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Insulin and growth factor effects on c-*fos* expression in normal and protein kinase C-deficient 3T3-L1 fibroblasts and adipocytes

(protooncogenes/transcription/blot hybridization/mRNA accumulation/hormonal control)

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ABSTRACT **We investigated the expression of the protooncogene c-*fos* in 3T3-L1 fibroblasts and adipocytes in response to a variety of growth-promoting agents in normal cells and in cells preincubated with phorbol esters to deplete them of protein kinase C. There was a rapid accumulation of c-fos mRNA in fibroblasts and adipocytes treated with phorbol 12-myristate 13-acetate, platelet-derived growth factor, fibroblast growth factor, fetal calf serum, bombesin, and insulin, especially in the adipocytes. Phorbol 12-myristate 13-acetate pretreatment abolished the increase in c-fos mRNA due to additional phorbol 12-myristate 13-acetate treatment and decreased but did not eliminate the ability of platelet-derived growth factor, fibroblast growth factor, fetal calf serum, bombesin, and insulin to stimulate c-fos mRNA. These data suggested that c-fos mRNA could be induced in serum-deprived 3T3-L1 fibroblasts and adipocytes by at least two separate pathways, one involving protein kinase C and the other independent of protein kinase C. In the very insulin-sensitive 3T3-L1 adipocytes, insulin rapidly and transiently increased c-*fos* expression (c-fos mRNA appeared by 15 min and disappeared after 60 min) via interaction with its own cellular receptor, rather than by interacting with receptors for one of the insulin-like growth factors. Cycloheximide treatment in combination with insulin or phorbol 12-myristate 13-acetate resulted in superinduction of c-fos mRNA. We conclude that insulin can rapidly stimulate c-fos mRNA accumulation in 3T3-L1 adipocytes and that part of the growth factor-stimulated increase in this mRNA that occurs in protein kinase C-deficient cells may be due to activation of a pathway similar or identical to that activated by insulin.**

Several polypeptide mitogens and growth factors, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), fibroblast-derived growth factor, bombesin, bradykinin, and others, appear to be able to activate protein kinase C in cultured fibroblasts (1–9). Activation of the kinase by these growth factors is thought to result from agonist-induced hydrolysis of membrane inositol phospholipids leading to the generation of diacylglycerols. In murine and human fibroblasts, insulin appears to be ineffective and epidermal growth factor to be minimally effective at activating protein kinase C (1, 3, 5, 8, 10, 11).

However, in fibroblasts made protein kinase C-deficient by preincubation with phorbol esters, several of the protein kinase C-activating growth factors can still exert biological effects on the cells, including stimulation of protein phosphorylation (3), mitogenesis (4, 12), increased ribosomal protein S6 kinase activity (3, 13–15), c-myc mRNA accumulation (4, 8), and induction of ornithine decarboxylase activity and mRNA levels (16). In the cases of stimulated protein phosphorylation and activation of the S6 kinase, the residual growth factor effects in protein kinase C-deficient cells were identical to the effects of insulin in the same cells, leading us to postulate an insulin-like pathway of growth factor action in addition to the activation of protein kinase C (3, 13).

However, other growth factor effects in protein kinase C-deficient fibroblasts have not been mimicked by insulin in normal cells, such as the rapid accumulation of c-myc mRNA, enhanced c-*myc*, c-*fos*, and β-actin gene transcription, and induction of ornithine decarboxylase (4, 16–18). In the present study, we considered the possibility that this lack of effect of insulin was due to the relative insensitivity of the fibroblasts to insulin; to this end, studies were performed in 3T3-L1 adipocytes, which contain many more insulin receptors per cell than their fibroblast precursors (19–21). The growth factor response evaluated was the rapid, transient accumulation of c-fos mRNA, which has been shown to occur in response to a variety of stimuli in several cell types (18, 22–32).

