Oncogenic Potential of a Dominant Negative Mutant of Interferon Regulatory Factor 3*

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Interferon regulatory factor 3 (IRF3) is activated in response to various environmental stresses including viral infection and DNA-damaging agents. However, the biological function of IRF3 in cell growth is not well understood. We demonstrated that IRF3 markedly inhibited growth and colony formation of cells. IRF3 blocked DNA synthesis and induced apoptosis. Based on this negative control of cell growth by IRF3, we examined whether functional loss of IRF3 may contribute to oncogenic transformation. IRF3 activity was specifically inhibited by expression of its dominant negative mutant. This mutant lacks a portion of the DNA binding domain like IRF3a, an alternative splice form of IRF3 in the cells. This dominant negative inhibition blocked expression of specific IRF3 target genes. Mutant IRF3 efficiently transformed NIH3T3 cells, as demonstrated by anchorage-independent growth in soft agar and tumorigenicity in nude mice. These results imply that IRF3 may function as a tumor suppressor and suggest a possible role for the relative levels of IRF3 and its dominant negative mutant in tumorigenesis.

Inhibition of cell growth is critical for the maintenance of normal tissue homeostasis. Disruption of this negative regulation can clearly contribute to the multistep process of tumorigenesis, as evidenced by the frequent mutation of tumor suppressor genes in human cancer (reviewed in Ref. 1). The p53 tumor suppressor gene is frequently mutated or functionally inactivated in human cancers of many different tissue types. The mechanisms by which p53 loss contributes to tumorigenesis have been substantiated by extensive studies of p53 function (2–4). For example, p53 plays a role in cell cycle checkpoint control by inducing cell growth arrest in response to DNA damage. Consistent with this function, cells lacking p53 fail to arrest in the cell cycle following DNA damage, which may lead to genetic instability. Thus, as a checkpoint regulator, p53 can directly and indirectly suppress tumor induction by preventing the propagation of oncogenic mutations in other genes.

Interferon regulatory factor 3 (IRF3)² is known to be induced by viral infection (reviewed in Ref. 5). We found that IRF3, like p53, can also be induced by DNA-damaging agents including doxorubicin (6–8). Recently, Weaver et al. (9) have shown that the DNA-damaging agent, etoposide, activates IRF3 and induces specific target genes including ISG54 in the absence of the action of interferon. Interestingly, Karpova et al. (10) demonstrate that IRF3 is an in vivo target of DNA-dependent protein kinase, which is involved in cell cycle checkpoint control in response to DNA damage. A similar regulatory mechanism was described for p53; DNA damage induced phosphorylation of Ser-15 in p53, and this serine site is the known target for the DNA-dependent protein kinase family of kinases (2–4). Furthermore, virus-inducible double-stranded RNA-dependent protein kinase, PKR, may be involved in the DNA damage-induced phosphorylation of p53 through regulating the activity of the DNA-PK family of kinases (11). These results, together with possible cross-talk between DNA damage and other stress response pathways, suggest that IRF3, like p53, may play a role in a variety of host defenses that have evolved to counter environmental stresses.

An interesting possibility emerging from these studies is that IRF3 can directly control cell growth and the functional loss of IRF3 may contribute to oncogenic transformation. In an effort to test this possibility and to determine the physiological function of IRF3, we induced IRF3 activity with its ectopic overexpression, and we inhibited IRF3 activity using a dominant negative mutant that contains a defective DNA binding domain like IRF3a, an alternative splice form of IRF3 in the cells (12, 13). These studies revealed the anti-oncogenic and oncogenic potentials of IRF3 and a dominant negative IRF3 mutant.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Human IRF3 was expressed under the control of the constitutive cytomegalovirus promoter in pcDNA3 (Amersham Biosciences). A deletion mutant for IRF3 lacking the DNA binding domain (IRF3Δ) was made by PCR amplification of the region encoding amino acid residues 58–427. This fragment of IRF3 (IRF3Δ) and an oncogenic version of Ha-ras (Ha-ras G12V) (14) were also cloned

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** The abbreviations used are: IRF3, interferon regulatory factor 3; IFN, interferon; GBP, guanylate-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; BrdUrd, 5-bromo-2′-deoxyuridine; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; 2′,5′-AS, 2′,5′-oligoadenylate synthetase.

