Characterization of two independent mechanisms by which interferon-induced gene expression is down regulated*

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Interferons (IFNs) induce the expression of a variety of cellular RNAs. Phorbol esters can inhibit IFN-induced expression of some of these RNAs, including ISG-54K. The actions of phorbol esters on IFN-activated ISG-54K transcription are cell specific and are reversed by inhibitors of protein synthesis. In those cell lines in which phorbol esters inhibit IFN-induced ISG-54K transcription, prolonged IFN exposure also induces a "desensitized state" such that further IFN exposure no longer induces ISG-54K expression. IFN-induced desensitization is also reversed by inhibitors of protein synthesis. Experiments are described to determine whether the mechanism by which phorbol esters inhibit IFN-activated ISG-54K expression is the same as the mechanism by which prolonged exposure to IFN makes cells refractory to further induction of ISG-54K expression. Cultured cells treated with 12-O-tetradecanoylphorbol 13-acetate (TPA) for 72 h are desensitized to phorbol esters such that further addition of phorbols does not inhibit IFN-induced ISG-54K expression. In both naive and TPA-desensitized human fibroblasts or WISH cells, prolonged IFN treatment induced a desensitized state that was reversible by cycloheximide. This observation suggests that the mechanisms by which prolonged IFN treatment and phorbol esters inhibit ISG-54K expression are independent.

Introduction

Interferons (IFNs)[1] are polypeptides that regulate a variety of biological effects, the most no-

table of which are antigrowth and antiviral activities (Pestka et al., 1987). Similar to other polypeptide hormones and neurotransmitters, both type I (α and β) and type II (γ) IFNs have specific cell-surface receptors. Among the events that occur within minutes after cells are exposed to IFNs, one of the earliest is the increased expression of several mRNAs (Friedman et al., 1984; Larner et al., 1984, 1986; Luster et al., 1985; Reich et al., 1987). Many IFN-induced RNAs have been shown to be activated via increased rates of transcription of their corresponding genes (Friedman et al., 1984; Larner et al., 1984). An understanding of the molecular mechanisms by which IFN activates gene transcription, which at present are ill defined, should provide insight into the signal transduction pathway(s) used by IFNs.

We have been investigating the gene ISG-54K, whose expression is activated by type I IFNs (Larner et al., 1984; Akai and Larner, 1989). In human cells, IFN induction of ISG-54K mRNA occurs by a rapid increase in the rates of transcription of the gene. Protein synthesis is not necessary for this response. In some types of cells, such as human diploid fibroblasts and WISH cells, long-term IFN exposure (6–8 h) down regulates the expression of IFN-inducible genes such that reexposure to fresh IFN will not reactivate expression. We call this process desensitization. Inhibitors of protein synthesis prevent IFN-induced desensitization and also enhance IFN-induced ISG-54K rates of transcription.

