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Interferon-γ-induced transcriptional activation is mediated by protein kinase C

(macrophages/protein kinase C inhibitors/major histocompatibility complex genes)

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ABSTRACT **Interferon-γ (IFN-γ) regulates a variety of biological functions and is the principal lymphokine known to activate macrophages. In studies of the molecular mechanisms by which these cells are regulated by IFN-γ, the transcriptional activation of an IFN-γ-inducible gene, γ.1, in human macrophage-like cell lines was examined. Transcription of this gene is rapidly induced by 0.1–1 unit of IFN-γ. In addition, γ.1 transcription is efficiently induced by phorbol 12-myristate 13-acetate, which is known to activate protein kinase C (PKC). Both stimulators of γ.1 transcription induce the translocation of PKC from the cytosol of a membrane fraction. Two selective inhibitors of PKC, H7 and sphingosine, suppressed not only the induction of γ.1 mRNA but transcription of HLA-DR by IFN-γ as well. These findings establish that PKC plays a significant role in the signal transduction pathway leading to transcriptional activation of some IFN-γ-regulated genes of cells of the mononuclear phagocyte lineage.**

Interferon-γ (IFN-γ) is the most potent known lymphokine for activating cells of the mononuclear phagocyte lineage (1). It augments expression of cell-surface molecules, including class I (2) and class II (3) antigens of the major histocompatibility complex and Fc receptors for IgG (4), enhances cytoxicity against tumor target cells *in vitro* (5), and induces both oxygen-dependent and -independent antimicrobial states (6, 7). IFN-γ exerts its action by binding to a unique receptor distinct from that for IFN-α/-β (8) and triggering processes requiring *de novo* RNA and protein synthesis. The mechanism(s) of signal transduction after binding of IFN-γ or, indeed, any species of IFN, to its receptor is at present unknown.

Protein kinase C (PKC) is known to be critically involved in signal transduction in many different types of cells in response to a wide variety of biologically active substances (reviewed in ref. 9). In previous studies on murine macrophage cell clones, we observed that stimulation of the respiratory burst and production of the superoxide anion and hydrogen peroxide by phorbol esters was preceded by specific phosphorylation of at least two proteins by an enzyme with the characteristics of PKC (10).

We have recently identified a human macrophage-specific gene, γ.1, which is rapidly induced in cells of the mononuclear phagocyte lineage, but not in B cells, T cells, or fibroblasts after stimulation with IFN-γ (11). The induction of this gene appears to be entirely due to transcriptional activation and it is 100 times more responsive to IFN-γ than to IFN-α. Because many specific macrophage functions are known to be regulated by both IFN-γ and phorbol esters, we explored the possibility that at least some IFN-γ-induced signals for transcriptional activation might be mediated by PKC.

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MATERIALS AND METHODS

Transcriptional Stimulators. Recombinant IFN-γ, generously provided by Genentech (South San Francisco, CA), had a specific antiviral titer of 2–4 × 10^7 units/mg against EMC virus in human lung carcinoma A549 cells. Phorbol 12-myristate 13-acetate (PMA) was obtained from LC Services (Woburn, MA).

PKC Inhibitors. H7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride] was obtained from Seikagaku America (St. Petersburg, FL). Sphingosine was purchased from Sigma. They were added to cells 3 min prior to addition of the stimulators.

Assays of mRNA Levels. U937 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum/streptomycin (100 μg/ml)/penicillin (100 units/ml)/nonessential amino acids (100 μM). THP-1 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum/streptomycin (100 μg/ml)/penicillin (100 units/ml). The cells (5 × 10^5 cells per ml) were treated with 100 units of IFN-γ or PMA (10 ng/ml) for various times, and RNA was isolated by the guanidine isothiocyanate method (12). In the inhibition experiments with sphingosine, the cells were maintained at 2 × 10^5 cells per ml. Total RNA (25 μg per lane) was electrophoresed on 1.2% agarose gels containing 1 M formaldehyde and transferred to nylon filters. The blots were hybridized with nick-translated cDNA inserts (specific activity, 2–3 × 10^8 cpm/μg at 2 × 10^6 cpm/ml) in 50% formamide/5× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.2% $NaDodSO_4$ denatured salmon sperm DNA (100 μg/ml) at 42°C. The blots were washed for 1 hr at room temperature, 1 hr at 50°C in 2× SSPE/0.2% $NaDodSO_4$, and then subjected to autoradiography with Kodak XAR film. Nuclear run-on assays of transcription were performed essentially as described (23).

