

# Modulation of Early Human Preadipocyte Differentiation by Glucocorticoids

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**Glucocorticoids provide an adipogenic stimulus that is most obvious in the truncal obesity of patients with Cushing's syndrome. Glucocorticoid treatment also strongly potentiates the differentiation of human preadipocytes in culture. However, the molecular basis of these stimulatory effects remains to be defined. In this study, we provide a detailed analysis of the specific contribution of glucocorticoid treatment to the differentiation of primary human preadipocytes cultured in chemically defined medium. Contrary to previous descriptions of glucocorticoids being required throughout the course of differentiation, our results show that glucocorticoid treatment is stimulatory only during the first 48 h of differentiation. Furthermore, stimulation by glucocorticoids and the peroxisome proliferator activator receptor- $\gamma$  agonist troglitazone is mediated sequentially. Several details of the early events in the differentiation of human preadipocytes and the**

**contribution of steroid to these events differ from the responses observed previously in murine preadipocyte models. First, glucocorticoid treatment stimulated the early accumulation of CCAAT enhancer binding protein- $\beta$  (C/EBP $\beta$ ) in primary human preadipocytes. Second, induction of C/EBP $\alpha$  in primary human preadipocytes was noted within 4 h of adipogenic stimulus, whereas C/EBP $\alpha$  induction is not detected until 24–48 h in the murine 3T3 L1 preadipocyte model. Remarkably, by contrast to human primary preadipocytes, which do not undergo postconfluent mitosis, 3T3 L1 murine preadipocytes stimulated to differentiate under chemically defined conditions required glucocorticoids to survive the clonal expansion that precedes terminal differentiation, revealing a novel signal imparted by glucocorticoids in this immortalized murine cell system. (*Endocrinology* 147: 5284–5293, 2006)**

**G**LUCOCORTICIDS PROVIDE A potent stimulus for adipogenesis. The manifestation of this stimulus is most obvious in the truncal obesity of patients with Cushing's syndrome, which is a direct consequence of prolonged hypercortisolemia (1). Furthermore, there are many similarities between the clinical features of non-Cushingoid obese and hypercortisolemic patients, including central fat accumulation, elevated blood pressure, insulin resistance with impaired glucose tolerance, and dyslipidemia (2).

The relationship between glucocorticoids and adipogenesis is complex. Circulating glucocorticoids contribute to white adipose tissue development, which in turn is supplemented by locally produced steroid, by mature white adipose tissue, as a result of the action of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1). In animal models, overexpression of 11 $\beta$ -HSD1 in mature adipocytes has provided a transgenic model for visceral obesity (3). Conversely, 11 $\beta$ -HSD1 knockout mice are resistant to visceral fat accumulation (4).

Although the physiological relevance of glucocorticoids in promoting adipogenesis is well accepted, definition of the specific stimulatory role of glucocorticoids in the transcrip-

tional cascade leading to preadipocyte differentiation has been largely limited to studies with established rodent preadipocyte cell lines such as murine 3T3 L1 cells (5, 6). Adipogenic stimulation of postconfluent 3T3 L1 cells cultured in the presence of calf serum initiates a 48-h period of clonal expansion and induction of a cascade of transcription factors beginning with CCAAT enhancer binding protein  $\beta$  (C/EBP $\beta$ ) and C/EBP $\delta$ . Commitment to differentiate is reached within 48 h of induction upon expression of C/EBP $\alpha$  and peroxisome proliferator activator receptor (PPAR $\gamma$ ) (5).

In these murine cells, glucocorticoid treatment is stimulatory only during the initial 48-h clonal expansion phase (7). Glucocorticoid stimulation has been ascribed to direct stimulation of C/EBP $\delta$  transcription (8), repression of the antiadipogenic preadipocyte factor 1 (pref-1) (9), and the depletion of a specific subcellular pool of histone deacetylase 1 (HDAC1) that represses the activation of C/EBP $\alpha$  transcription by C/EBP $\beta$  (10).

Significant differences between primary human preadipocytes and the 3T3 L1 murine model are that the human cells differentiate directly in the absence of clonal expansion, and differentiation is accomplished in serum-free medium. In this instance, early reports have suggested a requirement for glucocorticoids during the entire 14-d course of differentiation. Furthermore, differentiation is also dependent on a 4-d course of treatment with the PPAR $\gamma$  agonist troglitazone, which is dispensable for murine preadipocytes (11, 12).

In the present study, we have performed a detailed analysis of the contribution of glucocorticoids to the differentiation of primary human preadipocytes. Specific features of the differentiation were compared with the effects of steroid

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Abbreviations: aP2, Adipocyte fatty acid binding protein; C/EBP $\beta$ , CCAAT enhancer binding protein- $\beta$ ; dex, dexamethasone; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; 11 $\beta$ -HSD1, 11 $\beta$ -hydroxysteroid dehydrogenase type 1; MIX, methylisobutylxanthine; PPAR $\gamma$ , peroxisome proliferator activator receptor; TUNEL, terminal transferase dUTP nick end labeling.

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on the differentiation of murine 3T3 L1 cells stimulated to differentiate in chemically defined, serum-free medium. We report that the temporal effects of steroid are conserved between human and murine systems despite the absence of clonal expansion in human preadipocytes before terminal differentiation and that glucocorticoid and PPAR $\gamma$  agonist treatment act sequentially to promote differentiation. Significantly, in addition to the induction of C/EBP $\delta$  and the expected effects on C/EBP $\alpha$  and PPAR $\gamma$ , steroid treatment also modulated the rapid initial accumulation of C/EBP $\beta$ . Remarkably, glucocorticoid treatment was required for murine cells to survive the initial clonal expansion phase of differentiation in chemically defined serum-free conditions.

## Materials and Methods

### Chemicals and materials

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

### Culture and differentiation of human primary preadipocytes

Cryopreserved, sc human primary preadipocytes from female donors with a normal body mass index ( $22.5 \pm 0.2 \text{ kg/m}^2$ ) were purchased from Zen-Bio Inc. (Research Triangle Park, NC). Cells were maintained at 5% CO $_2$  in DMEM with 1.0 g/liter glucose, 20% calf serum (Life Technologies, Inc., Burlington, Canada), 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 U/ml nystatin. Cells were seeded into Nunc T75 flasks and expanded once before seeding for differentiation. Media was replaced every 2 d.

For differentiation, human preadipocytes were seeded at a density of approximately 50,000 cells per well in 12-well culture dishes. Upon reaching confluence (d 0), the media were replaced with serum-free DMEM/Ham's F12 (1:1, vol/vol) media supplemented with 33  $\mu\text{M}$  biotin, 17  $\mu\text{M}$  pantothenate, 10  $\mu\text{g/ml}$  transferrin, 0.2 nM triiodothyronine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 U/ml nystatin. To stimulate differentiation, the cells were treated with 100 nM insulin, 0.5 mM methylisobutylxanthine (MIX) from d 0–4, and 5  $\mu\text{M}$  troglitazone and 1  $\mu\text{M}$  dexamethasone (dex) (Steraloids, Newport, RI) as indicated. On d 4 and every 3–4 d thereafter, the media were replaced with serum-free media containing insulin until d 8, after which the media were not changed through d 14.