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into pDNA3 (Amersham Biosciences). The plasmid used as a negative control was an empty plasmid, pcDNA3, containing no corresponding gene in each experiment. To generate anti-human IRF3 antibody, recombinant glutathione S-transferase (GST)-IRF3 was expressed in Escherichia coli, purified by glutathione-Sepharose 4B (Amersham Biosciences), and then used to immunize rabbits.

Transfection—Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing fetal bovine serum or calf serum. All of the culture growth media contained 100 units/ml penicillin and 100 μg/ml streptomycin. Cells (typically 0.5–1 × 10^6; otherwise as indicated in the figure legends) were transfected with specific amounts of plasmids as indicated in the figure legends, using LipofectAMINE reagents (Invitrogen) in accordance with the manufacturer’s instructions. Transfection efficiency was analyzed by cotransfection with pEGFP-N1 (Clontech) followed by monitoring under a fluorescence microscope. To select transiently transfected cells, we utilized the Capture-Tec kit (Invitrogen) according to the manufacturer’s instructions. Cells were cotransfected with either control or IRF3 expression plasmids and phOs SVF expression plasmid (pHook-1, Invitrogen). Transfected cells were then selected through incubation with magnetic beads coated with hapten for 1 h as suggested (Invitrogen).

Cell Division Assay—Cells (0.5–1 × 10^6) were cotransfected with either control or IRF3 (and IRF3Δ) expression plasmids and GFP expression plasmid (pEGFP-N1, Clontech). After transfection, the number of GFP-expressing cells was monitored for 5 days under fluorescent microscopy. Cell division was observed as an increase in the number of aggregates of GFP-expressing cells.

Stable Colony Formation Assay—For stable colony formation assays, cells (1 × 10^6) were transfected with plasmids containing the neomycin resistance gene. At 24 h post-transfection, G418 (1 mg/ml) was added to the transfected cells and cells were cultured for 2 weeks. The plates were then stained with crystal violet in 20% ethanol, and colonies containing more than 30 cells were counted from representative fields.

BrdUrd DNA Synthesis Assay—DNA synthesis assay was performed with the 5-bromo-2′-deoxyuridine (BrdUrd) labeling and detection kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Cells were incubated with BrdUrd for 24 h. After the labeling, the medium was removed, the cells were fixed and the DNA was denatured by adding FixDenat (Roche Molecular Biochemicals). Then, anti-BrdUrd-POD antibody is added and BrdUrd incorporated into the newly synthesized DNA was detected by a substrate reaction (Roche Molecular Biochemicals). The DNA synthesis rate was quantified with a microplate reader (EGL&Gallic). Flow Cytometry—For fluorescence-activated cell sorter (FACS) analysis, cells were washed with phosphate-buffered saline. The harvested three cells were fixed with 70% ethanol for 1 h on ice. Cells were then washed three times with phosphate-buffered saline. After incubation with RNase (1 mg/ml), the DNA in fixed cells was stained with propidium iodide (10 mg/ml) for 30 min. Cells were analyzed by FACScaliber (BD Biosciences).

Western Blot—Whole cell extracts were prepared by lysis in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 50 mM NaF, and protease inhibitors. Following the centrifugation, proteins in the extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The membranes were blocked with 5% nonfat milk and probed with anti-human IRF3 antibody. Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and visualized using the enhanced chemiluminescence system (ECL; Amersham Biosciences).

Reverse Transcriptase PCR—Cells were incubated with IFN-β (1000 units/ml) or Sendai virus (200 hemagglutinin units/ml) as described previously (6–8). Total RNA was prepared from cells treated with Trizol reagent (Invitrogen). For each sample, 5 μg of isolated RNA was used for the reverse transcriptase reaction with the SuperScript System (Invitrogen) in accordance with the manufacturer’s instructions. The resultant cDNA was used for PCR with primers specific for IFN-β, 2′,5′-oligoadenylate synthetase (2′,5′-AS), and GBP-1 as described previously (15). Amplification was carried out for 28 cycles, and 5 μl of the resulting reaction mixtures were separated and analyzed on a 2% agarose gel.

