N-Ras–Induced Growth Suppression of Myeloid Cells Is Mediated by IRF-1

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Abstract

Activating mutations in ras oncogenes occur at high frequency in human malignancies and expression of activated ras in some myeloid cell lines has been shown to induce growth suppression associated with up-regulation of the cyclin-dependent kinase inhibitor p21CIP1/WAF1 in a p16INK4a, p15INK4b, and p53 independent fashion. The expression profile induced in growth-suppressed myeloid cells with that induced in myeloid cells, which are transformed by N-rasG13R. The expression profile induced in growth-suppressed cells was consistent with differentiation and included the up-regulation of the transcription factor IRF regulatory factor-1 (IRF-1), a known transcriptional activator of p21CIP1/WAF1 expression and a target of oncogenic mutations associated with myeloid leukemia. Antisense suppression of IRF-1 prevented N-rasG13R–associated growth arrest and up-regulation of p21CIP1/WAF1. These results define a novel tumor suppressive response to oncogenic signaling and provide a mechanistic link between growth suppression and differentiation in myeloid cells. (Cancer Res 2005; 65(3): 797-804)

Introduction

Activating mutations in the N-ras gene occur at high frequency in acute myeloid leukemia (AML) and the preleukemic condition myelodysplastic syndrome (1, 2), and expression of activated N-ras in hematopoietic stem cells can induce myeloproliferative disorders including leukemias in murine models (3). Whereas the oncogenic transforming abilities of ras genes in immortalized cell lines are well documented, expression of activated ras genes in primary human fibroblasts has been shown to induce a state of permanent growth arrest indistinguishable from replicative senescence. In these fibroblast cells, this effect has been shown to be mediated by p16INK4a, p15INK4b, p14ARF, and p53, and is associated with induction of the cyclin-dependent kinase inhibitor p21CIP1/WAF1 (4–6).

In myeloid cell lines, in contrast to other cell lines, ectopic expression of activated ras genes can induce either growth suppression or oncogenic transformation (7–10). In both primary CD34+ hematopoietic progenitor cells and some in immortalized myeloid cell lines, ectopic expression of activated ras has been shown to induce differentiation, growth suppression and/or apoptosis (8, 11). In particular, expression of activated N-ras and H-ras in the K562 and U937 cell lines respectively, has been shown to induce partial differentiation and growth suppression (7–9). In K562 cells, this was associated with accumulation of p21CIP1/WAF1 in a p16INK4a, p15INK4b, p14ARF, and p53 independent fashion (7, 12–14), implying the existence of an as yet undefined tumor suppressive pathway. By contrast, expression of activated N-ras in TF-1 myeloid cells induces growth factor independence, which is characteristic of oncogenic transformation (10). To elucidate the mechanism of ras–induced growth suppression in hematopoietic cells and to examine the potential link between growth suppression and differentiation in these cells, we have used cDNA array technology to analyze the transcriptional program induced by an activated N-ras (G-to-R mutation at amino acid 13, N-rasG13R) in K562, U937, and TF-1 cells. We report here that the expression profile induced by N-rasG13R associated growth suppression was indicative of differentiation, and included up-regulation of the transcription factor IRF regulatory factor-1 (IRF-1), a known activator of p21CIP1/WAF1 transcription. Moreover, antisense targeting of IRF-1 showed that N-rasG13R–induced growth suppression of these cells was dependent on IRF-1. This defines a novel tumor–suppressive pathway that is independent of the p53 and INK4 pathways, which have previously been shown to respond to oncogenic signaling (4).