Recently, acute treatment of cells with phorbol esters has been shown to inhibit transcriptional activation of certain IFN-sensitive genes such as ISG-54K and 2'5' oligo A synthetase (Akai and Larner, 1989; Yan et al., 1989). However, other IFN-responsive genes are minimally affected by phorbol esters or show enhanced expression (Fan et al., 1988; Akai and Larner, 1989; Erusalimsky et al., 1989; Faltynek et al., 1989). 12-O-tetradecanoylphorbol 13-acetate (TPA) inhibition of IFN-induced ISG-54K expression is reversed by inhibitors of protein synthesis (Akai and Larner, 1989). The acute
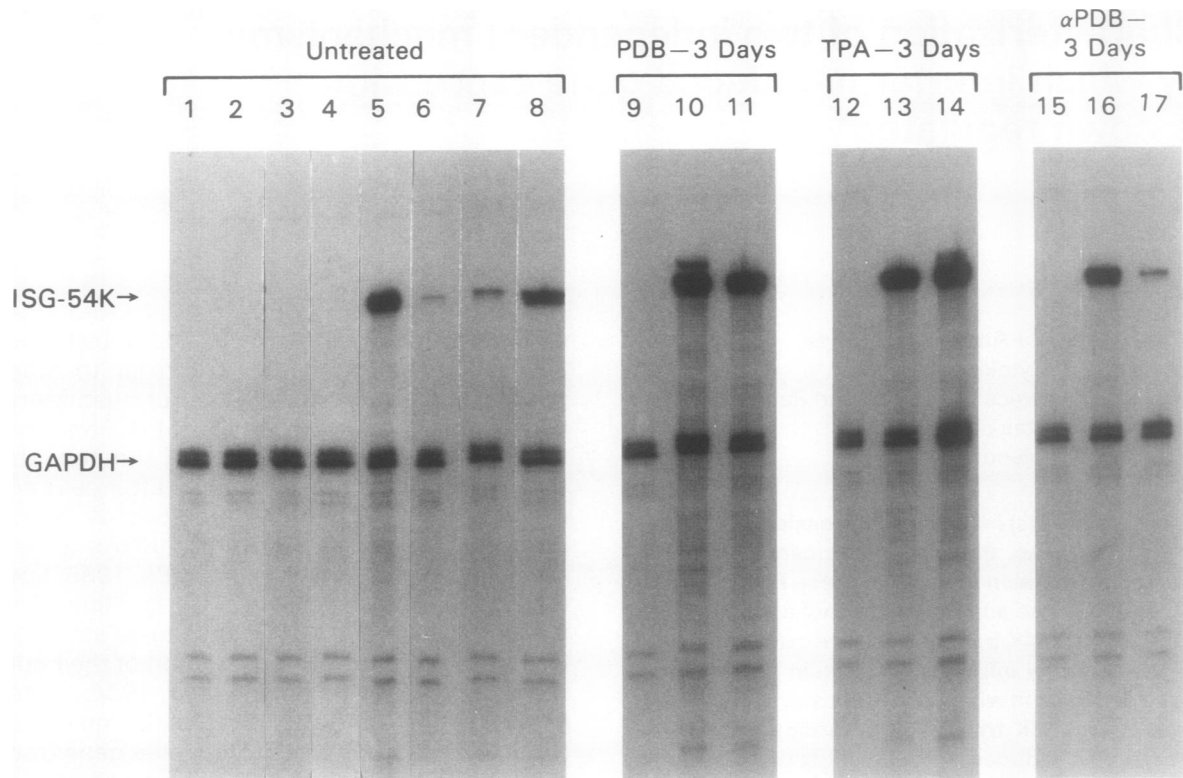
[1] Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; PDB, phorbol-12,13-dibutyrate; TPA, 12-O-tetradecanoylphorbol 13-acetate.

Figure 1. Pretreatment with phorbol esters reverses their inhibitory effects on IFN-induced ISG-54K RNA expression. Confluent human diploid fibroblasts were either untreated (lanes 1–8), pretreated with 10^{-7} M PDB for 3 d (lanes 9–11), pretreated with 10^{-8} M TPA for 3 d (lanes 12–14), or pretreated with 10^{-7} M α-PDB for 3 d (lanes 15–17). Cells were washed and treated with fresh PDB (10^{-7} M) or TPA (10^{-8} M) for 2 h before addition of IFN-α (250 U/ml) for 5 h. Total cellular RNA was isolated, and processed ISG-54K RNA and GAPDH RNA were assayed by solution RNase protection assays in the samples with ^{32}P-labeled antisense RNA probes (see Methods). Equal amounts (10 μg) of RNA as determined by A_{260} were used in each sample. The size of the protected fragment for ISG-54K is 367 bp and for GAPDH is 320 bp. Lane 1, untreated; lane 2, PDB; lane 3, TPA; lane 4, α-PDB; lane 5, IFN; lane 6, IFN and TPA; lane 7, IFN and PDB; lane 8, IFN and α-PDB; lane 9, PDB, 3 days; lane 10, IFN; lane 11, IFN and PDB; lane 12, TPA, 3 days; lane 13, IFN; lane 14, IFN and TPA; lane 15, α-PDB, 3 days; lane 16, IFN; lane 17, IFN and PDB.

actions of phorbol esters are also cell specific and are effective only in those cells in which cycloheximide prevents long-term IFN treatment from inducing a refractory state. These data suggest that a protein-kinase-C–dependent mechanism might also be responsible for IFN-induced desensitization.

