Phorbo1 Ester-mediated Down-regulation of an Interferon-inducible Gene*

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Interferons (IFNs) induce the expression of a variety of cellular RNAs and inhibit phorbo1 ester induction of other genes. Experiments reported here indicate that phorbo1 esters can also specifically inhibit the expression of an IFN-induced RNA (IFN-IND-1). Phorbo1 esters exert their effects by inhibiting IFN-induced transcription of the gene that encodes IFN-IND-1 (IGS-54K); inhibitors of protein synthesis reverse the effects of these compounds. The actions of phorbo1 esters are only seen in those types of cultured cells where cycloheximide in the presence of IFN prevents long-term IFN treatment of cells from inducing a "desensitized state." In desensitized cells, IFN is not able to induce the transcription of the RNA. Our results indicate that a protein kinase C-dependent pathway requiring protein synthesis may be one mechanism by which IFN is able to down regulate the transcription of genes whose expression it initially induces.

The interferons (IFNs) are a family of polypeptides that perform a variety of biological functions, among the most prominent of which are antiviral and antitumor effects (1). There are three general classes of IFNs (α, β, γ), which appear to initiate their effects by binding to two classes of plasma membrane receptors.

One of the more rapid actions of IFNs is to induce the expression of certain cellular RNAs (2–10). The expression of other RNAs, notably those of c-myc and c-fos, is decreased by IFNs (11, 12). It has been shown that the expression of some IFN-induced RNAs is regulated via rapid increases in the transcription rates of their corresponding genes (2–8).

To study the action of IFNs on gene expression, we have characterized two IFN-regulated mRNAs, IFN-IND-1 and -2 (3, 4). Treatment of human cultured cells with IFN-α or IFN-β induces a rapid (within 10 min) increase in the transcription of these RNAs that does not require the synthesis of new proteins. After 6–8 h of continuous treatment of cultured human fibroblasts with IFN, the transcription of these genes declines to 5–10% of maximal levels, and re-exposure of cells to fresh IFN does not re-stimulate the transcription of these genes. If cells are exposed to both cycloheximide and IFN for 6–8 h, the transcription rate of these genes remains nearly maximal. This result suggests that continuous exposure of cells to IFNs alone induces the synthesis of a protein(s) that specifically inactivates the transcription of these genes. An understanding of the mechanisms by which IFNs activate and then inactivate the transcription of certain genes will provide a better understanding of the signaling events that are regulated by IFNs.

We have recently demonstrated that a single class of type I IFN receptors can regulate both the inhibition of c-myc RNA levels and induction of IFN-IND-1 and -2 expression in cultured human melanoma cells. Since phorbo1 esters are known to induce the expression of both c-myc and c-fos, and since IFNs inhibit this action, it was of interest to determine if phorbo1 esters also have an effect on the expression of IFN-IND-1 and -2. Experiments presented here show that phorbo1 esters are able to selectively induce the synthesis of IFN-IND-1 by a mechanism that probably requires the synthesis of new proteins. These studies suggest that at least one mechanism by which transcription of this gene is repressed involves a protein kinase C-dependent process.

EXPERIMENTAL PROCEDURES

Cells and Culture—IFN-sensitive human melanoma SK-MEL-28 cells (SK-MEL-28/s) were passaged in vitro in minimum essential medium containing 10% fetal bovine serum (GIBCO) (13). Human fibroblasts and HeLa cells were grown and passaged in Dulbecco's modified Eagle's medium with 10% fetal calf serum (14). HL60 cells were grown in RPMI-1640 with 10% fetal calf serum (14).

IFNs and Reagents—Recombinant IFN-α was a generous gift from Hoffmann-La Roche. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma. Phorbol 12,13-dibutyrate (PDBu) and the α isomer were obtained from LC Services Corp.