MATERIALS AND METHODS

Sources of all materials were as described (3, 7, 13, 16), except that FGF (basic from bovine brain), PDGF, and multiplication stimulating activity (MSA) were from Collaborative Research (Waltham, MA), insulin-like growth factor I (IGF I) was from Amgen Biologicals (Thousand Oaks, CA), bombesin was from Peninsula Laboratories (Belmont, CA), and regular pork insulin was from Squibb-Novo.

3T3-L1 cells were grown to confluence, stimulated to differentiate into adipocytes, and pretreated with phorbol esters to make them protein kinase C deficient as described (3). All experiments were conducted in cells incubated for 18 hr in serum-free medium as described (3). Hormones and other agents were then added directly to the plates without change of medium.

Protein kinase C measurements were performed in cellular supernatant and particulate fractions as described (16, 69).

For RNA gel blot analysis, total cellular RNA was isolated and analyzed on formaldehyde/agarose gels as described (16). RNA gel blots were hybridized with 5×10^6 cpm of heat-denatured, ^{32}P-labeled v-*fos* [1 kilobase (kb) *Pst* I fragment of p*fos*-1 (ATCC No. 41040, American Type Culture Collection)] labeled to a specific activity of 2×10^9 cpm/μg using the Multiprime DNA labeling kit from Amersham.

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Abbreviations: FGF, fibroblast growth factor, basic from bovine brain; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; Me_2SO, dimethyl sulfoxide; IGF, insulin-like growth factor; MSA, multiplication stimulating activity; kb, kilobase(s).

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RESULTS

Induction of c-fos mRNA in Normal and Protein Kinase C-Deficient 3T3-L1 Fibroblasts. In confluent, serum-deprived 3T3-L1 fibroblasts, no c-fos mRNA could be detected (Fig. 1), in agreement with reports (18, 22–25). Treatment of fibroblasts with maximal concentrations of PMA, PDGF, FGF, fetal calf serum, or bombesin for 30 min resulted in the marked accumulation of c-fos mRNA (Fig. 1). In cells made protein kinase C deficient by preincubation with PMA (3), further short-term PMA exposure had no effect on c-fos mRNA levels; however, there were detectable increases in c-fos mRNA levels in response to all of the other growth factors in the protein kinase C-deficient cells (Fig. 1).

Effect of Preincubation with Phorbol Esters on Protein Kinase C Activity in 3T3-L1 Adipocytes. Fully differentiated 3T3-L1 adipocytes contained only about 25% of the soluble protein kinase C specific activity of confluent undifferentiated fibroblasts, and only 2% of the particulate activity of the fibroblasts (Fig. 2). Similar decreases in protein kinase C immunoreactivity during adipocyte differentiation have been noted (P.J.B., P. R. Girard, and J. F. Kuo, unpublished data). Preincubation of the fully differentiated adipocytes with 16 μM PMA for 16 hr resulted in the partial disappearance of protein kinase C from the cells (Fig. 2), compared to the virtually complete disappearance seen in fibroblasts (3, 16). This incomplete response may be due to the tendency of the phorbol esters to bind to the massive amounts of cellular lipid present, the low kinase activity in the cells, or other factors; however, the cells treated in this way were completely refractory to further PMA addition (see below).

Induction of c-fos mRNA in Normal and Protein Kinase C-Deficient 3T3-L1 Adipocytes. As in the fibroblasts, PMA, FGF, fetal calf serum, and bombesin caused the marked accumulation of c-fos mRNA in the differentiated adipocytes