Translocation of PKC. The cell pellet of 5 × 10^7 U937 cells was resuspended in 4 ml of ice-cold buffer A [5 mM Tris·HCl, pH 7.5/5 mM NaCl/0.5 mM $MgCl_2$/1 mM dithiothreitol/1× protease inhibitor mixture (10 μg of pepstatin per ml/25 μg of aprotinin per ml/10 μg of leupeptin per ml/10 μg of soybean trypsin inhibitor per ml)]. After homogenization and centrifugation at 100,000 × *g* for 1 hr, the supernates, designated as cytosol fraction, were kept on ice while the pellets were extracted in ice-cold buffer A containing 1% Triton X-100, recentrifuged at 100,000 × *g* for 1 hr, the supernate of which was designated as the membrane fraction. Samples of both cytosolic and particulate fractions were applied to a DEAE-cellulose column equilibrated with buffer B (20 mM Tris·HCl, pH 7.5/2 mM EDTA/5 mM EGTA/2 mM dithiothreitol).

Abbreviations: IFN-γ, interferon-γ; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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After the columns were washed with 10 vol of buffer B, the PKC was eluted with buffer B containing 50 mM NaCl. The samples were kept at 4°C in the presence of 1× protease inhibitors, and PKC activity was assayed within 24 hr (13).

RESULTS

Phorbol esters, such as PMA, that activate PKC do so by inducing a translocation of the enzyme from cytosol to membrane. This is true in the U937 macrophage cell line as well, and the kinetics of PKC translocation from cytosol to the membrane fraction after treatment of the cells with IFN-γ are shown in Fig. 1*A*. PKC translocation to the particulate fraction is detected as early as 1 min after exposure to IFN-γ, peaks at 10 min, and then gradually returns to unstimulated levels by 6 hr. As little as 0.1 unit of IFN-γ per ml can induce detectable PKC translocation, with maximum translocation requiring 10–100 units of IFN-γ per ml (Fig. 1*B*). Unlike PMA, which causes complete translocation of PKC to the particulate fraction (data not shown), IFN-γ caused only partial translocation of PKC. Under optimal conditions of 30 units of IFN-γ per ml for 10 min, approximately half of the cytosolic PKC was translocated to the particulate fraction. It is clear that IFN-γ induces a marked redistribution of PKC activity in these cells.

In studies on differential screening for genes of macrophages induced by IFN-γ (X.-d.F., G. Stark, and B.R.B., unpublished results), we obtained a cDNA clone designated γ.1, the mRNA of which appears to be entirely produced by transcriptional activation, as determined by nuclear run-on assays (Fig. 2*A Inset*). RNA blot analysis of total cellular RNA was consequently used to measure the degree of transcriptional activation. Fig. 2*B* shows the dose dependence of induction of γ.1 mRNA after 3 hr of treatment with IFN-γ. A detectable increase in γ.1 mRNA was seen at 0.1 unit/ml with maximal induction at 30 units of IFN-γ per ml. This dose range corresponds remarkably well to that which induced translocation of PKC to the membrane fraction (Fig. 1).

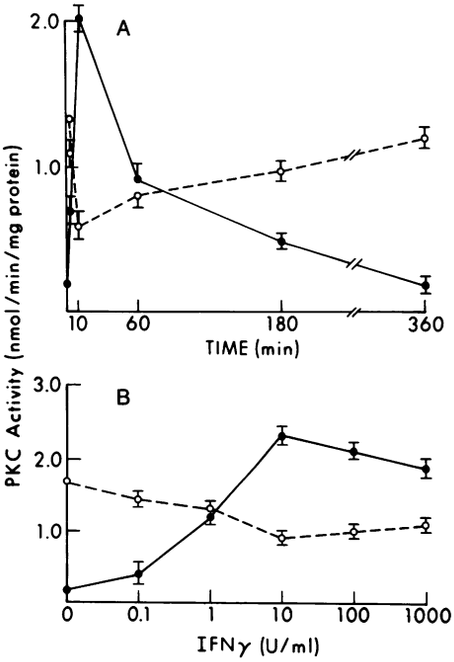

FIG. 1. PKC translocation in response to IFN-γ. (*A*) Kinetics of PKC translocation in response to IFN-γ at 100 units/ml. (*B*) Dose dependence of PKC translocation measured 10 min after IFN-γ stimulation. ●, PKC activity in the particulate fraction; ○, PKC activity in the cytosol fraction.