### Culture and differentiation of murine 3T3 L1 cells

3T3 L1 cells were maintained in DMEM with 1.0 g/liter glucose and 10% calf serum (Life Technologies) at 10% CO $_2$ . For differentiation experiments in serum-free media, at 2 d post confluence (d 0), the media were replaced with serum-free DMEM/Ham's F12 (1:1, vol/vol) media supplemented with 33  $\mu\text{M}$  biotin, 17  $\mu\text{M}$  pantothenate, 10  $\mu\text{g/ml}$  transferrin, 0.2 nM triiodothyronine, and 0.25 mg/ml fetuin. Differentiation was induced by treatment with 100 nM insulin, 0.5 mM MIX, and 250 nM dex as indicated for 48 h. Thereafter, media were replaced every 2 d with serum-free medium containing 100 nM insulin for 6 d. For 3T3 L1 cells differentiated in the presence of serum, 2 d postconfluency cells were induced to differentiate in the same media as used for maintenance and stimulated with induction cocktail as described above. Cells were differentiated for 7–8 d.

**TABLE 1.** Genes and primers sequences used for real-time PCR

Gene target	Accession no.	Forward primer (5'–3')	Reverse primer (5'–3')
C/EBP $\alpha$	Y11525	tggacaagaacagcaacgag	ccatggccttgaccaaggag
C/EBP $\beta$	BC005132	agaagaccgtggacaagca	gcttgaacaagttccgcagg
C/EBP $\delta$	BC094715	agaagttgggtggagctgtcg	ggtatgggtcgttctgtgagt
PPAR $\gamma$	BT007281	cataaagtccttcccgcgtga	gggctccataaagtcaccaa
aP2 (FABP4)	BT006809	catcagtgatggaatgggatg	gtggaagtgcagcctttcat
G3PDH	AY340484	accacagtcctatgccatcac	tccaccacctgttctgtgta

### Oil Red O staining of neutral lipid content in mature adipocytes and Western analysis of adipogenic factor expression

To assess neutral lipid content, mature adipocytes were washed with PBS, fixed with 10% formalin, stained with Oil Red O for 1 h, and then washed with water. Photomicrographs of cells were taken with a Leica MZ125 microscope and represent approximately 85% of a well in a 12-well dish. Images were quantified for relative Oil Red O staining using Image J software.

For Western analysis, cells were washed in PBS and lysed in IPH buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, and fresh 2 mM dithiothreitol and protease inhibitor cocktail]. The lysate was sonicated and cleared by centrifugation. Equal amounts of protein (30–50  $\mu\text{g}$ ) were resolved by SDS-PAGE and transferred to polyvinylidene membrane for Western analysis. Primary antibodies used were C/EBP $\beta$  (C-19), C/EBP $\delta$  (C-22), C/EBP $\alpha$  (14AA), PPAR $\gamma$  (H100), and pan-actin (H-300) from Santa Cruz Biotechnologies (Santa Cruz, CA) and adipocyte fatty acid binding protein [FABP4 (aP2)] from Cayman Chemical (Ann Arbor, MI).

Signal intensities of Western blots were quantified using ImageQuant software. Experiments were repeated with preadipocytes derived from three to four individual donors and a pool of five donor samples as indicated in the figure legends. For graphical analysis, data are presented as an average protein induction  $\pm$  SEM. Statistical significance was assessed using two-tailed, paired Student's *t* tests in Microsoft Excel.

### Real-time PCR analysis of mRNA expression

Total RNA was extracted from differentiating cells treated as indicated in individual experiments using an RNeasy kit (QIAGEN, Mississauga, Canada), and the samples were DNase I decontaminated. Total RNA (2–5  $\mu\text{g}$ ) was reverse transcribed using oligo-dT and Superscript II (Invitrogen, Burlington, Canada) as per the manufacturer's protocols. For real-time reactions, 2–4  $\mu\text{l}$  cDNA was amplified in a LightCycler (Roche, Indianapolis, IN) using a LightCycler FastStart DNA Master Sybr Green 1 kit (Roche). Primer pairs for each gene target are listed in Table 1. Reactions were standardized against glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (13). Data represent average mRNA abundance from a minimum of three differentiation experiments in primary preadipocytes from three individual donors  $\pm$  SEM. Statistical significance was determined using two-tailed, paired Student's *t* tests.

### Effect of inhibitors on the expression of C/EBP $\beta$

Human primary preadipocytes were treated with differentiation cocktails as described above in the presence of 10  $\mu\text{g/ml}$  actinomycin D, 20  $\mu\text{M}$  cycloheximide, 1  $\mu\text{M}$  MG132, or vehicle for the times indicated in individual experiments. For cycloheximide treatments, cells were pulsed for 15 min with 20  $\mu\text{M}$  cycloheximide before stimulation with differentiation cocktail. Cells were treated for 4 or 8 h with cocktail and then harvested and processed by Western analysis and quantified as described above.

### Analysis of DNA synthesis in human and murine preadipocytes by [ $^3\text{H}$ ]thymidine incorporation

Both human preadipocytes and 3T3 L1 cells were induced to differentiate under standard dex conditions as described above. Cells were

pulsed with 2  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine per well (human) or 4  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine per well (3T3 L1) for 12 h and then washed once with ice-cold PBS and incubated with ice-cold 5% trichloroacetic acid twice for 15 min each at room temperature. Wells were washed with PBS, and extracts scraped in 0.5 N NaOH/0.5% SDS. Thymidine incorporation was measured by measuring total dpm in a scintillation counter (Beckman Coulter, Mississauga, Ontario, Canada). Data are representative averages of two (human) or three (mouse) independent experiments each done in duplicate  $\pm$  SEM. Statistical significance was determined using two-tailed, paired Student's *t* tests.

*Analysis of cell death by in situ terminal transferase dUTP nick end labeling (TUNEL) assay*

Human and murine cells were seeded onto poly-L-lysine-coated coverslips and induced to differentiate for 48 h under serum-free conditions. Assay was performed using a Roche kit as per the manufacturer's instructions. In brief, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, rinsed in PBS, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. DNA ends were then labeled with fluorescein-dUTP using terminal transferase for 1 h at 37 C, rinsed with PBS, and analyzed using fluorescence microscopy. For positive controls, fixed cells were treated with 50–100 U DNase I for 10 min before labeling of DNA ends with fluorescein-dUTP.

