EBPα failed to induce p21/SDI-1 protein in clone HT2C4, which showed no growth inhibition. Cell extracts were isolated at different times after IPTG addition (*top*) and used in Western blotting with specific antibodies to p21/SDI-1 and to C/EBPα. (*Right*) The positions of C/EBPα (42 kD) and p21/SDI-1.

fected in growth-arrested cells. We would predict that C/EBPα activates the transcription of a gene whose product is responsible for prolonging the half-life of p21/SDI-1 protein as opposed to the direct interaction of C/EBPα with the CDK inhibitor. Macleod et al. (1995) reported post-translational regulation of the p21/SDI-1 protein in murine erythrsoleukemia cells, although protein stability was not examined. We are currently investigating both direct and indirect mechanisms for the specific effect of C/EBPα on the half-life of p21/SDI-1.

The pOP13–C/EBPα gene construct integrated into the chrsomosomal DNA of HT1080 cells, produced the full-length 42- and 40-kD C/EBPα isoforms. Both isoforms contain activation domains and have been shown to possess antimitogenic activity (Lin et al. 1993). In the clonally derived HT1 line, IPTG-induced C/EBPα was functionally active and stimulated the promoter from the human C3 gene, which contained a high affinity C/EBPα-binding site (Wilson et al. 1990; Juan et al. 1993). We did not detect expression of the 30-kD C/EBPα isoform in any experiments with HT1 or HT2 cells. This observation was unexpected because other investigators had reported a significant level of expression of the 30-kD C/EBPα in adipocytes and in the liver (Diehl and Yang 1993; Lin et al. 1993; Diehl et al. 1994). The 30-kD C/EBPα isoform was shown to be a translational product from the third in-frame AUG codon of the C/EBPα mRNA (Lin et al. 1993). This translational start is present in pOP13–C/EBPα but is apparently not used in HT1 cells. Calkhoven et al. (1994) have reported that a short 5′ open reading frame (ORF) dictated the use of the third AUG codon as a translational start within chicken and rat C/EBPα mRNA. A short 5′ ORF is also present in the human C/EBPα gene and in the integrated construct, yet expression of the 30-kD C/EBPα was not detectable in HT1 IPTG-induced cells. We suggest that alternative translation of the 30-kD C/EBPα isoform may also be dictated by the properties of the cell type under study or by the 3′- untranslated portion of the mRNA that is lacking in our inducible construct.

Growth arrest of HT1 and HT2 clones by C/EBPα was confirmed by three independent methods: colony growth assay, cell counting, and BrdU uptake. However, we have also identified clones of HT1080 cells transformed by the C/EBPα LacSwitch constructs that expressed levels of C/EBPα similar to HT1 and HT2 in response to IPTG but did not show significant growth arrest. These clonal isolates had elevated levels of liver inhibitory protein (LIP),

the 21-kD translation isoform from the C/EBPβ gene (N.A. Timchenko, unpubl.). Yeh et al. (1995) have described the ability of LIP to ablate C/EBPα activity. Freytag et al. (1994) also have observed that clonal mouse cell lines stably expressing high amounts of C/EBPα can be generated, although the expression of LIP was not examined. Lin and Lane (1994) reported the isolation of clonal lines that expressed moderate levels of C/EBPα but did not show growth arrest. These investigators suggested that the level of C/EBPα in these clones was not sufficient to block cell proliferation (Lin and Lane 1994). These multiple observations show that C/EBPα growth inhibition is complex and can be regulated by a number of factors including the level of C/EBPα protein, the C/EBPα isoform expressed, and the expression of LIP.

Because the human C/EBPα is a transcription factor, its growth inhibitory effect would be predicted to be attributable to the transcriptional activation of genes responsible for the inhibition. However, transcriptional activation of the p21/SDI-1 gene is minor and does not account for the sustained elevation of p21/SDI-1 protein in HT1 growth-arrested cells. Our results indicate that p21/SDI-1 protein is responsible for C/EBPα-mediated growth arrest. We suggest that human C/EBPα uses two different mechanisms for activation of p21/SDI-1. First, transcriptional activation is necessary for the initial events, but stabilization of the p21/SDI-1 protein is responsible for its high level at the later steps of growth arrest. The mechanism of p21/SDI-1 stabilization by C/EBPα is unknown. It may be that transcriptional activation by C/EBPα is important to produce a relatively high concentration of p21/SDI-1 protein, which then sets up a C/EBPα-independent pathway. For example, p21/SDI-1 could be involved in an autoregulatory loop. In such a case, a modest transcriptional induction would be a key element of p21/SDI-1 protein elevation.