Soft Agar Assay—Soft agar assays in 6-cm dishes were performed as previously described (16). Cells (1, 3, and 5 × 10^4) were seeded in 3.0% agarose solution in DMEM containing 10% calf serum as a bottom layer of 0.5% agarose solution also in DMEM growth medium. Cells were fed with fresh DMEM growth medium containing 0.3% agarose every week. After 3 weeks, plates were checked and photographed.

Tumor Growth in Nude Mice—Tumorigenicity assays were performed as previously described (16). Nude mice (BALB/c nu/nu) were injected subcutaneously with 1 × 10^6 cells suspended in 100 μl of phosphate-buffered saline. Injected mice were observed every 3 days. Subcutaneous tumors were grossly visible at the site of injection after 2–3 weeks. Each cell line was tested for tumorigenicity in 3–12 different mice.

RESULTS

To examine the function of IRF3 in cell growth, we analyzed the effect of its overexpression on cell growth (Fig. 1). To this end, HeLa cells were transfected with the IRF3 expression plasmid, together with the GFP expression plasmid as a marker of transfection. Using GFP, we were able to examine the division of live cells over time as evidenced by the increasing number of GFP-expressing cell aggregates. Examination by fluorescence microscopy for 5 days following transfection showed that IRF3-transfected cells failed to divide, whereas control-transfected cells continued to divide (Fig. 1A). In control-transfected cells, we found increased numbers of GFP-expressing cell aggregates over time, whereas only a few cells expressing GFP were observed in IRF3-transfected cells (Fig. 1B).

We also used a colony formation assay to evaluate the growth-inhibitory effect of IRF3 (Fig. 2). HeLa cells were transfected with the IRF3 expression plasmid with a neomycin resistance gene. After transfection, G418 was added and transfected cells were selected for 2 weeks. Ectopic expression of IRF3 markedly inhibited stable colony formation in HeLa cells (Fig. 2A). IRF3 inhibited by nearly 90% of the stable clones as compared with control-transfected cells (Fig. 2B). Furthermore, the average size of the colonies was much smaller when transfected with the expression plasmid for IRF3 (data not shown). Taken together with data from Fig. 1, these results
indicate that IRF3 inhibits cell division and stable colony formation in HeLa cells.

Next, we tried to gain insight into the mechanisms for the observed cell growth inhibition by IRF3. Ectopic expression of IRF3 induced chromatin condensation and fragmentation of the nucleus in a small percentage of transfected cells stained with 4',6-diamidino-2-phenylindole (data not shown). To test the significance of this observation, transfected cells were verified by cotransfection with GFP expression plasmids, and then cell survival was assayed by a trypan blue exclusion assay. Consistently, we detected a small decrease in the number of live cells upon expression of IRF3 (Fig. 3A). Thus, apoptosis is probably not the major cause for the observed growth-inhibitory effect of IRF3 under our conditions. Based on these results, we examined whether the IRF3-mediated growth inhibition was also due to a block in DNA synthesis (Fig. 3B). DNA synthesis was monitored by measuring BrdUrd incorporated into newly synthesized DNA. Ectopic expression of IRF3 dramatically reduced BrdUrd incorporation into DNA (Fig. 3B). These results indicate that IRF3 can inhibit cell growth by blocking DNA synthesis and, to some extent, inducing apoptosis.

To further examine the basis for IRF3-mediated cell growth inhibition, we analyzed the cell cycle progression upon the ectopic expression of IRF3 (Fig. 4). To this end, HeLa cells were transfected with the IRF3 expression plasmid, together with the phOx SFV expression plasmid as a marker of transfection. Transfected cells were stained with propidium iodide for FACS analysis. Western blot analysis showed the induction of IRF3 protein levels in the transfected cells (Fig. 4A). Under these conditions, IRF3 significantly increased the number of G1 cells, reducing that of cells in the S-phase (Fig. 4B). Consistent with the results shown in Fig. 3A, we detected no such marked increase in the number of apoptotic cells upon expression of IRF3. These results further support the conclusion from our results shown in Figs. 1–3 that IRF3 plays a negative role in cell division and growth.

