Differential Effects of Fibroblast Growth Factor and Tumor Promoters on the Initiation and Maintenance of Adipocyte Differentiation

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Abstract. Fibroblast growth factor (FGF) has been shown to inhibit the differentiation of myogenic and adipogenic cell lines without inducing a proliferative response. We have previously shown that agents capable of activating protein kinase C (PKC), such as FGF and the phorbol ester tetradecanoyl phorbol-13-acetate (TPA), inhibit the differentiation of the adipogenic cell line TA1, as measured by the rapid loss of adipocyte-specific RNAs. We report here that the treatment of fully differentiated TA1 adipocytes with FGF at 10 ng/ml induces the reversal of adipocyte differentiation, even in cells where PKC activity has been down-regulated by TPA pretreatment. In contrast, TPA or lower concentrations of FGF (1 ng/ml), both effective inducers of c-*fos* RNA in adipocytes, fail to reverse adipocyte differentiation. The adipocytes, however, will extinguish differentiation-specific functions in response to TPA by the addition of a calcium ionophore. Therefore, we propose that there are two FGF-sensitive pathways in TA1 cells: one responsible for the initiation of differentiation (TPA sensitive) and another required for maintenance of the adipose phenotype (TPA insensitive). These results suggest that activation of two distinct signaling pathways—one PKC and calcium dependent, the other FGF activated but PKC independent—are capable of inhibiting the biochemical events responsible for the maintenance of adipocyte differentiation.

It has become increasingly evident that the family of peptide hormones referred to as growth factors are locally acting intercellular messengers whose effects are not always related to stimulation of cell proliferation (Sporn and Roberts, 1988). The signal transduction pathways activated by interaction of these hormones with their cognate receptors remain poorly defined despite the realization that (at least) some of the receptors (e.g., for EGF and PDGF) harbor intrinsic tyrosine-specific protein kinase activity (Ullrich and Yarden, 1988). Activation of a phosphatidylinositol-specific phospholipase C (PLC)[1] has also been proposed as a primary event in signal transduction in response to several of these growth factors (Berridge, 1987). Indeed, the activation of protein kinase C (PKC) and the release of intracellular Ca^{2+} by the products of PLC action, diacylglycerol and inositol trisphosphate, respectively, have been implicated as intracellular events mediating the growth factor response (Hirasawa and Nishizuka, 1985).

A useful experimental paradigm in which proliferation-independent actions of growth factors can be investigated is their ability to inhibit the differentiation of determined stem cells. For example, EGF, fibroblast growth factor (FGF), and transforming growth factor β (TGF-β) have all been found to prevent conversion of myoblasts to myocytes without stimulating cell division (Florini et al., 1986; Gospodarowicz et al., 1976; Linkhart et al., 1981; Massagué et al., 1986; Miller et al., 1988; Spizz et al., 1986; Wang and Rubenstein, 1988). Particularly intriguing are studies of Lathrop et al. (1985*a*,*b*) documenting that FGF is also capable of reversing the myogenic conversion of BC3H1 cells as determined by the loss of expression of muscle-specific proteins and/or RNAs.

Analogous results with adipogenic cells lines indicate that growth factors may also modulate the differentiative potential of this mesenchymal lineage. Such cell lines resemble fibroblasts while growing at low density, but, upon reaching confluence, growth ceases and the cells take on the morphological and biochemical characteristics of mature adipocytes (Green and Kehinde, 1976). Using adipogenic cell lines, such as 3T3-L1, 3T3-F442A, *ob*17, and 3T3-T, numerous investigators have shown that the differentiation of these adipoblasts can be interfered with by exposure to FGF, PDGF, or TGF-β (Gaillard et al., 1984; Hayashi et al., 1981; Ignotz and Massagué, 1985; Sparks and Scott, 1986). Using the C3H-10T1/2 mouse embryo fibroblast-derived cell line TA1 (Chapman et al., 1984), we recently demonstrated that adipogenesis can be prevented by either FGF or the tumor-promoting phorbol ester tetradecanoyl phorbol-13-acetate (TPA) as reflected by the inability of the cells to undergo morphological differentiation or to accumulate adipocyte-specific RNAs (Muller et al., 1984). The effect of TPA is presumably mediated by activation of PKC (Castagna et al., 1982; Yamanishi et al., 1983)

1. *Abbreviations used in this paper*: FGF, fibroblast growth factor; LPL, lipoprotein lipase; PKC, protein kinase C; PLC, phospholipase C; TPA, tetradecanoyl phorbol-13-acetate; TNF, tumor necrosis factor.

although formal proof remains to be established. As in the case of myoblasts treated with FGF, the effects of the growth factor or TPA on TA1 cell differentiation are not due to stimulation of cell growth (Navre and Ringold, 1988).

