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Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα

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ABSTRACT **Skeletal muscle and adipose tissue development often has a reciprocal relationship *in vivo*, particularly in myodystrophic states. We have investigated whether determined myoblasts with no inherent adipogenic potential can be induced to transdifferentiate into mature adipocytes by the ectopic expression of two adipogenic transcription factors, PPARγ and C/EBPα. When cultured under optimal conditions for muscle differentiation, murine G8 myoblasts expressing PPARγ and C/EBPα show markedly reduced levels of the myogenic basic helix–loop–helix proteins MyoD, myogenin, MRF4, and myf5 and are completely unable to differentiate into myotubes. Under conditions permissive for adipogenesis including a PPAR activator, these cells differentiate into mature adipocytes that express molecular markers characteristic of this lineage. Our results demonstrate that a developmental switch between these two related but highly specialized cell types can be controlled by the expression of key adipogenic transcription factors. These factors have an ability to inhibit myogenesis that is temporally and functionally separate from their ability to stimulate adipogenesis.**

During mammalian development, the embryonic mesoderm gives rise to several highly specialized cell types, including skeletal myocytes, adipocytes, and chondrocytes (1–3). The development of distinct cell types from multipotent mesodermal precursors can be viewed as a two-step process (4, 5). In the first step, termed commitment or determination, the developmental potential of the cell becomes limited to one particular lineage, be it adipose, muscle, or cartilage. In the second step, terminal differentiation, the cell develops along its determined lineage to become a functional specialized cell.

Determination of the muscle and adipocyte lineages is thought to be controlled at the transcriptional level by a small number of tissue-specific transcription factors (6). In muscle, the basic helix–loop–helix (bHLH) proteins MyoD and myf-5 play an important role in lineage determination, while the related factors myogenin and MRF4 function to execute the differentiation program (7–11). In adipocytes, differentiation appears to be controlled by two major factors or groups of factors: PPARγ and the C/EBPs. PPARγ was recently cloned and identified as a component of the adipogenic transcription factor ARF6 (12) that bound to the aP2 promoter. PPARγ, like the other known PPARs (PPARα and PPARδ/Nuc-1), belongs to the nuclear hormone receptor family and is an "orphan" in that its ligand has not yet been identified (13, 14). However, a number of diverse lipids and lipid-like compounds can activate the PPARs when applied to cells (13, 15). These include fatty acids and synthetic arachidonate leukotrienes [such as 5,8,11,14-eicosatetraynoic acid (ETYA)] as well as drugs and plasticizers known to induce peroxisome proliferation (hence the name peroxisome proliferator activated receptors or PPARs). PPARγ is expressed primarily in adipose tissue and is induced very early in the process of adipose differentiation. When ectopically expressed in a number of different fibroblastic cell lines, it induces adipogenesis in a PPAR-activator-dependent manner (16). A second adipocyte-enriched factor, C/EBPα, has also been shown to promote the adipogenic program and is likely to play an important role in terminal adipocyte differentiation (17–19). Recently, McKnight and colleagues (20, 21) have demonstrated that in addition to C/EBPα, ectopic expression of C/EBPβ and -δ can also induce the adipocyte differentiation of fibroblasts. When expressed together, PPARγ and C/EBPα synergize to powerfully promote the adipocyte developmental program in fibroblastic cells (16).

In vivo, there is often an inverse relationship between skeletal muscle and adipose tissue development. For example, in Duchenne muscular dystrophy, the loss of viable myofibrils is accompanied by an expansion of adipose mass within the muscle (22, 23). A similar replacement of muscle mass by adipose cells is seen in mice carrying targeted disruptions of the myogenic bHLH proteins (24). In Prader–Willi syndrome, severe infantile myohypotonia accompanies early onset of childhood obesity (25, 26). *In vitro* observations have suggested that the skeletal muscle and adipose developmental programs are mutually exclusive (27). When treated with 5-azacytidine, the pluripotent mesodermal cell line 10T½ gives rise to clones that can differentiate into myocytes, adipocytes, or chondrocytes (2).