HeLa cells were utilized in various cell growth assays with IRF3 (Figs. 1–4). Next, we determined the inhibitory role of IRF3 in cell growth using other cell types (HepG2, NIH3T3, and REF52) (Fig. 5). These different types of cells were transfected with the IRF3 expression plasmid, together with the GFP expression plasmid as a marker of transfection. The cell growth was monitored by the increasing number of GFP-expressing cells under the fluorescence microscopy for 5 days after transfection. As in HeLa cells, IRF3-transfected cells failed to divide in all of the tested cells, whereas control-transfected cells didn’t.
fected cells continued to divide (Fig. 5, A (HepG2), B (NIH3T3), and C (REF52)). The slight decrease in the number of IRF3-transfected HeLa (Fig. 1 A), HepG2, and NIH3T3 (Fig. 5, A and B) cells may support the significance of IRF3-dependent apoptotic response demonstrated in Fig. 3 A (9, 17). Thus, IRF3 can inhibit cell growth in various kinds of cells.

Based on the function of IRF3 in negative control of cell growth, we next examined how the loss of IRF3 activity affects cell growth. To this end, we attempted to utilize mutant IRF3/H9004 lacking a portion of the DNA binding domain at its amino terminus like IRF3a, which is an alternative splice isoform of IRF3 in the cells (12, 13). As a first step, we examined the possible dominant negative effects of IRF3/H9004 on the observed IRF3-induced attenuation of cell growth (Fig. 6). The cell growth was monitored by the increasing number of GFP-expressing cells under fluorescence microscopy for 5 days after cotransfection with IRF3 and IRF3/H9004 expression plasmids. Consistent with our results (Figs. 1 and 5), IRF3 markedly attenuated the cell growth of NIH3T3 and HeLa. This attenuation was significantly inhibited by coexpression of IRF3/H9004 (Fig. 6). These results suggest that IRF3/H9004 can be functional as a dominant negative mutant of IRF3 in the cell growth.

To further examine the function of dominant negative mutant of IRF3 in cell growth, we generated NIH3T3 cell clones overexpressing IRF3/H9004. The plasmid pcDNA3, which expresses the human IRF3 protein (58–427 amino acid residues) without its DNA binding domain under the control of the constitutive cytomegalovirus promoter, was transfected into NIH3T3 cells. As a positive control, some cells were transfected with an expression plasmid for an oncogenic version of Ha-ras (Ha-ras G12V) in which glycine at amino acid position 12 was changed to valine (14). A control expression plasmid, pcDNA3, was transfected into NIH3T3 cells as a negative control. After selection for G418 resistance, we obtained several clones that expressed some levels of truncated human IRF3 protein. One such clone (2) was chosen for initial experiments because of its high level of expression (Fig. 7 A).

To address the specific effect of the IRF3 mutant, we assayed the induction of three endogenous genes having promoters that contain IRF binding sites: IFN-β, 2',5'-AS, and GBP-1 (Fig. 7B). These promoters are activated by viral infection through specific activation of IRF3 (18–25). IFN treatment also induces certain promoters but fails to stimulate transcriptional activity of IRF3; thus, IFN may induce the promoters through activation of the other IRF family factors (e.g. IRF1 and ISGF3) (26). Overexpression of a dominant negative IRF3 mutant (IRF3Δ) sharply reduced viral induction of three tested genes including IFN-β (Fig. 7B). In contrast, overexpression of IRF3a did not affect induction of the 2',5'-AS and GBP-1 genes in response to IFN treatment. Furthermore, under these conditions, expression of a control gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), was not affected by expression of IRF3Δ.

These results strongly suggest that expression of IRF3Δ inhibit-
its endogenous genes that are specifically regulated by IRF3 but not those regulated by other IRF family factors.