Whereas several studies have reported the ability of growth factors to inhibit adipogenesis only Torti et al. (1985) have demonstrated reversal of adipocyte differentiation with a peptide hormone. In that case, the macrophage product cachectin, now known to be tumor necrosis factor (TNF; Beutler et al., 1985), was shown not only to prevent the accumulation of adipocyte-specific RNAs in TA1 cells but to interfere with the maintenance of the differentiated state as seen by the coordinate inhibition of adipocyte-specific gene expression. Unfortunately, little if anything is currently known about the receptor for TNF or the signaling mechanisms by which it elicits biological responses.

Here we document that FGF at 10 ng/ml will not only prevent adipocyte differentiation but will rapidly reverse the expression of adipocyte-specific genes in fully differentiated TA1 cells even in adipocytes made PKC deficient by pretreatment with phorbol esters. In contrast, lower concentrations of FGF (1 ng/ml) or the phorbol ester TPA, though capable of inducing c-*fos* RNA, will block but not reverse adipocyte differentiation. However, TPA in the presence of a calcium ionophore (which has no effect on its own) extinguishes adipoycte-specific functions. This finding suggests that there are at least two FGF-sensitive pathways in TA1 cells: one responsible for the initiation of differentiation (TPA sensitive) and another required for maintenance of the adipose phenotype (TPA insensitive). We surmise that activation of two classes of intracellular messengers—a PKC-independent, FGF-activated pathway or a pathway requiring simultaneous activation of a PKC- and calcium-dependent second messenger signaling systems—are capable of interfering with the mechanisms responsible for the maintenance of adipocyte differentiation.

Materials and Methods

Cell Culture

TA1 cells (Chapman et al., 1984) were grown in Eagle's basal medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (lot no. MO131; M. A. Bioproducts, Walkersville, MD) or, in more recent experiments, 10% supplemented/defined calf serum (HyClone Laboratories, Logan, UT). All serum used was heat inactivated at 55°C for 30 min. Cultures were grown in 24-well plates or 100-mm dishes at 37°C in a humidified incubator at 5% CO_2 atmosphere.

Basic fibroblast growth factor was a gift of Dr. D. Gospodorowicz (University of California at San Francisco, San Francisco, CA). All dilutions of the peptide were made in 1 mg/ml BSA (Sigma Chemical Co., St. Louis, MO). EGF was from Collaborative Research (Bedford, MA). The phorbol ester TPA was from Sigma Chemical Co., and the calcium ionophore ionomycin was from Calbiochem-Behring Corp. (La Jolla, CA); they were each dissolved in DMSO at 1,000× concentrations so that the final DMSO concentration in the culture medium was never >0.2%. Dexamethasone and indomethacin (Sigma Chemical Co.) were made up at 1,000× concentrations of 1 and 30 mM, respectively, in 95% ethanol.

RNA Isolation

Total cellular RNA was isolated using the method of Chirgwin et al. (1979). Briefly, after dishes were washed with PBS, 3 ml of lysis buffer (guanidine-HCl, pH 5.2, 25 mM sodium citrate, 0.1% *N*-lauryl sarcosine) was added and the suspension was harvested by scraping and transferred to a sterile 15-ml polypropylene tube (Falcon Labware, Oxnard, CA). The DNA was sheared by passing the mixture through a 21-gauge needle 10 times, and 0.5 vol of absolute ethanol were added. The tubes were placed at −20°C overnight and then centrifuged at 7,500 *g* at 4°C for 20 min to pellet the RNA. The RNA pellet was resuspended in 600 μl of lysis buffer and 300 μl of cold (−20°C) absolute ethanol was added. The tubes were placed at −70°C for <1 h and the RNA was recovered by microfuging the pellet for 15 min. The pellet was resuspended in 200 μl of resuspension buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 50 mM NaCl, and 0.2% SDS), the mixture was extracted with an equal volume of phenol/chloroform, and the organic phase was reextracted once with 100 μl of water. RNA was precipitated with NaCl and ethanol. The RNA pellet was collected and its concentration was determined by reading absorbance at 260 nm.

Analysis of RNA

5–10 μg of total RNA was treated with 2.2 M formaldehyde in 50% formamide, 10 mM NaH_2PO_4, pH 7, at 55°C for 15 min. Samples were then subjected to electrophoresis in a 1.4% agarose-formaldehyde gel containing 2.2 M formaldehyde, 20 mM morpholine propane sulfonic acid, pH 7, 5 mM sodium acetate, and 1 mM EDTA (Lehrach et al., 1977). The gels were stained with 10 μg/ml ethidium bromide, and destained with several changes of 10 mM NaH_2PO_4, pH 7. RNA was then transferred to nitrocellulose or Nytran nylon membranes (Schleicher & Schuell, Inc., Keene, NH) using 20× SSPE (1× = 0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.7, 1 mM EDTA) as previously described (Danielsen et al., 1986).