To address the molecular basis of lineage determination and the potential plasticity of the determined state (28), we have examined the ability of PPARγ and C/EBPα to alter the developmental program of determined myoblasts. Our data demonstrate that ectopic expression of PPARγ, C/EBPα, or both transcription factors can strongly inhibit normal myogenesis induced by serum deprivation. Furthermore, when coexpressed in myoblasts, PPARγ and C/EBPα synergize powerfully to convert myoblastic cells into adipocytes upon hormonal stimulation. These results illustrate the plasticity of cellular determination states and demonstrate the dominant role for both PPARγ and C/EBPα in the adipocyte determination and differentiation processes.

MATERIALS AND METHODS

Plasmids. The retroviral expression vectors pBabe-PPARγ (29) and pLJ-C/EBPα (30) were constructed as described (16).

Virus Production and Infection. Bosc23 cells were used to generate virus (31). Transfection and subsequent infection were performed as described (16). Cells expressing both PPARγ and C/EBPα were established by infecting G8-PPARγ cells with virus containing the pLJ-C/EBPα expression vector.

Cell Culture and Induction of Myogenic and Adipogenic Differentiation. G8 myoblasts were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (vol/vol) fetal calf serum (FCS) as recommended by ATCC. After the establishment of G8 cells expressing PPARγ, C/EBPα, or both transcription factors, virally infected cells were cultured with-

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Abbreviations: ETYA, 5,8,11,14-eicosatetraynoic acid; FCS, fetal calf serum; bHLH, basic helix–loop–helix.

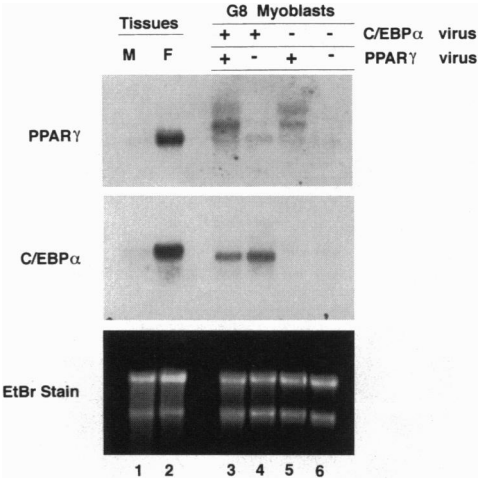

FIG. 1. Expression of exogenous PPARγ and C/EBPα in G8 myoblasts. Northern blot analysis of total RNA isolated from G8 myoblasts expressing retroviral vector only (lane 6), retroviral PPARγ (lane 5), retroviral C/EBPα (lane 4), or both PPARγ/C/EBPα (lane 3). The RNA was isolated 24 h after cells reached confluence. Viral transcript for PPARγ migrates slower than the endogenous mRNA and the viral C/EBPα transcript migrates slightly faster than the endogenous mRNA. Total RNA isolated from adult mouse fat (F; lane 2) and muscle (M; lane 1) are shown for comparison. Ethidium bromide (EtBr) staining of rRNA was used as control for RNA loading. Equivalence of RNA loading was also verified by hybridization to the ribosomal phosphoprotein 36B4 (data not shown).

out selection medium and the myogenic or adipogenic differentiation potential were examined for each of these cells. For myogenic differentiation, cells were cultured in DMEM/15% FCS to 80% confluence and were maintained in 15% FCS or treated with DMEM/15% FCS/ETYA (50 μM) for 7 days or with DMEM/2% (vol/vol) horse serum (BRL) for 4 days. At the end of treatment, cells were fixed and photographed. For adipogenic differentiation, various G8 cells were cultured to 80% confluence in DMEM/15% FCS. Prior to confluence, cells were refed with differentiation medium [DMEM/15% FCS/dexamethasone (1 μM)/insulin (5 μg/ml)/ETYA (50 μM)] [Dex/ETYA/insulin (DEI) induction]. Cells were maintained in this for 8–10 days, with a medium change every 48 h. After 10 days, cells were stained with oil red O as described (32).