Quantitation of RNA Using Antisense RNA Probes—RNA was prepared as described by Chirgwin (15). RNAs in all experiments were quantitated by RNase protection assays. Antisense RNA probes were synthesized with SP6 RNA polymerase (Bethesda Research Laboratories). Plasmid pIFN-IND-1 contains a 367-bp EcoRI restriction fragment of exon 2 of the gene (16) subcloned into pGEMI. The size of the protected fragment for pIFN-IND-1 is 367 bp. pIFN-IND-2 (3) contains a 653-bp PstI fragment of the cDNA also in pGEMI. The size of the protected fragment if 653 bp. To determine the relative rate of transcription of ISG-54K, a 32P-labeled antisense RNA probe containing 250 bp upstream of the cap site of the gene and 311 bp downstream was hybridized to 10 μg of total cellular RNA (see Fig. 4, left). The protected 311-bp fragment represents processed nuclear RNA (15) (the processed 78-bp product representing exon 1 was electrophoresed off the gel seen in Fig. 4, right).

Solution Hybridization and RNase Mapping—Five to ten micrograms of total cellular RNA were hybridized with 106 cpm of 32P-labeled antisense RNA probe in 80% (v/v) formamide, 40 mM Pipes, 0.4 M NaCl, and 1 mM EDTA at 56 °C for 16–20 h, and the protected RNA was analyzed as described (17). Unhybridized RNA was digested with RNases (Bethesda Research Laboratories) in 50 mM sodium acetate, 2 mM EDTA, and 100 mM NaCl at 30 °C for 2 h. Protected RNA was extracted with phenol-chloroform, precipitated by the addition of 2.5 M ammonium acetate and ethanol, evaporated, resus-

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The abbreviations used are: IFN, interferon; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol 12,13-dibutyrate; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); bp, base pair.

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were exposed to x-ray film at -70 °C using an intensifying screen.

RESULTS

It has been previously shown that both type I and II IFNs can inhibit phorbol ester-induced expression of c-myc (11, 12). We therefore wanted to know whether phorbol esters might also have an effect on the expression of IFN-IND-1 and -2. Treatment of human fibroblasts with phorbol ester alone did not induce the expression of IFN-IND-1 or -2 RNA (data not shown). However, pretreatment of cells with either PDBu or TPA for 30 min prior to the addition of IFN for 5 h substantially reduced (85-99%) the accumulation of IFN-IND-1 RNA (Fig. 1, lanes 1-5). PDBu or TPA had only minor effects (10-30% inhibition) on the accumulation of IFN-IND-2 RNA in fibroblasts (Fig. 1, lanes 6-10). The fact that both 32P-labeled RNA probes were used in the same hybridization serves as an internal control, assuring that differences in RNA concentrations between samples cannot account for the actions of these phorbol esters (Fig. 1, lanes 6-10). To further confirm the specificity of this phorbol ester activity, the α isomer of PDBu (α-PDBu), which does not activate protein kinase C (18), was tested to determine whether this compound inhibits the expression of either RNA. As shown in Fig. 1, lanes 5 and 9, this isomer did not significantly alter the accumulation of either IFN-IND-1 or -2. Inhibition is maximal at 10⁻⁷ M concentrations of PDBu and 10⁻⁸ M of TPA (data not shown). At submaximal IFN-α concentrations that stimulate the expression of IFN-IND-1 and -2 (10-50 units/ml), the effects of phorbol esters are more pronounced (Fig. 2). Cells were treated with 10, 20, or 50 units/ml of IFN-α in the presence of either PDBu or the inactive isomer α-PDBu. Over this entire concentration range, PDBu significantly inhibited the expression of IFN-IND-1 accumulation. At higher concentrations of IFN-α, there is some expression of IFN-IND-1 in the presence of PDBu (lane 5 versus 6). Again, the phorbol esters had little effect on the expression of IFN-IND-2 at any concentration of IFN.