Blots were prehybridized in buffer H (50% formamide, 5× SSPE, 0.1% SDS, 2× Denhardt's solution [1× Denhardt's solution is 0.02% wt/vol each of BSA, polyvinylporrolidone, and Ficoll] 0.5 mg/ml yeast RNA, 0.1 mg/ml denatured and sheared salmon sperm DNA) at 42°C for 1 h. Blots were hybridized in buffer H containing radioactive probe for 12–24 h at 42°C (riboprobes at 65°C). ^{32}P-Labeled probes were made using the random hexanucleotide priming method with the large fragment of DNA polymerase I (Feinberg and Vogelstein, 1983) or the riboprobe system (Melton et al., 1984). The probe for c-*fos* used was a gift of Dr. William Lee (University of California at San Francisco, San Francisco, CA). It contains a 220-bp Acc I–Stu I fragment derived from the 5′ end of exon 4 of the human genomic clone (van Straaten et al., 1983) inserted into the riboprobe vector sp64 (Promega Biotec, Madison, WI). The plasmid pmL5, a gift of Dr. Todd Kirchgessner (University of California at Los Angeles, Los Angeles, CA), was used to detect lipoprotein lipase (LPL) transcripts (Kirchgessner et al., 1987). Blots were washed at 50 (for DNA probes) or 65°C (for RNA probes) twice for 30 min in 0.2× SSPE, 0.1% SDS and autoradiographed. In all cases, equal amounts or RNA were loaded per lane (based on UV absorbance at 260 nm), and this was confirmed by observing equal fluorescence in all lanes of gels stained with ethidium bromide.

DNA Synthesis Assay

To determine whether cells were being stimulated to synthesize DNA, the method of Hovanessian and Wood (1980) was used to detect the incorporation of [^{3}H]thymidine. TA1 cells were seeded in 12- or 24-well dishes and allowed to reach confluence. Control wells were left unfed until the time of the experiment. Other wells were refed with media supplemented with dexamethasone and indomethacin on days 0 and 3, so that by day 5 they were >95% differentiated. On day 5, control and differentiated cells were treated with FGF (10 ng/ml) or left untreated. After 14 h, 0.5 μCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear) was added per well. 2 h later, the medium was removed, cells were washed with PBS, and cold 5% TCA was added. The wells were kept at 4°C for 30 min, then the TCA solution was aspirated off, 0.5 ml of 0.1 M NaOH was added, and the dish was maintained at 37°C for 30 min. The base was neutralized with 50 μl of 1 M HCl, and the entire mixture was counted with Aquasol scintillation fluid.

Results

Reversal of Adipocyte Differentiation by Various Agents

Upon reaching confluence, TA1 adipoblasts slowly differentiate into mature adipocytes, a process that can be accelerated by the addition of different hormones or pharmacological agents. Indomethacin, either alone at 125 μM (Knight et al.,

1987) or at 30 μM in the presence of 1 μM dexamethasone, will cause >95% of the cells in culture to undergo morphological differentiation in 4 d. The differentiation can also be documented by the rapid accumulation of adipocyte-specific mRNAs, as determined by RNA gel-blot hybridization (Northern) analysis (Fig. 1). Markers we have used include cDNAs for glycerolphosphate dehydrogenase (Spiegelman et al., 1983); aP2 (Hunt et al., 1986; Chapman et al., 1984 [formerly referred to as clone 28 by our group]); two cDNAs (clones 1 and 47) of unknown function (Chapman et al., 1984); and a cDNA clone of mouse LPL (Kirchgessner et al., 1987). The effects of exogenously added agents on adipocyte differentiation can be documented using these markers. For example, Torti et al. (1985) demonstrated the inhibition of glycerolphosphate dehydrogenase, clone 1 and 47 gene expression after exposure of TA1 adipocytes to TNF.

We recently reported that addition of 1 ng/ml FGF or 100 nM TPA to confluent cultures of TA1 cells supplemented with indomethacin blocks differentiation, as evidenced by the failure of these cells to undergo morphological conversion or to accumulate clone 1 and aP2 RNAs (Navre and Ringold, 1988). We wished to determine whether these same factors would reverse adipocyte differentiation as does TNF. This was accomplished by allowing TA1 cells to differentiate completely in the presence of dexamethasone and indomethacin for 5 d. The agents of interest were added on day 5 (5 d postconfluence), and RNA was isolated from the cells 18 h after treatment. Our initial studies indicated that these factors could not reverse differentiation (not shown). Additional experiments, however, showed that FGF is effective if used at a concentration of 10 ng/ml (Fig. 1 *A*, lane *2*). TPA, which has little or no effect on its own, will extinguish adipocyte-specific gene expression if the calcium ionophore ionomycin is included simultaneously (lanes *3–5*). Ionomycin also has little effect on its own (lane *3*). Analogous results have been obtained using the calcium ionophore A23187 at identical concentrations (not shown).