RNA Analysis. Total RNA was isolated from cultured cells and mouse tissue by guanidine isothiocyanate extraction (33). For tissue RNA, epididymal fat pad and leg muscle from adult mice 10 weeks old were obtained and total RNA was isolated. Electrophoresis of RNA and Northern blot analysis were as described (34).

RESULTS

Ectopic Expression of PPARγ and C/EBPα in G8 Myoblast Strongly Inhibits Myogenesis. Retroviral vectors were used to generate stable G8 myoblastic cell lines that expressed PPARγ, C/EBPα, or both factors. The myogenic bHLH proteins are expressed at significant levels in these cells and myogenesis can be induced readily by reduction of serum concentration (35). We chose to use G8 cells because they were more readily infected with our retroviral vectors than the better known C_2C_{12} or L6 myoblasts. A retroviral system was employed because it allows for the production of a large homogenous population of stably transfected cells (>1 × 10^5 individual clones) and thus minimizes clonal variability. Northern blot analysis was performed to examine the levels of transgene expression in each cell line (Fig. 1). G8 myoblasts normally expressed very low but detectable levels of PPARγ and C/EBPα (Fig. 1, lane 6). The G8-PPARγ cells expressed ≈20% of adipose

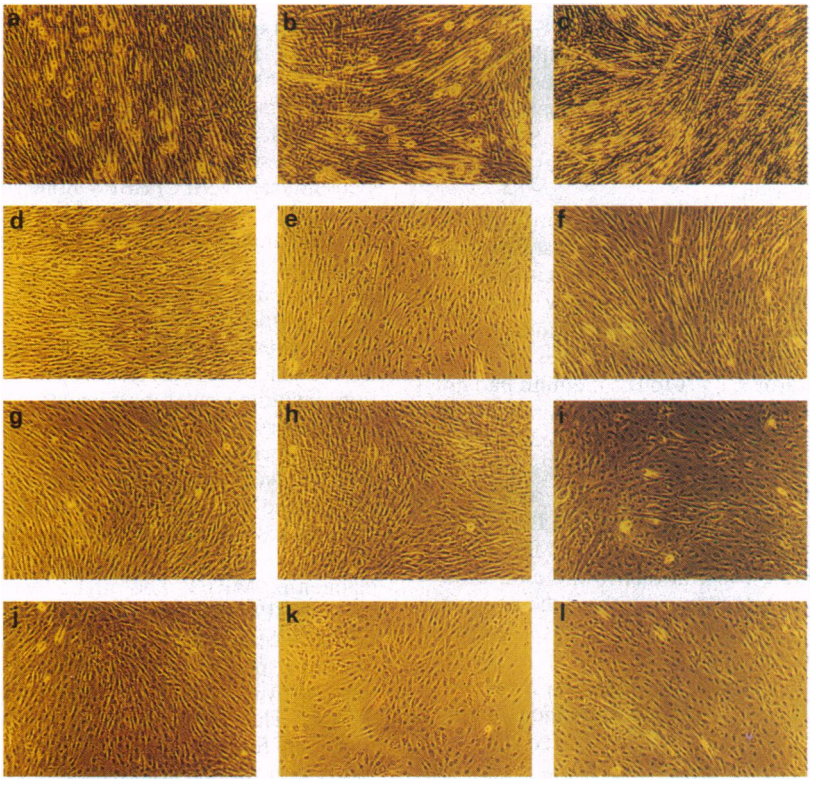

FIG. 2. Morphological alterations in G8 myoblasts expressing adipogenic transcription factors PPARγ, C/EBPα, or both PPARγ and C/EBPα. Stable G8-vector (*a–c*), G8-PPARγ (*d–f*), G8-C/EBPα (*g–i*), or G8-PPARγ/C/EBPα (*j–l*) cell lines were grown to 90% confluence in DMEM/15% FCS and were then treated with 15% FCS (4 days) (*a, d, g,* and *j*), 15% FCS/50 μM ETYA (4 days) (*b, e, h,* and *k*), or 2% horse serum (3 days) (*c, f, i,* and *l*). Cells were then fixed in Fresh-fix (Fisher) and examined. (×5.)

