Differentiation-dependent activation of interferon-stimulated gene factors and transcription factor NF- κ B in mouse embryonal carcinoma cells

(antiviral state/interferon sensitivity)

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ABSTRACT We have recently shown that the adenovirus *E1A* gene products block interferon- α -induced signal transduction and transcription factor NF- κ B-mediated gene induction. Here we report that the same responses are also blocked in undifferentiated F9 teratocarcinoma cells. The block was removed upon cellular differentiation and regained upon the introduction of viral *E1A* into the differentiated cells. In undifferentiated cells, interferon- β failed to induce the transcription of interferon-responsive genes because of a lack of activation of the cognate trans-acting factors. As a result, in these cells, virus replication was not inhibited by interferon. Similarly, in undifferentiated but not in differentiated F9 cells, tumor necrosis factor α failed to stimulate NF- κ B-mediated transcription of a reporter gene because of a failure in the activation of NF- κ B trans-acting factor. These results suggest that a cellular *E1A*-like activity, present in undifferentiated F9 cells, and adenoviral *E1A* use similar mechanisms for repressing the expression of specific cellular genes.

Interferons (IFNs) are multifunctional cytokines that are components of the host defenses against viral and parasitic infections and certain tumors. IFNs also have immunoregulatory functions and they affect cell proliferation and differentiation (1, 2). Postreceptor signaling by IFN- α/β is mediated by the activation of the IFN-stimulated gene factors (ISGFs). Once activated, these factors bind to the IFN-stimulated response elements (ISREs) of the IFN- α/β -inducible genes. Although murine ISGFs have not been well-characterized, several such factors have been identified in the human cells. Among these, the activation and functioning of one, ISGF3, closely correlates with the induced transcription of IFN-stimulated genes (ISGs) (3–7). ISGF3 is composed of two functional subunits, ISGF3 γ and ISGF3 α . Inactive ISGF3 α , present in the cytoplasm, gets activated by phosphorylation (8–15) within seconds after IFN- α/β binds to the receptor. Activated ISGF3 α (11–13) binds to ISGF3 γ (14) and the complex translocates to the nucleus where it binds to the ISRE-containing genes and stimulates their transcription. We (15) and others (16, 17) have shown that the adenoviral *E1A* proteins inhibit the IFN responses by blocking the activation of ISGF3. In a more recent study, we have shown (18) that the tumor necrosis factor α (TNF- α)-mediated induction of interleukin 6 (IL-6) gene, through an NF- κ B element, was also blocked by *E1A* gene products. Detailed analysis revealed that *E1A* alters the composition of the NF- κ B complex, which binds to its cognate element in the IL-6 gene (18).

Induction of IFN biosynthesis is dependent upon the state of cellular differentiation (19–21). In undifferentiated embryonal carcinoma (EC) cells, virus infection or double-stranded

RNA fails to induce IFN- β synthesis (19–21). This deficiency can be overcome by either inducing cellular differentiation or expressing the trans-acting factor interferon regulatory factor 1 (IRF-1), introduced by transfection, in the undifferentiated cells (21). Several studies have shown that the undifferentiated EC cells also do not respond to IFN- α/β (22–24), although others claimed that they respond partially (25). The undifferentiated F9 cells have been shown to contain an *E1A*-like activity that is lost upon retinoic acid-induced differentiation (26–28). However, ectopic expression of adenoviral *E1A* gene results in the reversal of the fully differentiated state (27). Concomitantly, expression of a large number of differentiation-specific genes was also reversed by *E1A*. In the present work, we report that the cellular *E1A*-like activity of F9 cells, like the viral *E1A*, inhibits the ISGF3-dependent and NF- κ B-dependent gene expression.

