MOLECULAR AND CELLULAR BIOLOGY, Apr. 1989, p. 1790–1793
0270-7306/89/041790-04$02.00/0

Vol. 9, No. 4

Induction of Tumor Promotor-Inducible Genes in Murine 3T3 Cell Lines and Tetradecanoyl Phorbol Acetate-Nonproliferative 3T3 Variants Can Occur through Protein Kinase C-Dependent and -Independent Pathways

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Received 31 October 1988/Accepted 6 January 1989

We isolated a group of genes that are rapidly and transiently induced in 3T3 cells by tetradecanoyl phorbol acetate (TPA). These genes are called TIS genes (for TPA-inducible sequences). Epidermal growth factor (EGF), fibroblast growth factor (FGF), and TPA activated TIS gene expression with similar induction kinetics. TPA pretreatment to deplete protein kinase C activity did not abolish the subsequent induction of TIS gene expression by epidermal growth factor or fibroblast growth factor; both peptide mitogens can activate TIS genes through a protein kinase C-independent pathway(s). We also analyzed TIS gene expression in three TPA-nonproliferative variants (3T3-TNR2, 3T3-TNR9, and A31T6E12A). The results indicate that (i) modulation of a TPA-responsive sodium-potassium-chloride transport system is not necessary for TIS gene induction either by TPA or by other mitogens and (ii) TIS gene induction is not sufficient to guarantee a proliferative response to mitogenic stimulation.

Tetradecanoyl phorbol acetate (TPA) is a potent biological response modulator with a diverse range of effects on cells, in vivo and in cell culture (27). TPA was first identified by using a mouse skin assay, as a powerful tumor promotor. It is also a potent mitogen for a variety of cell types, including murine 3T3 cells (2). TPA stimulates the differentiation of HL60 promyelocytic leukemic cells (20) but exerts an inhibitory effect on the differentiation of Friend erytholeukemic cells (29) and cultured myoblasts (5). TPA is thought to act primarily by binding to and activating the calcium- and phospholipid-dependent protein kinase C (13). To understand more fully the mechanisms by which TPA can modulate such a wide range of biological responses, we isolated, from mouse 3T3 cells stimulated with TPA in the presence of cycloheximide, a set of TPA-inducible primary response genes termed TIS genes (for TPA inducible sequences [10]). The TIS genes exhibit the following characteristics: (i) they are all induced as a primary response (28) to TPA stimulation, and no protein synthesis is necessary for the increase of TIS mRNAs; (ii) the induction is transient; (iii) TIS mRNAs are unstable; and (iv) TIS mRNA accumulation in the presence of TPA can be superinduced by cycloheximide.

TIS genes can be induced by other mitogens in 3T3 cells. We anticipated, when we isolated the TPA-inducible TIS genes, that at least some of them might be uniquely responsive to TPA. To determine whether epidermal growth factor (EGF) or fibroblast growth factor (FGF) could induce TIS gene expression in 3T3 cells and to compare induction kinetics of EGF and FGF with that of TPA, we analyzed the time courses of TIS gene induction by EGF and FGF. Like the TPA response (10), induction of the TIS genes (TIS 1, TIS 8, TIS 10, and TIS 11) in response to EGF or FGF generally peaked at about 30 to 60 min and returned to nearly uninduced levels by 3 to 5 h (Fig. 1). Induction of the TIS genes in 3T3 cells is thus not unique to TPA but will occur in response to other mitogens as well.

TIS genes can be induced through multiple pathways. The TIS gene cDNAs were isolated in response to a pharmacological agent, TPA, that directly activates the protein kinase C-mediated second messenger system of signal transduction. One might, therefore, expect that induction of the TIS genes by physiological inducers such as FGF or EGF would be mediated by the protein kinase C pathway. If such were the case, depletion of protein kinase C activity should abolish induction by EGF and FGF. Chronic exposure to TPA down regulates protein kinase C levels in 3T3 cells (16). The loss of enzyme activity is accompanied by the disappearance of immunologically cross-reactive material (23). The ability of EGF and FGF to induce TIS gene expression was examined in 3T3 cultures that had been pretreated with TPA (Chemsyn) for 24 h. Parallel cultures with no TPA pretreatment were used as controls. Since TPA reduces the binding affinity of the EGF receptor (7), we used a high dose of EGF (100 ng/ml) to saturate all available EGF-binding sites. The three TIS genes (TIS 1, TIS 8, and TIS 11) examined were still inducible by EGF and FGF in cultures pretreated with TPA, whereas induction by TPA in pretreated cultures was almost completely abolished (Fig. 2A). A quantitative analysis of the results from four experiments is shown in Fig. 2B. Although induction by EGF and FGF was somewhat diminished after TPA pretreatment, both peptide mitogens still induced substantial expression of the TIS genes. In contrast, TPA pretreatment of 3T3 cells essentially eliminated any subsequent induction of the TIS genes by TPA. Thus, even though they were isolated as TPA-inducible genes, the TIS genes can be activated through pathways that are distinct from, and independent of, protein kinase C activation. Similar findings have been reported for other genes whose expression is induced in response to mitogenic stimulation. For example, the stimulation by some peptide mitogens of