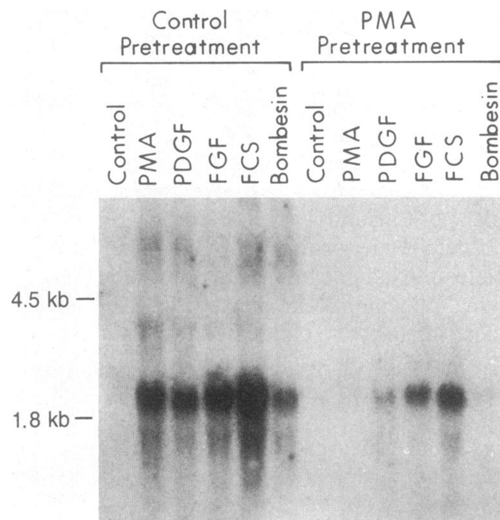

FIG. 1. Stimulation of c-fos mRNA accumulation in control and phorbol 12-myristate 13-acetate (PMA)-pretreated 3T3-L1 fibroblasts. Confluent, serum-deprived 3T3-L1 fibroblasts (ten 100-mm plates of cells for each condition) were pretreated with 0.1% (vol/vol) dimethyl sulfoxide (Me_2SO) (control pretreatment) or 16 μM PMA for 16 hr (PMA pretreatment) and subsequently incubated for 30 min with 0.01% Me_2SO (control)/1.6 μM PMA/PDGF (1.25 units/ml)/FGF (125 ng/ml)/10% (vol/vol) fetal calf serum (FCS)/10 nM bombesin. Total cellular RNA was isolated and 15 μg samples were fractionated on a 1.2% formaldehyde/agarose gel and transferred to nitrocellulose. The RNA gel blot was hybridized to 5×10^6 cpm of ^{32}P-labeled v-fos cDNA and then subjected to autoradiography. The markers 4.5 kb and 1.8 kb refer to the positions of the major species of ribosomal RNA (28S and 18S, respectively) on the stained gel.

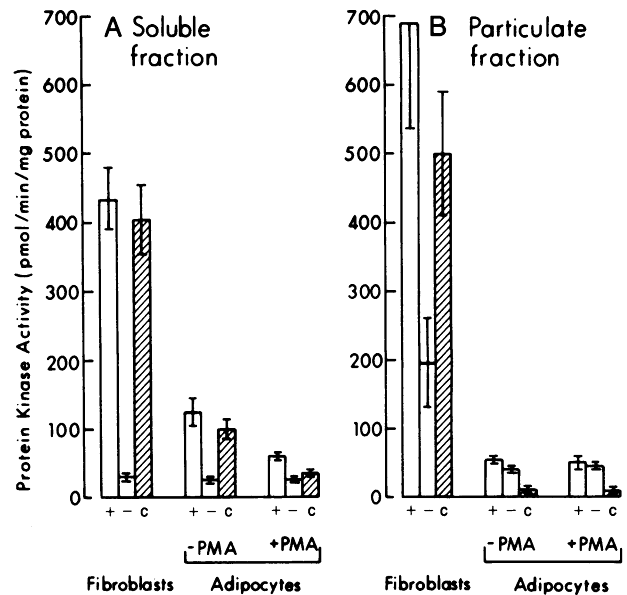

FIG. 2. Protein kinase C activities in 3T3-L1 fibroblasts and in normal and PMA-pretreated 3T3-L1 adipocytes. Confluent, serum-deprived 3T3-L1 fibroblasts, adipocytes, and adipocytes pretreated for 16 hr with 16 μM PMA were used for the measurement of protein kinase C-specific activities in soluble (*A*) and detergent-extracted particulate (*B*) cellular fractions. +, Protein kinase activity measured in the presence of phosphatidylserine, diolein, and Ca^{2+}; −, protein kinase activity in the absence of these cofactors; and c (and the hatched bars) refers to the difference between these two activities; that is, protein kinase C activity. Each bar represents the means ± SD of duplicate determinations from four plates of cells.

(Fig. 3). That activation of protein kinase C could lead to c-fos mRNA accumulation was further suggested by two types of studies. First, the active phorbol ester, 4β-phorbol 12,13-didecanoate (16 μM) was fully active in causing c-fos mRNA accumulation in the adipocytes, whereas its inactive epimer, 4α-phorbol 12,-13-didecanoate, was completely ineffective (data not shown). In addition, the synthetic diacylglycerol,