MATERIALS AND METHODS

Cells, Viruses, and Plasmids. For the present investigation, we employed F9 EC cells in three states of differentiation: undifferentiated (F9), terminally differentiated by treatment of cells with retinoic acid (dF9), and terminally differentiated cells stably transfected with the adenovirus *E1A12S* gene (dF9-E1A). Cells were cultured in Dulbecco's modified Eagle's medium (27). dF9 cells were obtained by growing the F9 cells in medium containing retinoic acid (0.1 μ M). mIFN- β and mIFN- γ were from Lee Biomolecular Laboratories (San Diego) and Genentech, respectively. Phorbol 12-myristate 13-acetate (PMA) and TNF- α were purchased from Sigma and Genentech, respectively. pRM-CAT, vesicular stomatitis virus (VSV), and encephalomyocarditis virus were described (15). IP10-CAT gene was provided by T. Hamilton (The Cleveland Clinic Foundation). IRF-1 and (2'-5')oligoadenylate synthetase cDNAs were as described (15, 21).

Antiviral Assays. Antiviral assays were carried out using VSV and encephalomyocarditis virus as described (15). After 30 h of infection, the plates were stained with methylene blue. The dye from the plates was extracted and the absorbance at 595 nm was measured. Average data from triplicate samples are presented.

Transcriptional Analysis. Procedures for Northern blot analyses, nuclear run-off transcription, DNA transfections, chloramphenicol acetyltransferase (CAT) assays, and electrophoretic-mobility-shift assays were described in our publications (3, 15).

Abbreviations: IFN, interferon; ISGF, IFN-stimulated gene factor; ISRE, IFN-stimulated response element; ISG, IFN-stimulated gene; TNF- α , tumor necrosis factor α ; IL-6, interleukin 6; EC, embryonal carcinoma; IRF-1, interferon regulatory factor 1; PMA, phorbol 12-myristate 13-acetate; VSV, vesicular stomatitis virus; CAT, chloramphenicol acetyltransferase.

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RESULTS

IFN- β Does Not Induce Antiviral State in Undifferentiated F9 Cells. To investigate the ability of IFN- β to induce the antiviral state, we employed F9 cells in three states (27). As shown in Fig. 1, treatment with various doses of mIFN- β did not protect the F9 cells against VSV infection. However, upon differentiation (dF9 cells), treatment with IFN- β protected these cells against VSV in a dose-dependent manner. These effects were abolished completely in dF9-E1A cells. Similar results were obtained using encephalomyocarditis virus infection (data not shown). These data demonstrate that the induction of antiviral state by IFN- β is dependent on the state of cellular differentiation and they agree well with the previous reports that undifferentiated EC cells do not acquire antiviral state upon IFN treatment (22–24).

Transcription of ISGs Is Inhibited in F9 Cells. Since the antiviral state is dependent on the products of ISGs, we examined whether the expression of ISGs was induced in these cells. As shown in Fig. 2, transcriptional induction of four ISGs by IFN- β was detected in dF9 cells but not in F9 or dF9-E1A cells. Transcription of the β -actin gene was readily detected in all cell types and it was not influenced by IFN treatment. Therefore, the inability of F9 cells and dF9-E1A cells to mount IFN- β -mediated antiviral responses is due to a failure in the transcriptional induction of ISGs.

We next determined whether the induction of specific genes by IFN- γ in these cells was also affected. Northern blot analysis revealed that two IFN- γ -inducible genes, IRF-1 and (2'-5')oligoadenylate synthetase, were induced in dF9 cells but not in F9 and dF9-E1A cells (Fig. 3). There was a basal level of (2'-5')oligoadenylate synthetase expression in F9 and dF9-E1A cells that was not further increased after IFN- γ treatment. All the lanes had comparable amounts of RNA as detected by a β -actin probe (data not presented).

Expression of IFN-Inducible CAT Genes Is Impaired in F9 Cells. Transcriptional induction of ISGs was also analyzed by transfecting F9 cells with chimeric CAT genes whose expression is driven by the ISG promoters. Fig. 4A shows the expression of pRMCAT, a CAT gene driven by the promoter elements derived from IFN- α/β -inducible 561 gene. No CAT gene induction by IFN- β was observed in F9 cells. In dF9 cells, the process of differentiation by itself slightly stimulated the expression of the CAT gene. This low level of CAT expression was increased by >20-fold upon IFN- β treatment of these cells. In dF9-E1A cells, a low level of CAT expression was detected only after IFN- β treatment. Similar results were obtained with two other IFN- α/β -inducible CAT genes driven by the promoters of 6-16 and (2'-5')oligoadenylate synthetase (data not shown). Similarly, IFN- γ -stimulated

FIG. 1. Induction of antiviral state by IFN- β . Cells were treated with different doses of mouse IFN- β for 16 h in the growth medium, followed by infection with VSV. Cells were stained with methylene blue; the dye bound to the plates was extracted and quantitated. A_{595} values for mock-infected and virus-infected cells without IFN treatment were 1.67 and 0.070, respectively.