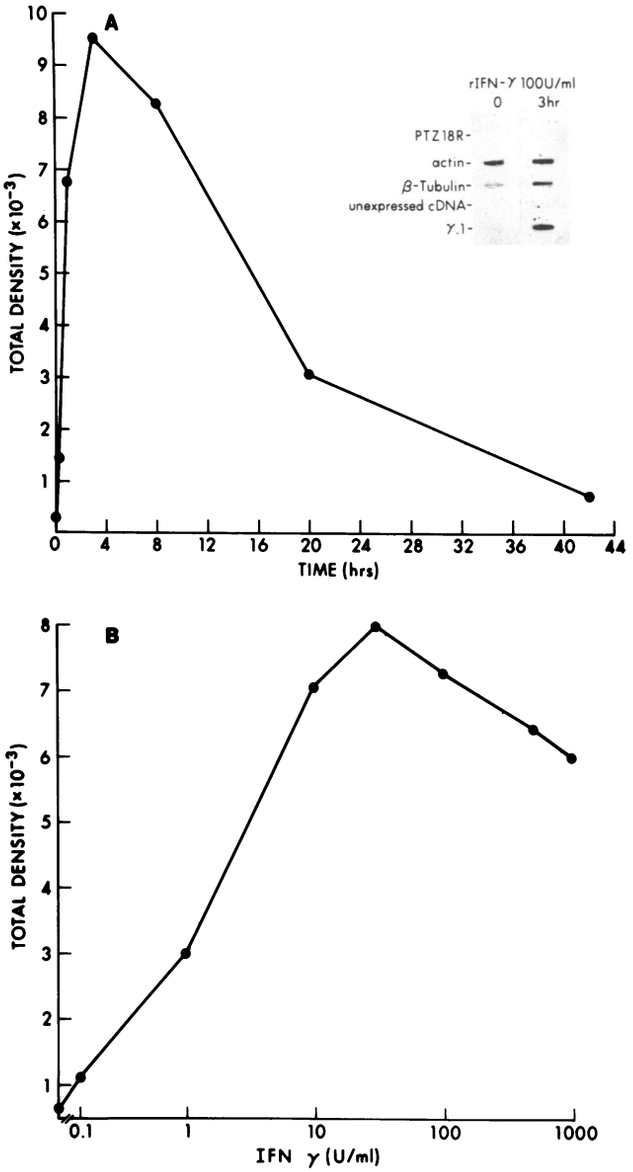

FIG. 2. Kinetics and dose–response of γ.1 mRNA accumulation in response to IFN-γ. (*A*) Densitometric scanning of an autoradiogram of an RNA blot analysis of the time course of accumulation of γ.1 mRNA in U937 cells treated with IFN-γ (100 units/ml). (*Inset*) Nuclear run-on assay of γ.1 transcription. rIFN-γ, recombinant IFN-γ. (*B*) Analysis of γ.1 mRNA accumulation in response to treatment for 3 hr with various concentrations of IFN-γ.

1). The kinetics of γ.1 induction by IFN-γ, shown in Fig. 2*A*, indicate that mRNA accumulation appears at 20 min, reaches maximum levels at 3 hr, and then slowly declines to basal level in 24–48 hr. Comparison of the kinetics of activation of γ.1 by IFN-γ with those on PKC translocation (Fig. 1*A*) indicates clearly that PKC translocation precedes the accumulation of γ.1 mRNA.

To analyze whether the correlation between activation of γ.1 transcription and PKC translocation is likely to be causal or adventitious, the effects of two known selective inhibitors of PKC on transcription of the γ.1 gene were examined. H7 is a potent inhibitor of PKC (and to some extent other kinases), and has been found to inhibit specific functions of viable cells (14–16). Sphingosine is a more specific inhibitor of PKC that has similarly been found to inhibit certain cell functions and differentiation processes (17–20). Both compounds inhibited the transcriptional activation of the γ.*1* gene induced by IFN-γ and by PMA in a dose-dependent fashion.

