IFI16 Mediates LIF/JAK/STAT-Induced Growth Arrest

IFI16 Is an Essential Mediator of Growth Inhibition, but not Differentiation, Induced by the Leukemia Inhibitory Factor/JAK/STAT Pathway in Medullary Thyroid Carcinoma Cells*

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1The abbreviations used are:
medullary thyroid carcinoma, MTC; leukemia inhibitory factor, LIF; small cell lung cancer, SCLC; calcitonin gene related peptide, CGRP
**SUMMARY**

Activation of Ras or Raf in the human medullary thyroid carcinoma (MTC) cell line, TT, induces growth arrest and differentiation via two parallel, yet independent pathways. One of these pathways is intracellular and the other is a cell-extrinsic, autocrine/paracrine pathway mediated by the leukemia inhibitory factor (LIF)/JAK/STAT pathway. Here, we show that IFI16 is a necessary and sufficient downstream effector for LIF effects in MTC cells, specifically required for the LIF/JAK/STAT pathway-induced growth inhibition in these cells. IFI16 was induced by Raf or LIF. Dominant negative STAT3 could block the induction, indicating that Raf can induce IFI16 only via the cell-extrinsic pathway. Knock-down of IFI16 using siRNA abrogated LIF-induced changes in cellular levels of E2F1, cyclin D1, and p21^{WAF/CIP1}, and cell cycle arrest. In addition, adenovirus-mediated overexpression of IFI16 was sufficient to induce growth arrest. In contrast to its essential role for LIF-mediated growth arrest, IFI16 was not required for differentiation induced by LIF. Knock-down of IFI16 could not block changes in differentiation markers of the MTC cells, including calcitonin, RET, and cell morphology. Our study identifies IFI16 as an essential growth-specific effector of the cell-extrinsic growth inhibitory pathway of Ras/Raf signaling in MTC cells.
INTRODUCTION

The key role of activated Ras in oncogenic transformation has been established in numerous studies. However, sustained activation of Ras or its downstream effector Raf induces growth arrest and premature senescence in primary normal cells; this response has been proposed to be an antioncogenic defense mechanism against aberrant Ras activation (1-11). Ras/Raf activation can also elicit growth arrest in a number of tumor cell lines, accompanied with differentiation (9,12-17). These tumor cell lines generally are derived from cell types that do not show dependency on dysregulated Ras/Raf signaling for cell growth, and mutation of Ras/Raf or elevated signaling of the pathway is rarely detected in these tumor types. This suggests that some tumor types may retain the ability to respond to Ras/Raf activation with growth arrest. Therefore, understanding the mechanism of Ras/Raf-induced growth arrest in these tumors may provide insight into the control of tumor growth.

Recently, we reported that sustained expression of activated Ras or Raf in the human medullary thyroid carcinoma (MTC) \(^1\) cell line, TT, induces growth arrest and differentiation via two parallel, yet independent pathways (18). One of these pathways is intracellular, similar to the Ras-mediated arrest of primary fibroblasts. The other pathway is a novel autocrine/paracrine pathway, in which Ras/Raf activation induces expression and secretion of leukemia inhibitory factor (LIF), a multifunctional cytokine of the interleukin-6 family (19-21). LIF then mediates a cell-extrinsic growth inhibitory signaling pathway, through the LIFR-gp130 receptor, via induction of JAK/STAT signaling. The pathway of Ras/Raf-mediated LIF expression and consequent activation of STAT3 is maintained in the small cell lung cancer (SCLC) cell lines NCI-H209 and DMS53 (18). However, intriguingly, these SCLC cells do not undergo growth arrest and differentiation in response to LIF, suggesting that the LIF-mediated growth inhibitory signaling in SCLC cells may be impeded at a step distal to STAT3 activation. Thus, it is important to identify
downstream components of the LIF/JAK/STAT-mediated growth inhibition and differentiation signaling to characterize the different responses of MTC and SCLC cells to LIF/JAK/STAT activation.

