C/EBPα induces adipogenesis through PPARγ: a unified pathway

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PPARγ and C/EBPα are critical transcription factors in adipogenesis, but the precise role of these proteins has been difficult to ascertain because they positively regulate each other's expression. Questions remain about whether these factors operate independently in separate, parallel pathways of differentiation, or whether a single pathway exists. PPARγ can promote adipogenesis in C/EBPα-deficient cells, but the converse has not been tested. We have created an immortalized line of fibroblasts lacking PPARγ, which we use to show that C/EBPα has no ability to promote adipogenesis in the absence of PPARγ. These results indicate that C/EBPα and PPARγ participate in a single pathway of fat cell development with PPARγ being the proximal effector of adipogenesis.

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Adipogenesis is the process by which undifferentiated precursor cells differentiate into fat cells. This has become one of the most intensively studied developmental processes for at least two reasons: the increasing prevalence of obesity in our society has focused attention on many aspects of fat cell biology, and the availability of good cell culture models of adipocyte differentiation has permitted detailed studies not possible in other systems. Experiments using these in vitro models of adipogenesis, which include the 3T3-L1 and 3T3-F442A lines, have illustrated the transcriptional cascade that promotes fat cell differentiation [Rosen et al. 2000]. Representatives of several transcription factor families have been implicated in this process, including the CCAAT/enhancer binding proteins C/EBPα, C/EBPβ, and C/EBPδ; the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ), and the basic helix-loop-helix protein ADD1/SREBP1c. Studies in adipogenic cell lines have shown that hormonal induction of differentiation is rapidly followed by expression of C/EBPβ and C/EBPδ (Cao et al. 1991; Yeh et al. 1995). Within the next day or so, levels of these proteins peak and then begin to drift downward, coincident with a rise in C/EBPα and PPARγ. These latter factors induce gene expression changes characteristic of mature adipocytes and remain elevated for the life of the cell. In the present model of the transcriptional cascade leading to adipogenesis, C/EBPβ and C/EBPδ induce low levels of PPARγ and C/EBPα, which are then able to induce each other's expression in a positive feedback loop that promotes and maintains the differentiated state. This model is consistent with gain-of-function data showing that the addition of either PPARγ or C/EBPα can promote adipogenesis in fibroblast cell lines [Lin and Lane 1994; Tontonoz et al. 1994].

Loss-of-function studies have shown convincingly that PPARγ is required for adipogenesis in vivo and in vitro, and cells lacking PPARγ express greatly reduced levels of C/EBPα [Barak et al. 1999; Kubota et al. 1999; Rosen et al. 1999]. Similarly, fibroblasts lacking C/EBPα have reduced adipogenic potential, and express reduced levels of PPARγ [Wu et al. 1999]. Importantly, adding PPARγ back to C/EBPα−/− fibroblasts with a retroviral vector restores their capacity to accumulate lipid and activate markers of adipogenesis, including the endogenous PPARγ gene [Wu et al. 1999]. These C/EBPα−/− adipocytes are normal in almost every way with the important exception that they do not show insulin sensitivity.

Importantly, because the converse manipulation has not been performed, it is not known whether the presence of exogenously applied C/EBPα is sufficient to promote adipogenesis in the absence of PPARγ. The reasons for this omission are largely technical, and relate to the fact that PPARγ−/− embryos die at embryonic day E9.5–E10, prior to the stage where the establishment of embryonic fibroblasts is generally considered feasible [Barak et al. 1999; Kubota et al. 1999]. Additionally, the protocols for differentiating adipocytes directly from ES cells are cumbersome [Dani et al. 1997; Rosen et al. 1999] and are not amenable to retroviral expression of C/EBPα or other factors, as the LTRs of most available retroviruses are rapidly and irreversibly silenced after ES cell infection [Cherry et al. 2000].

It has therefore been difficult to delineate the relationship between C/EBPα and PPARγ in adipogenesis. Two competing models consistent with the available data are illustrated in Figure 1. In Figure 1A, a model is depicted in which PPARγ and C/EBPα induce each other's expression and can each act independently to promote fat cell differentiation. Figure 1B presents an alternative model in which PPARγ is the direct regulator of adipogenesis, whereas the major role of C/EBPα is centered on maintaining expression of PPARγ and promoting full insulin sensitivity.