The demonstrated role of IRF3 in cell growth inhibition raised the possibility that its inactivation might play a role in oncogenic transformation in a manner analogous to mutations of the p53 tumor suppressor gene. NIH3T3 cells are highly susceptible to oncogenic transformation by inactivation of p53 or activation of Ha-ras. Indeed, microscopic examination of the cells expressing Ha-ras G12V revealed multilayer growth typical of transformed cells (Fig. 8A). Similar morphological changes were observed with expression of IRF3Δ. To assess their oncogenic potential, clones overexpressing IRF3Δ and Ha-ras G12V were tested for colony formation in soft agar (Fig. 8B). Anchorage-independent growth in soft agar often correlates with malignant transformation. At 3 weeks, we detected the formation of tight colonies in soft agar with expression of both IRF3Δ and Ha-ras G12V. In contrast, control-transfected control cells showed no colony formation under these conditions. Thus, cells overexpressing a dominant negative IRF3 mutant (IRF3Δ) displayed anchorage-independent growth in soft agar. Furthermore, whole populations of cells transfected with IRF3Δ were plated directly into soft agar, and the results confirmed the ability of IRF3Δ to form colonies (Fig. 8C).

To further confirm the tumorigenic potential of cells expressing IRF3Δ, we injected these cells and cells expressing Ha-ras G12V subcutaneously into nude mice (Fig. 8D). In all mice injected with cells overexpressing IRF3 or Ha-ras G12V, tumors developed within 2 to 3 weeks and continued to grow unrestrictedly (12 mice for IRF3Δ and 3 mice for Ha-ras G12V).

No tumors developed in nude mice injected with cells from control-transfected control cells during the same period of time. These results suggest that the expression of a dominant negative IRF3 mutant (IRF3Δ) imposes altered growth properties and tumorigenicity in NIH3T3 cells.

Finally, we analyzed the tumorigenic potentials of several other clones expressing some levels of truncated IRF3 protein (Fig. 9). Selected clones (4, 5, and 7) showed less amounts of truncated IRF3 mutant protein (IRF3Δ) than the clone (2) used for the experiments in Figs. 7 and 8 (Fig. 9A). Under these conditions, all of these clones facilitated tight colony formations in soft agar (Fig. 9B). Furthermore, tumors were developed within 2–3 weeks in all the mice injected with cells expressing IRF3Δ mutant protein (Fig. 9C). Alterations in growth properties were observed consistently in all of the tested clones with expression of a dominant negative IRF3 mutant (IRF3Δ), and therefore transformed phenotypes were not the result of clonal variations.

**DISCUSSION**

Our present studies have demonstrated the tumor suppressor function of IRF3 and the oncogenic potential of a dominant negative IRF3 mutant that specifically inhibits the expression of IRF3 target genes. Ectopic expression of IRF3 markedly attenuated cell growth in various kinds of cells including HeLa, HepG2, Ref52, and NIH3T3 cells. Expression of the IRF3 mutant (IRF3Δ) facilitated oncogenic transformation in NIH3T3 cells, and its tumorigenic potential was clearly demonstrated by anchorage-independent growth in soft agar and tumor formation in nude mice. Impaired IRF3 may be responsible for the observed transformation, because IRF3Δ inhibited expression of specific IRF3 target genes (Fig. 7) and ectopic

**FIG. 7.** A dominant negative IRF3 mutant inhibits specific IRF3 target gene expression. NIH3T3 cells were stably transfected with expression plasmids for a dominant negative mutant form of human IRF3 (IRF3Δ). One of the clones (2) was chosen for these experiments. A control expression plasmid was transfected as a negative control. A, IRF3 protein levels were measured by Western blotting with anti-human IRF3 antibody in whole cell extracts derived from control and an IRF3Δ cell line. B, control and an IRF3Δ cell line were control-treated, infected with virus, or stimulated with IFN-β (1000 units/ml). Total RNA was subjected to reverse transcriptase PCR analysis of IFN-β, 2′,5′-AS, GBP-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
expression of IRF3 suppressed the colony formation by IRF3Δ in NIH3T3 cells (data not shown).