FIG. 2. Transcriptional induction of ISGs. Nuclear run-off transcription reactions were performed using the nuclei from control (rows C) and IFN- β -treated (rows I) cells (500 units/ml). Total nuclear RNAs were hybridized to cDNA fragments of ISGs 561, 1-8, (2'-5')oligoadenylate synthetase [2',5' (A) syn], and 6-16 (3, 15). Phage λ DNA and β -actin cDNA were used as negative and positive controls, respectively.

expression of IP10-CAT was undetectable in F9 cells whereas its basal and IFN- γ -inducible levels rose sharply upon differentiation (Fig. 4B). CAT expression was significantly lower in dF9-E1A cells. Thus, both IFN- α/β - and IFN- γ -stimulated gene expression is repressed in F9 cells and dF9-E1A cells. These data and data in Fig. 2 demonstrate a clear transcriptional shut off of ISGs in the undifferentiated cells.

Transcriptional Repression of ISGs Is Due to the Absence of Active Transcriptional Factors in F9 Cells. In the next set of experiments, we investigated whether ISGF3 is activated in IFN- β -treated F9 cells. For this purpose, electrophoretic-mobility-shift assays were performed with a radiolabeled ISRE probe (3, 15). Four complexes were detected using a nuclear extract of IFN- β -treated dF9 cells (Fig. 5A, lane 4). Two of these, complexes c and d, were also detectable in the extract of untreated dF9 cells (Fig. 5A, lane 3). All four complexes could be competed out by unlabeled ISRE probe thus confirming their authenticity (data not shown). None of

FIG. 3. Status of IFN- γ -inducible gene expression. Total RNA (20 μ g) was analyzed on a Northern blot. RNA was extracted after 5 h (for detecting IRF-1 mRNA) and 18 h [for detecting (2'-5')oligoadenylate (2',5' A) synthetase mRNA]. Lanes: C, control; 1, IFN- γ (200 units/ml); 2, dF9; 3, dF9-E1A.

FIG. 4. IFN-mediated induction of CAT genes driven by ISG promoters. Cells were transfected with 5 μ g of pRM-CAT (A) or IP10-CAT (B). Forty hours after transfection, cells were treated with IFN- β (200 units/ml) in A or with IFN- γ (200 units/ml) in B for 16 h. CAT activity in the cell extracts was quantitated (3, 15).

these complexes was detectable in the extracts of IFN- β -treated or untreated F9 cells (lanes 1 and 2) or the extracts of dF9-E1A cells (lanes 5 and 6). The slowest complex, complex a, has the characteristics of the murine equivalent of human ISGF3 (3–7). It is inducible by IFN- β in dF9 cells (Fig. 5A) but not by IFN- γ in L929 cells (Fig. 5D, lane 1) or in dF9 cells (data not shown). Its induction is rapid (Fig. 5B), and unlike complexes c and d, it is present in the cytoplasm of IFN- β -treated cells (Fig. 5C). The identity of complex b is not apparent. It has many characteristics of complex a but seems to be inducible by IFN- γ as well (Fig. 5D, lane 1). Complexes c and d could be the murine equivalent of human ISGF2 and ISGF1, respectively, since they were formed in the nuclear extracts of both IFN- β -treated and untreated dF9 cells. These observations clearly indicate that the defect in the IFN- β response of F9 cells could be attributed to the failure of activation of a factor equivalent to ISGF3 upon IFN treatment of these cells. In addition, it is worth noting that the F9 and the dF9-E1A cells are also devoid of the constitutive factors that form the complexes c and d.