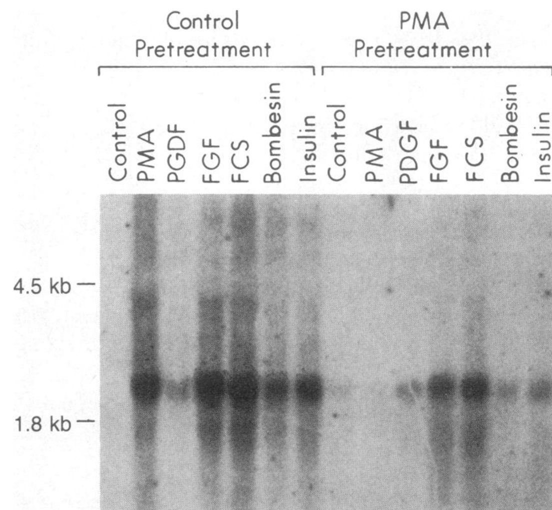

FIG. 3. Stimulation of c-fos mRNA accumulation in control and PMA-pretreated 3T3-L1 adipocytes. Confluent, serum-deprived 3T3-L1 adipocytes (five 100-mm plates of cells for each condition) were pretreated and treated with mitogens as described in the legend to Fig. 1, except that the second PMA concentration was also 16 μM, and the cells were also exposed to insulin (1 milliunit/ml). Total RNA was isolated, fractionated (15 μg per lane), transferred to nitrocellulose, and hybridized to ^{32}P-labeled v-*fos* as described in the legend to Fig. 1. FCS, fetal calf serum.

1,2-dioctanoyl-*sn*-glycerol (200 μM), which activates protein kinase C in these cells (3, 13), also caused an increase in c-fos mRNA accumulation (data not shown). In contrast to the fibroblasts, however, PDGF had a relatively modest effect, but insulin had a marked effect on c-fos mRNA accumulation (Fig. 3). In the PMA-pretreated cells, the further addition of PMA had no effect on c-fos mRNA accumulation (Fig. 3); however, the other growth factors and insulin were still effective at promoting c-fos mRNA accumulation in the protein kinase C-deficient adipocytes.

When the effects of PMA and insulin on c-fos mRNA accumulation in undifferentiated fibroblasts were compared to their effects in fully differentiated adipocytes, it was clear that PMA caused a marked accumulation of c-fos mRNA in both cases, which was abolished in the PMA-pretreated cells (Fig. 4). However, the effect of insulin, although detectable in the fibroblasts, was much more marked in the adipocytes (Fig. 4); it appeared to be of similar magnitude in control and PMA-pretreated cells.

Time-Course and Dose–Response of Insulin-Stimulated c-fos mRNA Accumulation. It has been shown that c-fos mRNA is rapidly induced and disappears within 1–2 hr after stimulation by growth factors (18, 22–25, 27, 29, 32). After exposure of serum-deprived 3T3-L1 adipocytes to insulin (1 milliunit/ml), c-fos mRNA was readily detected at 15 min, peaked at 30 min, and was not detectable at 60, 90, and 120 min (Fig. 5*A*). In other experiments, we have observed increases as early as 10 min after insulin addition (data not shown). We also observed a decrease in the apparent size of c-fos mRNA as a function of time (Fig. 5*A*); similar changes have been observed in serum-stimulated mouse fibroblasts (32), serum-stimulated human monomyelocytes (33), and nerve growth factor-treated PC12 rat pheochromocytoma cells (30) and have been attributed to a shortening of the mRNA prior to its complete degradation.

As shown in Fig. 5*B*, insulin stimulated c-fos mRNA expression at concentrations of 1 milliunit/ml (7 nM) and 10 milliunits/ml (70 nM), when evaluated after 30 min. Little or no effect was observed with lower insulin concentrations.