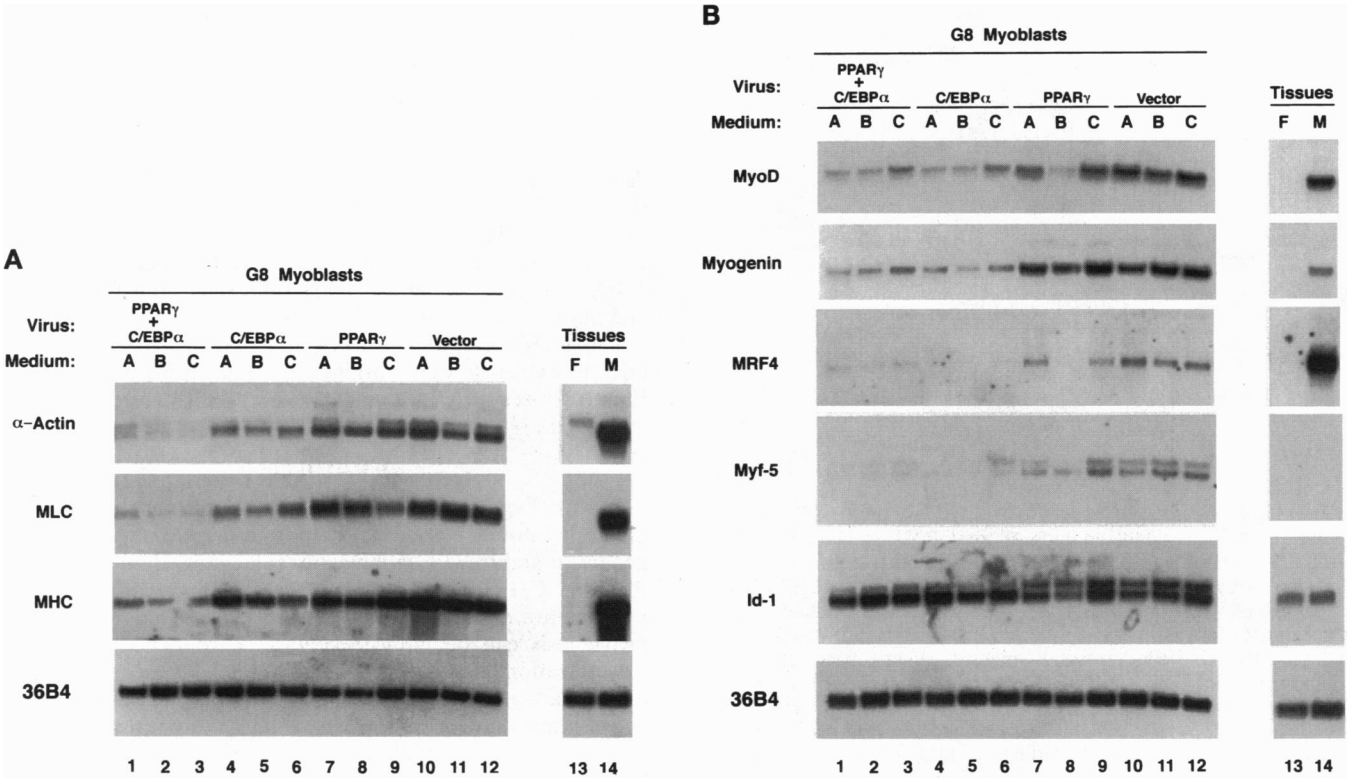

FIG. 3. Expression of muscle-specific genes in G8 myoblasts expressing PPARγ, C/EBPα, or PPARγ/C/EBPα. Cells were treated as in Fig. 2. Total RNA was isolated from these cells, and Northern blots were obtained for α-actin, myosin heavy chain (MHC), and myosin light chain (MLC) (*A*) and for myoD, myogenin, MRF-4, myf-5, and Id-1 (*B*). Lanes: 1–3, RNA samples from G8-PPARγ/C/EBPα cells; 4–6, from G8-C/EBPα cells; 7–9, from G8-PPARγ cells; 10–12, from G8-vector cells. RNA from mouse fat (lane 13) and muscle (lane 14) were controls. Treatments A, B, and C represent 2% horse serum, 15% FCS/ETYA (50 μM), and 15% FCS alone, respectively. Tissue RNA from adult mice fat (F) and muscle (M) were shown as controls. RNA (10 μg) was used for each sample and loading was equalized by EtBr staining as well as hybridization to ribosomal protein 36B4 (36).