The experiment shown in Fig. 5D documents that the absence of active ISGF3 in IFN-treated F9 cells is due to a failure to activate ISGF3 α . A complementation assay (3–6) was used to detect active ISGF3 α . Even when exogenous ISGF3 γ was supplied, a cytoplasmic extract of IFN- β -treated F9 cells failed to form the ISGF3 complex (Fig. 5D, lane 3). Such a complex was readily visible when similar extracts of IFN- β -treated L929 cells (lane 2) or dF9 cells (data not shown) were used. F9 cells do not appear to contain an inhibitor that directly prevents ISGF3 binding to ISRE because in a mixture of equal amounts of extracts of F9 and dF9

FIG. 5. Analyses of ISGF using electrophoretic-mobility-shift assays. (A) Cells were treated with IFN- β (200 units/ml) for 1 h and nuclear extracts were prepared. A 32 P-labeled ISRE oligonucleotide probe, derived from the human 561 gene, was incubated with nuclear extracts (0.68 μ g) and samples were analyzed as described (3, 15). Extracts were from the following cells. Lanes: 1 and 2, F9; 3 and 4, dF9; 5 and 6, dF9-E1A. Extracts in lanes 1, 3, and 5 were from untreated cells and those in lanes 2, 4, and 6 were from IFN- β -treated cells. (B) Kinetics of IFN-mediated activation of ISGFs in the nuclei of dF9 cells. Nuclear extracts were prepared after IFN- β (200 units/ml) treatments as follows. Lanes: 1, 0 min; 2, 5 min; 3, 15 min; 4, 30 min; 5, 60 min. (C) Kinetics of activation of ISGFs in the cytoplasm of dF9 and dF9-E1A cells. Cytoplasmic extracts (1.3 μ g) were incubated with ISRE probe and analyzed as above (3, 15). Lanes: 1 and 2, extracts from dF9-E1A cells; 3–7, extracts from dF9 cells. IFN- β treatments were as follows. Lanes: 1, 0 min; 2, 30 min; 3–7, correspond to the treatments in lanes 1–5 of B, respectively. (D) Complementation assay for ISGF3. Cytoplasmic extracts (1.0 μ g) from L929 cells and F9 cells were added to the binding reaction mixtures to detect ISGF3 α in F9 cells. Lanes: 1, IFN- γ -treated L929 cells; 2, extract in lane 1 plus extract of L929 cells treated with IFN- β plus cycloheximide; 3, extract from lane 1 plus extract from F9 cells treated with IFN- β . Cells were treated with IFN- β (200 units/ml) and cycloheximide (50 μ g/ml) for 30 min and with IFN- γ (200 units/ml) for 18 h where indicated. Bands a–d show the positions of four distinct ISGF complexes formed.

cells, the ISGF3 complex was efficiently formed (data not shown). It was not possible to experimentally test whether ISGF3 γ was induced in F9 cells by either IFN- β or IFN- γ

FIG. 6. Expression of NF- κ B-CAT. Cells were transfected with 10 μ g of a plasmid containing the NF- κ B motif of the IL-6 promoter placed upstream of a CAT gene driven by herpes simplex virus thymidine kinase promoter. After transfection, they were treated with either PMA (50 ng/ml in lanes 2, 5, and 8) or with mouse TNF- α (10 ng/ml in lanes 3, 6, and 9) and 50 μ g of cell protein was assayed for CAT activity. Lanes: 1, 4, and 7: untreated cells; 2–3, F9; 4–6, dF9; 7–9, dF9-E1A.

FIG. 7. Status of NF- κ B activation. Nuclear extracts (1.5 μ g of protein) from cells treated with mouse TNF- α (10 ng/ml) for 4 h (lanes 3, 5, and 7) or untreated cells (lanes 2, 4, and 6) were incubated with a 32 P-labeled IL-6 NF- κ B oligonucleotide and analyzed. Lanes: 1, probe alone; 2 and 3, F9; 4 and 5, dF9; 6 and 7, dF9-E1A. Only the relevant part of the autoradiogram is shown.

because of a lack of a source of murine ISGF3 α that is uncontaminated by ISGF3 γ .

