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Identification of c-*fos* Sequences Involved in Induction by Insulin and Phorbol Esters*

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We evaluated the mechanism of insulin and phorbol ester induction of the proto-oncogene c-*fos* in Chinese hamster ovary fibroblasts stably transformed with high levels of genes expressing normal or truncated human insulin receptors. Both insulin and the tumor-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) induced c-*fos* mRNA accumulation in cells expressing high numbers of normal human insulin receptors; PMA but not insulin was effective in the cells expressing the mutant receptor. Transient expression studies with plasmid constructions containing c-*fos* 5′-flanking sequences ligated to the bacterial chloramphenicol acetyltransferase gene indicated that sequences corresponding to the serum response element were required for induction of c-*fos* transcription by both insulin and PMA. The insulin-sensitive cells contained a nuclear factor, presumably a protein, which bound specifically to this sequence of the c-*fos* gene; the apparent affinity of this factor to the normal serum response element was not affected by prior treatment of the cells with insulin or PMA. This c-*fos* binding factor may prove to be important in the regulation of c-*fos* expression by insulin and activators of protein kinase C.

Insulin is known to increase (1–10) as well as decrease (11–15) the levels of certain cellular mRNAs after its addition to target cells. We recently reported that insulin stimulated the accumulation of mRNA for the proto-oncogene c-*fos* in 3T3-L1 adipocytes within 10 min, apparently acting through its own receptor (16). Similar induction of c-*fos* mRNA occurred in response to phorbol esters in these cells, but the insulin response appeared to occur through a protein kinase C-independent pathway. Responses of c-*fos* mRNA to both agents were transient, were superinduced by cycloheximide, and were completely inhibited by actinomycin D, suggesting that enhanced c-*fos* transcription was responsible for the mRNA accumulation.

In the current study, we attempted to determine whether the sequences in the c-*fos* 5′-flanking region that are known to be involved in the c-*fos* response to serum in fibroblasts (17–19) were the same as those involved in the responses to insulin and tumor-promoting phorbol esters. To do this, we transfected plasmid constructions containing normal and abnormal c-*fos* upstream sequences fused to the gene for chloramphenicol acetyltransferase into stable Chinese hamster ovary (CHO)[1] fibroblast lines expressing high levels of either normal or truncated human insulin receptors (20). Results of these studies indicate that the normal insulin receptor is necessary for the insulin induction of c-*fos* mRNA and that four bases in the serum-response element (SRE), located between −299 and −320 in the murine c-*fos* upstream region (17–19), are required for c-*fos* induction by both insulin and tumor-promoting phorbol esters.

EXPERIMENTAL PROCEDURES

Cell Culture—CHO, CHO.hIR, CHO.T, and CHO.T-t cells (20) were a generous gift from Dr. R. A. Roth, Department of Pharmacology, Stanford University, Palo Alto, CA 94305. They were grown in Ham's F12 medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). For experiments involving induction of c-*fos* mRNA, cells were grown to confluence and pretreated for 16 h with serum-free Dulbecco's modified Eagle's medium (DMEM) containing 1% (w/v) bovine serum albumin as described (16).

RNA Isolation and Northern Analysis—For gel blot analysis, total cellular RNA was isolated and analyzed on formaldehyde/agarose gels as described (21). Northern blots were hybridized with 5×10^6 cpm of heat-denatured ^{32}P-labeled v-*fos* as described (16).

Protein Kinase C Assay—Protein kinase C measurements were performed in 1% (v/v) Triton X-100 cellular extracts as described (22).

Plasmids—The plasmids used in the tranfection experiments were constructed as described (19), with modifications as noted below. The "wild type" plasmid −356 *fos*-chloramphenicol acetyltransferase contained murine c-*fos* sequences extending from −356 to +109 relative to the transcription initiation site ligated to the bacterial chloramphenicol acetyltransferase gene (19). The mutant plasmid (2–4 pM12) contained the identical construction in which four G residues in the SRE had been converted to C residues (bases −305 and −306 in the top strand, and −313 and −314 in the bottom strand). Details of these plasmid constructions will be published separately.[2]