Materials and Methods

Cell Culture. The PG13 viral producer cell lines were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Life Technologies), 0.2 mmol/L glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin. Human leukemia K562, U937, and TF-1 cells were grown in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Life Technologies), 0.2 mmol/L glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin. Human recombinant IL-3 (10 ng/mL) was added to the medium for growth of TF-1 cells. All cell lines were obtained from American Type Culture Collection (Rockville, MD). For induction of polyploidy and consequent growth arrest, K562 cells were treated with 80 ng/mL Nocodazole (Sigma, St. Louis, MO). For cell cycle analysis, 106 cells were incubated in 300 μL 0.1% Triton X-100 with 10 μg/mL propidium iodide and 20 units/mL RNase A for 30 minutes at room temperature, and analysis and cell sorting were done using a FACSort flow cytometer (Becton Dickinson, Mountain View, CA). For methylcellulose colony assays, cells were suspended at 200 cells/mL in 2% methyl cellulose/RPMI supplemented with 15% FCS, 0.2 mmol/L glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin, and 1 mL was plated into 12-well plate wells. Colonies were counted after 8 days. For two-color fluorescence-activated cell sorting analyses of cell-surface antigen expression, 106 U937 or K562 were stained with PE-conjugated anti-CD11b antibody or PerCP-conjugated anti-CD41 antibody (both from Becton Dickinson), respectively.

Retroviral Gene Transfer. The L9IGFP and L9NrGFP retroviral vectors, and the virus producer cell lines PG13/L9IGFP and PG13/L9NrGFP have been described previously (15). Virus containing media from these cells was harvested after a 16-hour incubation at 37°C and...
infection was done at a multiplicity of infection of 10 in the presence of 8 μg/mL Polybrene (Sigma). Transduced cells were identified and isolated using a FACSort Flow Cytometer (Becton Dickinson).

**Plasmid Construction and Transfection.** The IRF-1 antisense construct pcDNA3-IRF-1A was engineered by cloning the full-length IRF-1 gene from the SV40-IRF-1A construct (ref. 16, gift of L. Manzella, Department of Biomedical Sciences, University of Catania, Catania, Italy) into the HindIII and BamHI sites of pcDNA3 (Invitrogen, San Diego, CA) in the antisense orientation. The pcDNA3 and pcDNA3-IRF-1A constructs were delivered into K562 and U937 cells by electroporation using a Bio-Rad Gene Pulser at 250 V, 960 μF. Transfected cells were selected with 500 μg/mL G418.

**Expression Profiling.** Expression profiling of K562 cells was done using Atlas Human Cancer arrays expression profiling of U937 and TF-1 cells was done using Atlas Human Cancer 1.2 arrays (Clontech, Palo Alto, CA) according to the Manufacturers’ instructions. Briefly, RNA was extracted from cells using the Pure Total RNA kit (Clontech). 32P-radiolabeled cDNA was generated by reverse transcription and hybridized to arrays. Arrays were visualized using a Molecular Dynamics 445 SI phosphorimager and analyzed using Atlas 2.01 software (Clontech). To account for differences in signal intensity between arrays, the arrays were normalized using a global normalization algorithm.

**Reverse Transcription-PCR.** Relative expression of IRF-1, Egr-1, and p21CIP1/WAF1 were confirmed by semi-quantitative nested reverse transcription-PCR (RT-PCR). Each reaction comprised 200 μmol/L each deoxynucleotide triphosphate, 3 mmol/L MgCl₂, 0.2 μmol/L forward primer, 0.4 μmol/L reverse primer, 20 units RNasin, 2.5 units AmpliTaq Gold, 25 units MMLV-RT, 1 μg DNase treated total cellular RNA; cycling conditions of 1 hour at 55°C, 10 minutes at 94°C, then 10 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. One microliter of each reaction was used as template for nested PCR. Each reaction: 200 μmol/L each deoxynucleotide triphosphate, 3 mmol/L MgCl₂, 0.2 μmol/L each internal primer, 4 units AmpliTaq; Cycling conditions: 10 minutes at 94°C then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. A round of 25 cycles of amplification using primers specific for β-actin was used as a loading control (RT-PCR conditions as above). For quantification of β-actin amplicon DNA, triplicate RT-PCR reactions for each time point were run on an agarose gel and quantified using ImageQuant software (Amersham Biosciences, Arlington Heights, IL).