NF- κ B-Mediated Transcription Is Blocked in F9 Cells. We have recently shown that adenoviral E1A proteins inhibit the NF- κ B element-mediated transcriptional induction of the IL-6 gene in response to TNF- α or PMA (18). In the next set of experiments, we examined whether the cellular E1A-like activity present in F9 cells could do the same. As shown in Fig. 6, TNF- α or PMA could stimulate the expression of a NF- κ B-driven CAT gene only in dF9 cells. The failure of expression of this gene in F9 or dF9-E1A cells was due to a lack of TNF- α -induced activation of the NF- κ B factors in these cells (Fig. 7).

DISCUSSION

The results presented here indicate that the signaling by IFN- β is impaired in undifferentiated F9 cells due to a failure to activate an ISGF3 α -like factor. The characteristics of the putative cellular inhibitor of IFN signaling are very similar to those of the adenoviral E1A proteins (15–17). However, purified E1A or an E1A-containing cellular extract does not block ISGF3 complex formation *in vitro*, thereby suggesting that its effects are indirect (15). It is conceivable that the inhibitory effects of E1A are mediated by a cellular protein and this putative protein is present constitutively in undifferentiated F9 cells. Presumably, the process of differentiation shuts off its expression thereby enabling the cells to respond to IFN. For evading the antiviral action of IFN, adenoviruses may have evolved to take advantage of this cellular system by inducing the expression of the gene encoding the putative cellular inhibitor of IFN signaling, which is usually not expressed in differentiated cells. This hypothesis is consistent with the well-documented ability of E1A proteins to enhance transcription of specific cellular genes (29, 30). The presence of an E1A-like activity in undifferentiated F9 cells has been noted (26–28). This activity could support the growth of an adenovirus mutant, dl312, lacking the E1A gene and could enhance transcription from E1A-dependent promoters. A similar activity could also be detected in early mouse embryos (31). The E1A-like activity present in F9 cells is lost upon differentiation. Conversely, introduction of an adenovirus E1A gene into the differentiated cells results in a dramatic reversal of the fully differentiated state (27). The results presented in this report demonstrate that there is a concomitant loss of the IFN response as well. Like the viral E1A, the cellular E1A-like activity inhibited both IFN- β and IFN- γ -induced signaling.

We have reported (18) that expression of the IL-6 gene is inhibited by adenovirus E1A. TNF- α induces this gene through an NF- κ B element present in its promoter. In HeLa

cells, E1A repressed the NF- κ B-dependent CAT gene induction. Similarly, the NF- κ B-CAT gene failed to express in F9 and dF9-E1A cells. These results once again are in agreement with the supposition that cellular and viral E1A molecules act similarly. However, there were notable differences at the level of activation of the NF- κ B trans-acting factors. In F9 cells, there was no detectable NF- κ B-binding activity even after TNF- α treatment although there is no report, to our knowledge, suggesting that these cells lack TNF- α receptors. The absence of any NF- κ B binding proteins was also true for TNF-treated dF9-E1A cells. In contrast, in HeLa-E1A cells, TNF- α could induce NF- κ B binding activities, but the pattern of NF- κ B complexes formed in these cells was different from that in TNF- α -treated control cells (18). Further investigation is needed to explain this difference in the properties of the cellular and viral E1A molecules. It is reasonable to assume that the specific cellular backgrounds of F9 and HeLa cells play important roles as exemplified by the results with dF9-E1A cells.

Defects in the functioning of the IFN system in undifferentiated teratocarcinoma cells have been described (19–24, 32). Induction of IFN- β synthesis by double-stranded RNA is blocked in these cells. This block could be only partially overcome by expressing transfected IRF-1 protein, one of the trans-acting factors required for IFN- β gene expression (21). Our results indicate NF- κ B, the other factor needed for expression of the IFN- β gene (33), also fails to be activated in F9 cells thereby compounding the problem. The observed differences in IFN response between undifferentiated and differentiated cells are not due to differences in the number and affinity of IFN receptors present in these cells (34). Our results show that the defect is at the post-receptor level of trans-acting factor activation, the same step affected by the adenoviral E1A proteins. Further studies will be required to determine whether the viral E1A and cellular E1A-like activities use the same biochemical pathways for their actions.

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