As shown in Fig. 3*A*, the induction of γ.1 mRNA was partially inhibited by 10 μM H7, with complete inhibition produced at 30 μM, similar to the dose we found to inhibit PKC-mediated phosphorylation of histone type IIIS (data not shown). Sphingosine inhibited γ.1 mRNA induction at essentially the same concentration range (Fig. 3*B*). It should be noted that there is a variable but low level of constitutive expression of γ.1 mRNA in U937 cells.

To learn whether other protein kinases in addition to PKC, particularly cAMP- or cGMP-dependent kinases, were similarly capable of stimulating the induction of γ.1 mRNA, we examined the effects of: (*i*) cholera toxin, an activator of adenylate cyclase, and (*ii*) 8-bromo-cGMP, an activator of cGMP-dependent protein kinase (Fig. 3*C*). 8-Bromo-cGMP had no effect in this system. Significantly, however, stimulation of adenylate cyclase, the product of which is the activator of cAMP-dependent kinase, consistently inhibited the induction of γ.1 mRNA (Fig. 3*C*). The same effect was obtained by treatment with 8-bromo-cAMP (data not shown).

The question naturally arises whether PKC is involved in transcription only of the γ.1 gene or is more generally implicated in transcriptional activation of other genes by IFN-γ. Genes for the major histocompatibility complex, which play a pivotal role in the immune response, are well known to be regulated by IFN-γ. Because U937 cells do not express major histocompatibility complex class II antigens due to DNA methylation (21), we examined the effects of PKC inhibitors on induction of HLA-DR mRNA in another human macrophage cell line, THP1 (Fig. 4*A*) and on U937 cells treated with azacytidine (data not shown). H7 markedly inhibited the induction of HLA-DR mRNA by IFN-γ in both cell lines. In the same cells, transcription of another IFN-γ-inducible gene, *IP-30* (22), was similarly inhibited by the PKC inhibitor, H7 (Fig. 4*B*). *IP-10* was reported to be transcriptionally activated by IFN-γ (23). However, as shown in Fig. 3*B*, IP-10 mRNA was not induced by PMA nor was its induction by IFN-γ inhibited by sphingosine. These results indicate that PKC appears to be involved in the signal transduction pathway leading to transcription of many, but not all, IFN-γ-induced genes.

DISCUSSION

The present study provides three lines of evidence that PKC is involved in mediating the induction of γ.1 mRNA by IFN-γ: (*i*) PMA, whose principal action in cells is activation of PKC, effectively induces γ.1 expression; (*ii*) intracellular translocation of PKC from cytosol to particulate is induced by IFN-γ treatment prior to and at the same concentrations required for induction of γ.1 mRNA accumulation; and (*iii*) induction of γ.1 mRNA synthesis by IFN-γ or by PMA is blocked by inhibitors of PKC.

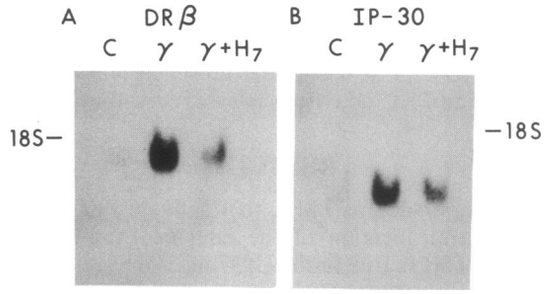

FIG. 4. Inhibitory effect of H7 (20 μM) on induction of HLA-DR and IP-30 mRNA in THP-1 cells by IFN-γ (100 units/ml). The RNAs were harvested 24 hr after IFN-γ addition. C, control.

A number of genes, however, are known to be transcriptionally induced by PMA, including the cellular protooncogenes c-*fos* (24), c-*myc* (25), c-*sis* (26), as well as genes encoding collagenase, stromelysin (27), and metallothionein IIa (28). Some of these genes can also be activated by hormonal stimulation, and the metallothionein II gene is induced by IFN as well (29). All these PMA-inducible genes contain a conserved cis-element, which is recognized by a PMA-modulated trans-acting factor, AP-1 (30). In mouse macrophages, it has been found that PKC plays a role in the transduction of signals resulting from the binding of colony-stimulating factor 1 to its receptor, leading to the rapid induction of the c-*fos* gene (31). The c-*fos* gene can also be induced by epidermal growth factor and PMA in HeLa cells, and the same upstream enhancer is responsible for responses to both stimuli (32).