To address this issue, we desensitized WISH cells and human fibroblasts to phorbol esters such that these tumor promoters were no longer effective in blocking IFN-induced ISG-54K expression. Under conditions in which phorbol esters failed to inhibit IFN-induced ISG-54K expression, prolonged IFN treatment still induced a desensitized state in which further stimulation by fresh IFN was unable to reactivate the transcription of ISG-54K. This result suggests that there are distinct mechanisms by which phorbol esters inhibit IFN-activated ISG-

54K expression and that long-term IFN exposure induces desensitization.

Results

It has been well documented that long-term treatment of cells with phorbol esters makes them protein-kinase-C deficient (Coughlin *et al.,* 1985; Stumpo and Blackshear, 1986). Conditions were defined in human fibroblasts in which pretreatment with TPA or phorbol-12,13-dibutyrate (PDB) rendered the cells insensitive to phorbol ester inhibition of IFN-induced ISG-54K RNA expression (Figure 1). Human diploid fibroblasts were incubated with 10^{-7} M PDB or 10^{-8} M TPA for 3 d and then washed and incubated with IFN alone or IFN and phorbol ester for 5 h. Total cellular RNA was harvested, and expression of processed ISG-54K RNA was as-
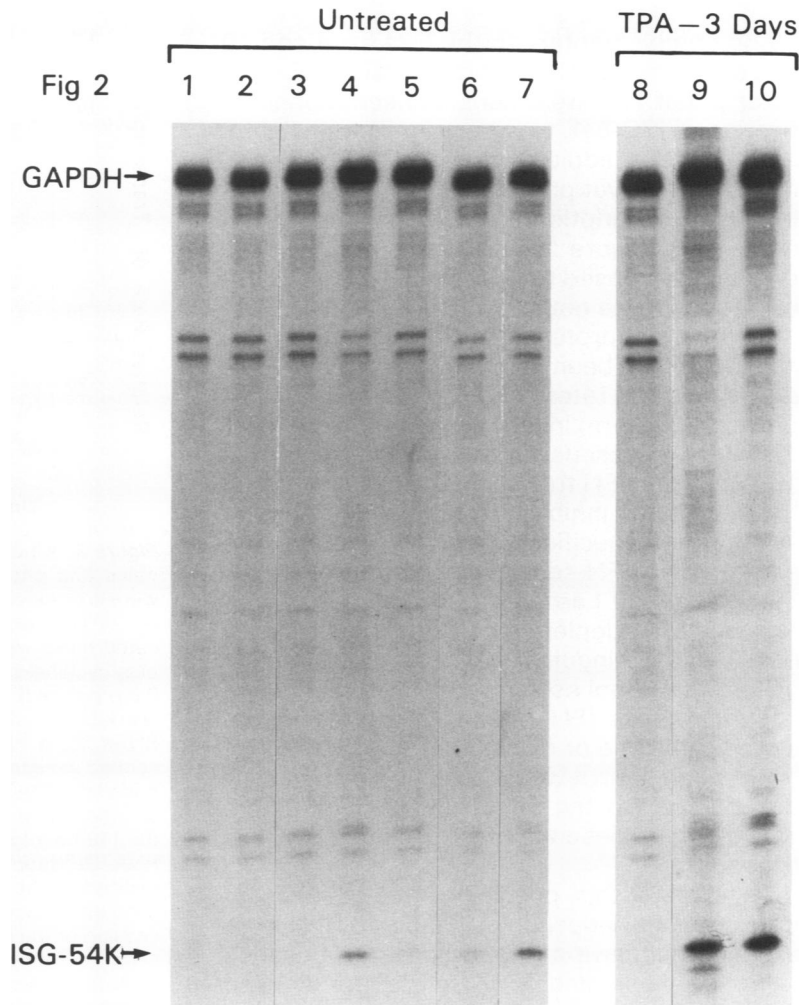
Figure 2. Extended phorbol ester exposure reverses its inhibitory actions on the transcriptional activation of ISG-54K by IFN. Human fibroblasts were either untreated (lanes 1–7) or treated with 10^{-8} M TPA for 3 d (lanes 8–10). The cells were washed and then exposed to fresh TPA (10^{-8}) for 2 h before addition of IFN-α for 90 min. Cellular RNA was isolated and assayed (10 μg/sample) by RNase protection with the use of a ^{32}P-labeled probe containing 250 bp upstream of the cap site and 118 bp 3' to the start site of transcription of ISG-54K and to ^{32}P-labeled GAPDH. The protected 118-bp fragment represents unprocessed nuclear RNA and reflects the relative rate of transcription of ISG-54K (Akai and Larner, 1989). Lane 1, untreated; lane 2, PDB; lane 3, TPA; lane 4, IFN; lane 5, IFN and PDB; lane 6, IFN and TPA; lane 7, IFN and α-PDB; lane 8, TPA 3 days; lane 9, IFN; lane 10, IFN and TPA.