DNA Transfections—CHO.T cells were plated the day before transfection at a density of 1.5×10^6 cells/100-mm plate in Hams' F12 supplemented medium without penicillin or streptomycin. Each plate was transfected with 10 μg of plasmid DNA which had been isolated by alkaline lysis and purified by two cesium-chloride-ethidium bromide gradient centrifugation steps (23). Transfections were carried out by a minor modification of the calcium phosphate procedure (24) followed by a glycerol shock (25), in which cells were treated with 10% (v/v) glycerol in phosphate-buffered saline for 2 min at room temperature. For each transfection experiment, 30 plates of cells were used. Equal volumes of the same calcium phosphate precipitate and plasmid were added to each of 15 plates. The plates were incubated at 37 °C in complete F12 medium for 48 h after the addition of DNA, at which time the cells were placed into serum-free Dulbecco's modified Eagle's medium for 16 h as described (26). Phorbol 12-myristate

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[1] The abbreviations used are: CHO, Chinese hamster ovary cells; PMA, phorbol 12-myristate 13-acetate; SRE, serum response element of c-*fos*; bp, base pair(s); EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

[2] M. Z. Gilman, manuscript in preparation.

13-acetate (PMA, 1.6 μM in 0.01% dimethyl sulfoxide) or insulin (10 milliunits/ml = about 70 nM) was added for 45 min, and then total RNA was isolated from 5 plates per condition as described above.

RNase Protection Assay—Plasmid p149 (19), which contains murine c-*fos* sequences from −56 to +109 cloned into pSP65 (27),[2] was used to quantitate transfected gene products essentially as described (27).

Gel Shift Analysis—Probes for gel shift analysis were prepared from −356 *fos*-chloramphenicol acetyltransferase or 2–4 pM12 as described (19). Nuclear extracts were prepared from CHO.T cells (and in one instance, 1321-N1 human astrocytoma cells; see Ref. 28) grown to confluence and pretreated for 16 h in serum-free medium as described above. Following the addition of hormones as indicated, the cells were washed three times with 4 ml of ice-cold phosphate-buffered saline and then used for the preparation of nuclear extracts as described (29). The nuclear extracts (4 μg of protein/reaction) were allowed to bind radiolabeled probes as described (29) and then subjected to nondenaturing electrophoresis on 7% acrylamide gels as described (18). The gels were then dried and subjected to autoradiography.

RESULTS

Exposure of the CHO.T cell line, which expresses approximately 5×10^5–10^6 normal human insulin receptors/cell, to 10 milliunits/ml insulin for 30 min resulted in the increased accumulation of c-*fos* mRNA when compared to control (Fig. 1). In contrast, insulin had no effect in identical experiments on the cell line CHO.T-t, which expresses similar numbers of insulin receptors that are missing the C-terminal 112 amino acids of the cytoplasmic β subunit. These truncated receptors bind insulin normally but lack insulin receptor tyrosine kinase activity (20). Similar studies in the line CHO.hIR, which expresses about 1.4×10^4 human insulin receptors/cell, showed slightly less c-*fos* mRNA accumulation than the CHO.T cells; in contrast, wild type CHO cells showed essentially undetectable c-*fos* mRNA accumulation after insulin treatment, similar to that seen in 3T3-L1 fibroblasts (16) (results not shown).

In contrast, the active tumor-promoting phorbol ester PMA caused a marked accumulation of c-*fos* mRNA after 30 min, in both the CHO-T and CHO-T-t cells (Fig. 1) as well as in the CHO.hIR cells and wild type CHO cells (not shown). These studies established that the c-*fos* gene was still inducible by a stimulus other than insulin in the CHO.T-t cells. Treatment of the CHO.T cells with 16 μM PMA for 16 h depleted the cells of more than 94% of protein kinase C enzyme activity (control cells: 318 pmol/min/mg protein; PMA-pretreated cells: 18 pmol/min/mg protein). In keeping with our previous results in 3T3-L1 adipocytes (16), insulin (but not further PMA) was still able to induce c-*fos* expression in the protein kinase C-depleted cells (not shown).