This study was performed to dissect the mechanism of the Ras/Raf-induced cell-extrinsic signaling pathways in MTC cells. Using a cDNA microarray, we have identified IFI16, an interferon-inducible transcriptional repressor, as a downstream target of the LIF/JAK/STAT pathway. We show that IFI16 is essential and sufficient for the LIF/JAK/STAT-induced growth arrest in MTC cells, but is not required for differentiation, which is also induced by the LIF/JAK/STAT pathway along with growth arrest. Thus, IFI16 appears to be specific for cell cycle regulation mediated by the cell-extrinsic LIF/JAK/STAT pathway, and separates the regulation of growth arrest from that of differentiation. We also show that, unlike in MTC cells, IFI16 cannot be induced by LIF in the SCLC cell line DMS53. However, IFI16 can induce growth arrest in DMS53 cells, suggesting that IFI16 may be a missing component for the LIF-induced growth inhibitory signaling in some SCLC cells. Our study defines IFI16 as an essential growth specific effector of the LIF/JAK/STAT-mediated cell-extrinsic pathway of Ras/Raf.
EXPERIMENTAL PROCEDURES

Cell Culture and Conditioned Media Treatment—The human MTC cell line TT, and the TTRaf cell line containing the activatable ΔRaf-1:ER construct, the catalytic domain of Raf-1 fused to the hormone binding domain of the human estrogen receptor, have been described previously (17). ΔRaf-1:ER was activated with 1 µM β-estradiol (Sigma, St. Louis, MO). TTRaf cells were maintained in phenol red-free RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 16% FBS, 100 U of penicillin and 100 µg of streptomycin per ml and 0.25 mg of G418 (Invitrogen) per ml for selection. DMS53 cells were maintained in Waymouth’s medium (Invitrogen) supplemented with 10% FBS.

Conditioned medium containing recombinant LIF was prepared from the HEK293LIFV5 cell line as previously described (18). Recombinant LIF (Chemicon, Temecula, Calif.) was used at a concentration of 4,000 U/ml. Interferon-α or γ was purchased from PeproTech (Rocky Hill, NJ), and used at 1,000 U/ml or 10 ng/ml respectively. For cell growth curves, cells were seeded in 24-well plates (Cellstar, Carrollton, Tex.) at a density of 5 × 10^4 cells per well, and counted using a hemocytometer.

Microarray Analysis—Microarray analysis was performed at the Johns Hopkins Comprehensive Microarray Core using glass arrays embedded with 20,000 human genes (Johns Hopkins Comprehensive Microarray Core). Briefly, 10 µg of total RNA was reverse-transcribed using oligo dT primer and Superscript II reverse transcriptase (Gibco/BRL), and labeled using a fluorescence DNA labeling system (Amersham, Piscataway, NJ). Glass arrays were then hybridized for 12–18 h with the probes. Data were analyzed using the Gene Spring 5.0 program (Silicon Genetics).

PCR—RT-PCR of IFI16 was performed by reverse transcription of 0.25 µg total RNA, and subsequent 30 cycles of polymerase chain reaction using Pfx polymerase (Invitrogen) and primers.
AAATTCAGATTGCTGACTTGA and CACAAAAAGATAATGTTTATTTA (IFI16 nucleotides 421–2477). The results were normalized for expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using primers CAGCGGAGCCACATCG and TGAGGCTTTGTCATACTTCTC. To measure calcitonin gene expression, real-time PCR was performed by reverse transcription of 0.25 µg total RNA, and subsequent polymerase chain reaction using Hot Star Taq DNA polymerase (Qiagen). For calcitonin, primers AGTGAGCTGGAGCAGGAGCAAGAG and TCAAATGATCAGCACATTTGAAG were used. For calcitonin-gene-related-peptide (CGRP), AGTGAGCTGGAGCAGGAGCAAGAG and CATTACCACATGTCCCCAGATGCC were used. The results were normalized for expression of phosphoglycerate kinase, using primers CAGTTTGGAGCTCCTGGAAG and TGCAAATCCAGGGTCCAGTG.

siRNA-Mediated Knock-Down of IFI16—The siRNA oligomer targeted to IFI16 UCAGAAGACCACAUCUAC (IFI16 nucleotides 1241–1259) and the control ACUCUAUCUGCAGCAGACUU were synthesized by Dharmacon (Lafayette, CO). Cells were seeded at 50% confluency 1 day prior to transfection, and transfected using LipofectAMINE 2000 (Invitogen) according to the manufacturer's protocol. For stable expression of small hairpin IFI16 RNA, GATCCCGTCAGAAGACCACAATCTACTTCAAGAGAGTAGATTGTGGTTTCTTGATTTTTGGAAA and AGCTTTTTCAAAAAATCAGAAGACCACAATCTACTTCAAGAGAGTAGATTGTGGTTTCTTGAA were annealed and ligated into the HindIII and BamHI sites of pSilencer 2.1-U6 hygro (Ambion, Austin, TX). Successful knock-down of IFI16 was confirmed by Western analysis of IFI16. The pSilencer-mediated knock-down of IFI16 in culture was compared to cells transfected with the pSilencer hygro Negative Control which was supplied with the pSilencer™ hygro kit (Ambion). The pSilencer hygro Negative Control plasmid is a
circular plasmid encoding a hairpin RNA whose sequence is not found in the mouse, human or rat genome database.