sayed by RNase protection. In cells not previously exposed to PDB, both PDB and TPA reduced the accumulation of ISG-54K RNA 93% by densitometry with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (Figure 1, lane 5 vs. 6 or 7). Incubation with the α-isomer of PDB, which does not activate protein kinase Cs, does not inhibit IFN-induced ISG-54K expression (lane 5 vs. 8). In fibroblasts pretreated with PDB or TPA for 3 d, further phorbol ester treatment had no effect on IFN-induced ISG-54K accumulation (Figure 1, lane 10 vs. 11 or 13 vs. 14). Incubation of cells with the α-isomer of PDB for 3 d failed to make the cells protein-kinase-C deficient, as indicated by the fact that PDB still inhibited IFN-activated ISG-54K RNA expression 95% compared with the IFN-treated sample. (Figure 1, lane 16 vs. 17). It should also be noted that no ISG-54K RNA is detected in untreated cells or cells treated with only phorbol esters (lanes 1–3, 9, 12, 15). The preincubation time required

for PDB or TPA to become ineffective in inhibiting IFN-induced ISG-54K RNA expression varied between 1 and 3 d (data not shown). However, cells were always desensitized to the inhibitory effects of phorbol esters after 3 d of exposure. Complete resensitization to the inhibitory effects of phorbol esters on IFN-induced ISG-54K expression after 3 d of pretreatment with PDB required 7 d or more of cells being cultured in media without phorbol ester (data not shown).

Phorbol esters inhibit ISG-54K expression at the level of transcription (Akai and Larner, 1989). We reasoned that the lack of phorbol ester inhibitory activity in protein-kinase-C–depleted cells might also be a transcriptional phenomenon. To test this possibility, a ^{32}P-labeled single-stranded complementary RNA probe that contains the first exon and part of the first intron of the ISG-54K gene was used (Figure 2). The 118-bp RNase-resistant fragment represents unprocessed nuclear RNA, because it contains

78 bp complementary to the first exon and 40 bp complementary to the first intron of ISG-54K. After 3 d of TPA treatment, transcriptional activation of ISG-54K was not significantly inhibited by further addition of fresh PDB (Figure 2, lane 9 vs. 10), yet phorbol esters inhibited IFN-induced transcription of ISG-54K by ~90% in naive cells (Figure 2, lane 4 vs. 5 or 6). Cells previously exposed to PDB for 3 d showed similar results (data not shown).