To test whether this inhibition represents a general phenomenon attributable to peptide growth factors, we tested the effect of EGF or 20% FCS on these cells. Our results indicate that these mitogenic agents do not reduce the levels of the adipocyte-specific RNAs in differentiated adipocytes (Fig. 1 *B*).

FGF at 1 ng/ml or TPA Are Capable of Inducing c-fos RNA

The requirement for higher FGF concentrations to reverse rather than simply block adipocyte differentiation could be due to a decrease in receptor number in TA1 cells during differentiation or to the activation of additional receptor/transduction pathways. Similarly, the inability of TPA to reverse differentiation could be due to down-regulation of PKC during adipogenesis. In fact, Stumpo and Blackshear (1986) have reported a reduction in PKC activity during the differentiation of 3T3-L1 adipocytes, although no loss of responsiveness to 160 nM TPA was detected. To begin addressing these possibilities, we chose to monitor the well-characterized induction of the protooncogene c-*fos* by growth factors or TPA (Greenberg and Ziff, 1984; Kruijer et al., 1984; Muller et al., 1984). Our initial studies (not shown) indicated that stimulation of TA1 adipocytes with 100 nM TPA resulted in a transient accumulation of c-*fos* RNA, which was

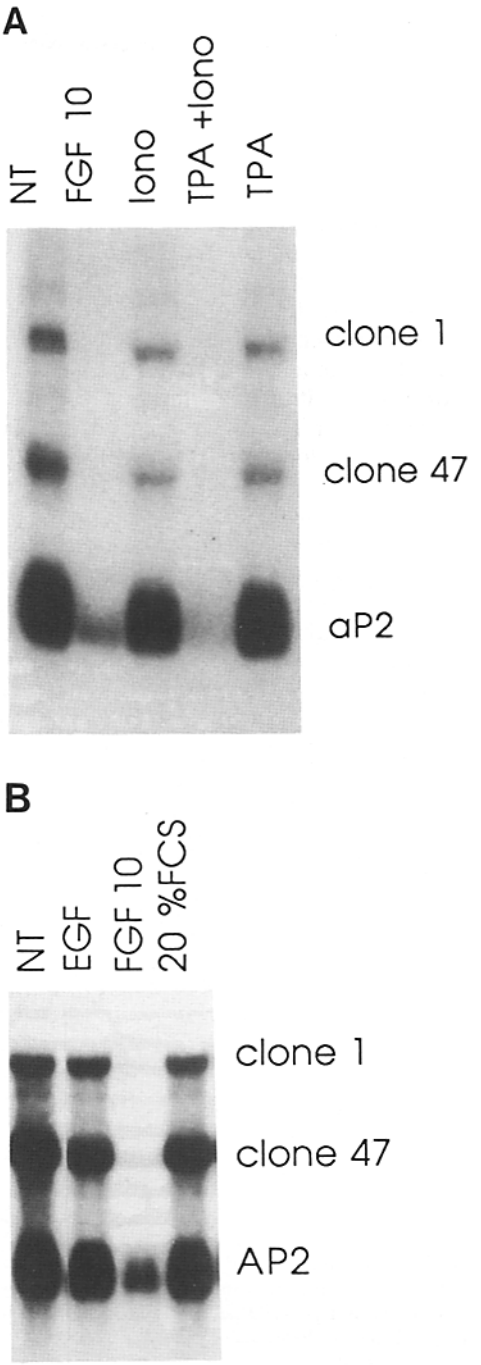

Figure 1. Loss of adipocyte-specific RNAs in TA1 cells induced by various agents. TA1 cells were grown to confluence and differentiated for 5 d in the presence of dexamethasone and indomethacin. The cells, which were refed in the same media on day 3, had generally become >95% fat by day 4. On day 5, the cells were treated with the agent(s) indicated and harvested after 18 h. RNAs from each sample were run on a 1.2% agarose gel, transferred to Nytran, and probed with ^{32}P-labeled cDNAs corresponding to clones 1 and 47 and AP2. (*A*) Adipocytes treated with (lane *NT*) control, no treatment; (lane *FGF 10*) 10 ng/ml FGF; (lane *Iono*) 1 μM ionomycin; (lane *TPA + Iono*) 100 nM TPA and 1 μM ionomycin; and (lane *TPA*) 100 nM TPA. (*B*) Adipocytes treated with (lane *NT*) control, no treatment; (lane *EGF*) 100 ng/ml EGF; (lane *FGF 10*) 10 ng/ml FGF; and (lane *20% FCS*) addition of 20% vol fresh FCS.