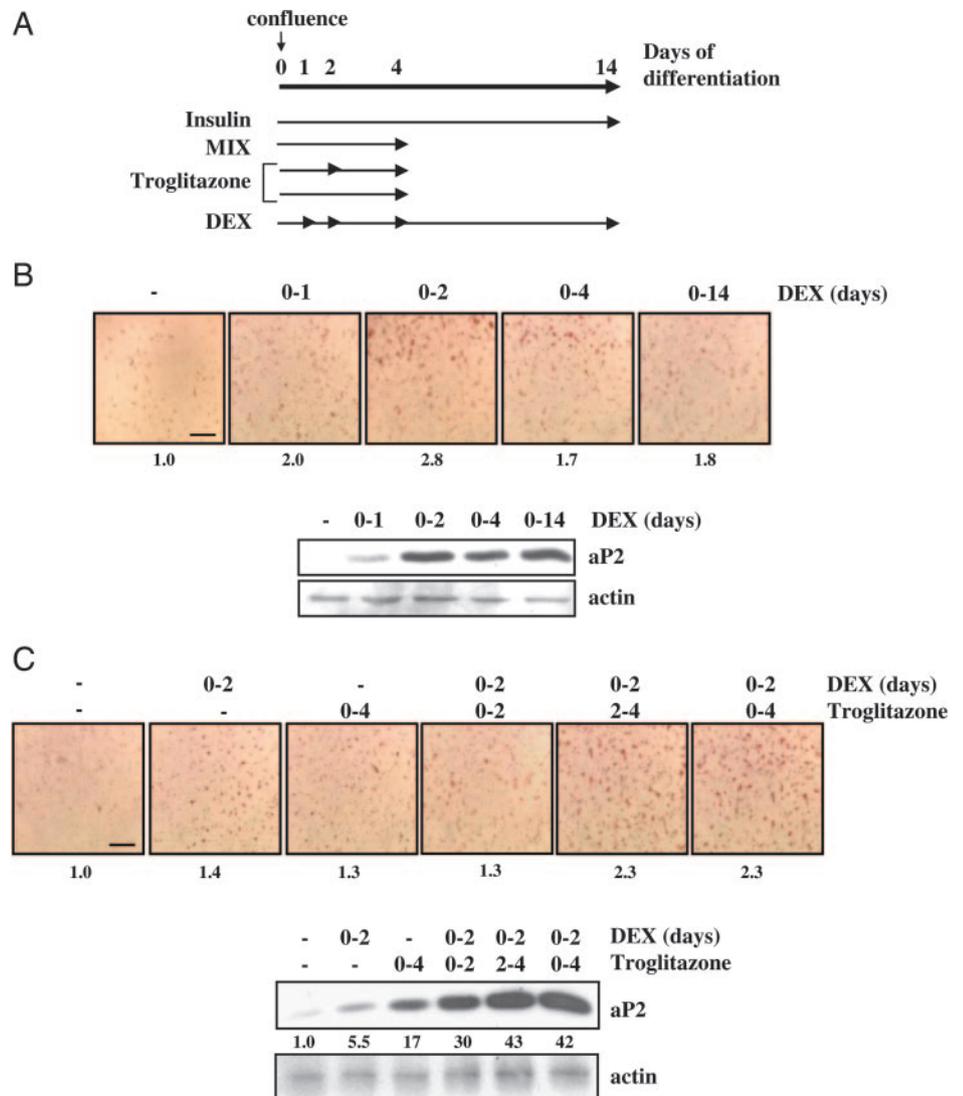
**Results**

*Dex and troglitazone sequentially stimulate the differentiation of primary human preadipocytes*

To assess the temporal contribution of glucocorticoid and troglitazone stimulation to the differentiation of human preadipocytes, we titrated the conditions required for optimal differentiation of sc human preadipocytes derived from donors with normal body mass index ( $22.5 \pm 0.2 \text{ kg/m}^2$ ). Cells were induced to differentiate upon reaching confluence (d 0), as summarized in Fig. 1A.

Dex treatment ( $10^{-6} \text{ M}$ ) provided a potent stimulus for differentiation, with its addition to the insulin/MIX/troglitazone differentiation cocktail inducing a marked increase in the levels of the adipogenic marker aP2 in the mature adipocytes on d 14. Lipid accumulation was also enhanced 2- to 3-fold by dex treatment as revealed by the increased staining intensity of neutral lipids in the mature adipocytes by Oil Red O (Fig. 1B). The stimulatory effect of dex was exerted early during the differentiation, with only 24 h of treatment being sufficient to provide significant increases in aP2 ex-

FIG. 1. Transient glucocorticoid and troglitazone treatments act sequentially to stimulate the differentiation of human preadipocytes. A, Schema of the timing of the insulin (100 nM), MIX (0.5 mM), troglitazone (5  $\mu\text{M}$ ), and dex (1  $\mu\text{M}$ ) treatments employed for the experiments shown in B and C through the 14-d course of differentiation of primary human preadipocytes cultured in chemically defined serum-free medium. Arrowheads denote the length of individual treatment times. B, The upper panel shows photomicrographs of Oil-Red-O-stained mature adipocytes 14 d subsequent to the induction of differentiation by treatment with insulin, MIX, and troglitazone for d 0–4 and dex as indicated. Scale bar, 1 mm. Numbers below the panels represent quantification of the amount of staining relative to the untreated condition as determined using Image J software. The lower panel shows corresponding Western analysis of aP2 and actin protein levels in duplicates of the upper panel. C, Analysis of lipid accumulation and aP2 expression, as in B for cells induced to differentiation with insulin and MIX for d 0–4, and dex and troglitazone as indicated (days). aP2 expression was quantified using ImageQuant software and is represented below the blot as relative aP2 induction.



pression and Oil Red O staining on d 14. Similar to previous studies with the murine 3T3 L1 model (7), inclusion of dex with the adipogenic cocktail for the first 48 h of stimulation of primary human preadipocytes provided the optimal stimulatory effect, with steroid treatment for longer periods providing no additional benefit.

Examination of the optimal timing for the combination of dex and troglitazone treatment revealed a sequential benefit of the two agonists on primary human preadipocytes (Fig. 1C). Little differentiation was observed when both dex and troglitazone were left out of the differentiation cocktail, with detection of an aP2 signal requiring prolonged Western blot exposure. Treatment with dex for 48 h or troglitazone for 4 d independently stimulated differentiation, with troglitazone being more effective than dex in eliciting aP2 expression (17-fold compared with 5.5-fold increase). However, the two treatments were most effective in combination, with dex

treatment for d 0–2 followed by troglitazone treatment from d 2–4 providing for optimal expression of aP2 and lipid accumulation. Differentiation was not further enhanced by including troglitazone in the cocktail for all 4 d, showing that steroid and troglitazone are contributing independently and sequentially to differentiation.