Inhibitors of protein synthesis such as cycloheximide have been shown to enhance IFN-induced ISG-54K rates of transcription as well as prevent long-term incubation with IFN from establishing a desensitized state (Larner et al., 1986). During IFN treatment, it is assumed that transcriptional inhibitory factor(s) are synthesized that specifically down regulate the expression of IFN-sensitive genes (Larner et al., 1986; Akai and Larner, 1989). The use of protein-kinase-C–depleted cells should make it possible to distinguish whether the mechanism by which phorbol esters and long-term IFN exposure repress IFN-induced ISG-54K expression is the same or different. If the mechanism is the same, then it would be expected that cells desensitized to the effects of phorbol esters would not be desensitized with long-term IFN exposure.

To explore this possibility, the kinetics of transcriptional induction of ISG-54K by IFN were examined in naive and phorbol-ester–desensitized fibroblasts and WISH cells (Figures 3 and 4). The effects of cycloheximide on IFN-activated ISG-54K expression were also measured. As shown previously (Larner et al., 1986) in human fibroblasts, maximal transcriptional activation of the ISG-54K gene was observed after 2–4 h of IFN-α incubation (Figure 3). After 6–8 h of continuous IFN, ISG-54K expression was 5–10% of maximal activity, and the cells were desensitized to further activation by fresh IFN (data not shown). In phorbol-ester–resistant cells, time-dependent IFN-induced transcriptional activity showed similar kinetics as untreated cells. After 8 h of continuous IFN treatment of TPA-desensitized cells, ISG-54K transcriptional activity also could not be reactivated by fresh IFN (data not shown). These findings indicate that, under conditions in which the protein kinase C pathway(s) is not functional, prolonged IFN treatment can still induce a desensitized state. In addition, no obvious differences were observed in the kinetics of induction of ISG-54K with IFN. Interestingly, IFN plus cycloheximide showed consistently elevated rates of IFN-induced ISG-54K transcription in phorbol-ester–desensitized cells. There is no obvious
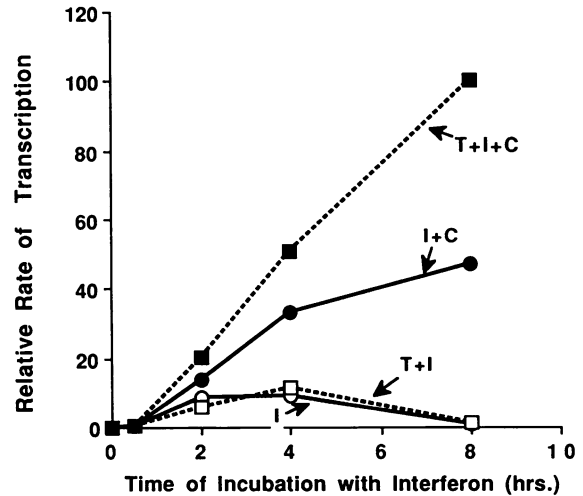

Figure 3. Kinetics of induction of ISG-54K expression in naive and phorbol-ester–desensitized fibroblasts. Cells were pretreated with 10^{-8} M TPA for 3 d (– – –) or untreated (———). They were then washed and incubated for the indicated times with either IFN (250 U/ml) or IFN (250 U/ml) plus cycloheximide (30 μg/ml). Cycloheximide was added 0.5 h before IFN. At the indicated time, total cellular RNA was isolated and hybridized (15 μg) to ^{32}P-labeled antisense RNAs. Both ^{32}P-labeled ISG-54K antisense probe as described in Figure 2 and ^{32}P-labeled GAPDH probe were hybridized to the same sample of RNA. Relative rates of transcription were determined by densitometric intensities of the 118-bp fragment corresponding to unprocessed nuclear RNA. Densitometric intensities of this fragment were normalized to the intensity of the GAPDH band (320 bp) to account for experimental errors in RNA yields or during processing of the RNase protection assay. The maximal transcription rate observed (8 h of IFN and cycloheximide in TPA-desensitized cells) was given an arbitrary value of 100. All other rates of ISG-54K transcription were normalized to this value. I, IFN alone; I + C, IFN plus cycloheximide; T + I, IFN alone in TPA-desensitized cells; T + I + C, IFN plus cycloheximide in TPA-desensitized cells.