**Plasmids and Recombinant Adenoviruses**—From pCMVSPORT6-IFI16 (Open Biosystems), IFI16 was subcloned into the KpnI and XbaI sites of pcDNA3.1(-). The adenovirus AdIFI16, expressing IFI16, was made using the AdEasy system (22). Briefly, IFI16 was subcloned into the KpnI and XbaI restriction sites of the pAdTrackCMV shuttle vector, and the resulting plasmid was recombined with the pAdEasy1 vector in BJ5183 bacterial cells. The dominant-negative STAT3 adenovirus AdSTAT3-DN and the control virus AdGFP (18), and the constitutively active Raf-BXB adenovirus (23) were described previously. The dominant-negative human STAT3 has a Y705F mutation (24). The viral titer was measured using an Adeno-X rapid titer kit (Clontech).

**Cell Cycle Analysis**—Cells were washed with ice cold 0.2% bovine serum albumin in phosphate-buffered saline (PBS) and resuspended in 250 mM sucrose–40mM citrate buffer (pH 7.6) containing 0.5% dimethyl sulfoxide. Nuclei were prepared, stained with propidium iodide (25), and analyzed with an LSR flow cytometer (Becton Dickinson, Franklin Lakes, N.J.) with a gate that selects single nuclei within a normal size range. The cell cycle parameters from 10,000 gated nuclei were determined by CellQuest software.

**Immunoblot Analysis**—Cells harvested at various times were lysed in 62.5 mM Tris (pH 6.8)-2% SDS with aprotinin, leupeptin, bestatin, pepstatin A, E-64 and 4-(2-aminoethyl)benzensulfonyl fluoride (Sigma), and briefly sonicated before determining the protein concentration using BCA reagents (Pierce, Rockford, Ill.). 50 to 100 µg of protein was resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane filter (Millipore), and stained with Fast Green reagent (Fisher Scientific, Pittsburgh, Pa.). Membrane filters were then blocked in 0.1 M Tris (pH 7.5)-0.9% NaCl-0.05% Tween 20 with 5% nonfat dry milk, and incubated with
appropriate antibodies. Antibodies were diluted as follows: IFI16, 1:1,000 (Santa Cruz Biotech, Santa Cruz, Calif); RET, 1:1,000 (Santa Cruz Biotech); E2F1, 1:1,000 (Neomarkers, Fremont, CA); Cyclin D1, 1:1,000 (Neomarkers) and GAPDH, 1:5,000 (Trevigen, Gaithersburg, MD). The Supersignal West Pico chemiluminescence kit (Pierce) was used for visualization of the signal.

**Colony Formation Assays**—Colony formation assays were performed as described previously (26). Briefly, cells were transfected with 4 µg of empty pcDNA3.1 vector or pcDNA3.1 harboring IFI16 gene, using LipofectAMINE 2000 (Invitrogen). Following 24 h of transfection, cells were split into two 100 mm plates and selected in G418 for about 2 weeks. Colonies exhibiting G418 resistance were stained with Coomassie brilliant blue before counting.

**Cell Proliferation Assay**—For the colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells infected with AdIFI16 or the control AdGFP in 24 well plates were treated with 50 µl of 1 mg/ml MTT (Sigma) in phenol-red free RPMI 1640 containing 10% FBS for 3 h at 37 °C. Then, the medium was replaced with 600 µl of DMSO, and shaken for 15 min prior to measuring optical density at 540 nm. A540 was measured every two days for 8 days.
RESULTS

*Ras/Raf Activation Induces IFI16 via the LIF/JAK/STAT-Mediated Autocrine/Paracrine Pathway*—Previously, we showed that Ras/Raf-induced growth arrest and differentiation in the human MTC cell line, TT, is mediated via two parallel, yet independent pathways (18). One is intracellular and the other is the LIF/JAK/STAT-mediated autocrine/paracrine pathway. These two pathways induce growth arrest accompanied by a differentiation program characterized by changes in morphology, expression of the neuroendocrine markers calcitonin and calcitonin-gene-related peptide (CGRP), and downregulation of RET, the proto-oncogene responsible for MTC development in the MEN 2 syndromes (17,27).