The primers used were (5’ to 3’): Egr1, forward primer GCACCCAAAGTGGCAAC, internal forward primer AGTGAGCATGACCAACCC, external reverse primer GGATCATGGAACCTGG, internal reverse primer GTACGGGCGCTGCC; IRF-1, external forward primer ATGCAGATTAATTCCAAC, internal forward primer GCCAAGCATGACCAACCC, external reverse primer GCTCTGGTCTTTCACCTC, internal reverse primer AAGTTGGCCTTCCACGTC; p21CIP1/WAF1, external forward primer GTACTGGAGCGCTGTCCC; IRF-1, external forward primer CACCC, external reverse primer GGGATCATGGGAACCTGG, internal reverse primer AGTGAGCATGACCAACCC, internal forward primer AGGTGAGCCTGCCAGTGTC; p21CIP1/WAF1, external forward primer AGGGGACAGCAGAGGAAG, internal forward primer GGAGGAGCGATAATCTTGATCTCATGGTGCT. Band intensities were quantified using ImageQuant software (Amersham Biosciences).

**Western Blotting.** Cells (10⁶) were lysed in radioimmunoprecipitation assay buffer and 25 μg of cell lysate was electrophoresed on a Novex 4% to 12% Bis-Tris gel (Invitrogen). Protein was transferred to a polyvinylidene difluoride membrane. Antibodies used were anti-WAF-1 mouse IgG (Ab-1; Oncogene Research, Uniondale, NY), anti-IRF-1 mouse IgG (Becton Dickinson), goat anti-mouse IgG horseradish peroxidase-conjugated (Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin mouse IgM (Calbiochem, La Jolla, CA), rabbit anti-mouse IgM (Calbiochem). Signals were detected using the enhanced chemiluminescence detection kit and Hyperfilm and quantified using ImageQuant software (Amersham Biosciences).

**Results**

**N-rasG13R Inhibits Proliferation of K562 and U937 Cells and Effects Tumor Progression in TF-1 Cells.** It has previously been shown that expression of an activated H-ras gene induced growth suppression and alterations in ploidy in the erythroleukemic/megakaryoblastic K562 cell line and growth suppression in the monoblastic U937 cell line. By contrast, activated H-ras induced growth factor independence in the TF-1 cell line (7–9). In myeloid leukemia, activating mutations in H-ras are far less frequent than activating mutations in N-ras (17, 18), and the effects of activated ras genes upon hematopoietic cells are known to be isofom specific (19). In previous studies, we have shown that expression of N-rasG13R from the L9NrIGFP vector (Fig. 1A) induced growth suppression in K562 cells and oncogenic transformation in TF-1 cells (15). To assess the effect of its expression upon the proliferation of U937 cells, the L9NrIGFP vector and the L9IGFP control vector (Fig. 1A) were separately introduced into these cells by retroviral infection. U937 cells transfected with the L9NrIGFP

![Figure 1. N-rasG13R inhibits U937 proliferation. A, schematic illustration of vectors. L9IGFP is a Moloney-based retroviral vector in which the 5’ long terminal repeat (LTR) drives expression of a transcript consisting of the internal ribosome entry site of cardiovitis virus (IRES) linked to the yellow-shifted green fluorescent protein topaz variant (GFP), L9NgFP is an identical vector with the N-rasG13R gene inserted upstream of the IRES. B, suppression of U937 proliferation by N-rasG13R. U937 cells were transfected in triplicate with either the N-rasG13R expressing L9NgFP vector (●) or the L9IGFP control vector (○). The proportion of GFP positive cells in each culture was determined by flow cytometry on the indicated days post-transduction. C, expression of differentiation-associated antigens on U937 and K562 cells. Cells were infected with the L9IGFP (white columns) and L9NgFP (black columns) vectors and expression of cell surface antigens (CD11b in U937 and CD41 in K562) was assayed in GFP-positive cells 48 hours after infection. D, cell cycle analysis of U937 cells expressing N-rasG13R. Purified populations of U937 cells expressing L9IGFP (white columns) or L9NgFP (black columns) were stained with propidium iodide and analyzed by fluorescence-activated cell sorting. Apoptotic cells were assessed by subdiploid DNA content (all experiments were done in triplicate; bars, SD).
vector showed marked growth suppression (as shown by the depletion of GFP-positive cells from a mixed population of transduced and untransduced cells) when compared with cells transduced with the L9IGFP control vector (Fig. 1B).