To ascertain which model is more likely, we have created a fibroblast cell line that lacks PPARγ. We use these cells to show that the adipogenic action of C/EBPα is entirely dependent on PPARγ.
Results and Discussion

Generation of immortalized cell lines heterozygous or null for PPARγ

Mice carrying floxed alleles of PPARγ were generated [data not shown]. The PPARγ null allele was generated by crossing PPARγ-floxed mice with the ELαCre transgenic mouse line as outlined previously [Hayhurst et al. 2001]. Offspring that lacked exon 2 were selected by Southern blotting, and the null allele was maintained in the heterozygous state with the active PPARγ-exon 2 allele. Matings were established between mice that were heterozygous null at the PPARγ locus (+/-) and animals carrying a single allele of PPARγ with loxP sites flanking exon 2 (flox/−). At E12.5, embryos were harvested, minced, and trypsinized to generate mouse embryonic fibroblasts (MEFs). Embryonic fibroblasts were plated, expanded, and infected with one of two different adenoviruses expressing Cre recombinase and green fluorescent protein (GFP) separated by an internal ribosome entry site (IRES) sequence, whereas the second virus expressed only GFP. After infection, cells were trypsinized and sorted by fluorescence detection; cells expressing GFP were collected, replated, and expanded. As shown in Figure 2B, Southern blotting showed that cells (flox/+ or flox/−) infected with the Cre-producing adenovirus completely lost their floxed alleles. This result was confirmed with genomic PCR (not shown), which showed the complete absence of a band associated with a residual floxed exon 2. Northern analysis of cells treated with conditions selected to induce adipogenesis [see Materials and Methods and below] shows the presence of PPARγ mRNA in −/− cells, but RT-PCR of this RNA shows that all of it is associated with loss of exon 2 [data not shown]. Western blot analysis of similarly treated cells reveals no PPARγ protein (Fig. 2C); the expected translation product of PPARγ mRNA lacking exon 2 is ~10 kD and does not appear on Western blotting, perhaps because of protein instability. One distinct advantage of this approach is that the PPARγ+/− and flox/− cells were immortalized as a single line prior to exposure to Cre recombinase. This ensures that any biological changes associated with transformation are present in both flox/− and −/− cells, which in turn increases our confidence that any differences noted between these cell lines specifically reflects the presence or absence of PPARγ.

Ectopic PPARγ can stimulate adipogenesis in PPARγ−/− fibroblasts, but C/EBPα cannot

Flox/− fibroblasts can be differentiated into adipocytes with low efficiency (<2%) in the presence of dexamethasone, methylisobutylxanthine, and insulin (DMI), in addition to the synthetic PPARγ agonist troglitazone. In contrast, however, the PPARγ+/− fibroblasts are not competent to undergo adipogenesis at all; we have never seen even a single fat cell develop in any experiment. This result is consistent with earlier observations made by our group and others showing that PPARγ is absolutely required for adipogenesis in vitro and in vivo [Barak et al. 1999; Kubota et al. 1999; Rosen et al. 1999].
We next examined whether the block in differentiation seen in PPARγ−/− cells is specifically caused by the lack of PPARγ by infecting them with a PPARγ2-expressing retrovirus. Ectopic expression of PPARγ in flox−/− cells resulted in a dramatic increase in adipogenic potential when DMI alone was used as the inducing cocktail. The effect was even more pronounced when troglitazone was added to the cells, suggesting that at some level of PPARγ expression, the amount of endogenous ligand for PPARγ becomes limiting. Importantly, retroviral introduction of PPARγ was able to rescue the adipogenic potential of PPARγ−/− cells, indicating that the lack of adipogenesis seen in vector-treated cells truly reflects the absence of this nuclear hormone receptor, and not some unanticipated disruption of the adipogenesis machinery at a site distinct from PPARγ.

To ascertain the adipogenic potential of C/EBPα in the absence of PPARγ, this transcription factor was added to the PPARγ flox−/− and −/− cells using the same retroviral delivery system. C/EBPα enhanced the extent of adipogenesis in PPARγ flox−/− cells, consistent with the gain-of-function effects seen in the past with this protein [Fig. 3A,B]. C/EBPα, however, failed to induce any lipid accumulation whatsoever in the PPARγ−/− cells. This lack of adipogenic action is not caused by any impairment of C/EBPα expression in these cells, because immunoblot analysis reveals high levels of the 42-kD C/EBPα protein [Fig. 4A].