tissue levels of PPARγ mRNA (Fig. 1, lane 5); the G8-C/EBPα cells expressed ≈30% of adipose tissue levels of C/EBPα mRNA (Fig. 1, lane 4); the doubly infected G8 cells expressed both transgenes at ≈20–30% of adipose tissue mRNA levels (Fig. 1, lane 3). Interestingly, a slight increase in PPARγ mRNA was detected in G8-C/EBPα cells, raising the possibility that C/EBPα could regulate PPARγ expression.

To assess the effects of PPARγ and C/EBPα expression on the myogenic program, the stable cell lines were first cultured under conditions permissive for myogenesis. When cultured in 15% FCS, G8 myoblasts differentiate spontaneously, adopting a spindle-shaped morphology and eventually undergoing membrane fusion to form multinucleated myotubes (Fig. 2). Addition of the PPAR activator ETYA to the medium had no effect on differentiation (Fig. 2*b*), while differentiation was accelerated if the cells were cultured in 2% horse serum (Fig. 2*c*). In contrast, G8 myoblasts expressing either PPARγ or C/EBPα exhibited a marked reduction in their ability to undergo myogenesis (Fig. 2 *d–i*). When cultured in 15% FCS, the G8-PPARγ and G8-C/EBPα cells maintained a fibroblastic morphology (Fig. 2 *d* and *g*), in contrast to the elongated tubular shape of G8 cells that contain vector without the inserted gene (Fig. 2*a*). For the G8-PPARγ cells, this effect was enhanced when ETYA was included in the culture medium (Fig. 2*e*). Even under the most favorable differentiation conditions for myogenic differentiation (2% horse serum), myotube formation in the G8-PPARγ and G8-C/EBPα cells was greatly inhibited (Fig. 2 *f* and *i*). G8 myoblasts infected with both PPARγ and C/EBPα expression vectors exhibited almost no capacity to undergo myogenesis (Fig. 2 *j–l*). It is important to note that under these conditions the myogenic program was inhibited in the absence of terminal adipocyte differentiation.

To characterize the differentiation state of these cells at a molecular level, Northern blot analysis was performed to examine the expression of skeletal muscle genes. As shown in Fig. 3*A*, muscle-specific mRNAs encoding α-actin, myosin light chain, and myosin heavy chain were greatly reduced in G8 cells expressing both PPARγ and C/EBPα (Fig. 3*A*, lanes 1–3). Cells infected with C/EBPα alone showed a slight reduction of these mRNAs (Fig. 3*A*, lanes 4–6). It is interesting to note that although myotube formation was severely compromised in all three cell lines, some expression of muscle-specific genes persisted in each line. Thus, while ectopic expression of PPARγ and/or C/EBPα in myoblasts did not completely abolish muscle-specific gene expression, it was strongly inhibitory to the morphological aspects of muscle cell differentiation, including myotube formation.

In theory, inhibition of myogenesis could be accomplished by the repression or inactivation of positive myogenic factors such as MyoD, myf-5, myogenin, or MRF4 or by the induction of negative regulators such as Id-1 (37, 38). To address these possibilities, the expression of known myogenic regulators was examined. As shown in Fig. 3*B*, the expression of MyoD, myogenin, MRF4, and myf-5 was significantly reduced in G8-C/EBPα cells (Fig. 3*B*, lanes 4–6) and the G8-PPARγ/C/EBPα cells (Fig. 3*B*, lanes 1–3) under all culture conditions. In cells expressing PPARγ, repression of MyoD and MRF4 was clearly dependent on the presence of the PPAR activator ETYA. This observation suggests that the transcriptional activation function of PPARγ is required for this repression. Expression of the negative myogenic regulator Id-1 was also examined in all cell lines. Although there was little overall change in total mRNA level for Id-1, the adipogenic factors appeared to cause a decrease in the Id-1 mRNA of lower mobility (39). The functional significance of this is currently unknown. Thus, these