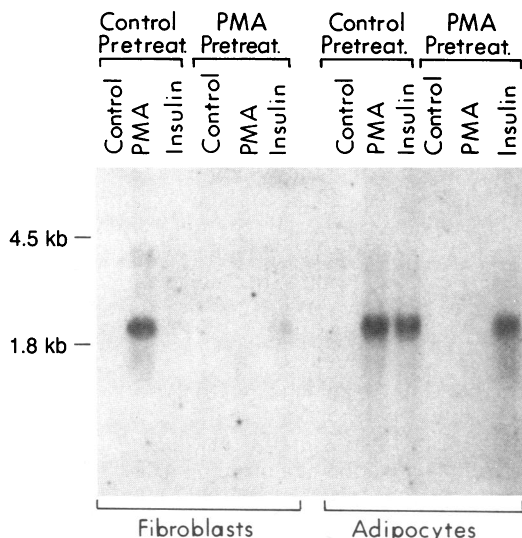

FIG. 4. Comparison of the effects of PMA and insulin in control and PMA-pretreated 3T3-L1 fibroblasts and adipocytes. Confluent, serum-deprived cells were treated for 16 hr with 0.1% Me_2SO (control pretreatment) or 16 μM PMA (PMA pretreatment). Following pretreatment, 3T3-L1 fibroblasts were incubated for 30 min with 0.01% (vol/vol) Me_2SO (control), 1.6 μM PMA, or insulin (1 milliunit/ml). The adipocytes were incubated for 30 min with 0.1% (vol/vol) Me_2SO (control), 16 μM PMA, or insulin (1 milliunit/ml). RNA was isolated and analyzed (15 μg per lane) as described in the legend to Fig. 1.

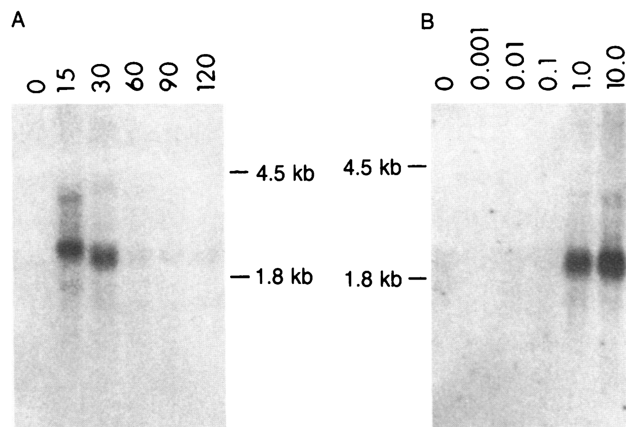

FIG. 5. Time course and dose–response of c-fos mRNA induction in confluent, serum-deprived 3T3-L1 adipocytes treated with insulin. In *A*, RNA was isolated at the times (in min) indicated following treatment with insulin (1 milliunit/ml). RNA samples (15 μg) were analyzed as described in the legend to Fig. 1. In *B*, serum-deprived, confluent adipocytes were treated for 30 min with water alone (0) or the indicated concentrations of insulin in milliunits/ml. RNA was isolated and analyzed (15 μg per lane) as described in the legend to Fig. 1.

Maximal concentrations of insulin (10 milliunits/ml) and PMA (16 μM), added together to the cells for 30 min, had an additive effect on c-fos mRNA accumulation when compared to PMA added alone (data not shown).

Effect of Insulin-Like Growth Factors on c-fos mRNA Induction. These cells contain high levels of receptors for insulin-like growth factors (IGF) type I and II; the former, similar to the insulin receptor, appears to increase in number during adipocyte differentiation (34). To ascertain that insulin was inducing c-fos mRNA through the insulin receptor and not through the IGF I or II receptors, we examined the effect of 7 nM and 70 nM insulin, IGF I, and MSA [equivalent to rat IGF II (35)] on c-fos mRNA accumulation (Fig. 6). In this experiment, exposure of the 3T3-L1 adipocytes to both 7 nM and 70 nM insulin stimulated c-fos mRNA accumulation, whereas MSA at 7 nM and 70 nM and IGF I at 7 nM did not result in accumulation of c-fos mRNA. However, 70 nM IGF I caused an increase in c-fos mRNA accumulation, although to a lesser extent than either 7 or 70 nM insulin.