To further elucidate the mechanism of the growth inhibition mediated by Ras/Raf-induced cell-intrinsic or extrinsic signaling, we sought to identify downstream effectors of these parallel pathways. For this, we examined changes in gene expression induced by these signaling pathways using microarray analysis techniques. Information on genes regulated by the cell-extrinsic growth inhibitory pathway was collected by examining total RNA extracted from TT cells treated with LIF, in the absence or presence of a dominant negative STAT3, and subsequently selecting genes induced in a STAT3-dependent manner. We have shown that STAT3 is essential for the LIF-mediated growth inhibitory response in TT cells (18). We then examined gene expression upon Raf activation, which represents both intracellular and the autocrine/paracrine pathways. This result was then compared with the LIF-regulated gene expression profile. In this way, we could identify genes specifically regulated either by cell-intrinsic signaling or by the LIF/JAK/STAT-mediated extrinsic pathway.

We were particularly interested in the genes regulated by LIF in a STAT3-dependent manner, since they represent potential downstream targets specific for the cell-extrinsic pathway. Among these STAT3-induced genes in TT cells, IFI16, which has been shown to inhibit growth in
other cell types (28), was a fascinating candidate to mediate LIF-induced growth arrest. We further analyzed the LIF/JAK/STAT-dependent induction of IFI16 by RT-PCR and Western blotting. Indeed, increase in mRNA level of IFI16 could be detected upon Raf activation or LIF treatment, while the induction was blocked in cells infected with a dominant-negative STAT3 adenovirus (Fig. 1A and B). A similar pattern of STAT3-dependent induction was also observed at the protein level (Fig. 1C). Since STAT3 is activated and utilized only by the cell-extrinsic pathway, these data indicate that Raf induces IFI16 via the LIF/JAK/STAT-mediated cell extrinsic pathway, but not via intracellularly mediated signaling pathways.

IFI16 Is Necessary and Sufficient for Growth Inhibition, but Is Not Required for Differentiation Induced by LIF—To investigate the functional relevance of IFI16 to LIF-mediated cell growth arrest and differentiation, we knocked down IFI16 expression during LIF treatment, and also overexpressed IFI16 in TT cells. First, to determine whether IFI16 is necessary for LIF-mediated growth inhibition, we transfected TT cells with siRNA targeted to IFI16. The siRNA treatment could effectively block IFI16 induction by LIF (Fig. 2A). For a more durable knock-down, we stably transfected TT cells with pSilencer 2.1-U6 hygro vector harboring a sequence to express small hairpin IFI16 RNA. We then measured cell growth in the presence of LIF. LIF suppressed growth of control TT cells, whereas TT cells expressing the small hairpin IFI16 RNA could still proliferate in the presence of LIF (Fig. 2B). Consistent with this result, the TT cells expressing the small hairpin IFI16 RNA could undergo cell cycle progression despite LIF treatment (Fig. 2C). IFI16 is known to regulate the expression of E2F1 and p53, and, thus, affect cellular levels of p21^{WAF/CIP1} (29,30). Thus, we measured the cellular level of these proteins and the cell cycle regulator, cyclin D1. Knock-down of IFI16 could block downregulation of E2F1 and cyclin D1, and upregulation of p21^{WAF/CIP1} by LIF (Fig. 2A). However, p53 expression was not affected by LIF, and IFI16 knock-down did not induce any additional effect on p53 expression.
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in TT cells (data not shown). LIF also induces differentiation in TT cells, with changes in cell morphology, downregulation of the RET receptor tyrosine kinase, and increased expression of the calcitonin/CGRP gene (18). In contrast to its effects on LIF-mediated cell growth arrest, IFI16 knock-down did not block RET downregulation, expression of calcitonin/CGRP, or the typical morphology change induced by LIF (Fig. 3, A to C), indicating that IFI16 may not be required for the differentiation program induced by LIF in TT cells. These data show that, in TT cells, IFI16 is an essential mediator specific for cell cycle arrest, but not for differentiation, induced by the LIF/JAK/STAT pathway.