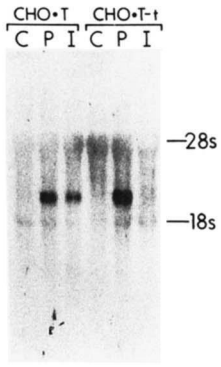

FIG. 1. **Levels of c-*fos* mRNA in CHO.T or CHO.T-t cells exposed to PMA or insulin.** Serum-deprived CHO.T or CHO.T-t cells were exposed to control conditions (*C*), PMA (*P*; 1.6 μM in 0.01% dimethyl sulfoxide), or insulin (*I*; 10 milliunits/ml (about 70 nM)) for 30 min. Total cellular RNA was then prepared from the cells, and 15 μg were loaded into each gel lane. The RNA was transferred to nitrocellulose and used for Northern analysis of c-*fos* mRNA levels as described in the text. The *markers* indicate the positions of the major ribosomal RNA species on the gel.

We next investigated the effects of insulin and phorbol esters on CHO-T cells which had been transfected with plasmid chloramphenicol acetyltransferase constructions containing either the normal murine c-*fos* upstream sequences from −356 to +109 (−356 *fos*-chloramphenicol acetyltransferase) or the same sequence in which G residues in positions −305 and −306 in the top strand, and −314 and −313 in the bottom strand had been changed to C residues by site-directed mutagenesis (2–4 pM12). These G residues are thought to be in close contact with a nuclear factor that binds to this site (18, 19). Expression of these fusion genes was evaluated in transient expression experiments using an RNase protection assay (Fig. 2*A*). Each *lane* represents pooled RNA from 5 of 15 dishes exposed to the same precipitate, thereby minimizing possible differences in transfection efficiency. As shown in Fig. 2*A*, both PMA and insulin stimulated the expression of the fusion gene containing the normal c-*fos* sequences; quantitation of these changes by densitometry revealed increases of approximately 3-fold in response to 45 min of treatment with PMA or insulin (Fig. 2*B*). However, the fusion gene containing the mutated bases in the SRE responded minimally to both stimuli (Fig. 2, *A* and *B*). Similar results were obtained in a second identical transfection experiment. Averages from the two experiments were as follows: plasmid −356 *fos*-chloramphenicol acetyltransferase, 3.3-fold stimulation with PMA, 3.0-fold stimulation with insulin; plasmid 2–4 pM12, 31% stimulation with PMA, 3% stimulation with insulin. No protected fragments were noted in RNA from insulin- or PMA-stimulated nontransfected CHO.T cells. However, several protected bands of larger apparent size shown in Fig. 2*A* were noted in RNA from the transfected and nontransfected cells as well as in mouse RNA.

To ensure that the transfection protocol did not adversely affect hormonal stimulation of endogenous c-*fos* mRNA accumulation, we evaluated total cellular RNA from both sets of transfected cells by Northern blotting, using a v-*fos* probe as described (16). Both the undetectable c-*fos* mRNA levels in the basal state and the increased accumulation of c-*fos* mRNA in response to PMA and insulin were equivalent in both sets of cells transfected with either the normal or mutant plasmids (Fig. 2*C*).

Nuclear extracts from the CHO.T cells contained factors, presumably proteins, which bound to the ^{32}P-labeled inserts containing 83 bp of either the normal or mutant c-*fos* sequences, as assessed by gel shift analysis. However, only a single slowly migrating species bound uniquely to the normal plasmid insert when compared either to the insert from the mutant plasmid or a slightly larger fragment from the vector, pUC 13 (Fig. 3). This experiment showed that the G residues required for biological activity were also required for binding of this nuclear factor. Evaluation of this factor from control CHO.T cells or those treated with either insulin or EGF revealed no apparent increase or decrease in the amount of ^{32}P-labeled insert shifted into the position of the unique band (Fig. 3). In other experiments, treatment of the cells with either insulin (1 milliunit/ml) or PMA (1.6 μM) for 30 min did not affect the intensity of the specific shifted band on the autoradiographs (data not shown). A similar protein could be detected in nuclear extracts from 1321-N1 human astrocytoma cells, in which c-*fos* is also rapidly induced by various stimuli (28) (Fig. 3).