Superinduction of c-fos mRNA by Cycloheximide. It has been demonstrated that c-fos mRNA can be superinduced by

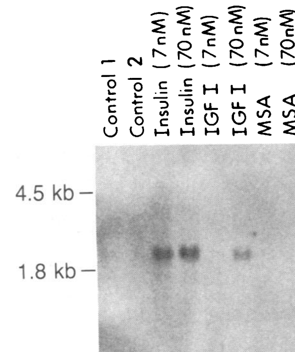

FIG. 6. The effect of the insulin-like growth factors on c-fos mRNA induction in confluent, serum-deprived 3T3-L1 adipocytes. Cells were treated for 30 min with vehicle alone (Control 1, H_2O, for insulin additions; Control 2, 20.3 μM acetic acid for IGF I and MSA additions); or with 7 nM and 70 nM insulin (equivalent to 1 miiliunit/ml and 10 milliunits/ml, respectively), IGF I, and MSA. RNA was isolated and analyzed (15 μg per lane) as described in the legend to Fig. 1.

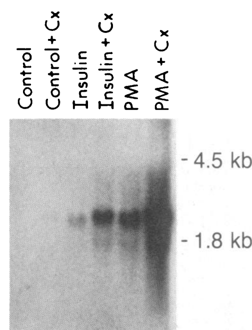

FIG. 7. The effect of cycloheximide on the accumulation of c-fos mRNA in 3T3-L1 adipocytes. Cells were pretreated with or without 0.1 mM cycloheximide (Cx) for 1 hr before exposure to H_2O (control), insulin (1 milliunit/ml or 7 nM), or PMA (16 μM) for 30 min. Total cellular RNA was isolated and analyzed (15 μg per lane) as described in the legend to Fig. 1.

cycloheximide in several cell types (22, 23, 25, 29–33, 36). In 3T3-L1 fibroblasts, 0.1 mM cycloheximide inhibited protein synthesis by approximately 90% (D. L. Halsey, and P.J.B., unpublished data). When confluent, serum-starved 3T3-L1 adipocytes were exposed to 0.1 mM cycloheximide for 1 hr and then stimulated with 7 nM insulin or 16 μM PMA, c-fos mRNA was induced to a greater extent than was seen with insulin or PMA alone (Fig. 7). Cycloheximide treatment alone resulted in a small stimulation of c-fos mRNA (Fig. 7).

DISCUSSION

The principal finding of this study is that insulin, apparently acting through its own cellular receptor, stimulated the rapid but transient accumulation of c-fos mRNA in 3T3-L1 adipocytes, both in normal and protein kinase C-deficient cells. The effect of insulin was much more marked in the adipocytes than in the undifferentiated fibroblasts; this may be due to the greatly increased number of insulin receptors on the adipocytes (19–21), with concomitant increases in cellular insulin sensitivity (20, 21, 37). As in studies of growth factor induction of c-fos mRNA in these and other cell types, the effect of insulin was magnified in the presence of cycloheximide, suggesting that c-fos mRNA levels may be regulated by a labile repressor protein(s) (22, 23, 25, 29–33, 36). Other explanations are possible; for example, cycloheximide has been shown to potentiate the ability of insulin to activate the S6 kinase in 3T3-L1 adipocytes (38).

The mechanism by which insulin increased c-fos mRNA levels in the 3T3-L1 adipocytes is not known. By analogy with the effects of other growth factors and mitogens (18, 27, 32), insulin may well stimulate c-*fos* gene transcription. Support for this mechanism comes from our laboratory, in which exposure of 3T3-L1 adipocytes to actinomycin D completely prevented c-fos mRNA accumulation in response to insulin or PMA (unpublished data). Previous studies failed to show stimulation by insulin of c-fos mRNA accumulation in primary cultures of adult rat hepatocytes (31) or PC12 rat pheochromocytoma cells (28); insulin also failed to stimulate c-*fos* gene transcription in murine fibroblasts (18). However, Greenberg *et al.* (27) have shown that insulin stimulated c-*fos* gene transcription in PC12 pheochromocytoma cells, as assessed by the nuclear run-off technique; these authors used high concentrations of insulin (862 nM), however, and it is conceivable that this effect of insulin was due to interaction with one or more types of IGF receptors in these cells.