explanation for this phenomenon. WISH cells showed findings similar to the fibroblasts (Figure 4A). As in fibroblasts, prolonged treatment of WISH cells with IFN and cycloheximide precluded down regulation of transcription of ISG-54K. However, it is interesting to note that TPA-desensitized WISH cells showed consistently greater sensitivity to IFN and IFN plus cycloheximide than naive cells. This result implies that there is a basal level of protein kinase C activity in WISH cells that ordinarily inhibits IFN-induced ISG-54K transcription. (Unstimulated transcription rates of ISG-54K in naive and phorbol-ester–desensitized cells were the same). Figure 4B exhibits relative transcriptional rates of ISG-54K in untreated phorbol-ester–sensitive and TPA-treated (3 d) phorbol-ester–resistant WISH cells. In untreated cells, short-term (2 h) TPA pretreatment inhibited ISG-54K transcriptional activity induced by short-term (3
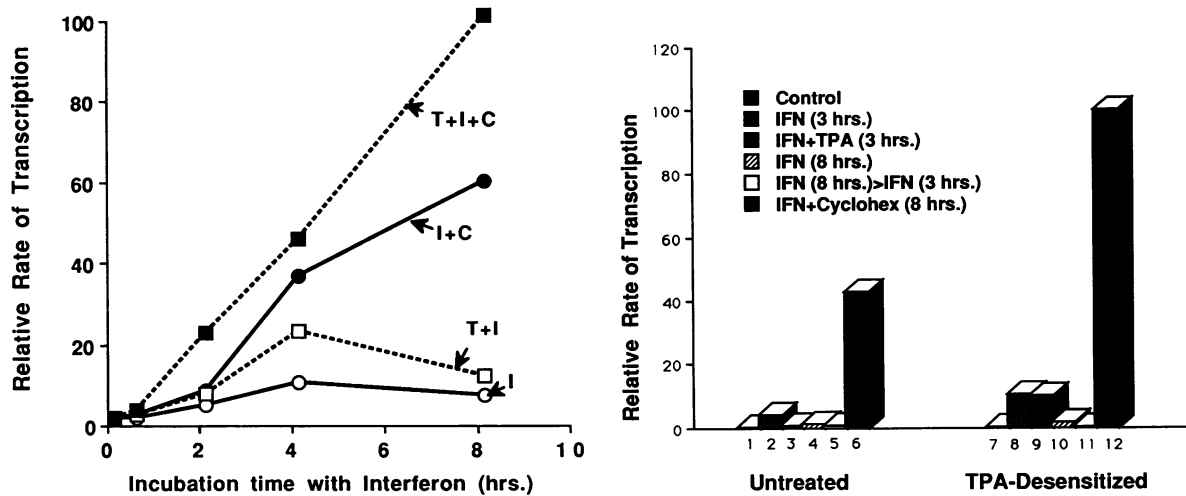
Figure 4. Effect of phorbol esters and extended IFN treatment on ISG-54K expression in WISH cells. (A) Kinetics of induction of ISG-54K expression in naive or TPA-desensitized WISH cells. See legend to Figure 3. (B) Relative rates of transcription of ISG-54K in untreated and TPA-desensitized WISH cells. Rates of transcription were determined as described in the legend to Figure 3. Columns 1–6, naive cells; columns 7–12, cells pretreated with 10^{-8} M TPA for 3 d before IFN treatment. TPA-desensitized cells were washed two times with DMEM before initiating the protocol. Columns 1 and 7, untreated; columns 2 and 8, IFN (250 U/ml) 3 h; columns 3 and 9, IFN and TPA (10^{-8} M) (cells were preincubated with TPA 2 h before addition of IFN); columns 4 and 10, IFN 8 h; columns 5 and 11, IFN 8 h followed by 3 h more of fresh IFN; columns 6 and 12, IFN and cycloheximide (30 μg/ml) 8 h. Cells were exposed 0.5 h to cycloheximide before addition of IFN.

h) IFNα treatment (Figure 4B, column 2 vs. 3). In contrast, phorbol-ester–desensitized cells did not show reduced transcriptional activity after further fresh TPA treatment, so that the value was almost equal to that of cells treated with IFNα alone (Figure 4B, column 8 vs. 9). Eight hours of continuous treatment with IFNα reduced the transcription rate of ISG-54K to ~20% of maximal activity, and additional treatment with fresh IFNα for 3 h was ineffective in restimulating the transcription rate of ISG-54K in both phorbol-ester–sensitive and –resistant cells (column 4 vs. 5 and column 10 vs. 11).