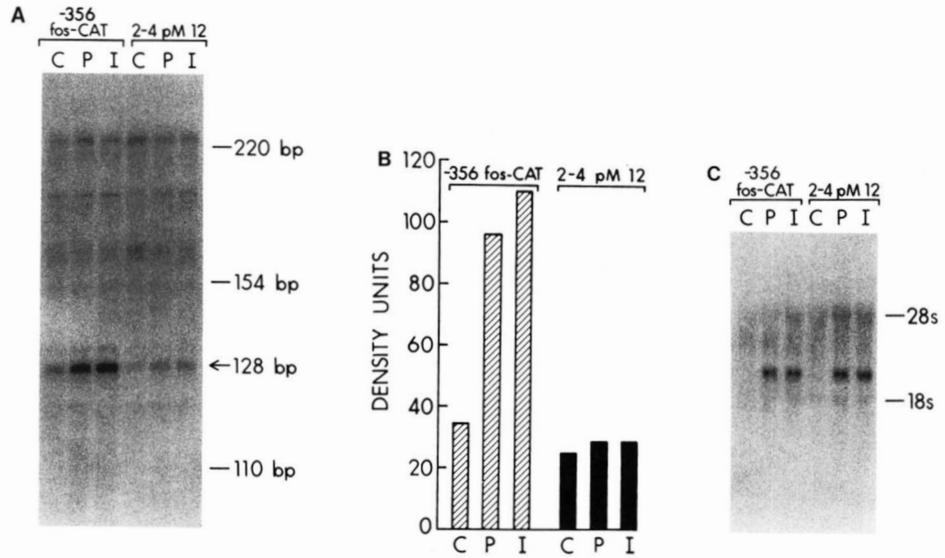

FIG. 2. **Induction of *fos*-chloramphenicol acetyltransferase fusion genes by PMA or insulin in CHO.T cells.** CHO.T cells were tranfected with plasmids containing either normal murine c-*fos* sequences (−356 *fos-CAT*) or the same region of the *fos* gene carrying point mutations as described in the text (2–4pM12). Forty-eight hours after transfection the cells were deprived of serum for 16 h and then exposed to control conditions (*C*), PMA (*P*; 1.6 μM in 0.01% dimethyl sulfoxide), or insulin (*I*; 10 milliunits/ml) for 45 min. Total RNA was then prepared from five plates of cells for each condition and used for the analysis of induced transfected genes. In *A* is shown the RNase protection assay, in which the protected fragment from the *fos*-chloramphenicol acetyltransferase constructions is indicated with the *arrow* at its expected apparent size position of 128 bp. The positions of other size markers are indicated. RNase protection of murine RNA in the same assay revealed a major protected band of endogenous c-*fos* mRNA at 110 bp, where there is no protected hamster species. Similarly, no RNA species of 128 bp from nontransfected CHO.T cells was protected by the probe used. In *B* is shown a graphic representation of the densitometry tracing of the 128-bp protected fragment; abbreviations are the same as in *A*. In *C* is shown Northern analysis of endogenous c-*fos* mRNA levels in the same RNA samples as used for the RNase protection assay described in *A* and *B*; 15 μg of total cellular RNA was loaded into each gel lane. Abbreviations are the same as in the legend to Fig. 1 and in *A* above.

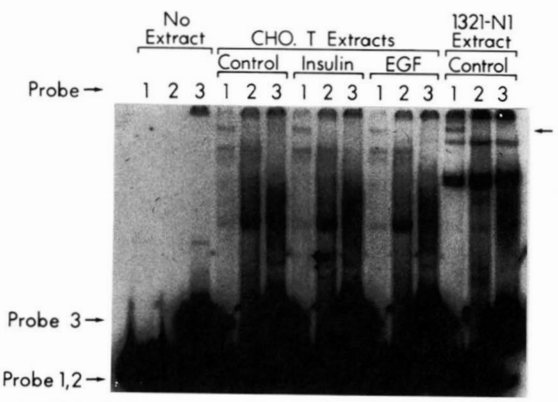

FIG. 3. **Gel shift analysis of a nuclear factor from CHO.T cells which binds to the normal but not the mutated region of the c-*fos* 5′ flanking element.** CHO.T cells and 1321-N1 human astrocytoma cells were deprived of serum and then exposed to control conditions, insulin (10 milliunits/ml), or EGF (100 ng/ml) for 30 min. Nuclear extracts were then prepared and used for DNA binding gel shift analyses as described in the text. Probe *1* represents the probe derived from plasmid −356 *fos*-chloramphenicol acetyltransferase, and probe *2* represents the probe of identical size from plasmid 2–4 pM12; both migrated to an identical position on the gel, as indicated by the *arrow*. Probe *3* is a 140-bp fragment of the non-*fos* portion of plasmid −356 *fos*-chloramphenicol acetyltransferase, which contains only sequences of the pUC 13 vector; its migration position is indicated by the *arrow*. The *numbers* above each gel lane indicate the probe used to bind to the nuclear extract in that lane. The *arrow* to the right of the autoradiograph points to a single band in both cell types which appears to bind specifically to probe 1 but not to probe 2 or 3.