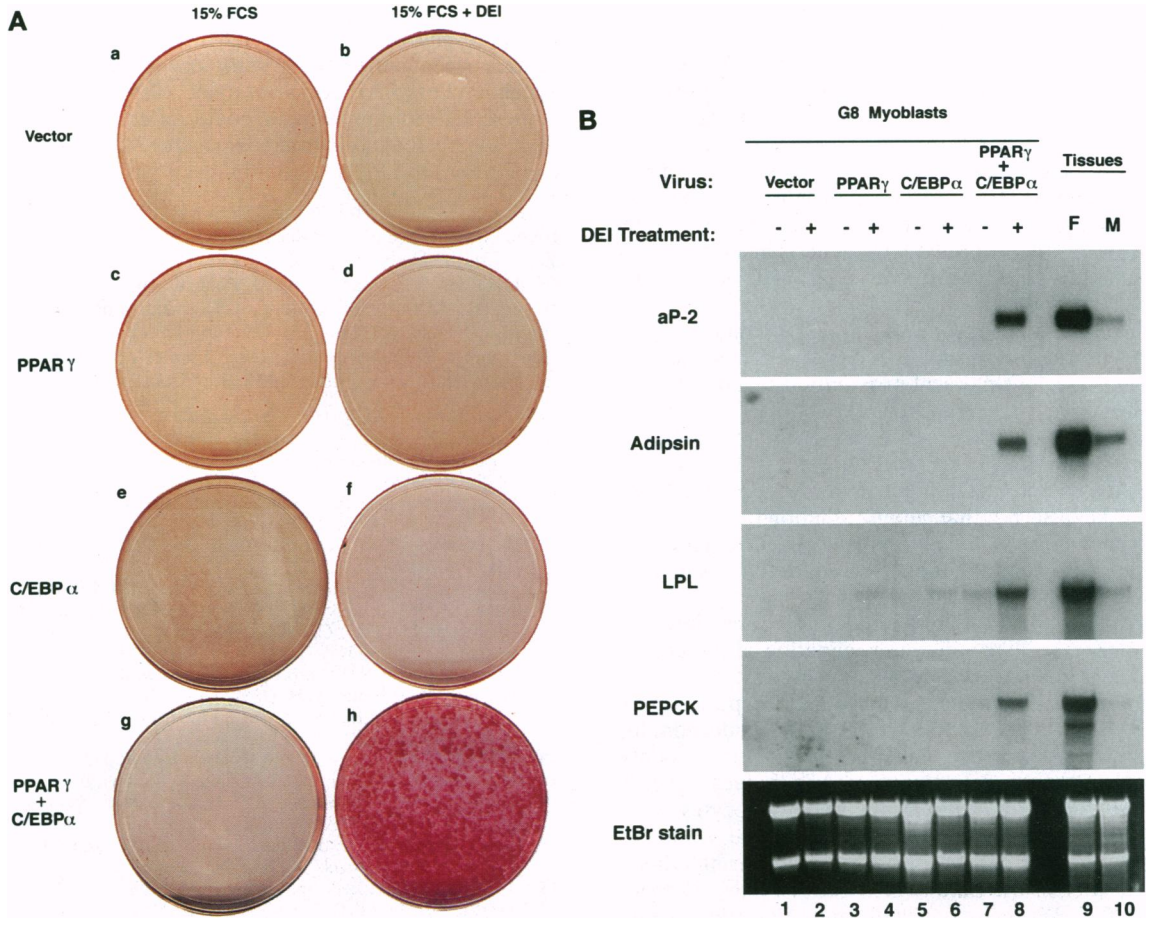

FIG. 4. Adipogenesis in G8 myoblasts expressing PPARγ and C/EBPα. (*A*) G8 myoblasts infected with retroviral vector alone or retroviruses expressing PPARγ, C/EBPα, or both PPARγ/C/EBPα were grown to confluence and induced for adipogenic differentiation. Oil red O-stained dishes of uninduced (15% FCS) or induced (15% FCS/DEI) G8-vector (plates a and b), G8-PPARγ (plates c and d), G8-C/EBPα (plates e and f), or G8-PPARγ/C/EBPα (plates g and h) cells are presented. (*B*) RNA was isolated from induced (+DEI) or uninduced (−DEI) G8 myoblasts infected with virus containing empty vector (lanes 1 and 2), PPARγ (lanes 3 and 4), C/EBPα (lanes 5 and 6), or both PPARγ/C/EBPα (lanes 7 and 8). Mouse tissue RNAs from fat (F; lane 9) and muscle (M; lane 10) were used for comparison. Expression of mRNA for LPL, PEPCK, adipsin, and aP-2 was examined. RNA (10 μg) was loaded for each lane. Equivalence of RNA loading was verified by EtBr staining and 36B4 hybridization (data not shown).

data suggest that the mechanism underlying myogenic inhibition in G8-PPARγ and C/EBPα cell lines may involve reduction of the expression of the myogenic bHLH proteins.

Transdifferentiation of G8 Myoblasts into Adipocytes. We have demonstrated (16) that ectopic expression of PPARγ stimulates adipose differentiation of uncommitted fibroblast cell lines. Similar observations have also been made for C/EBPα (17), and the two factors act synergistically when coexpressed in fibroblasts (16). The ability of these factors to activate the adipogenic program in cells already committed to another lineage has not been explored. To test the possibility that C/EBPα, PPARγ, or a combination of these factors could alter the developmental potential of determined myoblasts, G8-PPARγ, G8-C/EBPα, and G8-PPARγ/C/EBPα cell lines were cultured under conditions permissive for adipogenesis. G8-vector cells cultured in the presence of FCS, dexamethasone, insulin, and ETYA differentiated normally to form multinucleated myotubes (Fig. 4*A*, plate b). Under similar culture conditions, G8 cells expressing PPARγ or C/EBPα alone did not undergo significant muscle or adipocyte differentiation (Fig. 4*A*, plates d and f). In contrast, G8 cells expressing both PPARγ and C/EBPα underwent a dramatic