#### Dex treatment enhances the early accumulation of C/EBP $\beta$

Western analysis of C/EBP $\beta$ , C/EBP $\delta$ , C/EBP $\alpha$ , PPAR $\gamma$ , and aP2 accumulation in primary human preadipocytes (Fig. 2) revealed both similarities and striking differences to the responses seen previously in murine cells. C/EBP $\beta$  levels were induced in the human cells within 4 h of treatment with adipogenic cocktail lacking dex (Fig. 2, A and B), consistent with results from 3T3 L1 cells in which C/EBP $\beta$  is also induced immediately (8, 14). However, whereas in murine

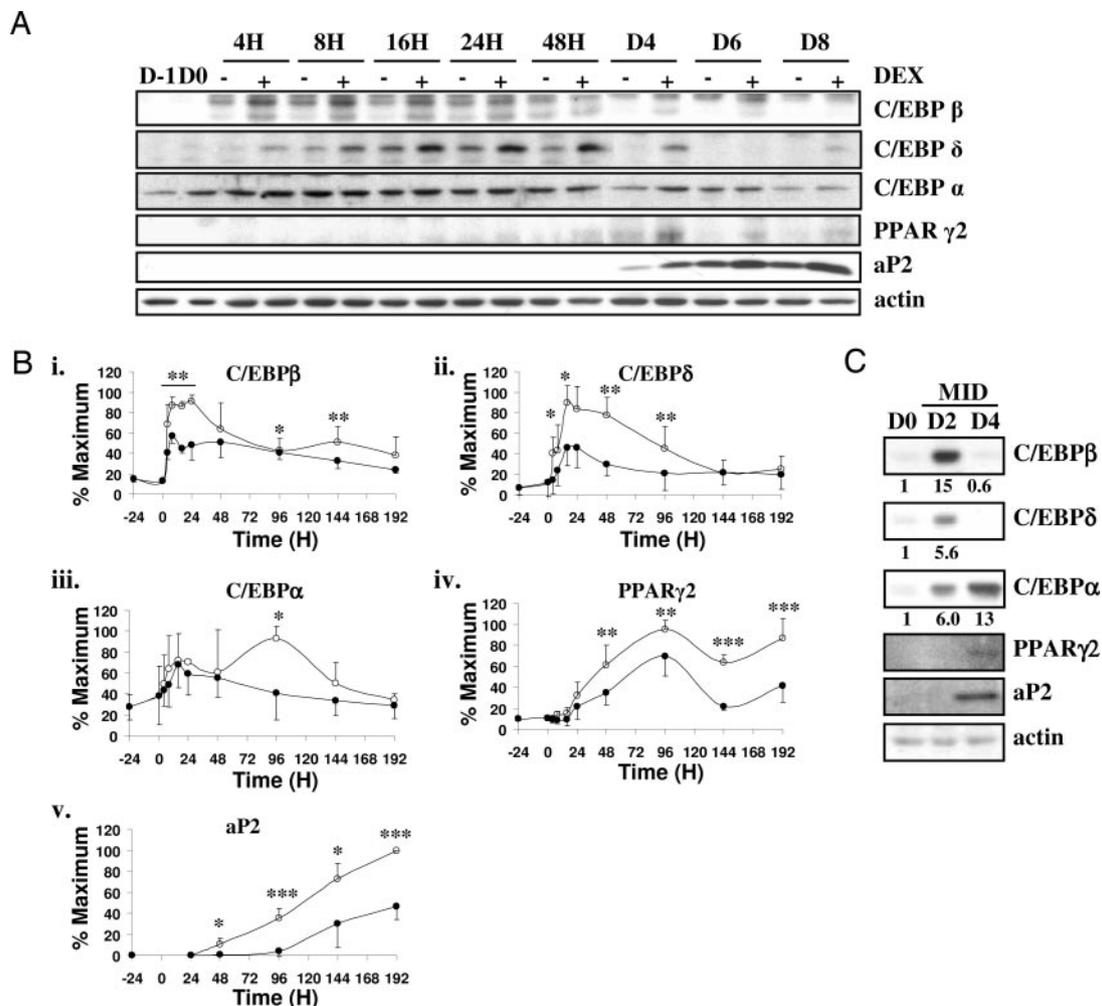


FIG. 2. Glucocorticoid treatment stimulates accumulation of adipogenic factors. A, Western analysis of protein expression during the course of human preadipocyte differentiation. Preadipocytes were harvested 24 h before treatment (d -1) and at the times indicated during treatment with insulin (d 0–14), MIX, and troglitazone (d 0–4) in the presence or absence of  $10^{-6}$  M dex (d 0–2). B, Graphical presentation of the protein expression profiles as a function of percentage of maximal protein expression ( $\pm$ SEM). ●, insulin, MIX, and troglitazone (MIT); ○, insulin, MIX, troglitazone, and dex (MITD); n = 3 to 5 and was comprised of up to four individual experiments with different cells derived from different donors and one pool of cells derived from another five donor samples. Student's paired *t* tests were performed for each time point to compare MIT and MITD expression levels. \*,  $P \leq 0.10$ ; \*\*,  $P \leq 0.05$ ; \*\*\*,  $P \leq 0.01$ . C, Western analysis of adipogenic factors expressed at indicated times in 3T3 L1 cells differentiated under serum-free conditions as described in *Materials and Methods*. Signals were quantified as described and represent relative induction.

cells the induction of C/EBP $\beta$  is dex independent (8, 14), in the human cells, the early accumulation of C/EBP $\beta$  was reproducibly enhanced 2- to 2.5-fold by dex treatment during the first 24 h. This steroid-mediated enhancement of C/EBP $\beta$  accumulation was reproduced over a series of five independent trials including a total of nine different donor samples.

The increase in C/EBP $\beta$  due to dex treatment was transient, with C/EBP $\beta$  levels declining in dex-treated cells to the level observed in MIX/insulin-treated cells by 48 h. (Fig. 2B, panel i). C/EBP $\beta$  levels remained readily detectable thereafter from d 2–8. By contrast, a limited analysis of the response of 3T3 L1 cells to adipogenic stimulus in serum-free chemically defined medium showed a more dramatic decline in C/EBP $\beta$  levels than reported previously in the presence of serum (8, 14), with expression declining to near undetectable levels by d 4 (Fig. 2C).

The induction of C/EBP $\delta$  in primary human preadipocytes closely paralleled its induction in murine cells (8, 14). Accumulation was rapid, dex dependent, and sustained through 96 h (Fig. 2, A and B, panel ii).

The detection of C/EBP $\alpha$  protein expression in pre-confluent cells (d -1) and in cells as they reach confluence (d 0) contrasted with previous reports in human preadipocytes in which its expression was detected only subsequent to an adipogenic stimulus (15). However, it is consistent with results from murine preadipocytes where modest levels of C/EBP $\alpha$  expression before differentiation is thought to be linked to the growth arrest of confluent cultures (16–18).