We next tested whether IFI16 could be a sufficient effector for the growth arrest induced by the LIF/JAK/STAT pathway. To test this, we made an adeno viral construct containing the IFI16 gene, and observed the effect of IFI16 overexpression in TT cells. TT cells infected with this virus showed a substantial increase of IFI16, relative to the parental cells or the control virus infected cells (Fig. 4B). Cells infected with the adenovirus containing IFI16 gene showed significantly retarded growth, indicating that IFI16 is sufficient to induce growth arrest in TT cells (Fig. 4A). Consistently, cells infected with the IFI16 virus showed a substantial decrease in S-phase population and increase in G0/G1-phase, relative to the control virus-infected TT cells (Fig. 4C). No significant differences in growth were observed between the parental cells and the control virus infected cells. Along with this, we observed a decrease in the level of E2F1 and cyclin D1, and a modest increase in p21WAF/CIP1 level (Fig. 4B). The growth inhibitory effect of IFI16 was also confirmed by a colony formation assay (Fig. 4D). TT cells transfected with the expression vector pcDNA3.1 harboring the IFI16 gene formed a significantly reduced number of colonies relative to the cells transfected with the empty vector. Consistent with our findings using IFI16 siRNA described above, IFI16 overexpression did not affect the differentiation of TT cells. Thus,
contrary to its sufficiency for cell growth arrest, IFI16 did not induce RET downregulation, calcitonin/CGRP, and morphological changes in TT cells (Fig. 5, A to C).

Taken together, these data show that IFI16 is necessary and sufficient to mediate growth arrest induced by the LIF/JAK/STAT pathway, but it is not required for differentiation that is also induced by the LIF/JAK/STAT pathway in TT cells.

IFI16 Is Not Induced in the SCLC Cell Line DMS53 by LIF—We previously showed that Ras/Raf activation also induced LIF expression and consequent activation of STAT3 in the SCLC line DMS53 (18). However, DMS53 cells did not undergo growth arrest and differentiation in response to LIF, suggesting that this SCLC cell line may be impeded in its ability to respond to LIF at a step distal to STAT3 activation. Therefore we questioned whether the expression status of IFI16 might be correlated with this lack of LIF-mediated growth arrest in DMS53 cells. We examined whether IFI16 could be induced in DMS53 cells by LIF treatment. The basal level of IFI16 was low in DMS53 cells. LIF treatment or Raf activation could not induce IFI16 expression, as tested by Western blotting, indicating that signaling between STAT3 and IFI16 may be disabled in DMS53 cells (Fig. 6). Nevertheless, IFI16 expression could be induced by the known inducers of IFI16, interferon-α or γ (Fig. 6), indicating that the endogenous IFI16 gene is competent to be expressed in DMS53 cells.

Overexpression of IFI16 Leads to Growth Arrest in DMS53 Cells—We then asked whether expression of IFI16 was able to induce growth arrest in DMS53 cells. Infection of DMS53 cells with the IFI16 adenovirus resulted in a substantial increase of IFI16 level (Fig. 7B), and inhibition of growth and cell cycle progression (Fig. 7, A and C). We further confirmed this growth inhibitory effect of IFI16 in DMS53 cells by a colony formation assay. DMS53 cells transfected with the expression vector pcDNA3.1 harboring the IFI16 gene formed significantly reduced number of colonies relative to the cells transfected with the empty vector (Fig. 7D).
Consistent with these data, downregulation of E2F1 and cyclin D1 level, and upregulation of p21\(^{WAF/CIP1}\) level were observed in DMS53 cells infected with the IFI16 virus (Fig. 7B). Together, these data indicate that some SCLC cells, like MTC cells, are sensitive to IFI16-mediated growth arrest, but these cells may be impeded in their ability to undergo growth arrest in response to LIF, due to lack of IFI16 inducibility by the LIF/JAK/STAT pathway.
DISCUSSION