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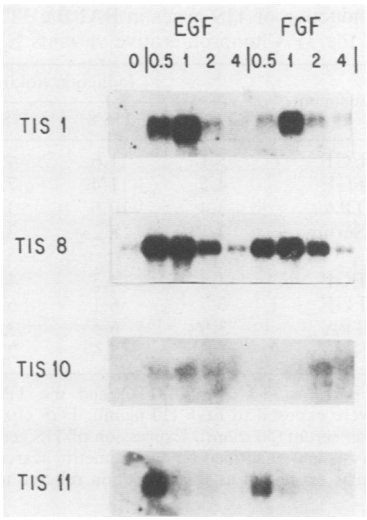

FIG. 1. Induction of TIS genes by EGF and FGF in Swiss 3T3 cells. Confluent Swiss 3T3 cells (2) were treated with either EGF (20 ng/ml) or FGF (10 ng/ml) for the times indicated (in hours). RNAs were prepared (3) and analyzed for TIS gene expression by Northern (RNA) gel analysis (26). Each lane had 10 μg of total cellular RNA. EGF was purified by the method of Savage and Cohen (22). FGF was purchased from Collaborative Research.

proto-oncogenes c-*fos* (a member of the TIS gene family [10]) and c-*myc*, as well as the stimulation of virus-related VL30 genes, can proceed through protein kinase C-independent pathways (6, 11, 17).

TIS gene expression in Swiss 3T3 TPA-nonproliferative variants 3T3-TNR2 and 3T3-TNR9. One of our primary interests is the identification of causal steps in the mitogenic response. To this end, we have established mitogen-specific TPA-nonproliferative mutants of 3T3 cells (2). TIS mRNAs accumulate in TPA-nonproliferative 3T3-TNR9 cells treated with TPA plus cycloheximide (10). Stabilization of mRNA due to the presence of cycloheximide, however, could have obscured more subtle differences in the TPA inducibility of TIS genes between the 3T3-TNR9 variant and the parental Swiss 3T3 cells. To examine more extensively the question of the inducibility of the TIS genes in our TPA-nonproliferative variants, we have (i) compared the time course and extent of TIS gene expression in response to TPA stimulation in 3T3-TNR9 TPA-nonproliferative cells with the response to serum (a competent mitogen [2]) and (ii) analyzed TIS gene induction in 3T3-TNR2, a second, independently-isolated TPA-nonproliferative Swiss 3T3 variant (2). One of the TIS genes (TIS 8) was induced, by both TPA and serum, in both TPA-nonproliferative variants in a manner that is indistinguishable from that observed in the parental 3T3 cells (Fig. 3). The levels of induction of TIS 8 and three other TIS genes (TIS 1, TIS 10, and TIS 11) observed after 1 h of serum or TPA treatment were quantified and are summarized in Table 1. While quantitative differences in the levels of the induction of the TIS genes varied somewhat from experiment to experiment, it is clear that all the TIS genes examined (TIS 1, TIS 8, TIS 10, and TIS 11) were induced by TPA in the Swiss 3T3 TPA-nonproliferative variants.

We conclude that TIS gene induction by mitogen is not sufficient to guarantee a proliferative response. It is possible that induction of the TIS genes is extraneous to the TPA-induced mitogen response pathway. Alternatively, the induction of TIS gene expression may be necessary to mount a mitogenic response, but the defect(s) in the TPA-nonproliferative variants may reside either in more distal steps in

TABLE 1. Induction of TIS genes in Swiss 3T3 cells and the TPA-nonproliferative variants 3T3-TNR2 and 3T3-TNR9

Cell line	Treatment	Induction (fold) of[a]:			
		TIS 1	TIS 8	TIS 10	TIS 11
3T3	Serum	13.3	21.2	23.1	10.6
	TPA	16.2	37.0	11.7	12.8
TNR2	Serum	5.1	44.3	14.1	6.4
	TPA	5.6	25.9	15.8	4.3
TNR9	Serum	3.9	21.8	9.1	4.1
	TPA	3.3	26.6	4.9	4.7

[a] Cultures of 3T3, 3T3-TNR2, and 3T3-TNR9 cells were exposed to TPA (50 ng/ml) or to fetal calf serum (50 μl/ml). Expression of TIS genes was analyzed by Northern analysis and qualified by densitometric scanning of autoradiographs. Induction is expressed as fold induction relative to that of control (untreated) cells.