C/EBP $\alpha$  expression in the primary human preadipocytes appeared to be biphasic. There was a modest, but reproducible 1.5- to 2-fold dex-independent induction in response to the differentiation stimulus within the first 4 h of treatment

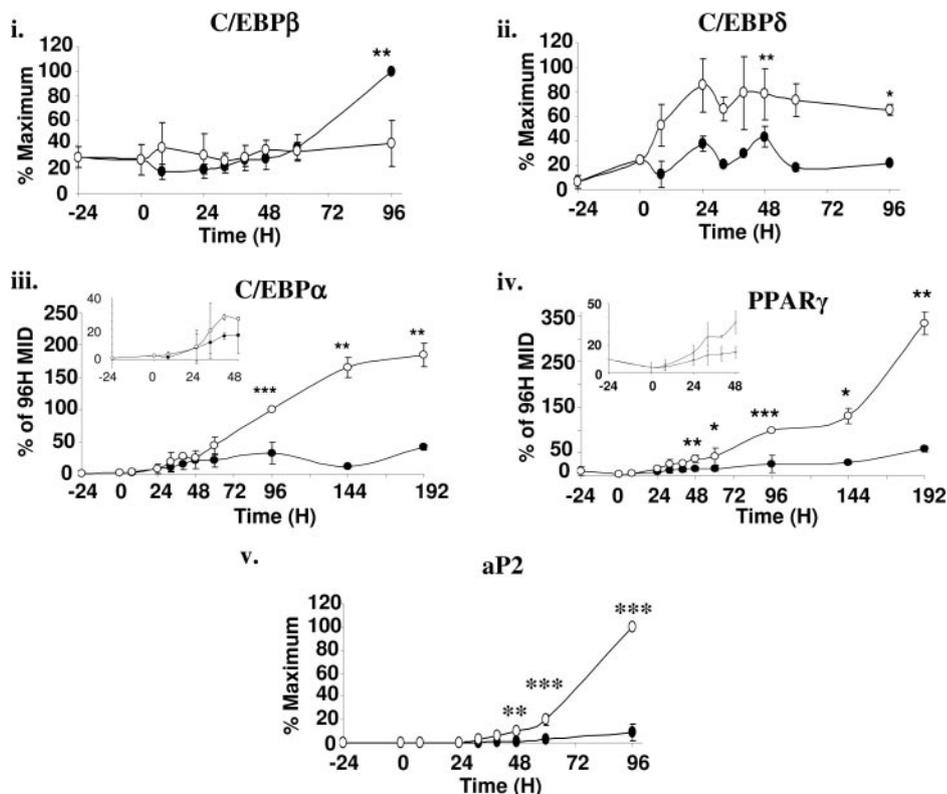
that was sustained for 48 h. By contrast, in murine cells, the basal level of C/EBP $\alpha$  is rapidly suppressed upon exposure to adipogenic cocktail and becomes reinduced only between 24 and 48 h into differentiation (Fig. 2C). When dex was included in the adipogenic cocktail, C/EBP $\alpha$  accumulation was enhanced (Fig. 2B, panel iii) and was maintained at maximal levels to d 4, before declining thereafter to the levels seen in MIX/insulin-treated cells (Fig. 2B, panel iii).

By contrast to C/EBP $\alpha$ , PPAR $\gamma$  expression was first detected at 48 h, peaked at 96 h, and was enhanced throughout the course of its expression by the initial presence of dex in the differentiation cocktail (Fig. 2 B, panel iv). This profile parallels what has been observed in 3T3 L1 cells differentiated in both the presence and absence of serum (18, 19).

Lastly, upon MIX/insulin dex treatment, aP2 was detected by d 2 and accumulated essentially linearly through d 8 (Fig. 2B, panel v). When dex was omitted from the differentiation cocktail, aP2 accumulation was decreased, and aP2 expression reached our limit of detection at d 4. This parallels the effect of dex seen on the induction of aP2 in murine systems such as 3T3 L1 cells (20).

To determine whether the changes in the protein levels of adipogenic factors observed correlated directly with differences in mRNA level, we performed real-time PCR analysis of their expression in parallel with the Western analysis (Fig. 3). Interestingly, the rapid accumulation of C/EBP $\beta$  protein in our primary human preadipocyte cultures was not accompanied by the dramatic cAMP (MIX)-dependent increase in C/EBP $\beta$  mRNA that has been reported for murine cell lines (8, 14). Indeed, there was no significant difference between the C/EBP $\beta$  levels at 24 h, regardless of the treatment (Fig. 3, panel i). Curiously, 4 d into the treatment of the

FIG. 3. Effects of glucocorticoid treatment on mRNA induction during preadipocyte differentiation. RNA was extracted from primary human preadipocytes 24 h before treatment (-24 h) and at the indicated times during treatment with insulin (d 0–14), MIX, and troglitazone (d 0–4) in the absence (●) or presence (○) of  $10^{-6}$  M dex (d 0–2). Each expression profile has been standardized against control G3PDH mRNA levels. Data are presented as the average of the percentage of maximal mRNA levels for each gene over a minimum of three repeats performed with cells derived from different donor samples, except for C/EBP $\alpha$  and PPAR $\gamma$  mRNA, where data are presented as percentage of 96-h insulin, MIX, and troglitazone. The insets in C/EBP $\alpha$  and PPAR $\gamma$  graphs highlight the scale for data points between -24 and 48 h. Data are plotted  $\pm$  SEM. Student's paired *t* tests were performed for each time point to compare insulin, MIX, and troglitazone with insulin, MIX, troglitazone, and dex: \*,  $P \leq 0.10$ ; \*\*,  $P \leq 0.05$ ; \*\*\*,  $P \leq 0.01$ .



human preadipocytes with insulin, MIX, and troglitazone, conditions under which only modest preadipocyte differentiation was observed (Fig. 1), there was a consistent and reproducible 3- to 4-fold spike in C/EBP $\beta$  mRNA. This induction was not observed upon the inclusion of dex from d 0–2 of differentiation and did not appear to be translated into an increase in C/EBP $\beta$  protein levels (Fig. 2, A and B). Additional mRNA analysis indicated that this change in C/EBP $\beta$  mRNA levels, although reproducible, also was transient and returned to baseline levels by d 5 of treatment (data not shown). By contrast, induction of C/EBP $\delta$  mRNA occurred similarly to the induction of C/EBP $\delta$  protein, with an early response that was strongly enhanced by dex and that was sustained for 4 d (Fig. 3, panel ii).

Before differentiation, mRNAs for C/EBP $\alpha$  and PPAR $\gamma$  were expressed at very low levels (Fig. 3, panels iii and iv). The early 4-h induction of C/EBP $\alpha$  observed at the protein level (Fig. 2, A and B) was not reflected by detectable induction of mRNA synthesis, suggesting that the early up-regulation of C/EBP $\alpha$  expression that was unique to the primary human preadipocytes is regulated through nontranscriptional mechanisms. Induction of both C/EBP $\alpha$  and PPAR $\gamma$  mRNAs was detectable by 24 h, and their levels continued to increase thereafter. Induction was also stimulated by early dex treatment, with both mRNAs being 5-fold higher on d 4 in the presence of dex. Interestingly although C/EBP $\alpha$  protein levels declined after d 4 and PPAR $\gamma$  levels had reached their peak, the mRNA levels for both factors continued their strong dex-dependent increase through d 8 of differentiation.

Lastly, aP2 mRNA expression paralleled protein accumulation in both the timing of its appearance and in the enhancement of expression by dex (Fig. 3, panel v).