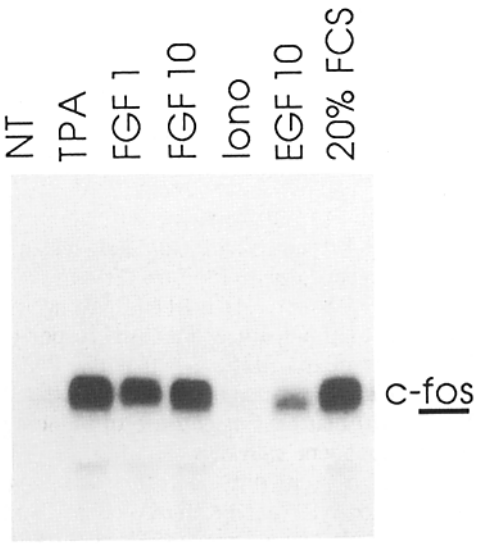

Figure 2. Induction of c-*fos* RNA in TA1 adipocytes. Day-5 adipocytes, as in Fig. 1, were treated with various agents and harvested 30 min after treatment. RNAs were analyzed as in Fig. 1, but were probed with ^{32}P-labeled RNA corresponding to exon 4 of the human c-*fos* gene. (Lane *NT*) No treatment; (lane *TPA*) 100 nM TPA; (lanes *FGF 1* and *FGF 10*) 1 and 10 ng/ml FGF, respectively; (lane *Iono*) 1 μM ionomycin; (lane *EGF 10*) 10 ng/ml EGF; and (lane *20% FCS*) addition of 20% vol fresh FCS.

maximal after 30 min of treatment, consistent with observations in other systems. As shown in Fig. 2, both TPA (100 nM) or low concentrations of FGF (1 ng/ml) are capable of inducing the accumulation of c-*fos* RNA in differentiated adipocytes. Thus, although these factors do not affect the maintenance of differentiation, at these concentrations they are still capable of inducing cellular responses. Similarly, both FCS and EGF, which also are ineffective as reversal agents, induce c-*fos* RNA. In contrast to its effects on other cell types (Moore et al., 1986; Tsuda et al., 1986), ionomycin does not induce any detectable c-*fos* expression in TA1 adipocytes. Of particular interest, these experiments also demonstrate that the repression of adipocyte-specific gene expression by 10 ng/ml FGF or TPA plus ionomycin cannot be attributed solely to the transient induction of the c-*fos* gene.

Requirements for FGF-mediated Reversal of Differentiation

To better characterize the increased requirement for FGF by TA1 cells for reversal of differentiation, we investigated the levels of FGF required to elicit the reversal of differentiation or the inhibition of the onset of differentiation. Differentiation reversal was monitored as discussed above. The inhibition of differentiation by FGF was accomplished by treating adipoblasts with FGF on days 0, 1, and 2 and harvesting on day 3 (Navre and Ringold, 1988; Fig. 3). The results of this experiment (Fig. 3) show that, whereas inhibition of differentiation is essentially complete at 3 ng/ml, the reduction of adipocyte-specific RNA in fat cells is only minimally effected at this concentration, consistent with our previous findings that the inhibition of differentiation-maintenance functions requires higher levels of FGF. Although in this figure it appears that clone 47 is somewhat more sensitive to FGF than aP2 or LPL, this effect is variable and sometimes LPL is more completely inhibited by FGF than is clone 47. We do not know the cause of this variability, although it may be related to changes in RNA stability.

In their study of the reversal of BC3H1 myocyte differentiation, Spizz et al. (1986) demonstrated that the FGF repression of creatine kinase RNA could be blocked by concurrent addition of cycloheximide. Likewise, we have found that the addition of this protein synthesis inhibitor will block most of the growth factor- or TPA plus ionomycin-mediated repression of the adipocyte RNAs (Fig. 4). This result suggests that the reversal process requires either de novo protein synthesis or the activity of a labile protein.