The physiological relevance of a dominant negative IRF3 mutant (IRF3Δ) can be supported by the identification of IRF3α in the cells (10, 12, 13). IRF3α is generated by alternative splicing of IRF3 pre-mRNA. As demonstrated with IRF3Δ, IRF3α can selectively and potently inhibit virus-induced activation of specific target genes including IFN-β and it contains defective DNA binding domain. The expression of IRF3α is ubiquitous, but the levels of IRF3α, compared with those of IRF3, can vary in different cellular contexts. Such regulated production of the IRF3α protein would result in the controlled inhibition of IRF3 activity at the specific target promoters. Because the present studies indicate the role of IRF3 in the cell growth control, it is important to elucidate the mechanisms showing how the ratio of IRF3α to IRF3 is maintained and controlled in response to various signals and that the ratio can be deregulated to alter cell growth control during tumorigenesis.

The ability of an IRF3 mutant to transform cells was nearly as great as that of the oncogenic Ha-ras G12V. Furthermore, mutational inactivation of the p53 tumor suppressor gene can also facilitate oncogenic transformation under similar conditions (27–30). One interesting possibility emerging from the present studies is that expression of mutant IRF3 protein may confer tumorigenic phenotype upon the cells, similar to the gain-of-function associated with p53 mutants (31). The p53 mutant loses its tumor suppressor function as a consequence of the functional inactivation of p53. However, certain types of p53 mutations exert additional gain-of-function phenotypes with dominant negative effects on wild type p53. The mechanisms for these gain-of-function of p53 mutants have not been well characterized. The relevance of these mechanisms remains to be determined in IRF3, with further elucidation of how tumorigenesis may be enhanced by IRF3Δ.

It is well known that most spontaneously immortalized rodent cells such as NIH3T3 cells can be transformed efficiently by activation of one oncogene (or inactivation of one tumor suppressor gene) alone, in contrast to primary rodent and human cells. Interestingly, NIH3T3 cells were shown to contain the deleted p16 tumor suppressor gene, which plays a critical role in Rb regulation pathways (32). Thus, inactivation of IRF3 might cooperate with other oncogenic events for tumorigenesis in normal cells. To understand these tumorigenesis mechanisms (including gain-of-function effects), it is critical to determine which genes play a role in the inhibition and promotion of cell growth with up- and down-regulation of IRF3, respectively.
Related to this concern, the profiling of genome activities is in progress with conditional expression of IRF3 and IRF3a using DNA microarray methods.

Another member of the IRF family, IRF2, blocks the activation of promoters containing IRF binding sites. When IRF2 was overexpressed in NIH3T3 cells, the cells became transformed and displayed enhanced tumorigenicity in nude mice (33). Consistently, overexpression of IRF2 impaired the activation of genes that are controlled by IRF family factors (e.g. IRF1 and ISGF3). Based on our results, we infer that the ability of IRF2, like an IRF3 mutant, to induce cell transformation might also be due to suppression of the transcriptional activity of IRF3. Indeed, ectopic expression of IRF2 can inhibit IRF3-dependent transcriptional activation (data not shown). Thus, several IRF family members including IRF3a might interact functionally to tightly modulate cell growth, and alterations in their regulation could contribute to oncogenic transformation.

Thus far, the involvement of IRF3 in human cancer has not been studied extensively, and no mutation in the IRF3 gene has been reported in a human cancer. However, several viral oncoproteins, including human papillomavirus 16 E6 and adenovirus E1A, can inhibit the activation of IRF3 as well as p53 (18, 34). The v-IRF protein from Kaposi’s sarcoma-associated herpes virus is shown to inhibit IRF3 transcriptional activity by blocking the interaction of CBP (CREB-binding protein/p300 coactivator with IRF3 (35). On the basis of the present studies, the interaction of these oncoproteins with IRF3 and the inhibition of its transcriptional activation function could contribute directly to the oncogenic potential of these viruses by altering cell growth control pathways. Quite interestingly, and consistently with our idea of the tumor suppressing function of IRF3, ectopic expression of IRF3 has recently been found to suppress the growth of B16 melanoma tumors in the context of gene therapy (36).

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