#### Early accumulation of C/EBP $\beta$ is dependent on C/EBP $\beta$ transcription

Although C/EBP $\beta$  protein levels in the primary human preadipocytes increased within 4 h of MIX/insulin treatment and were further enhanced by the addition of dex, C/EBP $\beta$  mRNA levels were not significantly different from basal levels at 8 and 24 h after stimulation (Fig. 3, panel i). This suggested that the early C/EBP $\beta$  protein accumulation in primary human preadipocytes was mediated through a mechanism other than the transcriptional regulation described for 3T3 L1 cells. Therefore, we examined the early accumulation of C/EBP $\beta$  in greater detail (Fig. 4).

In the first instance, we tested the effect of the proteasome inhibitor MG132 on the accumulation of C/EBP $\beta$  at 4 h after adipogenic stimulation (Fig. 4A). In untreated cells and cells treated with MIX/insulin/dex for 4 h, inclusion of MG132 in the culture had no effect on the accumulation of C/EBP $\beta$ . Similarly, although addition of MG132 to the treatment with MIX/insulin alone appeared to have a slight effect on C/EBP $\beta$  levels, it did not reach statistical significance over the course of the three repetitions performed on individual patient samples. This indicated that protein stabilization through inhibition of 28S proteasome-mediated degradation was unlikely to account for the up-regulation of C/EBP $\beta$  expression mediated by dex. A similar lack of stabilization

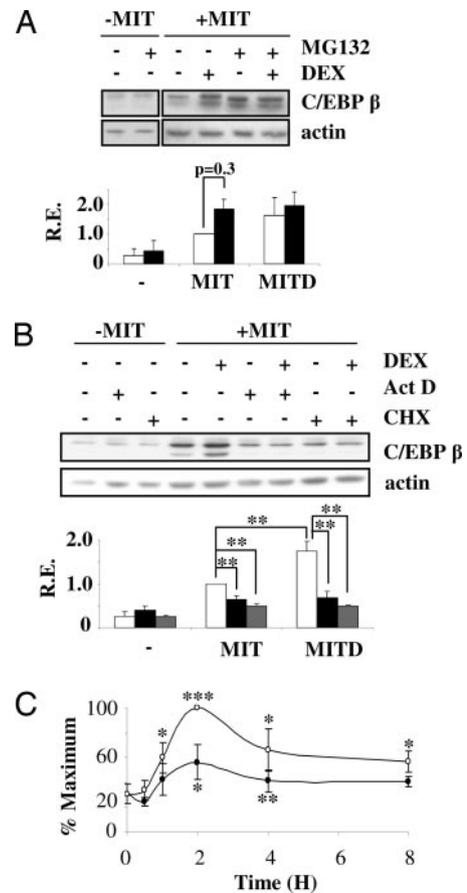


FIG. 4. Enhancement of C/EBP $\beta$  expression by glucocorticoid is mediated through transcription. A, Western analysis of C/EBP $\beta$  expression after 4 h stimulation in the presence or absence of insulin, MIX, and troglitazone (MIT), dex, and 1  $\mu$ M MG132 as indicated. *Bottom*, Average fold induction relative to the MIT condition  $\pm$  SEM from three independent experiments performed in duplicate with cells derived from different donors. *White bars*, No treatment; *black bars*, MG132 treatment. The *P* value was determined using Student's paired *t* test. B, *Top*, Western analysis of C/EBP $\beta$  expression after 4 h stimulation as described in A with 10  $\mu$ g/ml actinomycin D (Act D) or 20  $\mu$ g/ml cycloheximide (CHX) as indicated. *Bottom*, Average fold induction as described in A. *White bars*, No treatment; *black bars*, Act D treatment; *gray bars*, CHX treatment. C, Real-time PCR analysis of C/EBP $\beta$  mRNA levels during early differentiation. Cells were induced to differentiate in either the absence (●) or presence (○) of  $10^{-6}$  M dex. Data are plotted as average mRNA abundance as percentage of maximum  $\pm$  SEM where *n* = 3. Significance of the difference of each data point was assessed relative to d 0 as described in A: \*, *P*  $\leq$  0.10; \*\*, *P*  $\leq$  0.05.

was observed at 8 h post stimulation, although MG132 treatment was observed to stabilize the glucocorticoid receptor (GR) over this time period (data not shown).

By contrast, addition of the protein synthesis inhibitor cycloheximide significantly inhibited the induction of C/EBP $\beta$  by MIX/insulin and MIX/insulin/dex at both 4 h (Fig. 4B) and 8 h (data not shown), demonstrating that the induction of C/EBP $\beta$  depended on ongoing C/EBP $\beta$  translation. Addition of actinomycin D, which inhibits the initiation of new transcription, also prevented the induction of C/EBP $\beta$  protein, demonstrating a requirement for new transcription subsequent to the adipogenic stimulus.

To determine whether the adipogenic stimuli induced a

rapid pulse of transcription not apparent in our initial mRNA analyses, we refined our RT-PCR analysis to examine mRNA induction between 0 and 8 h post stimulation (Fig. 4C). This revealed a rapid 4-fold induction of C/EBP $\beta$  mRNA by MIX/insulin/dex treatment that peaked at 2 h ( $P = 0.005$ ). Although induction receded rapidly thereafter, C/EBP $\beta$  mRNA levels were still modestly elevated at 8 h ( $P = 0.1$ ). Treatment with MIX/insulin alone induced a modest 2-fold induction of C/EBP $\beta$  mRNA at 2 h ( $P = 0.1$ ), and C/EBP $\beta$  mRNA returned to baseline by 4 h. Notably, the addition of dex to the MIX/insulin treatment provided for a significant elevation of C/EBP $\beta$  mRNA beyond the effect of MIX/insulin alone from 4 h ( $P = 0.05$ ) through 8 h ( $P = 0.1$ ). These results indicate that the induction of C/EBP $\beta$  transcription, although brief, is required for the accumulation of C/EBP $\beta$  by MIX/insulin and its further stimulation by dex.

#### *Dex communicates a survival signal to murine preadipocytes*

Previous data have suggested that human preadipocytes in defined medium differentiate directly in response to stimulus (21, 22). By contrast, murine preadipocytes differentiated in the presence of serum undergo one to two rounds of cell division in progressing to a commitment point beyond which they are irrevocably committed to differentiation (5). To determine whether this difference reflected the difference in culture conditions, we monitored [ $^3$ H]thymidine incorporation into primary human preadipocytes and 3T3 L1 cells over the first 4 d after the stimulation of differentiation in defined medium (Fig. 5). Furthermore, because dex treatment is known to be antimitotic in other systems (23–25) and to retard the proliferation in undifferentiated primary preadipocytes (15), we specifically monitored the effect of dex treatment in this assay.