Whatever the mechanism by which insulin stimulated c-fos mRNA accumulation, its occurrence at 10–15 min after insulin addition represents the most rapid effect of insulin-stimulated mRNA accumulation of which we are aware (39–50). However, similar rapid effects of insulin to inhibit cAMP-induced transcription of the phospho*enol*pyruvate carboxykinase gene have been known for some time (49–53). It is interesting that the dose–response relationship for insulin-stimulated c-fos mRNA accumulation more closely resembled those for insulin receptor occupancy and insulin-stimulated receptor phosphorylation than those for more distal insulin effects, such as activation of glycogen synthase, induction of tyrosine aminotransferase, and stimulation of amino acid uptake (54); taken together with the very rapid insulin effect on c-fos mRNA levels, the data suggest a close linkage between insulin receptor occupancy and increased c-fos mRNA levels, without intervening amplification steps.

The c-*fos* protooncogene is the cellular homologue of the transforming oncogene from the FBJ murine osteosarcoma virus (55, 56), which encodes a M_r 55,000 nuclear phosphoprotein of unknown function. The rapid increases in c-fos mRNA accumulation and gene transcription seen after exposure of a variety of cells to mitogens (18, 22–32) and during differentiation (27, 28, 36, 56–60) have led to suggestions that the expression of this gene may be important in cell proliferation and differentiation. There is evidence that the c-fos protein itself may act as a trans-acting regulator of subsequent gene transcription (55, 56, 61). What then might be the function of insulin- and PMA-induced increases in c-*fos* gene expression in 3T3-L1 adipocytes, which are capable of neither further differentiation nor division? The answer to this question is not known. However, both PMA and insulin stimulate lipogenesis in adipose tissue (62–64), in part through induction of lipogenic enzymes (41, 43, 45, 48), and it is interesting to speculate that the increases in c-fos mRNA and presumably protein seen after exposure of the cells to PMA or insulin represent an early or perhaps first step in the induction of the lipogenic program.

As shown previously in the case of c-*myc* induction by growth factors in protein kinase C-depleted fibroblasts (4, 8), several polypeptide growth factors, as well as insulin, induced c-fos mRNA accumulation in the cells preincubated with PMA. The PMA pretreatment decreased the effect of the growth factors somewhat when compared to their effects in the normal cells, presumably due, at least in part, to the protein kinase C deficiency induced by these means. However, we cannot exclude other possible effects of the PMA pretreatment on the cells, such as effects on the phosphorylation state and behavior of the growth factor receptors themselves (65–68); such effects may account for the occasional variability noted in the responses after PMA pretreatment. In addition, we cannot exclude a contribution to the residual growth factor effects by the small amount of protein kinase C remaining in the PMA-pretreated cells. This seems unlikely, however, since all experiments were conducted in the continuous presence of high (16 μM) concentrations of PMA, and re-added PMA had no effect on c-fos mRNA accumulation.

The present study was intended to determine whether the effects of certain polypeptide growth factors to stimulate c-fos mRNA accumulation in protein kinase C-deficient cells could be due to activation of a protein kinase C-independent pathway by the growth factors, similar to the pathway activated by insulin. Since these growth factors stimulate protein phosphorylation and the ribosomal protein S6 kinase in a manner similar to insulin in protein kinase C-deficient cells (3, 13–15) and since insulin can clearly induce c-fos mRNA accumulation in both normal and protein kinase C-deficient cells, it seems likely that at least one of the protein kinase C-independent pathways of c-fos mRNA accumulation induced by growth factors is the same as the insulin pathway. The nature of this pathway is not known. However, it is interesting that the increase in insulin's ability to stimulate c-fos mRNA accumulation that occurs during

differentiation of the cells into adipocytes is accompanied by marked decreases in the specific activity and specific immunoreactivity of protein kinase C. It may be that these changes reflect an increasing relative importance of the insulin-activated pathways compared to protein kinase C in the mature adipocyte.

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