The regulation of transcriptional activation of cellular genes by IFNs is the subject of intense current study (33–35), but only a few genes induced preferentially by IFN-γ have been studied (36, 37). Of particular interest are recent findings (38) indicating that the *H-2D*^{d} gene contains several transcription factor AP-1 binding sites and an IFN response sequence. Although it is not known whether the binding of nuclear factors from MPC11 cells to the IFN response

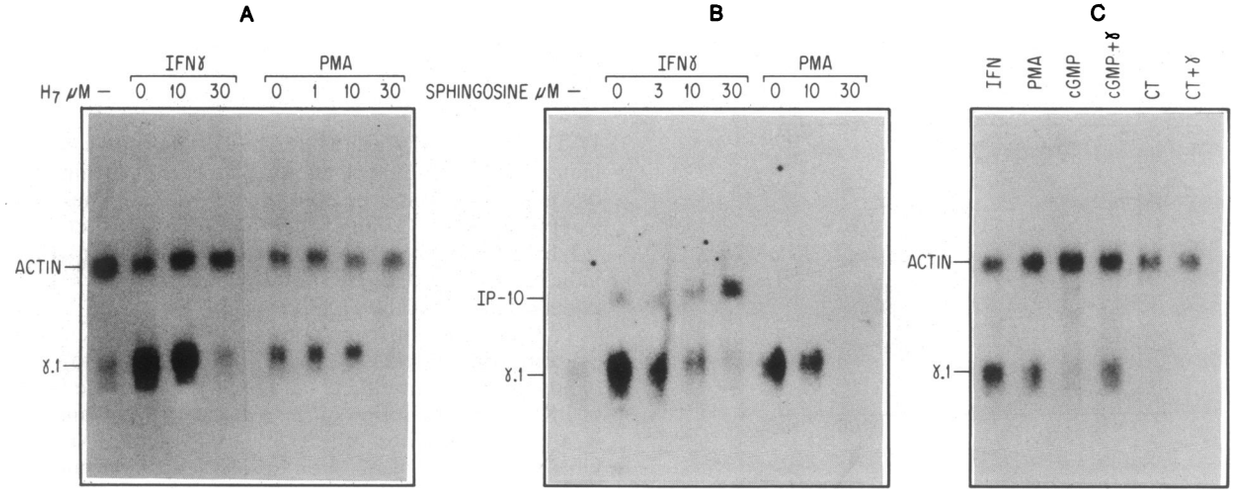

FIG. 3. Effects of inhibitors of PKC and activators of protein kinases A and G on the induction of γ.1 mRNA. (*A*) Inhibitory effect of different concentrations of H7 on induction of γ.1 by IFN-γ (10 units/ml) and PMA (10 ng/ml) in U937 cells. (*B*) Effect of different concentrations of sphingosine on the induction of γ.1 and IP-10 mRNAs (see text). (*C*) Effect of cholera toxin (CT; 100 ng/ml) and 8-bromo-cGMP (40 μM) on the induction of γ.1 mRNA. The RNAs were harvested 3 hr after IFN-γ addition.

sequence and AP-1 sites is involved in transcriptional activation induced by IFN-γ, IFN response sequences are conserved in several IFN-inducible genes, including the IFN genes themselves (29, 38, 39). The finding that IFN response sequence and AP-1 binding activities were present in extracts of MPC11 cells not treated with IFN is consistent with the possibility that IFN-induced regulation of transcription could result from modification or activation of latent specific positively acting or repressive DNA binding factors, possibly by PKC-mediated phosphorylation.

We hope that elucidation of the genomic structure of the *γ.1* gene will provide insights at the molecular level into the mechanisms of IFN-γ-specific transcriptional activation in macrophages and the regulatory role of protein kinases in that process. Of interest, our preliminary data on the 5′ genomic sequence of the *γ.1* gene suggest the presence of two putative AP-1 binding sites and a putative IFN response sequence. Since IFN-γ is the most potent known biological inducer of macrophage activation, the present findings that transcriptional regulation of several IFN-γ-induced genes is mediated through PKC may suggest a possible approach to pharmacological intervention in clinical situations involving immunological tissue damage.

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