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Evidence for involvement of protein kinase C in the cellular response to interferon α

(signal transduction/stimulated gene expression/DNA-binding factors/antiviral activity)

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ABSTRACT **Phospholipid/Ca^{2+}-dependent protein kinase (protein kinase C; PKC) appears to be involved in the signal-transduction pathway mediated by human leukocyte interferon (IFN) in HeLa cells. IFN treatment results in a rapid increase in [^{3}H]phorbol 12,13-dibutyrate binding to intact cells, indicating an activation of PKC. In addition, inhibitors of PKC (H7 and staurosporine) block the induction of antiviral activity by IFN against vesicular stomatitis virus. PKC inhibitors also block the accumulation of IFN-stimulated mRNAs in the cytoplasm of HeLa cells and suppress the transcriptional induction of IFN-stimulated genes. Activation of IFN-stimulated genes is mediated through a DNA response element that is necessary and sufficient for the transcriptional response to IFN. IFN treatment induces the appearance of several DNA-binding factors that specifically recognize the response element, and the appearance of these factors is suppressed by PKC inhibitors. This observation provides evidence that PKC activity is involved during IFN-stimulated signal transduction. Although activation of PKC appears to be required for the response to IFN, agonists of PKC activity alone do not turn on expression of IFN-stimulated genes.**

Type I interferons (IFN α/β) induce a variety of physiological responses that include antiviral activity and inhibition of cellular proliferation (1, 2). IFNs elicit these effects by binding to specific cell surface receptors and transducing a signal to the nucleus that results in the transcriptional stimulation of a responsive set of genes. These genes possess an IFN-stimulated response element (ISRE) that functions as an inducible enhancer and is itself sufficient for transcriptional activation by IFN (3–8). Several DNA binding factors that recognize the ISRE have been identified and characterized (4–9). The signal-transduction pathway of IFN appears to involve the activation of a latent transcription factor resident in the cytoplasm and its subsequent translocation to the nucleus (10, 11). However, the nature of the receptor-mediated signals, the mechanism of activation of the latent cytoplasmic factor, and the influence of IFN-induced DNA binding factors on transcriptional regulation remain to be determined.

Since protein phosphorylation plays a major role in a variety of signal-transduction pathways, the possible involvement of protein kinases in the expression of IFN-stimulated genes (ISGs) was investigated. In the present study, inhibitors of the protein kinase C (PKC) enzymes were found to block both ISG transcriptional activation and mRNA cytoplasmic accumulation in HeLa cells. The PKC family of enzymes can be activated by receptor-mediated production of diacylglycerol or, alternatively, by tumor-promoting phorbol esters (12). Activation of PKC correlates with increased binding of the phorbol ester phorbol 12,13-dibutyrate (PDBu) to intact cells, which was found to occur within minutes of IFN-α treatment of HeLa cells. However, activation of PKC enzymes by phorbol esters in the absence of IFN is not sufficient to activate the transcription of ISGs.

Previous studies have shown that IFN-α treatment induces two ISRE-binding factors in the nuclei of cells (4–9). These induced DNA-binding factors are believed to play a role in the transcriptional regulation of the ISGs. The protein PKC inhibitor staurosporine was found to abrogate the appearance of the IFN-α-induced binding factors. The ability of staurosporine to block activation of these DNA-binding factors correlates with a block in transcriptional stimulation of the ISGs.

MATERIALS AND METHODS

Cell Cultures. HeLa S3 cell cultures were maintained as monolayers in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal bovine serum. Recombinant human IFN-αA was generously provided by Hoffmann–LaRoche and used at 1000 units/ml. The protein kinase inhibitors, H7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride], H8 {*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride}, and HA1004 [*N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride], were obtained from Seikagaka America (Saint Petersburg, FL). Staurosporine was obtained from Kamiya Biomedical (Thousand Oaks, CA). [^{3}H]PDBu (19 Ci/mmol; 1 Ci = 37 GBq) was obtained from DuPont/NEN.

mRNA Analyses. The levels of specific cytoplasmic mRNAs were measured by solution hybridization with antisense RNA probes as described (3). The ISG15 probe was generated from a genomic *Taq* I second exon fragment (3, 13); the ISG54 probe was from a genomic *Eco*RI second exon fragment (14); the γ-actin probe was from a murine cDNA clone (15). Nuclear run-on transcription assays were performed with specific DNA test plasmids of ISG15, ISG54, chicken β-actin, and pBR322 as described (3, 16).