The HT1080 cells from which HT1 was derived, do not express endogenous C/EBPα mRNA or protein. The size of the endogenous C/EBPα mRNA is 2.7 kb, whereas the transcript from pOP13–C/EBPα is 1.5 kb, enabling us to detect activation of the endogenous 2.7-kb C/EBPα mRNA in growth-arrested HT1 cells. The activation of endogenous C/EBPα is probably caused by the induction of its own promoter by the C/EBPα protein. We showed previously that human C/EBPα can activate its own promoter through upstream stimulatory factor (USF), a ubiquitous nuclear factor (Timchenko et al. 1995). Lin and Lane (1994) also observed activation of endogenous murine C/EBPα in stable transformants overexpressing the mouse C/EBPα protein. It is interesting to note that IPTG-induced C/EBPα (42 kD) could not activate the expression of the endogenous 2.7-kb C/EBPα mRNA in the HT2C4 clone that was resistant to C/EBPα growth inhibition (data not shown). In HT1 cells, IPTG-induced C/EBPα increased expression of both p21/SDI-1 and endogenous C/EBPα mRNA. Activation of p21/SDI-1 mRNA by C/EBPα in growth-arrested HT1 cells was transient with a maximal level at 4 hr after IPTG stimulation. Elevation of the p21/SDI-1 promoter (550 bp) by C/EBPα in stable transformants suggests that the elevation in p21/SDI-1 mRNA is caused by an elevation in transcription of the gene. Induction of the endogenous 2.7-kb C/EBPα mRNA species correlated with the level of the 1.5-kb C/EBPα mRNA. Thus, two endogenous genes (p21/SDI-1 and C/EBPα) were activated in C/EBPα growth-arrested HT1 cells. Both genes are known to be involved in inhibition of cell proliferation. C/EBPα is a transcription factor and may well activate several genes during growth arrest. Our results demonstrate that the inhibition by C/EBPα is mediated through p21/SDI-1 because expression of p21/SDI-1 antisense mRNA abolished C/EBPα-mediated growth arrest indicating that p21/SDI-1 is critical to inhibition of cell proliferation by C/EBPα. Two clones, HT2C4 and HT1b9—not subject to arrest by C/EBPα—had no increased expression of p21/SDI-1, consistent with the role of p21/SDI-1 in C/EBPα-mediated growth arrest. The mechanism by which these clones are protected from C/EBPα action is unknown, but it is clear that C/EBPα cannot elevate p21/SDI-1 in these cells.

Our results show that C/EBPα influences p21/SDI-1 expression in HT1 cells in two ways: (1) a modest and transient transcriptional induction of the message level and post-translational stabilization of the protein that resulted in a significant increase in p21/SDI-1 levels, and (2) in inhibition of cell proliferation. p21/SDI-1 is also induced by MyoD during growth arrest and differentiation of skeletal muscle cells (Halevy et al. 1995) and by p53 during p53-mediated growth arrest (El-Deiry et al. 1993). The involvement of p21/SDI-1 in growth arrest by several DNA-binding proteins, both tissue specific and ubiquitous, suggests that p21/SDI-1 is a universal cell cycle regulator, functioning in growth arrest associated with differentiation, DNA damage response pathways, and cellular senescence.

Materials and methods

Cell culture conditions

HT1080 human fibrosarcoma cells were cultured in M/M (three parts Eagle's minimal essential medium, one part Waymouth MAB 87/3) plus 8% bovine serum and 2% fetal bovine serum. General tissue culture methods are as described (Wilson et al. 1990). HT1 and HT2 clonal derivatives were cultured in medium that also contained 250 μg/ml of G418 and 100 μg/ml of hygromycin. To induce expression of 42-kD C/EBPα, 10 mM IPTG was added to the culture medium. Control cells received 10 mM glucose. Most experiments were carried out with low-density (<50% confluence) HT1 cells. The functional activity of IPTG-induced C/EBPα was tested by using a plasmid C3luc 200 that contained a 200-bp DNA fragment of the human third component of complement C3 promoter that has a high affinity C/EBP-binding site (Wilson et al. 1990). The C3luc 200 plasmid was electroporated into HT1 cells or into HT1080 cells, and luciferase activity was measured 18–24 hr after IPTG addition.

For investigation of p21/SDI-1 protein stability, HT1 cells were incubated overnight with 10 mM IPTG or with 10 mM glucose. CHX was then added to a final concentration of 20 μg/ml of medium. WCEs were isolated at 0, 40, and 80 min after CHX addition.

BrdU uptake was measured by counting the number of im-

munopositive cells after a 2-hr incubation with medium containing BrdU (Amersham).

Generation of clonal derivatives containing the human C/EBPα gene under Lac repressor control

The LacSwitch-inducible promoter system (Stratagene) was used for conditional expression of C/EBPα. The coding region of C/EBPα was cloned into the pOP13CAT vector (Stratagene) by replacing the CAT gene with 1.3 kb of the C/EBPα gene (pOP13–C/EBPα). This 1.3-kb DNA fragment also was used for the generation of a CMV–C/EBPα-expressing vector described previously (Timchenko et al. 1995). The vector containing the *lac* repressor gene, and pOP13–C/EBPα were coelectroporated into HT1080 human fibrosarcoma cells in a ratio of 10:1. Clones resistant to G418 and to hygromycin were tested for C/EBPα induction by the addition of 10 mM IPTG. Based on the results of electrophoretic mobility-shift assays (EMSAs), Western and Northern analyses, we selected several clones that did not express a detectable amount of C/EBPα in the absence of IPTG but were highly induced for C/EBPα in the presence of IPTG. Two clones, HT1 and HT2, were used for study of C/EBPα-mediated growth arrest.