To determine whether phorbol ester inhibition of IFN-IND-1 RNA accumulation is reversed by inhibitors of protein synthesis, cells were treated with cycloheximide prior to addition of phorbol ester and IFN. As seen in Fig. 3, cycloheximide reverses the inhibition by PDBu or TPA. As noted previously, in human fibroblasts cycloheximide in the presence of IFN causes a superinduction of IFN-IND-1 RNA expression (4), which is why the concentration of IFN-IND-1 in the presence of cycloheximide and phorbol esters is greater than that in the presence of IFN alone. Although there have been several phorbol ester-inducible genes described (19-25) and at least one gene whose transcription is repressed by phorbol esters (21), we are not aware of any phorbol ester-repressible gene for which inhibitors of protein synthesis reverse the actions of these compounds. These experiments suggest, but do not prove, that the synthesis of new proteins is necessary for the actions of phorbol esters. Since cycloheximide is known to cause a superinduction of the expression of these genes in the presence of IFN, it is also possible that this effect is potent enough to negate the actions of phorbol esters. It has not been possible to detect any basal level of transcrip-
tion of IFN-IND-1, so these two possibilities cannot be distinguished.

Phorbol ester inhibition of IFN-induced IFN-IND-1 expression could be regulated by a transcriptional or post-transcriptional mechanism. To determine whether the transcription rate of IFN-IND-1 (ISG-54K) was being inhibited in the presence of phorbol esters, a 52P-labeled single-stranded complementary RNA probe that contains the first exon and part of the first intron of ISG-54K was employed (see Fig. 4). Cells were treated with or without IFN and phorbol esters for 90 min, at which time the transcription of ISG-54K is maximal (4), and total RNA was isolated. The 311-bp RNase-resistant fragment represents unprocessed nuclear RNA, since it contains 78 bp complementary to the first exon and 233 bp complementary to the first intron of ISG-54K. The intensity of this band reflects the relative rate of transcription of this gene at the time cellular RNA is isolated. Fig. 4, right, demonstrates that there is a selective inhibition of the 311-bp product in cells treated with a combination of IFN and PDBu compared with IFN alone or IFN and a-PDBu (compare lanes 2, 4, and 5). In concurrence with the measurements for processed IFN-IND-1 RNA, the effects of phorbol esters were reversed by pretreatment with cycloheximide (Fig. 4, right, lane 6). The magnitude of the phorbol ester inhibition is similar to that seen with the probe that measures only the processed RNA species assayed in Figs. 1 and 2. If phorbol esters were acting to inhibit the expression of cellular RNA by inhibiting the processing of transcripts, this would be reflected in this assay by an increase in the amount of the 311-bp product. Thus, these results confirm that phorbol ester inhibition of IFN-induced ISG-54K occurs primarily via a decrease in the transcriptional activity of this gene.

Previous experiments studying the effects of cycloheximide on expression of IFN-IND-1 and -2 have shown that in certain types of cells (i.e., HL60 and HeLa cells), cycloheximide in the presence of IFN has no effect on either the transcription rate of these genes or the accumulation of the RNAs (26). In other types of cells (i.e., human fibroblasts and melanoma cells) cycloheximide in the presence of IFN causes a super-induction of the transcription of these RNAs (4). Since the inhibitory effects of phorbol esters are reversed by cycloheximide, we wanted to see if phorbol esters were active in other cell lines where cycloheximide does or does not enhance the IFN-induced response of IFN-IND-1. The effects of IFN alone, IFN and cycloheximide, or IFN and PDBu, were examined in human fibroblasts, human melanoma cells, HeLa cells, and HL60 cells with or without prior differentiation with TPA (Table I). Within this limited survey there is a good correlation between cell lines where cycloheximide can augment the accumulation of IFN-IND-1 and those that are also sensitive to phorbol esters, while in cell lines where cycloheximide has no effect, phorbol esters are also without effect. It is also interesting to note that there is a correlation between the relative efficacies of cycloheximide enhancement of IFN-IND-1 expression and PDBu inhibition of this RNA. In melanoma cells, neither cycloheximide nor PDBu is as efficacious as in fibroblasts.