DNA-Protein Binding. Nuclear cell extracts were prepared and employed in gel-retardation electrophoresis to analyze specific protein–DNA complexes (5, 8). An ISRE-containing *Eco*RI fragment of ISG15-TK-109 (3) was employed as the specific DNA probe.

Antiviral Assay. HeLa cells were treated with various agents for 30 min prior to the addition of IFN-α (250 units/ml). After overnight incubation with IFN, the cells were washed with saline and infected with vesicular stomatitis virus at a multiplicity of infection of 0.1 plaque-forming unit per cell. After viral adsorption, cells were washed and

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Abbreviations: IFN, interferon; ISRE, IFN-stimulated response element; ISG, IFN-stimulated gene; ISGF, IFN-stimulated gene factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate.

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incubated with fresh medium. Twenty-four hours later the virus was harvested after a freeze-thaw cycle and yield was assayed on mouse L cells.

[^{3}H]PDBu Binding Assay. Binding of [^{3}H]PDBu was determined as described (17, 18).

RESULTS

Influence of Protein Kinase Inhibitors on the IFN-Induced Antiviral Response. One of the main physiological responses to IFN is the establishment of resistance to viral infection. To determine whether pathways involving known second messengers were necessary for the development of the antiviral state induced by IFN, HeLa cells were pretreated with agents that affect some of these pathways. The effect of these agents on the replication of vesicular stomatitis virus was determined in IFN-α-treated cells. Agents that affect the intracellular level of cAMP or cGMP (dibutyryl cAMP or cGMP, forskolin, pertussis toxin, or cholera toxin) did not affect the induction of the antiviral state in these cells (data not shown). In contrast, pretreatment of cells with inhibitors of PKC blocked the induction of antiviral activity by IFN-α.

Isoquinolinesulfonamide derivatives have been shown to inhibit the action of distinct protein kinases differentially (19). Among these derivatives H7 is the most potent and selective PKC inhibitor. H8 is a more effective inhibitor of cAMP- and cGMP-dependent protein kinases, and HA1004 is a weak PKC inhibitor and, therefore, the best control for H7 action. These inhibitors were employed to assess the involvement of protein kinases in the induction of antiviral activity by IFN-α (Table 1). Viability of the cells was >90% after 24 hr in the presence of the various agents tested. Any growth inhibition noted was reversible upon removal of the agent. H8 and HA1004 did not appear to influence the antiviral activity of IFN-α in HeLa cells. However, the PKC inhibitor H7 dramatically impaired the antiviral response to IFN-α at relatively low doses (ID_{50} = 10 μM). Staurosporine, a more potent inhibitor of PKC, was also employed in these studies (20). Staurosporine blocked the IFN-mediated response even when used at low concentrations (ID_{50} = 10 nM). In addition, pretreatment of cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA), which down-regulates PKC (21), blocked induction of the antiviral activity of IFN, although PMA alone is not antiviral. Depletion of PKC activity by inhibitors or down-regulation of PKC with PMA suggests that an active PKC system is necessary for the development of the antiviral state in IFN-α-treated cells.

Table 1. Effect of protein kinase inhibitors on vesicular stomatitis virus replication in IFN-α-treated HeLa cells

Inhibitor	μM	Virus titer, plaque-forming units $\times 10^{-3}$/ml		Reduction in virus yield, fold
		− IFN-α	+ IFN-α	
None	0	39,000	13	3000
H7	10	38,000	30	1266
	30	21,000	3100	7
	50	11,000	4900	2
H8	50	35,000	14	2500
HA1004	50	20,000	8	2500
Staurosporine	0.01	37,000	33	1120
	0.03	33,000	250	132
	0.1	25,000	2600	10
	0.3	5,100	1700	3
PMA	0.1	23,000	930	25

Data were compiled from the average of three experiments with a standard error of <15%.