Discussion

IFN-activated gene expression is a complex phenomenon that involves both positive and negative regulatory pathways. Transcriptional induction of several genes by IFN occurs rapidly and does not require the synthesis of new proteins. Although much information has been obtained in recent years concerning the DNA sequence elements and transcription factors that are necessary for IFN to activate the expression of cellular genes, an understanding of the signal transduction pathways by which cellular genes are activated and subsequently repressed by long-term IFN exposure is still not complete. Some evidence has been reported that both type I and type II IFNs can increase intracellular diacylglycerol and inositol tris-phosphate con-

centrations (Yap et al., 1986a,b). However, it is clear from our results that IFN-induced increases in these second messengers probably does not correlate with IFN-regulated gene expression because neither PDB or TPA alone activates expression of ISG-54K (Figure 1, lanes 2 and 3).

Results presented in these experiments and elsewhere indicate that there are at least two mechanisms that inhibit IFN-activated ISG-54K expression (Larner et al., 1984; Akai and Larner, 1989). One mechanism occurs after long-term exposure of cells to IFN, and the other occurs in the presence of phorbol esters. These regulatory pathways have characteristics in common. Inhibitors of protein synthesis reverse the actions of both phorbol esters and long-term IFN exposure. Phorbol ester inhibition of ISG-54K expression and long-term IFN-induced desensitization are seen only in certain cell types. The types of cells in which phorbol esters repress IFN-induced ISG-54K expression are the same cells in which IFN-induced desensitization occurs (Akai and Larner, 1989).

To determine whether each inhibitory mechanism is regulated by the same pathway, we depleted cells of protein kinase C by long-term treatment with phorbol esters, a well-documented method used by numerous investigators (Coughlin et al., 1985; Stumpo and Blackshear, 1986). If phorbol esters and extended IFN exposure down regulate IFN-induced ISG-54K

transcription by similar pathways, then phorbol-ester–desensitized cells will not display long-term IFN repression of ISG-54K expression. Data in this report demonstrate a completely opposite result (Figures 3 and 4). Phorbol-ester–desensitized cells displayed the same kinetics of IFN-induced ISG-54K transcription. In addition, both cell types can be desensitized with long-term exposure to IFN even after being desensitized to the inhibitory actions of phorbol esters. Cycloheximide prevents the desensitization process resulting from prolonged exposure to IFN in both naive cells and cells pretreated with phorbol esters. These results prove that the regulatory mechanisms by which phorbol esters and extended IFN exposure inhibit IFN-induced ISG-54K expression are distinct. The data presented here also argue against the idea that rapid IFN-induced increases in diacylglycerol (Yap et al., 1986a,b) account for the desensitization process that occurs with long-term exposure of cells to IFN (see Figures 3 and 4).

It remains to be determined whether the DNA sequence elements that regulate phorbol ester and long-term IFN inhibition of ISG-54K expression are the same or different. Preliminary studies with transient transfection assays suggest that the elements that mediate transcriptional repression by phorbol esters and long-term IFN exposure are also distinct and neither appears to involve the IFN-stimulated response element. This is in contrast to the results of Levy et al. (1988), who suggested that the negative element that turns off ISG-54K expression by long-term exposure to IFN lies within the IFN-stimulated response element, which is present in the promoters of all IFN-inducible genes thus far characterized. However, definitive results that characterize these transcriptional repressors will require the development of permanent cell lines so that actual rates of transcription can be determined to confirm those results seen in transient expression assays. Other approaches, such as DNase I hypersensitivity studies and exonuclease protection assays, are also being pursued to provide more information regarding the definition of the phorbol-ester–inhibitory element. When this element is defined, it will then be possible to characterize how phorbol esters inhibit expression of IFN-inducible genes in molecular and biochemical terms, a phenomenon that may or may not be similar to the much better defined actions of phorbol esters to activate the expression of a variety of transcription units (Rabin et al., 1986; Angel et al., 1987; Chui et al., 1987; Fisch et al., 1987; Harrison et al., 1987). These studies should also