Gene expression analysis confirms the findings shown at the level of cell morphology and lipid accumulation, that is, that C/EBPα can support adipocyte-specific gene expression only when PPARγ is also present. The adipocyte-selective fatty-acid-binding protein aP2 is a direct target gene of PPARγ. Ectopic high-level expression of PPARγ is able to promote aP2 expression at day 0, before fat cell differentiation has even begun [Fig. 4B]. The lack of aP2 expression in PPARγ−/− fibroblasts in the presence of C/EBPα reflects the lack of adipogenesis in these cells. A similar pattern is observed for adipsin, another fat-cell-specific protein induced during adipogenesis. Recent data indicate that C/EBPα is a major contributor to the expression of adipsin in fat cells (Chen et al. 2000), the fact that we do not detect adipsin message in PPARγ−/− cells even when high levels of C/EBPα are present indicates that other factors present in mature adipocytes must be required as well. PPARγ itself is unlikely to be the missing factor, in part because we do not see induction of adipisin message in PPARγ-expressing cells before the onset of adipogenesis [Fig. 4B], and because activation of PPARγ by thiazolidinedione drugs has actually been shown to decrease adipisin expression in mature fat cells. PPARγ also must be present in order for C/EBPα to maximally induce its own mRNA expression.

**Figure 3.** PPARγ restores adipogenesis in PPARγ−/− cells, but C/EBPα does not. [A] Dishes containing PPARγ flox−/− or −/− cells were infected with retroviruses expressing PPARγ2, C/EBPα, or vector only. Cells were exposed to a prodifferentiative regimen with or without troglitazone, and stained with oil red O after 7 d. [B] Microscopic view of cells in A.

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An enhanced model of the adipogenic transcriptional cascade

Studies using NIH-3T3 fibroblasts have indicated that expression of either PPARγ or C/EBPα is sufficient to induce adipogenesis (Lin and Lane 1994; Tontonoz et al. 1994). Regardless of which factor is employed, the phenotype of the differentiated cells appears to be very simi-
lar. The mechanistic interpretation of this result is difficult, however, because ectopic expression of either factor results in enhanced endogenous expression of the other. Fibroblasts that lack C/EBPα have been generated previously, and have been shown to be deficient in both PPARγ expression and adipogenic potential, when PPARα is replaced, however, these cells are competent to undergo nearly all aspects of fat cell differentiation with the exception of insulin sensitivity [El-Jack et al. 1999; Wu et al. 1999]. This result opened the possibility that PPARα and C/EBPα are largely redundant in adipogenesis. In the present study, PPARγ−/− fibroblasts were generated and used to show that such is not the case.

In light of the data presented above, what role does C/EBPα play in adipogenesis? Clearly, C/EBPα is required for adipogenesis as shown by loss-of-function studies in vivo and in vitro. The fact that C/EBPα null fibroblasts undergo adipogenesis when PPARγ is replenished, however, strongly supports the argument that the role of C/EBPα in fat cell differentiation is limited to the induction and maintenance of PPARγ levels. Indeed, this is consistent with observations that C/EBPα can induce PPARγ2 expression by direct binding to specific sites in the PPARγ promoter [Elberg et al. 2000]. Interestingly, recent studies on C/EBPα null mice indicate that the development of white adipose tissue (WAT), but not brown adipose tissue (BAT), is dependent on C/EBPα (Linhart et al. 2001); we would hypothesize based on our data that PPARγ levels in WAT are more dependent on C/EBPα than PPARγ levels in BAT.

In addition to maintaining PPARγ expression in developing adipocytes, C/EBPα is critical in the establishment of insulin sensitivity. This effect is mediated in part by direct transcriptional induction of insulin receptor and IRS-1 levels, and in part by a poorly characterized post-receptor mechanism [Wu et al. 1999]. Additionally, other genes typical of the differentiated state [e.g., adipin and leptin] are strongly promoted by the direct actions of C/EBPα (Chen et al. 2000). C/EBPα, then, plays several important roles in adipocyte function, despite not being directly responsible for the process of adipogenesis per se.

Another possibility is that PPARγ may be permissive for C/EBPα action, perhaps directly through protein–protein interactions [although no evidence exists for such an interaction] or alternatively through induction of a cofactor critical for C/EBPα. We believe that the PPARγ−/− fibroblasts will be invaluable to screen for these and other factors that act downstream of PPARγ in adipogenesis, such studies are already underway.