Stimulatory Effect of IFN-α on PDBu Binding. Since the response to IFN-α seemed to require functional PKC activity, this suggested that IFN-α might activate PKC. The activation of PKC activity has been correlated with a translocation of PKC from the cytosol to cellular membranes, and a subsequent increase in the binding of [^{3}H]PDBu to intact cells (17, 18). Therefore, [^{3}H]PDBu binding was used to determine the effect of IFN-α treatment on the activation of PKC. Addition of IFN-α for 15 min was found to increase the binding of PDBu to intact HeLa cells. Scatchard analysis of the binding of PDBu indicated a resultant 93% increase in the number of high-affinity PDBu binding sites after IFN-α treatment (0.54–1.04 pM bound per 10^6 cells) without an effect of IFN-α on the affinity of the receptors for PDBu (50 nM) (Fig. 1). The increase in PDBu binding sites induced by IFN-α likely represents a redistribution of PKC from a loosely associated component that binds PDBu at low affinity to a more tightly associated form.

Inhibitors of PKC Suppress IFN-Stimulated Gene Expression. The requirement of functional PKC for the IFN-mediated antiviral response suggested that blockage of PKC would impair the expression of ISGs. For this reason kinase inhibitors were tested for their ability to influence the appearance of cytoplasmic ISG mRNAs.

HeLa cells were treated with various concentrations of the protein kinase inhibitors H7 or HA1004 for 10–15 min prior to the addition of IFN-α. After 2 hr of IFN treatment, cytoplasmic mRNA was isolated and hybridized to specific antisense RNA probes, and the ribonuclease-resistant products were displayed on sequencing gels (Fig. 2). The expression of two ISGs, ISG15 and ISG54, was assayed in IFN-α-treated cells (3, 13, 14). At 30 μM, H7 was found to abrogate the induction of ISG15 and ISG54 mRNAs (compare lanes 2 and 7). The accumulation of ISG15 mRNA was unaffected by pretreatment with HA1004 even at 70 μM (lane 5). Although the appearance of ISG54 mRNA was slightly reduced with HA1004, the extent of inhibition was not comparable to that produced by H7. The slight effect of HA1004 on ISG54 expression ensured that this inhibitor was active in HeLa cells and that it was appropriate to use as a control. To ascertain whether H7 led to generalized instability of mRNA,

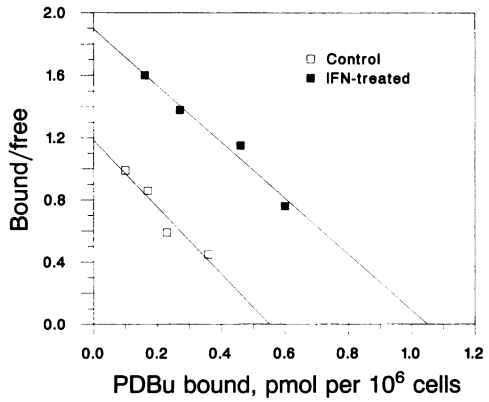

FIG. 1. Effect of IFN-α on [^{3}H]PDBu binding to HeLa cells. HeLa cells were seeded into individual wells of 24-well multiwell plates. After 1 day the medium was removed and the cells were refed with 0.25 ml of medium containing [^{3}H]PDBu (20–80 nM) and IFN-α (1000 units/ml). Control cultures received no IFN. After a 15-min incubation, the cells were washed three times with saline and solubilized in 0.5% SDS/1 mM EDTA/0.1 M NaOH, and radioactivity was quantitated by liquid spectrometry. Specific PDBu binding represents the difference between binding in the absence and presence of 10 μM unlabeled PDBu. The data from four experiments performed in duplicate were averaged (standard error, <20%), corrected for cell numbers, expressed as specific PDBu binding to cells [(Bound/free) $\times 10^{-2}$], and plotted according to the method of Scatchard.