Consistent with the initial reports, the stimulation of newly confluent primary human preadipocytes with differentiation cocktail led to an immediate cessation of DNA synthesis (Fig. 5A). Moreover, this effect was independent of the inclusion of dex in the differentiation cocktail. By contrast, for 3T3 L1 cells, addition of differentiation cocktail in the presence of dex stimulated DNA replication that initiated between 12 and 24 h post treatment and reinitiated between 60 and 72 h, consistent with two rounds of cell division over that period (Fig. 5, B and C). Thus clonal expansion of 3T3 L1 murine preadipocytes appears to be an inherent step in the differentiation pathway, which is distinct from the behavior of primary human preadipocytes and does not simply reflect a serum response. Notably, in the presence of serum, two rounds of replication, reflected by two well-distinguished peaks of [ $^3$ H]thymidine incorporation, were detected in the 3T3 L1 cells, whereas under serum-free conditions, replication progressed in an apparently less coordinated manner, with thymidine incorporation seen through the period from 12–72 h.

Unexpectedly however, treatment of postconfluent, serum-free 3T3 L1 cells with insulin and MIX in the absence of dex restricted DNA replication to 12–24 h after the onset of treatment, with an almost complete cessation of replication thereafter (Fig. 5B). Although this could indicate that dex

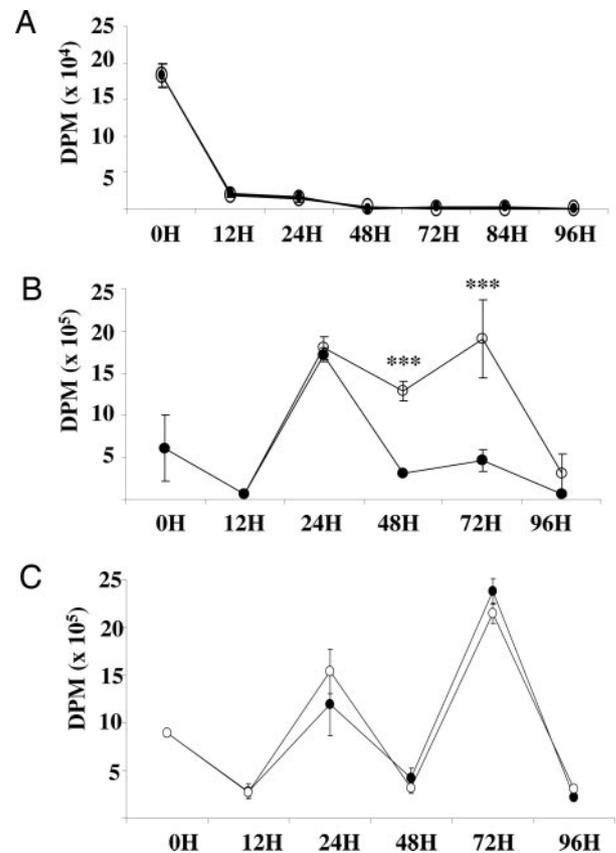


FIG. 5. Dex is required for 3T3 L1 cells to complete clonal expansion in the absence of serum. A, [ $^3$ H]Thymidine incorporation into primary human preadipocytes over 96 h after the stimulation of differentiation by treatment with insulin, MIX, and troglitazone in the presence (○) or absence (●) of  $10^{-6}$  M dex (d 0–2). Approximately  $2.5 \times 10^5$  cells were pulsed with [ $^3$ H]thymidine for 12 h before harvesting. B and C, [ $^3$ H]Thymidine incorporation into 3T3 L1 cells over 96 h after the stimulation of differentiation of 2-d postconfluent cells by treatment with insulin and MIX in the presence (○) or absence (●) of  $0.25 \mu\text{M}$  dex (d 0–2) differentiated in the absence (B) or presence (C) of serum. There were approximately  $8 \times 10^5$  cells upon reaching confluence at d 0. Data are plotted as dpm values  $\pm$  SEM for three independent experiments performed in duplicate. Student's paired t tests were performed for each time point in A and B: \*\*\*,  $P \leq 0.01$ .

contributes directly to DNA replication of 3T3 L1 cells during the clonal expansion phase, microscopic tracking of the cells over the period of the experiment revealed massive lifting of the 3T3 L1 cells from the plate so that few cells remained in culture at 96 h (data not shown).

Microscopic comparison of mature primary human adipocytes on d 14 and 3T3 L1 cells on d 7, respectively, differentiated under serum-free conditions in complete cocktail, revealed that whereas the human cultures remained confluent and featured a mixture of lipid-laden and undifferentiated cells, the 3T3 L1 cells were subconfluent and consisted almost entirely of lipid-laden cells (Fig. 6A). The sparseness of the 3T3 L1 cells was a specific consequence of the absence of serum because cells differentiated in the presence of serum remained confluent and consisted of a mixture of lipid-laden and undifferentiated cells (Fig. 6A). Phase-contrast photomicrographs taken on d 0 in Fig. 6B confirm that the cells were confluent upon induction of differentiation.

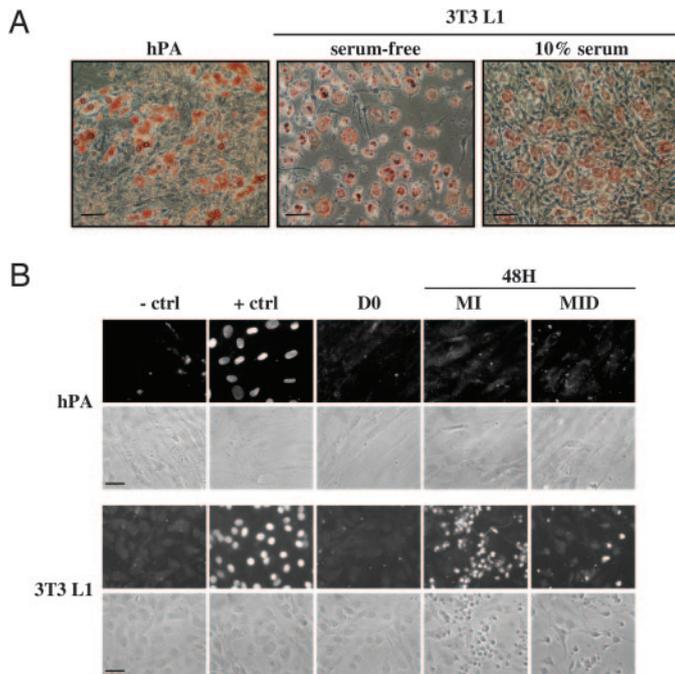


FIG. 6. Dex provides a signal that allows committed 3T3 L1 cells to survive to terminally differentiate. A, Phase-contrast photomicrographs of Oil-Red-O-stained human primary cells (hPA) on d 14 of differentiation and 3T3 L1 cells differentiated in the absence (serum-free) or presence of 10% serum on d 7 as indicated; B, *in situ* TUNEL assay analysis of cell death (top) and phase-contrast images (bottom) in confluent human primary (d 0) (top two panels) and 2-d postconfluent murine 3T3 L1 preadipocytes (d 0) (bottom two panels) and cells differentiated in the absence of serum 48 h after treatment with either MIX and insulin alone (MI) or with dex (MID). The negative control (– ctrl) represents assay conditions lacking terminal transferase, whereas the positive control (+ ctrl) represents cells that have been treated with DNase I before end labeling. Scale bar, 40  $\mu$ m.