Stable clones containing the p21/SDI-1-CAT promoter

HT1 cells were cotransfected with plasmids −550–p21/SDI-1–CAT and pPGK–puromycin coding for the puromycin resistance gene. Clones resistant to three antibiotics—G418, hygromycin, and puromycin—were selected and tested by Southern hybridization with a probe derived from the CAT gene. Several populations of −550–p21/SDI-1–CAT stable clones were induced by IPTG or by glucose. Activation of the p21/SDI-1 promoter was investigated by using Northern analysis with a CAT-specific probe and by detection of enzymatic CAT activity.

Colony growth assay

HT1, HT2, and HT1080 (800 cells) were plated in petri dishes with 10 mM IPTG or with 10 mM glucose. Seven days after IPTG stimulation, cells were fixed and stained with hematoxylin.

RNA extraction and Northern analysis

Total RNA was isolated by Stat-60 (TEL-TEST "B," INC.). Total RNA (15 μg) was loaded on 1% agarose/2.2 M formaldehyde gel, transferred onto a nylon filter (Zeta probe), and hybridized with specific probes as described in the supplier's manual. For detection of p21/SDI-1 mRNA, a 2.1-kb full-length cDNA probe was used (Noda et al. 1994). C/EBPα mRNA was detected by a 228-bp fragment obtained after digestion of the C/EBPα DNA by *Bsm*aI (1085) and *Xho*I (1.313). The 228-bp probe corresponds to the 3′ end of the coding region. The p21/SDI-1 and C/EBPα probes were labeled by random priming (Pharmacia) to high specific activity. After hybridization with these probes, the membrane was stripped and rehybridized with an oligonucleotide complementary to 18S RNA (5′-GCCGTGCGTACTTAGACATGCATG-3′) used as a control for sample loading. The hybridization solution for this short oligonucleotide contained 40% formamide, 5× SSPE, 7% SDS, and 100,000 cpm/ml of ^{32}P-labeled oligonucleotide. The filter was washed twice with 2× SSC, 0.1% SDS, for 30 min at room temperature.

Protein isolation and Western assay

Low-density HT1 cells were induced by 10 mM IPTG or 10 mM glucose. WCEs were isolated at different times after induction. Cells were scraped in PBS, centrifuged for 5 min at 5000 rpm, and resuspended in 100 μl of buffer B [10 mM Tris-HCL (pH 7.5), 0.420 M NaCl, 1.5 mM $MgCl_2$, 1 mM DDT, 0.5 mM EDTA, and 25% sucrose]. Cells were homogenized by shearing the suspension through a 23-gauge needle and incubated on ice for 10 min. After centrifugation, the supernatant (WCE) was frozen and stored at −80°C. Extracted proteins (80–100 μg) were loaded on a 0.1% SDS–12% polyacrylamide gel (Laemmli 1970), electrophoresed, and electroblotted onto NitroBind membranes (Micron Separations, Inc.). To equalize the loading of proteins, a preliminary filter was stained with Coomassie blue. The filter was blocked by 10% dry milk, 2% BSA, prepared in TTBS (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.05% Tween 20) buffer saline. For estimation of p21/SDI-1 protein, we used three different specific antibodies: monoclonal anti-p21 (Santa Cruz Biotechnology, Cat. no. SC-817), monoclonal anti-SDI-1 6B6 (Pharmingen), and a polyclonal anti-SDI-1 (Pharmingen). All antibodies showed identical results with little or no difference in sensitivity. Antibodies to C/EBPα were obtained by immunization of rabbits with bacterially expressed full-length human C/EBPα. These antibodies are specific to C/EBPα and do not cross-react with C/EBPβ or C/EBPδ in Western assay (data not shown). Antibodies to PCNA, p16, p27, CDK2, CDK4, CDC2, and c-Fos were obtained from Santa Cruz Biotechnology. Anti-p53 monoclonal antibody was from Oncogene Science. Immunoreactive proteins were detected by the enhanced chemiluminescent (ECL) protocol (Amersham). Each filter was reprobed three to five times with different antibodies according to the recommended procedure (ECL system).

EMSA

Binding activity of C/EBPα was determined by bandshift assay as described previously (Timchenko et al. 1995). A ^{32}P-labeled basic leucine zipper (bZIP) oligonucleotide was used as the probe (Wilson et al. 1990). Unlabeled bZIP oligonucleotide (100-fold excess) or antibodies were added to cell protein extracts before probe addition.

Transient transfection of an antisense p21/SDI-1 expression vector

The CMV–As–p21/SDI-1 plasmid contained 1–165 nucleotides of p21/SDI-1 cDNA in the opposite orientation. CMV–stop (control) and CMV–As–p21/SDI-1 were cotransfected with CMV–β-Gal plasmid into HT1 cells using calcium phosphate precipitation. IPTG was added to the cells 18 hr after plasmid delivery. Cell growth was measured by counting cell clusters of one, two, three, and more cells at day 1 and 3 after addition of IPTG.

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