Activated H-ras induced growth suppression of U937 cells was shown to be associated with increased expression of differentiation-associated cell-surface antigens (8). To assess whether N-rasG13R would also increase expression of such antigens, U937 cells were infected with the L9IGFP or L9NrIGFP vectors, stained with a phycoerythrin-conjugated anti-CD11b antigen and the proportion of cells expressing both GFP and CD11b was assessed by two-color fluorescence-activated cell sorting. Two days after infection, U937 cells expressing N-rasG13R were more than 30% positive for CD11b expression, as compared with ~10% of cells expressing the control vector (Fig. 1C). A similar analysis was done on K562 cells using an anti-CD41 antibody. K562 cells expressing N-rasG13R showed increased expression of the megakaryocytic marker CD41 (Fig. 1C). This shows that, as has been shown for activated H-ras (8), the growth suppression induced by N-rasG13R in K562 and U937 cells seems to be part of a differentiation program.

We had previously shown that growth suppression induced by N-rasG13R in K562 cells was associated with an aberrant cell cycle profile, characterized by polyplody (a feature of megakaryocytic differentiation) and apoptosis (15). To assess the effect of N-rasG13R expression on the cell cycle of U937 cells, purified populations of U937 cells expressing either the L9IGFP or L9NrIGFP vectors (>90% GFP positive, data not shown) were obtained by fluorescence activated cell sorting. Propidium iodide staining of these cells revealed that around 10% of cells expressing N-rasG13R were apoptotic, as assessed by subdiploid DNA content (Fig. 1D). There was also a significant (P < 0.00005) decrease in the proportion of viable cells in the S-G2-M phases of the cell cycle (Fig. 1D). This suggests that in U937 cells N-rasG13R both induces apoptosis and inhibits progression through the cell cycle.

Expression Profiling. In order to investigate the basis of the growth inhibitory effect of N-rasG13R in U937 and K562 cells, expression profiling was done and the transcriptional programs induced in these two growth-suppressed cell lines compared with that induced by N-rasG13R in TF-1 cells where N-rasG13R induces transformation rather than growth suppression. The L9NrIGFP and L9IGFP constructs were separately introduced into K562, U937 and TF-1 cells by retroviral gene transfer. Seventy-two hours after transduction, GFP positive cells were isolated by flow cytometry and following culture for 24 hours, RNA was extracted for expression profiling. In all cases, cell populations were >90% GFP positive after sorting (data not shown). In order to minimize background signal, each infection was done in five separate experiments and samples were not pooled until after RNA extraction. Triplicate expression profiles were then generated from separate pooled samples (i.e., 15 individual infection cultures per treatment per cell line in total).

The changes in gene expression induced by N-rasG13R in each of these three cell lines were found to be distinctly different (Supplementary Tables S1-S6). No genes were identified which showed significant (>2-fold) up-modulation or down-modulation across all three cell lines. Of the 588 genes assayed in K562 cells, 44 (7.5%) were up-regulated and 105 (17.9%) down-regulated. Of the 1076 genes assayed in TF-1 and U937 cells, 35 (3.3%) were up-regulated in TF-1 and 38 (3.5%) were down regulated; in U937, 88 (8.2%) were up-regulated and 43 (4.0%) were down-regulated.

The analysis sought to identify genes that showed changes in expression specific to the growth inhibitory phenotype. To achieve this, the expression profiles of L9NrIGFP-transduced cells were compared with L9IGFP-transduced cells to generate a differential expression ratio for each gene (differential expression ratio = ras signal divided by control signal). Comparison of the differential expression ratios for K562 and U937 cells (Fig. 2A) revealed three genes which showed significant (>2-fold) up-regulation in both cell lines. These genes were the transcription factor IFN regulatory factor-1 (IRF-1), rac2 (a small GTPase) and the CDK inhibitor p21CIP1/WAF1 (which was previously shown to be up-regulated during N-ras-associated growth arrest of K562 cells; ref. 7). Comparison of K562 and U937 differential expression ratios with TF-1 differential expression ratios showed that expression of these three genes was not altered in response to ectopic N-rasG13R expression in TF-1 cells (Fig. 2B and C) indicating that up-regulation of these three genes was characteristic of N-rasG13R induced growth suppression.