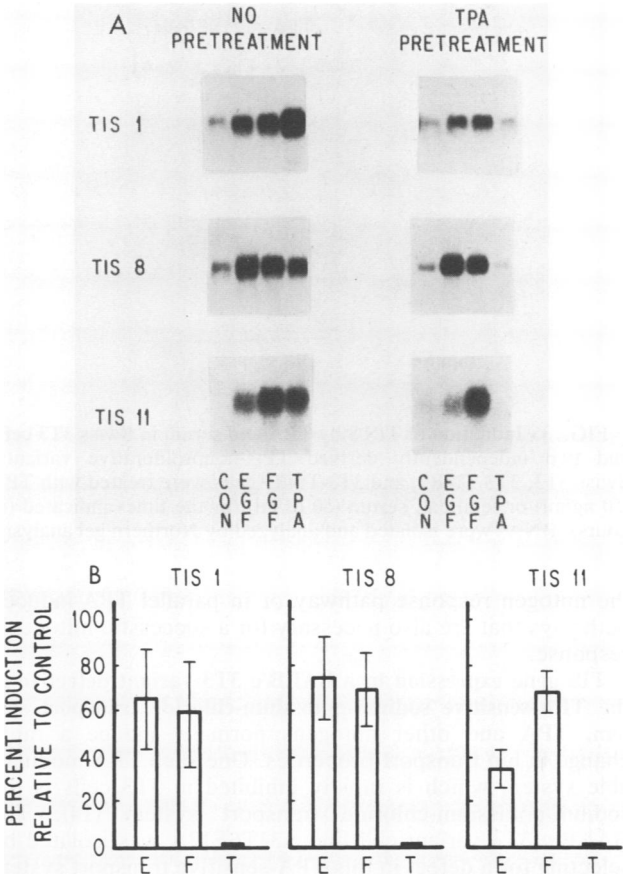

FIG. 2. Induction of TIS genes in TPA-pretreated 3T3 cells. (A) Swiss 3T3 cells cultures either were treated for 24 h with TPA (50 ng/ml) or were left untreated. Cells were then exposed to EGF (100 ng/ml), FGF (10 ng/ml), or additional TPA (50 ng/ml) or left without further treatment (control [CON]). RNAs were isolated 1 h later and analyzed by Northern gel analysis for TIS gene expression. (B) Levels of TIS gene expression were quantified by densitometry from Northern gel analysis of four independent experiments similar to those described above. Induction by EGF (E), FGF (F), and TPA (T) in TPA-pretreated cells was determined and expressed as a percentage of the induction observed in similarly treated cells with no previous TPA exposure.

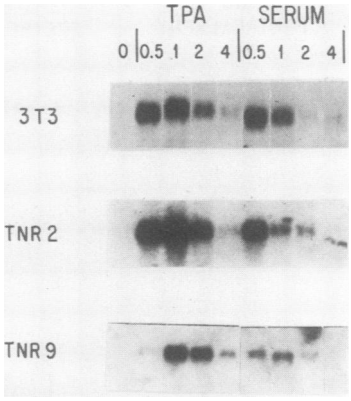

FIG. 3. Induction of TIS 8 by TPA and serum in Swiss 3T3 cells and two independently derived TPA-nonproliferative variants. Swiss 3T3, 3T3-TNR2, and 3T3-TNR9 cells were treated with TPA (50 ng/ml) or fetal calf serum (50 μl/ml) for the times indicated (in hours). RNAs were isolated and analyzed by Northern gel analysis.

the mitogen response pathway or in parallel TPA-induced pathways that are also necessary for a successful mitogenic response.

TIS gene expression in a BALB/c 3T3 variant defective in the TPA-sensitive sodium-potassium-chloride transport system. TPA and other mitogens normally induce a rapid change in ion transport properties. One such TPA-modulatable system which is rapidly inhibited in 3T3 cells is the sodium-potassium-chloride transport system (14). The BALB/c 3T3 variant cell line A31T6E12A was isolated by selecting for a defect in this TPA-sensitive transport system (25). Like 3T3-TNR2 and 3T3-TNR9, this variant does not proliferate in response to TPA (15). To determine (i) whether the sodium-potassium-chloride transport system is involved in TIS gene expression and (ii) whether TPA-nonproliferative variants isolated by a different selection protocol might be defective in TIS gene expression, we examined the TPA inducibility of the TIS genes in BALB/c 3T3 cells and the A31T6E12A variant. Our results indicate that TPA, like other competent BALB/c 3T3 mitogens, induced TIS gene expression equally well in BALB/c 3T3 cells and in the E12A variant. An example of the analysis is shown in Fig. 4. Note that the basal level of TIS 8 expression in the BALB/c 3T3 cells and its variant is greater than that observed in Swiss