conversion to mature adipocytes. Lipid accumulation was apparent in ≈75–85% of the cells after 10 days of culture in differentiation medium (Fig. 4*A*, plate h). The differentiated cells were mononucleate, and their morphology was similar to that of normal adipocytes seen in 3T3-L1 and 3T3-F442A cells. The kinetics of overt adipose differentiation for G8-PPARγ/C/EBPα cells was very similar to that of established preadipocytes such as 3T3-L1 and 3T3-F442A. Furthermore, adipose differentiation of the G8-PPARγ/C/EBPα cells was strongly dependent both on hormonal stimulation with dexamethasone and insulin and on the potency of the PPAR activator (data not shown).

Northern blot analysis was performed to characterize further the phenotype of the G8-PPARγ/C/EBPα lipid-containing cells. As shown in Fig. 4*B*, the differentiated G8-PPARγ/C/EBPα cells expressed the characteristic repertoire of tissue-specific fat cell mRNAs, including PEPCK, LPL, aP2, and adipsin. Thus, PPARγ and C/EBPα can act synergistically to activate an apparently authentic adipocyte differentiation program in a determined myoblast cell line.

DISCUSSION

Embryonic development is accompanied by the restriction of cell lineages to distinct pathways leading to the formation of functional differentiated cell types. These pathways tend to be exclusive, and only rarely do they allow "transdifferentiation" from one cell type to another. However, examples of this do exist, particularly in avian and amphibian tissues in which regeneration is important (ref. 40 and reference therein). Transdifferentiation has also been observed when cells are

transplanted to ectopic sites where the environmental cues impose new constraints (41–43).

In cultured cell systems, it has been possible to observe the activation of inappropriate programs of gene expression through certain experimental manipulations. It has been shown that the nuclei from muscle cells can cause the extinction of liver-specific gene expression and activation of muscle genes when these nuclei share cytoplasm in heterokaryons (44, 45). Recently, ectopic expression of the muscle regulatory gene MyoD has been shown to stimulate myogenesis in a number of cell lines including fibroblasts, liver cells, neuroendocrine epithelial cells, and preadipocytes (27).