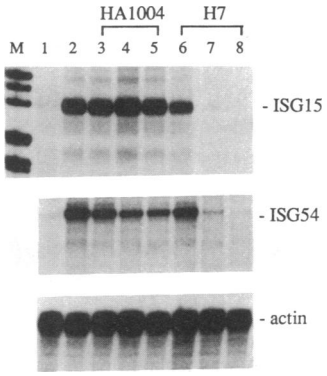

FIG. 2. Effect of protein kinase inhibitors on cytoplasmic accumulation of ISG mRNAs. HeLa cells were untreated (lane 1) or treated with IFN-α (1000 units/ml) for 2 hr in the absence (lane 2) or presence of protein kinase inhibitors. HA1004 (10 μM, 30 μM, and 70 μM, lanes 3–5, respectively) or H7 (10 μM, 30 μM, and 70 μM, lanes 6–8, respectively) were added to cell cultures 15 min prior to IFN. Cytoplasmic mRNA was hybridized to the specific antisense probes for ISG15, ISG54, or γ-actin, as indicated, and analyzed by gel electrophoresis.

the levels of γ-actin mRNA were analyzed and were found to remain constant during the various treatments.

To examine whether inhibition of PKC resulted in the cytoplasmic instability of ISG mRNAs, the levels of ISG mRNAs were determined by adding H7 after IFN-α treatment of cultures. H7 had little or no effect on the accumulation of ISG mRNAs when it was added after ISG mRNA induction (data not shown).

Since H7 blocked the appearance of cytoplasmic ISG mRNAs but had little effect on mRNA stability, nuclear run-on transcription assays were performed to determine whether PKC activity was required for transcriptional activation by IFN-α. Nuclei were isolated from untreated cells or cells treated with IFN-α for 10, 30 or 90 min in the presence of protein kinase inhibitors. Nascent RNA was radiolabeled *in vitro* by incubating the nuclei in the presence of [α-^{32}P]UTP for 10 min and was then hybridized to DNA samples fixed to nitrocellulose (Fig. 3). The addition of HA1004 (30 μM) prior to a 10-min treatment with IFN did not block the activation of the ISGs by IFN. In contrast, the PKC inhibitor H7 (30 μM) did repress IFN-stimulated transcription of both ISG15 and ISG54. After 10 min of IFN treatment, transcription was reduced by $\approx$80% and by 60% after 30 min with respect to transcription of the control actin gene (as determined by densitometric scanning of the autoradiogram within a linear range of exposure). Although 30 μM H7 reduced the transcriptional induction of the ISGs, it did not have the dramatic effect seen on the accumulation of steady-state ISG mRNA. It appears that this kinase inhibitor blocks the pathway leading to transcriptional activation as well as the pathway leading to the posttranscriptional appearance of ISG mRNAs in the cytoplasm of HeLa cells.

H7 did not inhibit the transcriptional induction of a control gene (*hsp70*) by heat shock. Although the cytoplasmic appearance of hsp70 mRNA was impaired by H7, nuclear run-on assays showed no effect of H7 on transcriptional induction (data not shown).

The more potent PKC inhibitor staurosporine was also tested for its ability to block ISG expression. The cytoplasmic appearance of ISG15 mRNA was analyzed in the presence of increasing concentrations of staurosporine (Fig. 4 *Left*). Complete abrogation of the IFN-α-induced appearance of ISG15 mRNA was observed with 1 μM staurosporine (lane 5). The inhibitory activity of staurosporine appears to result solely from its effect on transcriptional activation by IFN-α. Nuclear run-on experiments performed in the presence of

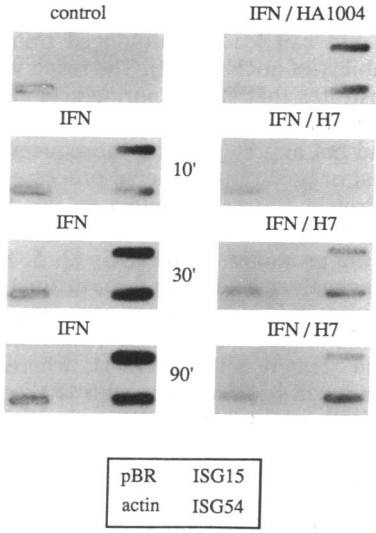

FIG. 3. Transcriptional analysis of ISGs in the presence of protein kinase inhibitors. Nuclear run-on assays were performed, as indicated, with control cells, cells treated with IFN-α for 10, 30, or 90 min, cells treated with IFN-α for 10 min in the presence of 30 μM HA1004, or cells treated with IFN-α for 10, 30, or 90 min in the presence of 30 μM H7. The position of test DNAs on the nitrocellulose filter is shown at the bottom of the figure.

staurosporine demonstrate a dose-dependent inhibition of ISG transcription. Transcription is reduced at 10 nM by 25%, at 100 nM by 45%, and at 1 μM by 100% with respect to actin gene transcription. This inhibition correlates with the impairment of ISG mRNA appearance in the cytoplasm (Fig. 4 *Right*). The effect of staurosporine was not reversed in the presence of the protein synthesis inhibitor cycloheximide.