This study shows that IFI16 is an essential and sufficient mediator specific for the growth inhibition induced by the Ras/Raf-mediated cell-extrinsic signaling pathway in MTC cells (Fig. 8). IFI16 is a member of the interferon-inducible HIN200 nuclear protein family that shares a structural hallmark of a 200 amino acid repeat (31-33). IFI16 has been shown to have transcriptional repressor activity (34), and also has been shown to inhibit tube morphogenesis and proliferation of primary human endothelial cells in a p53 and Rb dependent manner (35). In addition to its originally suggested role in the control of myeloid differentiation (32), recent studies suggest that IFI16 may have a role in cancer biology. IFI16 can bind to p53 and modulate expression of its target genes in the control of cell cycle regulation (30,36), while loss of IFI16 can result in deregulation of p53-mediated apoptosis, leading to cancer development (37). IFI16 can also interact with BRCA1 through its Pyrin domain, and mediate DNA damage-induced apoptosis in mouse embryonic fibroblasts in a p53-dependent manner (38). Our data show that overexpression or depletion of IFI16 can affect cellular level of E2F1, cyclin D1 and p21\textsuperscript{WAF/CIP1}, suggesting that IFI16 regulation of these cell cycle control proteins may play a role in LIF-mediated growth arrest.

Interestingly, IFI16 is an effector specific only for growth arrest but not differentiation, in response to the LIF-mediated cell extrinsic pathway upon Ras/Raf activation in TT cells. LIF mediates a cell-extrinsic growth inhibitory signaling pathway, through the LIFR-gp130 receptor, via induction of JAK/STAT signaling. Negative regulation of the gp130/JAK/STAT3 pathway can be achieved by protein tyrosine phosphotases such as SHP-1, by proteolytic degradation, and by other regulators such as SOCs and PIAS (39). Activation of the Ras/Raf/MEK signal transduction pathway induces TT cells both to undergo growth arrest and to display changes in differentiation markers, including morphology, calcitonin/CGRP ratio, and downregulation of
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RET and hASH expression (16,17,27,40). These differentiation changes, except for hASH downregulation, are also induced by the LIF-mediated cell extrinsic pathway of Ras/Raf signaling (18). Thus, these signal transduction cascades appear to induce a coordinated program of differentiation and growth arrest (Fig. 8). Identification of IFI16 as an effector specific for growth arrest, in the cell-extrinsic signaling pathway, clearly demonstrates that the downstream effectors of Ras/Raf/MEK-mediated growth arrest can be separated from those for differentiation (Fig. 8).

In the LIF-induced cell extrinsic pathway, STAT3 activation is required for both differentiation and growth arrest, including induction of IFI16 (Fig. 8). Identification of the signaling components linking STAT3 activation and IFI16 expression may further elucidate the divergent mechanisms of growth arrest and differentiation signaling.

Previously, we have shown that the SCLC cell lines, NCI-H209 and DMS53, could be growth arrested by Raf activation (12,13). We have also shown that these SCLC cells produce LIF in response to Ras/Raf activation (18). The level of LIF production was high enough to activate STAT3 in the SCLC cell lines at a similar level as in TT cells, and to induce growth arrest of TT cells. However, LIF could not induce growth arrest in these SCLC cells. Therefore, we hypothesized that the LIF-induced growth inhibitory signaling is disabled at a step distal to STAT3 in these SCLC cell lines while their cell intrinsic pathway for growth inhibitory signaling by Ras/Raf is intact. In this study, we have found that IFI16 could not be induced by LIF in DMS53 cells, although overexpression of IFI16 could lead to growth arrest of the cell line. Thus, it appears that the inability of the LIF-mediated cell extrinsic pathway to elicit growth arrest in some SCLC cells may be due to the failure to induce IFI16 in response to LIF, and that SCLC cells may be impeded in a signaling step between STAT3 and IFI16 (Fig. 8). We speculate that IFI16 induction may determine the ability of some cells to undergo growth arrest in response to LIF. The prostate carcinoma cell line LNCaP undergoes growth arrest in response to Raf activation (14).
We have observed that LNCaP could not respond to LIF, although it could produce LIF and activate STAT3 in response to Ras/Raf activation (our unpublished data). IFI16 could not be induced in LNCaP by LIF, while overexpression of IFI16 was sufficient to induce growth arrest of the cell line (data not shown). IFI16-mediated growth arrest in LNCaP has also been observed in another study (29).

The inability of SCLC cells to respond to LIF does not appear to be due to DNA methylation-induced silencing of the IFI16 gene. We considered the potential involvement of DNA methylation, since SCLC cells undergo substantial epigenetic changes during tumorigenic development (41,42). However, treatment of the SCLC cells with the inhibitor of DNA methylation, 5-Aza-2-dC, did not elicit a cellular response to LIF (data not shown), suggesting that DNA methylation is not responsible for the inability of IFI16 induction in these cells. In addition, IFI16 could be induced by interferon α or γ in DMS53 cells (Fig. 6), indicating that the IFI16 gene is not silenced in the cells. Further elucidation of the mechanism by which LIF activates IFI16 in TT cells will be important to understanding the biology of Ras/Raf-mediated growth arrest in neuroendocrine cancer cells.