DISCUSSION

These studies established several facts about the induction of c-*fos* by insulin and phorbol esters in these cells. First, the studies with the CHO.T and CHO.T-t cells indicated that the presence of normal insulin receptors was required for the insulin induction of c-*fos* mRNA. Induction did not occur in cells with similar numbers of truncated insulin receptors, which exhibit similar amounts of insulin binding but essentially no insulin-stimulated receptor tyrosine kinase activity (20). As in our previous studies (16), the insulin effect occurred in normal cells and in those almost completely depleted of protein kinase C. Second, although they appear to act by different initial mechanisms and their responses are additive in 3T3-L1 cells (16), insulin and PMA required the same intact sequences in the c-*fos* SRE (bases −299 to −320) in order to induce c-*fos* transcription. Mutation of four guanine residues in this region that contact a nuclear factor (18, 19) abolished induction by both stimuli. These mutations, or similar ones, also reduce c-*fos* induction by serum in 3T3 fibroblasts[2] (30). We also demonstrated the existence of a nuclear factor, presumably a protein, from CHO.T cells that interacted with the SRE with similar specficity. However, we could not demonstrate that exposure of the cells to insulin, EGF, or PMA caused changes in the apparent affinity of this factor to the c-*fos* sequences, in keeping with previous studies of serum-induced c-*fos* expression in HeLa cells (18, 29) but differing from recent studies involving EGF-induced c-*fos* expression in A431 cells (29). The reasons for these differences are not clear but may be resolved when purified SRE binding proteins and antibodies to them become available.

To our knowledge, insulin-stimulated c-*fos* transcription is

the earliest stimulatory transcriptional response to insulin yet reported. In addition to the present findings and our previous studies in 3T3-L1 adipocytes, we have noted insulin-stimulated c-*fos* induction in two other insulin-responsive cell types, H4IIE rat hepatoma cells and BC3H1 mouse muscle cells.[3] Similar findings in Reuber H35 rat hepatoma cells have been reported recently (31). Taken together, these observations suggest that the insulin induction of c-*fos* might be an early physiological response in many insulin-sensitive tissues, perhaps affecting later insulin-stimulated transcriptional responses, since data identifying the c-*fos* protein as a *trans*-acting regulator of gene transcription are fairly strong (32–35). For these reasons, we believe that the protein(s) involved in the insulin-stimulated transcription of c-*fos*, possibly the same as those involved in the response to activators of protein kinase C, will prove to be important early targets of some insulin-stimulated cellular pathway, perhaps through phosphorylation-dephosphorylation reactions.

Previous studies (17–19, 36) established that a region of dyad symmetry with properties of a viral enhancer located between −320 to −299 relative to the transcription initiation site in the human c-*fos* gene were required for serum induction of the c-*fos* gene in 3T3 fibroblasts. However, a sequence from −351 to −337 in the human c-*fos* gene has recently been shown to bind a PDGF-inducible factor in BALB/c-3T3 cells, a factor which was not induced by insulin or PMA (37). Binding sites in the c-*fos* gene for other nuclear factors have been identified (19, 38).

Serum is a complex mixture of potentially inducing compounds including PDGF, which can activate c-*fos* expression by protein kinase C-dependent and -independent pathways (16) as well as by cyclic AMP-dependent pathways (39), and insulin, which appears to act in 3T3-L1 cells through a protein kinase C- and cAMP-independent pathway (16, 26). It appears from the present work that at least two components of serum, insulin and PDGF, can induce c-*fos* transcription through involvement of the SRE, with PDGF acting, at least in part, through activation of protein kinase C in this case. Whether other PDGF-activated pathways, such as activation of the cAMP-dependent protein kinase, will induce c-*fos* expression through other sequences (37) remains to be determined.

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