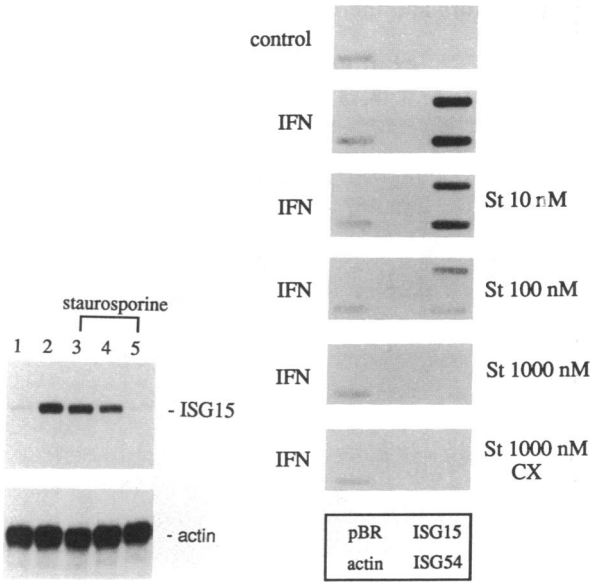

FIG. 4. Suppression of IFN-induced gene expression by staurosporine. (*Left*) Cytoplasmic levels of ISG15 and actin mRNAs. Cells were untreated (lane 1) or treated with IFN-α 1000 units/ml for 2 hr in the absence (lane 2) or presence of staurosporine (10 nM, 100 nM, or 1000 nM, lanes 3–5, respectively). mRNA analyses were performed by hybridization to specific antisense probes and gel electrophoresis. (*Right*) Transcriptional induction by IFN-α. Nuclear run-on experiments were performed with control cells or IFN-treated cells (30 min) in the absence or presence of increasing concentrations of staurosporine (St) as noted in the figure. The effect of cycloheximide (CX) is shown as indicated. The pattern of test DNAs is shown at the bottom of the figure.

Protein synthesis inhibitors do not impair IFN-α-induced transcription but can lead to prolonged transcription and increased stability of mRNAs (22). The result with cycloheximide demonstrates that staurosporine acts at a step in the signal-transduction pathway that leads to ISG transcriptional activation and not at a step in the subsequent regulation of transcriptional or posttranscriptional processes.

Effect of Protein Kinase Inhibitors on the Appearance of ISRE-Binding Factors. The ISRE functions as an inducible enhancer in the promoter of the ISGs (3, 5, 8). This DNA sequence is recognized by at least three distinct DNA binding proteins, designated IFN-stimulated gene factors (ISGFs) (5, 8). One of the factors is present constitutively in both untreated and treated cells (ISGF-1), whereas two other factors are induced by treatment with IFN-α (ISGF-2 and ISGF-3). ISGF-2, whose appearance is dependent upon new protein synthesis (5), is identical to interferon regulatory factor-1 (IRF-1), a DNA binding factor involved in the regulation of the IFN-β gene (23, 24). In contrast to ISGF-2, the appearance of ISGF-3 does not require new protein synthesis and appears in the cytoplasm within minutes of IFN treatment before its appearance in the nucleus (5, 10, 11). Since IFN-α stimulation does not require ongoing protein synthesis (22), ISGF-3 is likely to be a latent positive regulator of transcription. The finding that PKC inhibitors impair the ability of IFN-α to activate transcription of the ISGs suggests that they might function by blocking the expression of the IFN-α-induced ISGFs.