These results suggested that dex treatment of 3T3 L1 preadipocytes stimulated in the absence of serum provided a survival signal for cells that committed to differentiate. TUNEL analysis revealed that in the absence of dex, treatment of 3T3 L1 cells with insulin and MIX in the absence of serum resulted in massive cell death within 48 h (Fig. 6B). Cell death was strongly reduced but not completely eliminated by the inclusion of dex in the cocktail. By contrast, little evidence of cell death was observed in primary human preadipocytes regardless of the inclusion of dex in the treatment. These results reveal a fundamental difference in the role of dex in the differentiation of primary human preadipocytes and murine 3T3 L1 cells.

### Discussion

Previous studies have indicated that C/EBP family members and PPAR $\gamma$  are up-regulated during human primary preadipocyte differentiation (15, 26, 27). However, this is the first report that provides a detailed examination of the expression profiles of these factors during early differentiation and is also the first to specifically examine the impact of glucocorticoids on this process. Titration of dex treatment indicated that the action of glucocorticoids are restricted to the first 48 h of differentiation and are reflected thereafter by

increases in transcription factor and adipogenic marker levels. Furthermore, the primary effect of steroid appears to be to facilitate the decision for the preadipocyte to differentiate, rather than to enhance differentiation within individual cells, because dex treatment resulted in more Oil-Red-O-positive cells.

A first notable difference in the profile of factor expression in primary human preadipocytes compared with what is known in the 3T3 L1 model was an immediate up-regulation of C/EBP $\alpha$  that was accomplished within 4 h of exposure to insulin and MIX. By contrast, in murine cells, the basal level of C/EBP $\alpha$  detected in confluent preadipocytes is abolished upon stimulation with differentiation cocktail. This has been shown to be a specific requirement for clonal expansion, given the antimutic properties of C/EBP $\alpha$  (16, 18). Thus, the immediate induction of C/EBP $\alpha$  may contribute to that lack of clonal expansion in human preadipocytes. It also could, however, reflect that the human preadipocytes have completed clonal expansion before isolation from donors, thereby allowing for the direct induction of a factor that contributes to commitment in response to adipogenic stimulation. Most interestingly, the early induction in C/EBP $\alpha$  protein levels occurred without an obvious early increase in its mRNA, suggesting a mechanism of control of C/EBP $\alpha$  that may be unique to human cells.

Subsequently, between d 2 and 4, C/EBP $\alpha$  and PPAR $\gamma$  were induced in a manner that correlates with induction of their mRNAs and that was enhanced by the inclusion of steroid for the first 48 h of stimulation. Thus steroid treatment shows a memory effect, with early short-term treatment translating into accentuated downstream effects on secondary targets after the withdrawal of steroid. Interestingly, after d 4, the pattern of C/EBP $\alpha$  and PPAR $\gamma$  accumulation diverged, with PPAR $\gamma$  protein levels remaining elevated in concert with a continuing induction of PPAR $\gamma$  mRNA, whereas C/EBP $\alpha$  levels declined from their peak on d 4, despite the continued elevation of C/EBP $\alpha$  mRNA. This difference may reflect a predominant role of PPAR $\gamma$  over C/EBP $\alpha$  in the mature adipocyte. It also reinforces a future need to investigate nontranscriptional mechanisms that may control C/EBP $\alpha$  expression that appear to occur specifically in the primary human preadipocyte.

The early induction and temporal expression profile of C/EBP $\beta$  in 3T3 L1 cells has been suggested to reflect a requirement for C/EBP $\beta$  for mitotic clonal expansion (5, 28, 29). In the human preadipocytes, the early induction of C/EBP $\beta$  protein was remarkably similar to that in 3T3 L1 cells despite a lack of clonal expansion. This suggests that the C/EBP $\beta$  profile is an inherent characteristic of the early transcriptional cascade that drives differentiation and that its induction reflects more than a requirement for clonal expansion.

Whereas in murine cells, C/EBP $\beta$  induction is dependent on MIX treatment alone, is mediated prominently through the induction of transcription, and is independent of glucocorticoid treatment (8, 14), the induction of C/EBP $\beta$  in primary human preadipocytes was strongly dependent on dex and involved only a modest and shorter-term induction of transcription. Investigation of additional potential mechanisms for regulating C/EBP $\beta$  excluded interference with

the targeting of C/EBP $\beta$  to the 28S proteasome and demonstrated a requirement for transcription. Although the actinomycin D results highlight a need for new transcription for the induction of C/EBP $\beta$ , given the low initial levels of C/EBP $\beta$  mRNA, they do not exclude direct effects on the RNA or on the efficiency of translation. Indeed, the interaction of RNA binding proteins, such as CUG repeat binding protein 1, with C/EBP mRNAs has been reported in other systems as a regulatory mechanism for their expression (30, 31).

In chemically defined differentiation media, 3T3 L1 cells required dex to progress through the second of two rounds of postconfluence mitosis that occurred between 48 and 96 h (Fig. 5). Although it has been previously shown that this still occurs in 3T3 L1 cells differentiated in the absence of serum, this is the first evidence that glucocorticoids are required for this process (32). Furthermore, we demonstrated by TUNEL staining that in the absence of dex, there was extensive cell death at the onset of the second round of mitosis at 48 h (Fig. 6). In the presence of glucocorticoids, the majority of cells that survived were committed adipocytes. Although we cannot exclude the possibility that committed cells also died, we suggest that glucocorticoids are required for the survival of committed preadipocytes, because complete cell death was observed by d 8 in the absence of steroid, conditions under which there was no terminal differentiation.

Glucocorticoids have primarily been characterized as antimitotic and proapoptotic stimuli, although there are also reports of GR-mediated survival signals in other cell systems (33–35). The survival signal contributed by dex is a novel function for GR in the regulation of 3T3 L1 adipogenesis. This signal may be linked to supporting progression through mitotic clonal expansion, because the human preadipocytes, which do not undergo clonal expansion, did not die in the absence of dex when differentiated under similar chemically defined conditions. The effect of dex persisted beyond its presence in the culture because the second round of mitosis was initiated after dex withdrawal at 48 h. We note that one potential candidate mediator of this signal may be the growth-arrest-specific gene product Gas6, a direct immediate-early target of GR in differentiating 3T3 L1 cells that is required for establishing dex-dependent postmitotic growth arrest (36). Gas6 has also been shown to promote the survival of oligodendrocytes (37).

In summary, our study demonstrates that whereas glucocorticoids appear to promote the decision of a preadipocyte to enter the differentiation pathway, the specific effects of the steroid appear to be mediated at multiple levels. They also exert distinct effects on primary human preadipocytes and immortalized murine cells, which emphasizes the need to focus future studies on the human cell system. Although our study focused on pharmacological doses of steroid and preadipocytes derived from sc tissue from female donors, both regional and gender differences in GR distribution have been described. The elevation of GR levels in male visceral preadipocytes correlates with their predisposition to visceral adipose tissue (38). It will be therefore important to determine the extent to which these differences in GR expression affect the potential of the steroid to stimulate adipogenesis.

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