**DISCUSSION**

Although the mediators that initiate the transcriptional activation of IFN-IND-1 and -2 are undefined, experiments presented here and elsewhere (4) suggest that the factors that inactivate the transcription of these gene products require the synthesis of new proteins. At least one pathway that inhibits the transcriptional activation by IFNs of ISG-54K and, presumably, IFN-IND-2 to a much lesser extent (i.e. 10-30%), can be selectively induced by treatment of cells with phorbol esters. This pathway probably requires the synthesis of a new protein(s) and presumably acts through a protein kinase C-dependent mechanism.

Phorbol esters exert their effects only in those types of cells where cycloheximide is present in the presence of IFN enhances the accumulation of IFN-IND-1 RNA (Table I). This correlation might suggest that the regulatory sequences in the gene that allow long term IFN treatment to inhibit IFN-induced transcription of ISG-54K may be the same as those that allow phorbol esters to inhibit the IFN-induced expression of this gene. However, the fact that IFN-IND-2 RNA accumulation and transcriptional activity are enhanced by cycloheximide in fibroblasts to the same extent as IFN-IND-1 (4), yet phorbol esters have a much less dramatic effect on the accumulation of IFN-IND-2 argues against this hypothesis. This question can be resolved by transfection studies in which the DNA elements that are necessary for cycloheximide to prevent inhibition of ISG-54K expression after prolonged IFN exposure are mapped and compared to those DNA sequence...
elements necessary for phorbol ester inhibition of IFN activation. Studies are presently being performed to resolve this issue. Such studies should also permit us to determine if those transcriptional factors that have already been characterized (AP1/AP2, c-fos) (27, 28, 30) and are known to be regulated by phorbol esters are the same as those factors that allow phorbol esters to inhibit IFN-induced expression of ISG-54K. From the results presented here, it appears that these factors may not be involved in this action of phorbol esters, since in human fibroblasts and melanoma cells phorbol esters inhibit the transcriptional activation of ISG-54K by a mechanism that is reversed by an inhibitor of protein synthesis.

The results of the experiments described here not only indicate the complexity of the mechanism by which IFNs regulate the expression of specific genes, they also emphasize the multiple mechanisms by which phorbol esters regulate gene expression. Although phorbol esters have been shown to activate the expression of a number of cellular RNAs and inhibit the expression of other gene products (21), we are unaware of any other gene whose transcriptional activity is inhibited by phorbol esters through a mechanism that can be reversed by inhibition of protein synthesis. The inhibition of IFN-induced ISG-54K expression is relatively rapid, with the maximal inhibition occurring within 30 min of pretreatment by PDBu (data not shown). These compounds cause differentiation and cell attachment of HL60 cells, a fact that emphasizes the multiplicity of independent cellular responses that are regulated by phorbol esters. However, phorbol esters are not able to inhibit IFN-induced gene expression of ISG-54K in HL60 cells under the conditions that induce differentiation and cell attachment (see Table I).

One conclusion from these results is that TPA and PDBu act on at least two independent pathways, both of which may be mediated by different protein kinase Cs (i.e. one involved in HL60 differentiation and one involved in the regulation of IFN-activated genes). This is a reasonable assumption, since it is known that protein kinase Cs are products of a multigene family (29). Alternatively, there may be an initial common protein kinase C-mediated pathway that later diverges. Recent experiments from this laboratory indicate that a single class of IFN receptors can control events that appear to be regulated by separate transducing signals (i.e. inhibition of c-myc expression and activation of these IFN-induced RNAs). It is therefore also possible that phorbol esters may exert multiple regulatory actions through a single species of protein kinase C, and that one of its actions selectively inhibits the expression of an IFN-activated gene as described in this report. The information from these experiments provides a framework for understanding the relationships between the signal transduction mechanisms by which interferons and phorbol esters are able to co-regulate the expression of specific genes.

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