contribute to our general understanding of transcriptional repression in eukaryotes (Levine and Manley, 1989), an area in which considerably less is understood than selective activation of eukaryotic promoters.

Methods

Cells and cultures

Human fibroblasts and WISH cells were grown and passaged in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum.

IFNs and reagents

Recombinant IFN-α was a generous gift from Hoffmann LaRoche (Nutley, NJ). TPA was purchased from Sigma (St. Louis, MO). PDB and the α-isomer were obtained from LC Services (Woburn, MA).

Quantitation of RNA using antisense RNA probes

RNA was prepared as described by Chirgwin et al. (1979). RNAs in all experiments were quantitated by RNase protection assays. Antisense RNA probes were synthesized with SP6 RNA polymerase (Bethesda Research Laboratories, Gaithersburg, MD). Plasmid pIFN-IND-1 contains a 367-bp EcoRI restriction fragment of exon 2 of the gene (Akai and Larner, 1989) subcloned into pGEMI. The size of the protected fragment for pIFN-IND-1 is 367 bp. A 1400-bp PSTI fragment of rat GAPDH (Piechaczyk et al., 1984) was subcloned into pGEMI. The insert was linearized with Sau 3A; the size of the protected fragment is 320 bp. To determine the relative rate of transcription of ISG-54K, ^{32}P-labeled antisense RNA probes containing 250 bp upstream of the cap site of the gene and 118 bp downstream were hybridized to 10 μg of total cellular RNA. The protected 118-bp fragment represents unprocessed nuclear RNA (Levy et al., 1986). (The processed 78-bp product representing exon 1 was electrophoresed off the gels shown in Figure 2).

Solution hybridization and RNase mapping

Five to 10 μg of total cellular RNA were hybridized with 10^5 cpm of ^{32}P-labeled antisense RNA probe in 80% (v/v) formamide, 40 mM piperazine-N,N-bis[2-ethanesulfonic acid] (PIPES), 0.4 M NaCl, and 1 mM EDTA at 56°C for 16–20 h, and the protected RNA was analyzed essentially as described (Akai and Larner, 1989), except that T1 RNase was used instead of T2 RNase. T1 RNase (130 U) was added in a 330-μl digestion buffer containing 200 mM NaCl and 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4. Samples were incubated 1 h at 37°C, and protected RNA was extracted with phenol:chloroform; precipitated by the addition of ethanol; evaporated; resuspended in the loading buffer containing 94% (v/v) formamide, 10 mM EDTA, 0.05% (w/v) xylene cyanol, and 0.05% (w/v) bromophenol blue; and electrophoresed through 5% (w/v) acrylamide gel containing 42% (w/v) urea, 40 mM Tris, 40 mM boric acid, and 1 mM EDTA. The gels were exposed to X-ray film at −70°C with the use of an intensifying screen. The relative rates of transcription shown in Figures 3 and 4 were determined by densitometry of the 118-bp RNase-resistant fragment (see Figure 2). The relative intensity of the protected fragment was normalized to the 320-bp protected fragment corresponding to GAPDH, which was assayed simultaneously in the same hybridizations. In all other experiments, phorbol ester inhibition of IFN-induced ISG-54K RNA expression was quantitated by densitometry using GAPDH as an internal control;

the results are stated in the text. In general, phorbol esters inhibited IFN-induced ISG-54K expression from 85% to >99% in human fibroblasts.

Acknowledgment

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