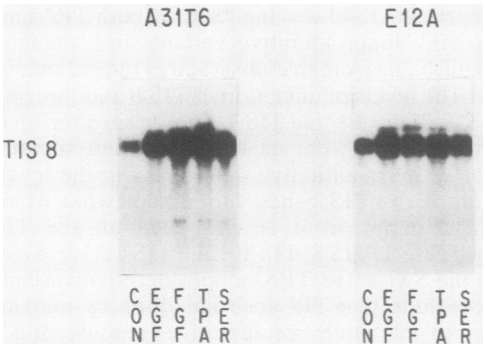

FIG. 4. Induction of TIS 8 in BALB/c 3T3 cells and the TPA-nonproliferative variant E12A. BALB/c 3T3 cells (A31T6) and variant cells (E12A) were treated for 1 h with EGF (10 ng/ml), FGF (10 ng/ml), TPA (50 ng/ml), or serum (50 μl/ml) before RNA isolation. TIS gene expression was analyzed by Northern analysis.

TABLE 2. Induction of TIS genes in BALB/c 3T3 A31T6 cells and the TPA-nonproliferative variants E12A

Cell line	Treatment	Induction (fold) of[a]:			
		TIS 1	TIS 8	TIS 10	TIS 11
A31T6	EGF	3.1	11.6	4.9	6.1
	FGF	4.5	11.4	7.8	12.0
	TPA	3.4	10.6	3.5	4.3
	Serum	3.1	8.2	2.6	2.9
E12A	EGF	4.4	4.3	4.4	4.5
	FGF	6.7	6.1	6.6	9.4
	TPA	3.9	6.5	3.7	7.9
	Serum	7.5	5.6	7.8	6.8

[a] Cultures of BALB/c 3T3 cells (A31T6) and the TPA-nonresponsive variant (E12A) were exposed to EGF (10 ng/ml), FGF (10 ng/ml), TPA (50 ng/ml), or fetal calf serum (50 μl/ml). Expression of TIS genes was analyzed by Northern analysis and quantified by densitometric scanning of autoradiographs. Induction is expressed as fold induction relative to that of control (untreated) cells.

3T3 cells and its variants (compare Fig. 4 and Fig. 3). This is true for all the TIS genes examined and accounts for the generally lower TIS gene induction levels observed for BALB/c 3T3 cells (Table 2) relative to Swiss 3T3 cells (Table 1). The lack of a TPA-sensitive sodium-potassium-chloride transport system did not interfere with TPA-induced expression of TIS genes. We conclude that the presence of an intact TPA-sensitive Na-K-Cl transport system is not required for TIS gene expression in response either to TPA or to peptide mitogens.

A comparison of TIS genes and other transiently induced sequences. Several TIS genes can also be induced in PC12 cells by TPA, EGF, and nerve growth factor (8). The TIS genes may therefore include genes identified in response to peptide mitogens or nerve growth factor (1, 4, 9, 12). The TIS 11 cDNA sequence does not show similarity to any sequences in GenBank, NBRF-PIR, EMBL, or SWISS-PROT data bases (B. C. Varnum, R. W. Lim, V. P. Sukhatme, and H. R. Herschman, Oncogene, in press). The proto-oncogene c-*fos*, a member of the TIS gene family (TIS 28 [10]), is a nuclear protein (19) that interacts with the transcription factor c-*jun*/AP-1 (21). Another TIS-like gene (c-*jun* B) exhibits extensive homology to AP-1 (18). Sequence data on a partial cDNA clone (Varnum et al., unpublished) demonstrates that TIS 8 is homologous and probably identical to Egr-1, a serum-inducible primary response gene (24). This gene has also been isolated as a nerve growth factor-inducible gene in PC12 cells (12). The TIS 8/Egr-1 gene contains zinc finger domains (24). Thus, at least some TIS genes may regulate transcription of secondary response genes during biological responses to extracellular signals. Each of six TIS genes has a distinct developmental profile (26), suggesting that these genes have unique regulatory characteristics as well as common responses.

This work was supported by Public Health Service grants GM24797 (H.R.H.) and CA36353 (T.G.O.) from the National Institutes of Health and contract DE FC03 87ER 60615 between the Department of Energy and the Regents of the University of California (H.R.H.). B.C.V. was supported by Public Health Service training grant T32 GM07104 from the National Cancer Institute.

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