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FIGURE LEGENDS

FIG. 1. Ras/Raf induces IFI16 via LIF/JAK/STAT in MTC cells. (A) Expression of IFI16 was analyzed by RT-PCR using total RNA from TTRaf cells treated with β-estradiol (E₂) to activate Raf or TT cells treated with LIF for 2 days. β-estradiol alone had no effect on IFI16 expression (data not shown). (B and C) TTRaf or TT cells were infected with the STAT3 dominant negative adenovirus AdSTAT3-DN (STAT3DN) or the control virus AdGFP for 2 days prior to Raf activation or LIF treatment. After 2 days, expression of IFI16 was analyzed by RT-PCR (B), or by Western blotting (C). The results were normalized for expression of GAPDH. Experiments were repeated at least three times with similar results.

FIG. 2. IFI16 is essential for growth inhibition induced by LIF. (A) TT cells transiently transfected with siRNA targeted to IFI16 (si-IFI16) were treated with LIF for 2 days. Cell lysates were then analyzed for expression of IFI16, E2F1, cyclin D1, and p21WAF1/CIP1 by Western blotting. Cells were also transfected with scrambled siRNA control (control). GAPDH was measured as a protein loading control. (B and C) TT cells stably transfected with pSilencer 2.1 containing sequences for expression of small hairpin RNA for IFI16 (si-IFI16) were treated with LIF for growth curve (B), or cell cycle analysis after 3 days (C). Control cells were stably transfected with the empty pSilencer 2.1 vector. Data (mean ± standard error) are from a representative experiment performed in triplicate. Experiments were repeated at least three times with similar results.

FIG. 3. IFI16 is not required for LIF-mediated differentiation. TT cells stably transfected with pSilencer 2.1 containing sequences for expression of small hairpin RNA for IFI16 (si-IFI16) were treated with LIF for 2 days and observed for expression of RET by Western blotting (A), expression of calcitonin/CGRP by real time PCR (B), and morphological changes (C).
cells were stably transfected with the empty vector. Data (mean ± standard error) are from a representative experiment performed in triplicate. Experiments were repeated at least three times with similar results.

**FIG. 4.** IFI16 is sufficient to induce growth inhibition in MTC cells. TT cells were infected with the adenoviral vector encoding IFI16 (AdIFI16), or the control AdGFP. Cells were then tested for growth for 8 days (A), expression of IFI16, E2F1, cyclin D1, and p21\(^{WAF/CIP1}\) by Western blotting after 2 days (B), and cell cycle analysis after 4 days (C). Similar infection ratio was ascertained by GFP expression (data not shown; see also Fig. 5C). (D) For colony formation assay, TT cells were transfected with pcDNA3.1 harboring the IFI16 gene or the empty vector, and selected with G418 for 2 weeks. Data (mean ± standard error) are from a representative experiment performed in triplicate. Experiments were repeated at least 3 times with similar results.

**FIG. 5.** IFI16 cannot induce differentiation in MTC cells. TT cells were infected for 2 days with AdIFI16, and the control AdGFP. Cells were then tested for expression of RET by Western blotting (A), expression of calcitonin/CGRP by real time PCR (B), and morphological changes (C). TT cells were also treated with LIF for 2 days as the positive control for morphological changes. Similar infection ratio was ascertained by GFP expression (bottom panel). Data (mean ± standard error) are from a representative experiment performed in triplicate. Experiments were repeated at least 3 times with similar results.

**FIG. 6.** LIF cannot induce IFI16 in the SCLC cell line DMS53. DMS53 cells were treated for 2 days with LIF, interferon-\(\alpha\) (IFN\(\alpha\)), or interferon-\(\gamma\) (IFN\(\gamma\)). For Raf activation, cells were

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infected for 2 days with the Raf-BXB adenovirus (Raf) or the control AdGFP (control). Cell lysates were then analyzed for expression of IFI16 by Western blotting. Experiments were repeated at least 3 times with similar results.