IRF-1 has been shown to be involved in the earliest response of murine myeloid cells to differentiation stimuli and is a direct activator of p21CIP1/WAF1 expression (20, 21). Up-regulation of rac2 has also been shown to be associated with myeloid differentiation (22), although, unlike IRF-1, there is no evidence to suggest that rac2 has a causative role in this process. Thus, the expression array data indicated that the p53-independent growth arrest of K562 and U937 cells in response to activated N-rasG13R was part of a normal differentiation process. In support of this conclusion, the expression array data showed that several genes known to be associated with myeloid differentiation were found to be up-regulated in response to N-rasG13R expression in at least one of these cell lines (K562 or U937) but not in TF-1 cells (Table 1). Of particular interest were the early growth response-1 (Egr-1) gene and MYD88 gene, both of which (along with IRF-1) have been shown to be up-regulated during the primary response of myeloid cells to differentiation stimuli (23), and are causally implicated in myeloid differentiation. MYD88 (24) and several other known IRF-1–responsive genes [i.e., p47-phox (25), (2'-5') oligoadenylate synthetase 2 (26), and CD40 (27)] showed alteration of expression in these cell lines (see Table 1). Thus, N-rasG13R induced growth inhibition in K562 and U937 cells seemed to be mediated by IRF-1 and p21CIP1/WAF1 as part of a differentiation program, involving early induction of Egr-1 (K562) and MYD88 (U937).

Specificity of the N-rasG13R response. Whereas cDNA nylon arrays of the type we used are quantitative (28), the analysis of data generated by such arrays is confounded by the statistical problem of multiple testing (29, 30), which may lead to a large number of false positive signals. To confirm the N-rasG13R induced transcriptional up-regulation of the genes which are most strongly implicated as being causal of myeloid differentiation and consequent growth arrest (i.e., IRF-1 and p21CIP1/WAF1 in K562 and U937 cells, and Egr-1 in K562 cells),"semiquantitative" RT-PCR was done (Fig. 3). Although this technique is not useful for quantifying the magnitude of alterations in transcription, it is sufficient for evaluating trends (i.e., up-regulated, down-regulated, or unaltered expression) in gene expression, and, as such, is an appropriate technique for the validation of array data (30, 31). To ensure even RNA loading, RT-PCR using primers specific for β-actin (expression of which was not affected by N-rasG13R expression in any of the three cell lines, data not shown) was done. For this control, 23 cycles of amplification were done, since this number of cycles was experimentally determined to correspond to an early stage of linear amplification (Fig. 3E).
In all cases, the RT-PCR data correlated well with the expression array data (compare Fig. 3D with Fig. 3A, B, and C). p21CIP1/WAF1 expression was only detected in K562 and U937 cells expressing N-rasG13R. IRF-1 also showed clear up-regulation in these two samples when compared with K562 and U937 cells expressing the control vector. Egr-1 was only detected in K562 cells expressing N-rasG13R.

In TF-1 cells, the basal level of IRF-1 expression was high relative to U937 and K562 cells (Fig. 3A), yet TF-1 cells do not seem to show impaired proliferation. In primary AML samples, it has been shown that expression of IRF-1 is often higher than in normal hematopoietic tissues (32). However, primary AML samples have also been shown to have relatively high expression of the IRF-1 antagonist IRF-2 (33). Despite the increase in IRF-1 expression observed in AML, the ratio of IRF-1/IRF-2 is generally lower in AML than in normal tissues (33), providing an explanation for the ability of these cells to proliferate in the presence of high IRF-1 expression. To assess whether increased expression of IRF-2 might account for the ability of TF-1 cells to proliferate in the presence of high levels of IRF-1, Northern analysis was done using RNA extracted from parental TF-1, K562 and U937 cells (Fig. 3F). IRF-2 was expressed more strongly in TF-1 cells than in U937 or K562 cells, and the IRF-1/IRF-2 ratio in TF-1 cells was intermediate between the ratio observed for U937 and K562 cells. This provides an explanation for the ability of TF-1 cells to proliferate despite high basal IRF-1 expression.