FGF Does Not Stimulate Mitogenesis in Adipocytes

The reduction of steady-state levels of adipocyte RNAs by FGF would be of less interest if it was merely due to the reentry of the adipocytes into the cell cycle. To address this issue, we measured [^{3}H]thymidine incorporation to test whether FGF-treated adipocytes are not stimulated to initiate DNA synthesis. Previous work showed that confluent, undifferentiated TA1 cells begin to incorporate [^{3}H]thymidine 12–16 h after serum stimulation (data not shown). In the experiment shown in Table I, TA1 adipocytes were stimulated with 20% FCS or 10 ng/ml FGF and labeled with [^{3}H]thymidine 14 h later. As the results indicate, while the serum causes some stimulation of growth, FGF alone has no effect. To show that the FGF was functional, we also treated undifferentiated adipoblasts (also 5 d postconfluence) with FGF. Under these conditions, the adipoblasts do increase their uptake of [^{3}H]thymidine (Table I), indicating that the preparation of FGF was active and, under the proper conditions, can

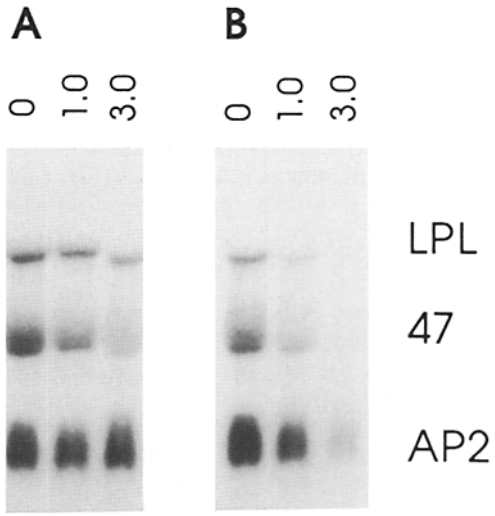

Figure 3. TA1 cell responses to FGF. (*A*) For the reversal of differentiation, TA1 adipocytes on day 5 were treated with varying concentrations of FGF (0, 1, and 3 ng/ml), harvested after 18 h, and analyzed for LPL, clone 47, and aP2 RNA levels as in Fig. 1. (*B*) For the inhibition of differentiation, TA1 adipoblasts on day 0 were refed with media supplemented with dexamethasone and indomethacin and treated with the indicated concentration of FGF on days 0, 1, and 2. Cells were harvested on day 3 and analyzed for LPL, clone 47, and aP2 RNA levels as above.

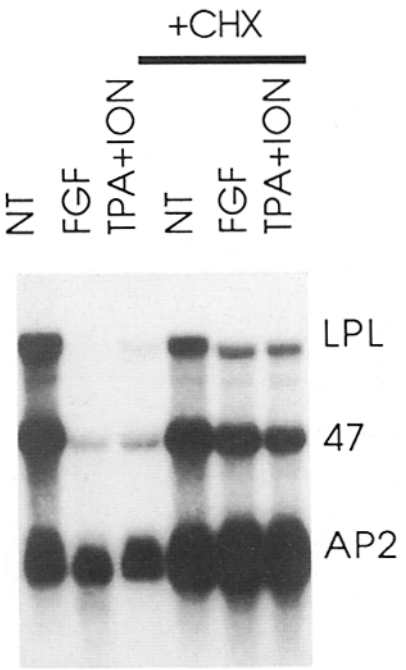

Figure 4. Cycloheximide effects on the reversal of differentiation. TA1 adipocytes on day 5 were treated with 10 ng/ml FGF or TPA plus ionomycin as in Fig. 1, but in the presence or absence of 1 μg/ml cycloheximide. RNAs were harvested as described, and the blot was probed with ^{32}P-labeled RNAs corresponding to LPL, clone 47, and AP2.

stimulate mitogenesis. The experiment shows, however, that the treatment of differentiated adipocytes with high concentrations of FGF is not mitogenic. Similar results have been observed up to 20 h after treatment (data not shown).

FGF Reversal of Differentiation Is Independent of PKC

FGF receptor-linked stimulation of PLC, which results in the accumulation of diacylglycerol (an activator of PKC) and inositol phosphates (calcium-mobilizing agents), is one of the proposed routes for the generation of FGF receptor–induced second messengers (Kaibuchi et al., 1986; Tsuda et al., 1985). The finding that TPA plus ionomycin, but neither alone, can effect reversal of adipocyte differentiation, suggests that PLC activation might be the mediator of FGF-stimulated reversal. To test this hypothesis, we pretreated adipocytes on day 4 with a very high concentration of TPA (10 μM), which has been shown to cause a depletion of PKC in 3T3-L1 adipocytes (Stumpo and Blackshear, 1986). To confirm that the TA1 adipocytes treated in this manner are no longer capable of undergoing a PKC-mediated response, 100 mM TPA was added 18 h after pretreatment and the cells were harvested after 30 min to assay for c-*fos* induction. As shown in Fig. 2 *A* and Fig. 4 *A*, adipocytes exhibit a vigorous induction of c-*fos* RNA in response to FGF or TPA. In contrast, cells pretreated with 10 μM TPA for 18 h have no response at all to TPA, yet maintain a near-maximal response to FGF (Fig. 5 *A*). This demonstrates (*a*) that the PKC-mediated response is greatly attenuated in the cells pretreated with 10 μM TPA; and (*b*) that FGF is capable of inducing c-*fos* through PKC-independent pathways.