**FIG. 7. IFI16 can induce growth arrest in DMS53 cells.** (A to C) DMS53 cells were infected with AdIFI16, or the control AdGFP. Cells were then tested for growth for 8 days (A), expression of IFI16, E2F1, cyclin D1, and p21WAF1/CIP1 by Western blotting after 2 days (B), and cell cycle analysis after 4 days (C). (D) For colony formation assay, DMS53 cells were transfected with pcDNA3.1 harboring the IFI16 gene, or the empty vector, and selected with G418 for 2 weeks. Data (mean ± standard error) are from a representative experiment performed in triplicate. Experiments were repeated at least 3 times with similar results.

**FIG. 8. Signal transduction pathway for Ras/Raf-mediated growth arrest.** The potential growth-inhibitory signaling by Ras/Raf is depicted. Activation of Ras/Raf in TT cells induces IFI16 via the LIF/JAK/STAT pathway. IFI16 is necessary and sufficient to mediate growth arrest signaling via the cell-extrinsic growth inhibitory pathway of Ras/Raf, but it is not required for differentiation signaling, or for the cell intrinsic growth inhibitory pathway. DMS53 and LNCaP cell lines can not induce IFI16 upon activation of the LIF/JAK/STAT pathway although IFI16 can induce growth arrest in these cell lines.
### A

| TTRaf | TT |
|-------|----|
| -     | + (E<sub>2</sub>) |
| -     | + (LIF) |

- IFI16
- GAPDH

### B

| Raf | LIF |
|-----|-----|
| GFP | STAT3DN |
| GFP | STAT3DN |

- IFI16
- GAPDH

### C

| Raf | LIF |
|-----|-----|
| GFP | STAT3DN |
| GFP | STAT3DN |
| GFP | STAT3DN |
| GFP | STAT3DN |

- IFI16
- GAPDH
Fig. 2

A

LIF

Normal Normal Control si-IFI16

IFI16 E2F1 CyclinD1 p21^{WAF1} GAPDH

B

C

G2/M S G0/G1

% cells

Control Control + LIF si-IFI16 si-IFI16 + LIF

(G0/G1) 78.7 ± 0.32 88.3 ± 1.10 81.5 ± 1.11 80.0 ± 1.19

(S) 13.8 ± 0.48 4.62 ± 0.12 11.5 ± 0.97 13.4 ± 0.92
Fig. 3
**Fig. 4**

**A**

- Normal
- AdGFP
- AdIF16

10^4 cells/well

1 2 3 4 5 6 7 8 (d)

**B**

- IFI16
- E2F1
- CyclinD1
- p21
- WAF1
- GAPDH

**C**

|          | Normal       | AdGFP        | AdIF16       |
|----------|--------------|--------------|--------------|
| G2/M     | 78.3 ± 0.18  | 79.4 ± 0.15  | 87.9 ± 0.32  |
| S        | 15.6 ± 0.04  | 14.7 ± 0.18  | 7.54 ± 0.12  |
| G0/G1    | 79.4 ± 0.15  | 87.9 ± 0.32  |              |

**D**

- pcDNA3.1
- pcDNA3.1-IFI16

% colony formation 23.5 ± 6.03
Fig. 5

A

| Normal | AdGFP | AdIFI16 |
|--------|--------|---------|
| RET    |        |         |
| GAPDH  |        |         |

B

![Bar graph showing fold changes for Calcitonin and CGRP](image)

C

| Control | LIF |
|---------|-----|
| AdGFP   | AdIFI16 |

![Images showing control and LIF conditions](image)
| Control | Raf | LIF | IFNα | IFNγ |
|---------|-----|-----|------|------|

- **IFI16**
- **GAPDH**
Fig. 7

A. Graph showing A<sub>540</sub> values over time (0 to 8 days) for Normal, AdGFP, and AdIFI16 conditions.

B. Western blot analysis showing levels of IFI16, E2F1, CyclinD1, p21<sup>WAF1</sup>, and GAPDH proteins in Normal, AdGFP, and AdIFI16 conditions.

C. Bar graph showing cell cycle distribution (G0/G1, S, G2/M) for Normal, AdGFP, and AdIFI16 conditions.

D. Bar graph showing percentage of colony formation for pcDNA3.1 and pcDNA3.1-IFI16.
Fig. 8
IF16 is an essential mediator of growth inhibition, but not differentiation, induced by the leukemia inhibitory factor/JAK/STAT pathway in medullary thyroid carcinoma cells

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