Materials and methods

Generation of adenoviral constructs
To create the Ad-Cre-IRES-GFP virus, the pLIE plasmid (Wang et al. 2000) was cleaved with HindIII and NotI, and a cDNA encoding Cre recombinase [kindly provided by M. Murakawa, MGH Nessel Gene Therapy Center] was inserted. A second fragment, encoding the internal ribosomal entry site sequences derived from the encephalomyocarditis virus and a codon optimized green fluorescent protein [kindly provided by E.C. Park, MGH Nessel Gene Therapy Center] was inserted at the NotI site [Fukumura et al. 1998]. This plasmid and the plasmid pREP7 were both cleaved by PstI-PspI, ligated to each other using DNA ligase, and then packaged in phage packaging extracts (MaxPlax, Epicentre Technologies) as described (Wang et al. 2000). Cosmid DNA was isolated from Escherichia coli transduced with the packaged DNA and human 293 embryonic kidney cells were transfected with 10 µg of this DNA follow-

ing cleavage by 1-CelI. Virus propagation, purification, and plaque assay were performed using established adenoviral protocols (Graham and Prevec 1991).

Generation of PPARγ flox/− and −/− cell lines
PPARγ flox/− mice were mated to PPARγ +/+ mice. At E12.5, embryos were harvested. Primary MEFs were generated by removing the heads of the embryos (saved for PCR genotyping), scrapping out the visera with a forceps, and trypsinizing the bodies after mincing. The resulting slurry was plated in 75-cm² flasks. Cells derived from flox/− and flox/+ embryos were passaged by plating 3 × 10⁶ cells per 60-mm dish every 3 d as described (Todaro and Green 1963). After crisis and expansion, cells were split into two aliquots and infected with adenovirus Ad-Cre-IRES-GFP or Ad-X-IRES-GFP at an m.o.i. of 100,000:1. Forty-eight hours after infection, cells were flow-sorted on a Cytomation Mo-Flo by exciting cells with a 488-nm laser and collecting at 530/480 bp. The most intensely GFP-expressing cells were replated, expanded, and frozen.

Cell culture
Cell cultures were cultured in DME with 10% FBS at 10% CO₂. After retroviral infection and selection (see below), cells were allowed to grow to confluence in either 100-mm dishes or 6-well plates. Once confluence was reached, cells were exposed to a pro-differentiative regimen including dexamethasone [1 µM], insulin [5 µg/mL], and isobutylmethylxanthine [0.5 mM] with or without 10 µM troglitazone. After 2 d, cells were maintained in medium containing insulin until ready for harvest at day 7.

Retroviral infections
Retroviruses were constructed in pMSCV vectors (Clontech) using either puromycin or hygromycin selectable markers. Viral constructs were transfected into 293EBNA cells using FuGene [Roche] along with plasmids expressing gag-pol and the VSV-G protein. Supernatants were collected after 48 h, and either used immediately or frozen at −80°C for later use. Viral supernatants were added to PPARγ flox/− or −/− cells for 4 h, with selection with puromycin [2 µg/mL] or hygromycin [175 µg/mL] started 48 h later. Cells were selected, expanded, and studied immediately or frozen for later use.

Northern and Western blots
For Northern analysis, cells were grown to confluence and treated with a pro-differentiative regimen as noted above. Cells were lysed in Trizol and processed according to the manufacturer’s instructions. For each sample, 10 µg of total RNA was loaded onto formaldehyde-agarose gels, transferred onto nylon membranes, and hybridized with the appropriate 32P-labeled probe in Ultrahyb (Ambion). Blots were stripped in boiling 1% SDS between different probes.

For Western analysis, cells were grown to confluence and treated with a pro-differentiative regimen as noted above. Lysis buffer [PBS with 1% Triton X-100 and complete protease inhibitor tablets, Boehringer Mannheim] was added, and the cells were triturated in Eppendorf tubes on ice. After shaking at 4°C for 10 min, lysates were spun at 14,000g, and supernatants were collected and snap-frozen in liquid nitrogen. Protein concentrations were determined with a BioRad assay, and 200 µg of total protein was TCA-precipitated and run on 10% SDS-PAGE. Protein was transferred onto nitrocellulose membranes, which were blocked in 5% milk, and probed with antibodies against PPARγ (Santa Cruz). After washing in TBST and exposing to secondary antibody, blots were stripped in boiling 0.1% SDS between different probes.

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