To determine whether PKC activity is required for the FGF-mediated loss of adipocyte-specific RNAs, control and TPA-pretreated cells were challenged with FGF or with TPA plus ionomycin as above. As shown in Fig. 5 *B*, pretreatment with TPA has little effect on FGF reversal of differentiation, whereas the reversal induced by TPA plus ionomycin is virtually eliminated by PKC down-regulation. Similar results (not shown) were obtained even when we did not add the very small (100 nM) dose of TPA at 18 h, indicating that the initial 10 μM TPA pretreatment was capable of repressing PKC levels throughout the 36 h of the experiment.

Table I. Effect of FGF on [^{3}H]Thymidine Incorporation in TA1 Fibroblasts and Adipocytes

	[^{3}H]Thymidine incorporation*		
Cell type	No addition	With FGF	With FCS
Adipocyte‡	0.97	0.46	5.8
Adipoblast§	3.2	20	ND

* Incorporation into cells measured in cpm ($\times 10^{-3}$), over a 2-h period, 14 h after addition of FGF.
‡ Cells were seeded on day −5 and refed with media supplemented with dexamethasone and indomethacin on days 0 and 3, so that by day 5 they were >95% differentiated.
§ Cells were seeded on day −5 and left unfed until the time of the experiment (day +5). Less than 10% of these cells show the morphology of differentiated adipoblasts.

Discussion

Many recent studies have clearly demonstrated that peptide growth factors can have effects on cellular metabolism that are not directly related to proliferation (Sporn and Roberts, 1988). One aspect of these effects that has received a modicum of attention is the inhibition by these factors of myogenic and adipogenic differentiation. In nearly all cases, the investigations have focused on the ability of the factor in question to block the conversion of the determined stem cell to its mature phenotype. In only two cases have the effects of growth factors on already differentiated cells been tested. The best series of studies have been those using the BC3H1 cell line, which loses its muscle-specific markers in the presence of purified FGF. Lathrop et al. (1985*a*,*b*) have clearly shown that these cells extinguish myogenic gene expression without reentering the cell cycle, suggesting that FGF is capable of moving differentiated cells into an uncharacterized compartment of the G1 portion of the cell cycle in which differentiated functions cannot be expressed. Similarly, Torti et al. (1985) reversed the differentiation of TA1 adipocytes with TNF/cachectin, which causes the cells to lose their adipose phenotype, also without reentering the cell cycle.

The goal of the current work has been to further characterize the ability of growth factors to reverse adipocyte differentiation. We are especially interested in understanding the nature of the physiologically relevant second messengers generated by these peptide hormones and determining the mechanisms by which they affect the expression of differentiation-specific genes. Our previous work showed that either FGF or TPA treatment would block the differentiation of TA1 adipoblasts (Navre and Ringold, 1988), suggesting that activation of the PKC pathway alone is sufficient to block the conversion. In contrast, treatment of fully differentiated