Gel-retardation analyses were employed to assess the appearance of these DNA binding factors. Cell extracts were prepared from untreated or IFN-α-treated cells in the presence of H7, HA1004, or staurosporine. Protein extracts were incubated with a radiolabeled ISRE probe and specific protein–DNA complexes were analyzed by gel-mobility-shift electrophoresis (Fig. 5). The appearance of the constitutive ISRE-binding factor, ISGF-1, was not affected by the protein kinase inhibitors and thereby served as an internal control.

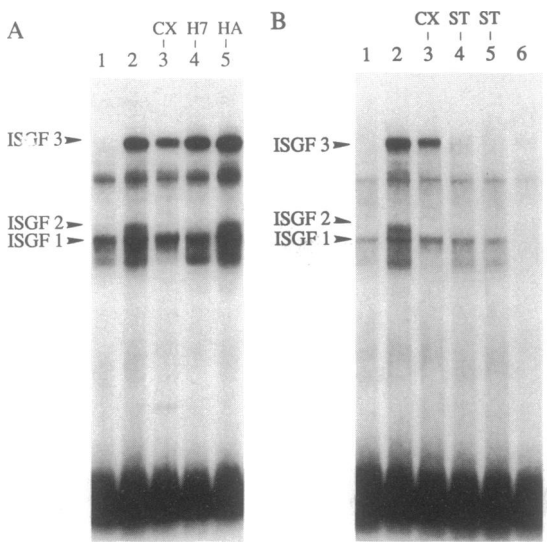

FIG. 5. Effect of protein kinase inhibitors on the appearance of ISRE-binding factors. (*A*) Gel-retardation analyses were performed with nuclear extracts isolated from untreated cells (lane 1), cells treated with IFN-α for 2 hr (lane 2), cells treated with IFN-α in the presence of cycloheximide (CX; 50 μg/ml; lane 3), 30 μM H7 (lane 4), or 30 μM HA1004 (lane 5). (*B*) Gel-retardation analyses of extracts prepared from control cells (lane 1), IFN-α-treated cells (lane 2), IFN-α-treated cells in the presence of cycloheximide (CX; lane 3), 200 nM staurosporine (ST; lane 4), or 1 μM staurosporine (lane 5) are shown. Competitor ISG15 DNA containing the ISRE (100-fold excess) was included in the binding reaction of IFN-α-treated cell extracts to demonstrate ISGF specificity (lane 6).

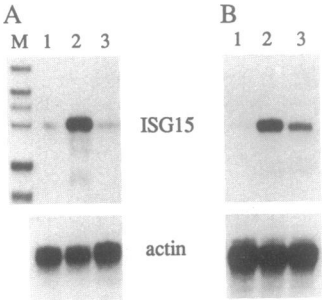

FIG. 6. Effect of phorbol esters on expression of ISG15 mRNA. (*A*) Cytoplasmic mRNA was isolated from control cells (lane 1) or cells treated with IFN-α (1000 units/ml for 2 hr; lane 2) or PDBu (100 nM for 2 hr; lane 3). Specific ISG15 or actin transcripts were quantitated by hybridization to antisense probes and analyzed by gel electrophoresis. (*B*) mRNA analysis of control cells (lane 1), IFN-α-treated cells (lane 2), or IFN-α-treated cells that were pretreated with PDBu (100 nM) for 30 hr (lane 3).

However, the induction of ISGF-2 was abolished when cells were treated with the PKC inhibitors H7 or staurosporine (Fig. 5 *A*, lane 4, and *B*, lane 4 and 5) but was unaffected with HA1004 (Fig. 5*A*, lane 5). Since ISGF-2 is induced transcriptionally by IFN (23), this result was not unexpected because PKC inhibitors block the appearance of cytoplasmic ISG mRNA (Figs. 2 and 4). However, when the potent PKC inhibitor staurosporine was used at 200–1000 nM, the appearance of the ISGF-3–DNA complex was abolished (Fig. 5*B*, lanes 4 and 5). The ability of staurosporine to inhibit the appearance of ISGF-3 correlates with its ability to inhibit transcriptional activation by IFN-α. Cytoplasmic extracts of staurosporine–IFN-treated cells do not reveal the presence of ISGF-3, indicating that the PKC inhibitor blocks the activation of latent ISGF-3 by IFN-α and not merely its translocation to the nucleus (data not shown).