The latter result is of particular interest in that fat and muscle have several interesting connections. These tissues often occur in very close physical proximity. Indeed, fat cells can pervade muscle tissue, particularly in obesity. Muscle atrophy, whether due to disuse or disease (Duchenne muscular dystrophy or Prader–Willie syndrome), often results in replacement of the muscle mass by fat tissue (22, 23, 25). Most recently, it has been shown that genetic ablation of the muscle regulatory proteins MyoD and myf-5 results in a dramatic loss of muscle development and the replacement of this tissue with adipose tissue (24).

Recent advances in the understanding of the transcriptional basis of adipogenesis allow a direct examination of the ability of myogenic cells to enter the adipogenic pathway. PPARγ is an orphan member of the nuclear hormone receptor superfamily that is specifically expressed in fat (12). In addition, its expression is induced very early in several cell culture models of adipogenesis. Ectopic expression of PPARγ induces several fibroblastic cell lines to differentiate into adipocytes in a PPARγ-activator-dependent manner (16). C/EBPα, while expressed in many tissues *in vivo*, is also induced during adipogenesis, albeit later in the time course than PPARγ. Ectopic expression of C/EBPα can trigger differentiation in preadipocytes and cause adipogenesis in several fibroblastic cell types (17). When C/EBPα mRNA is expressed at or near fat cell levels, it powerfully synergizes with PPARγ to stimulate adipogenesis (16). Both PPARγ and the C/EBPs are known to be direct transcriptional activators of several fat cell genes, and the best characterized adipocyte-specific regulatory sequences have been shown to contain binding sites for both factors (12, 46).

We demonstrate here that PPARγ and C/EBPα have profound effects on the process of myogenesis. Their expression is sufficient to block muscle differentiation and cause a "transdifferentiation" of myoblasts to fat cells. Importantly, the myogenic inhibitory activity of these factors is temporally and functionally separable from their ability to stimulate overt adipogenesis. When both factors are expressed in G8 cells in the absence of PPAR activators and other adipogenic hormones, there is no morphological or biochemical evidence of fat development. However, expression of these factors clearly interferes with the expression of myogenic transcriptional regulators and myofibrillar proteins. Moreover, the ability to form myotubes is completely lost. When a PPAR activator and adipogenic hormones are applied, these cells differentiate into adipocytes as efficiently as established fat cells lines such as 3T3-L1 and 3T3-F442A. Efficient stimulation of adipogenesis in the cell lines developed here occurs with the expression of both adipogenic regulators at or below the levels of mRNA seen in adipose tissue. Similar results have been observed (16) for the differentiation of adipocytes from fibroblasts. Inhibition of myogenesis and/or suppression of myogenic regulatory proteins can be accomplished by expression of C/EBPα alone, to a certain extent. While expression of PPARγ alone was not effective in blocking the expression of muscle end-product genes in response to the PPAR activators used here, it does significantly reduce the expression of myogenic regulators MyoD and MRF4.

Do these results have physiological relevance? While it is unlikely that myoblasts are readily converted to fat in a healthy organism, it is possible that adipogenesis may be triggered when the normal pathways of myogenesis are blocked, such as in myodystrophic and myohypotonic disease. Furthermore, obesity is often accompanied by a hyperlipidemic state that may provide a high level of PPAR activators to all cells. Thus, proliferation of adipose cells in muscle tissue could reflect a certain degree of susceptibility to lipid-induced adipogenesis through the low level of PPARγ and C/EBPα seen in myoblasts.

We dedicate this paper to the memory of Dr. Harold Weintraub, a friend and a source of great inspiration. We acknowledge helpful discussions with members of our laboratory. We also thank Dr. S. McKnight (Tularik), Dr. A. Lassar (Harvard Medical School, Boston), and Dr. N. Rosenthal (Massachusetts General Hospital, Charlestown, MA) for various cDNAs used here. E.H. was supported by a postdoctoral fellowship from Dana–Farber–Sandoz Drug Discovery Program. This work was funded by National Institutes of Health Grant DK31405 to B.M.S.

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