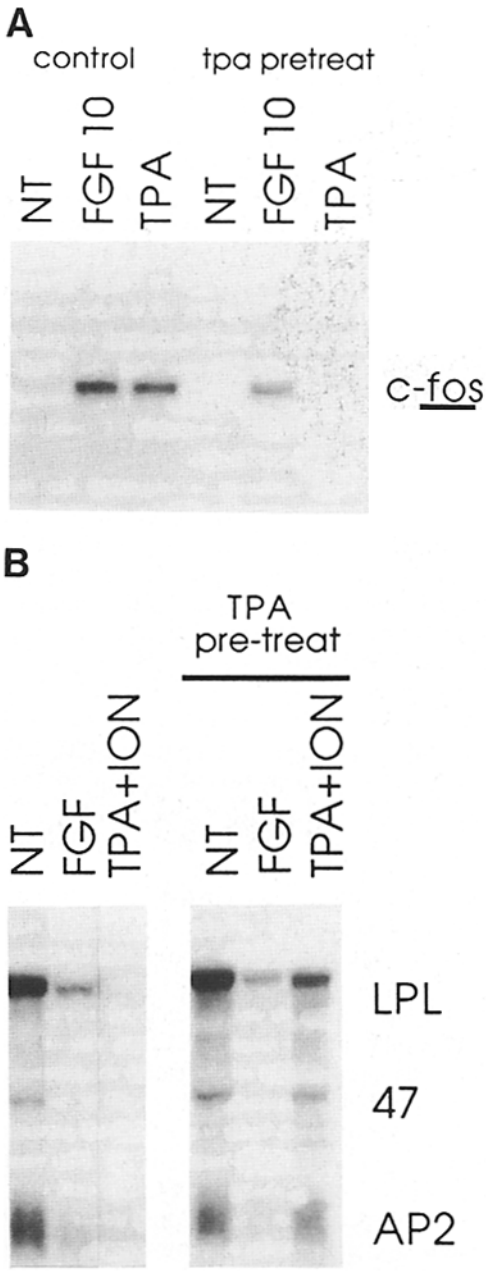

Figure 5. Effects of down-regulation of PKC. TA1 adipocytes on day 4 were either left untreated or were pretreated with 10 μM TPA. On day 5, 18 h after pretreatment, the cells were then challenged with: (lane *NT*) no challenge; (lanes *FGF 10* and *FGF*) 10 and 1 ng/ml FGF, respectively, for *A* and *B*, respectively; and (lanes *TPA* and *TPA + IONI*) 100 nM TPA and 100 nM TPA plus 1 μM ionomycin, respectively, for *A* and *B*, respectively. Cells were then harvested after 30 min (*A*) or 18 h (*B*). RNA blots were probed with c-*fos* (*A*) as in Fig. 2 or LPL, clone 47, and AP2 (*B*) as in Fig. 3.

adipocytes with identical levels of these agents had no effect on the expression of adipocyte-specific RNAs. This suggests that once the triggering of adipocyte differentiation has occurred (an event sensitive to the presence of FGF at 1 ng/ml or TPA), mechanisms required for the maintenance of the adipose phenotype begin to function; these mechanisms are refractory to FGF (at 1 ng/ml) or TPA treatment. We have found that the failure of these agents to inhibit the maintenance pathway is not due to the inability of adipocytes to respond to them, as demonstrated by the induction c-*fos* RNA expression by both FGF (at 1 ng/ml) and TPA (Fig. 2).

Additional studies demonstrated that the maintenance pathway can be rendered sensitive to FGF or TPA under certain conditions. Specifically, we found that using higher concentrations of FGF (≥10 ng/ml) or treatment with TPA concurrently with a calcium ionophore (which is ineffective when used on its own; Fig. 1) will potently inhibit the function of the maintenance pathway(s). These results suggest a model in which one or more substrates must be acted upon to shut down adipocyte-specific gene expression.

The ability of the combined actions of the PKC and calcium pathways to suppress differentiation-specific functions suggests that hormones that activate these pathways, for example, by stimulation of a phosphatidylinositol-specific PLC, should have similar effects. Since FGF (at 10 ng/ml) is capable of inhibiting adipocyte-specific gene expression it seemed prudent to determine whether FGF was, in fact, operating via such a pathway. We therefore tested whether or not the activation of PKC was required. Not surprisingly, we found that PKC down-regulation blocked the effect of TPA plus ionomycin. The loss of PKC activity, however, had little, if any, effect on the ability of FGF to reduce the expression of the RNAs tested. This result strongly suggests that FGF elicits its effect on adipocyte-specific genes through a PKC-independent pathway, and that it is unlikely that activation of a classical PKC plays a role in mediating the FGF-induced reversal of differentiation. One must conclude that the action(s) of other, as yet uncharacterized, signal transduction pathways are involved, and that, in TA1 adipocytes, they are not activated until FGF concentrations reach 10 ng/ml. It is important to note, however, that PKC-independent FGF pathways must also be functional at 1 ng/ml FGF since c-*fos* RNA is inducible by the growth factor at this concentration in PKC-deficient cells (data not shown).

Our studies clearly show that two classes of signal transduction pathways—one induced by concurrent PKC and Ca^{2+} activation and a second induced by FGF in a PKC-independent manner—can inhibit the maintenance of adipocyte-specific gene expression. FGF may be functioning in BC3H1 myocytes as it does in TA1 adipocytes given that the effect in both cell types is blocked by cycloheximide (Spizz et al., 1986) and that in neither is the reversal of differentiation dependent on mitogenic stimulation (Lathrop et al., 1985*a*,*b*; Spizz et al., 1986). The results obtained here do not reveal, however, whether the effects of FGF and TPA plus ionomycin are directly on adipocyte gene transcription or on mRNA stability. We are currently beginning experiments to address this issue, including nuclear run-on assays and heterologous gene fusions to delineate the mechanism(s) of the inhibition. Elucidation of the signal transduction pathways activated by FGF in these experimental paradigms may shed light on the biochemical processes involved in maintaining the differentiated state.

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