Influence of Phorbol Esters on ISG Expression. Although these studies provide evidence that functional PKC is required for the IFN-stimulated response in HeLa cells, activation of PKC by phorbol esters does not induce expression of the ISGs (Fig. 6*A*). Treatment of cells with PDBu for 2 hr does not increase the cytoplasmic appearance of ISG15 mRNA (lane 3) nor block its appearance in the presence of IFN-α (data not shown). However, depletion of functional PKC by prolonged exposure of cells to PDBu (30 hr) reduced the IFN-stimulated appearance of ISG15 mRNA (60% of control; Fig. 6*B*, compare lanes 2 and 3). This treatment did not affect viability of the cells.

DISCUSSION

IFNs are mediators of a primary host defense mechanism and can confer cellular resistance to viral infections. The cellular response to IFN is triggered by a receptor-mediated pathway that transduces a signal from the plasma membrane to the nucleus stimulating specific gene expression. The studies presented here provide evidence that PKC may be involved in the relay of information from the IFN-α receptor to the transcriptional machinery of a HeLa cell.

Inhibition of PKC activity by H7 or staurosporine blocks the ability of IFN-α to establish an antiviral response in HeLa cells (Table 1). This blockage appears to be the result of a suppression of ISG expression. The cytoplasmic accumulation of specific ISG mRNAs is impaired in the presence of PKC inhibitors and, more significantly, nuclear run-on experiments demonstrate a suppression of the transcriptional activation of ISGs (Figs. 3 and 4). The PKC inhibitors did not have any effect on IFN-α binding to its cell membrane receptor (data not shown).

The transcriptional response to IFN-α is dependent upon the presence of an ISRE that is recognized by the induced DNA-binding factors ISGF-2 and ISGF-3 (4–8). Gel-retardation analyses revealed a block in the IFN-induced appearance of ISGF-2 in cells treated with either PKC inhibitor H7 or staurosporine (Fig. 5). Moreover, the potent PKC inhibitor staurosporine blocked IFN-α activation of the latent cytoplasmic factor ISGF-3 (10, 11). The negligible effect of 30 μM H7 on induction of ISGF-3 may be due to the lower potency of this agent or possible differential effects on the various PKC isozymes leading to a decrease in transcriptional activity but not a decrease in DNA binding. Since H7 has an additional inhibitory influence on the post-transcriptional appearance of ISG mRNAs in the cytoplasm, this agent may exert more complex effects upon gene expression. However, a signal that mediates the activation of ISGF-3 is impaired in the presence of the PKC inhibitor staurosporine. This result suggests that a phosphorylation event induced by IFN-α is required for ISGF-3 activation.

The requirement for PKC in the IFN-α-stimulated response does not involve PMA-inducible enhancers. Phorbol esters such as PMA or PDBu do not induce expression of the ISGs (Fig. 6*A*). However, PDBu binding studies demonstrate that IFN-α treatment leads to increased binding to intact cells that correlates with activation of PKC (12). Recent immunochemical analyses have demonstrated a specific translocation of the β isoform of PKC from the cytosol to the particulate fraction of HeLa cells after IFN-α treatment, while other isoforms of PKC remain unaffected (25). In addition, IFN-α induces a transient increase in diacylglycerol, an activator of PKC (25, 26). The results described herein thereby suggest that the increase in PDBu binding sites induced by IFN-α treatment reflect the selective translocation of the β isoform of PKC. Depletion of cellular PKC by prolonged exposure to phorbol esters has been reported to decrease the level of IFN-α-induced gene expression in certain cell lines (27, 28). We have seen a similar decrease in ISG mRNA expression after extended exposure of HeLa cells to PDBu (Fig. 6).

The utilization of protein kinase inhibitors by other investigators has provided evidence for a role of phosphorylation in the expression of IFN-α-induced genes (29–32). Although the possible involvement of another protein kinase cannot be ruled out, the results reported in this study indicate that inhibition of PKC suppresses ISG transcriptional activation. The evidence presented suggests that functional PKC is required, but not sufficient, for IFN-α-induced gene expression. The future determination of physiological substrates of PKC in IFN-α-mediated signal transduction should lead to an elucidation of its role in this pathway.

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