**Suppression of IRF-1 Inhibits N-rasG13R–Induced Growth Arrest.** We next analyzed the up-regulation of IRF-1 in response to N-rasG13R expression and its association with p21CIP1/WAF1 up-regulation during the growth arrest of U937 and K562 cells. For these experiments, an antisense gene suppression strategy was used to inhibit IRF-1 expression. A full-length IRF-1 antisense expression plasmid (pcDNA3-IRF-1A) was introduced into K562 and U937 cells by electroporation, and transduced cells were selected by treatment with G418. These cultures were then transduced with the L9IGFP or L9NrIGFP vectors, and the proportion of GFP-positive cells was monitored by flow cytometry. U937 cells expressing the IRF-1 antisense construct were completely resistant to the growth suppressive effect of N-rasG13R as shown in TF-1 cells expressing either K562 and/or U937 but not in TF-1 cells.

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### Table 1. Myeloid differentiation-associated genes demonstrating modulation of expression (>2-fold) in response to N-rasG13R in either K562 and/or U937 but not in TF-1 cells

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<th>Gene</th>
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IRF-1 Mediates N-ras Induced Growth Suppression

Figure 3. Confirmation of N-rasG13R induced gene up-regulation. A-C, histogram plots of the expression level of IRF-1, Egr-1, and p21^{CIP1/WAF1} in TF-1, K562, and U937 cells expressing either N-rasG13R (L9NrlGFP) or the control vector (L9GFP) as determined by array analysis. Ordinate axis, gene expression intensity (Atlasimage arbitrary units, AAU); bars, 99% confidence intervals. D, RT-PCR done on the same samples using primer sets specific for IRF-1, Egr-1, p21^{CIP1/WAF1}, and β-actin. E, quantification of β-actin amplicon DNA amplified from 1 μg U937 total cellular RNA. Points, mean of three experiments; % SD for each point was <2%. (F) expression of IRF-1 and IRF-2 in parental cell lines. Expression of IRF-1 and IRF-2 was determined in parental TF-1, K562, and U937 cells by Northern analysis. β-Actin was used as a loading control.

evidenced by the stability of the GFP expressing population (Fig. 4A). K562 cells expressing the IRF-1 antisense construct showed inhibition of the N-rasG13R induced growth arrest at 4 days after transduction, but the GFP expressing population decreased at approximately the same rate between days 4 and 6 in both of the N-rasG13R expressing cultures (Fig. 4B), suggesting that in these cells, either the antisense suppression of IRF-1 is less effective than in U937 cells or that an alternative growth suppressive pathway is activated.

Purified populations of each cell type (K562 or U937), transduced with each combination of vectors (pcDNA3 + L9GFP, pcDNA3 + L9NrlGFP, pcDNA3-IRF-1A + L9GFP, and pcDNA3-IRF-1A + L9NrlGFP) were isolated by flow cytometry, to generate populations that stably expressed the N-rasG13R transgene (>90% GFP positive, data not shown). Growth of these cells was assessed in liquid culture (Fig. 4C and D). As in the mixed populations, proliferation of both K562 and U937 cells seemed to be suppressed by N-rasG13R expression. Expression of IRF-1 antisense seemed to completely restore proliferation of U937 cells and partially restore proliferation of K562 cells. Colony assays done with these pure populations showed that expression of N-rasG13R in U937 cells completely abolished colony formation in methylcellulose culture and that suppression of IRF-1 expression restored normal colony formation (Fig. 4F-I). In K562 cells the number of colonies observed in methylcellulose culture was unaffected by expression of N-rasG13R; however, the average size of each colony was smaller in N-rasG13R expressing cultures, and this was partially offset by inhibition of IRF-1 expression (Fig. 4E).

To confirm that IRF-1 expression was inhibited by the antisense construct, protein was extracted from the purified cell cultures and analyzed by Western blot (Fig. 5). In both cell lines, there was a clear increase in the quantity of IRF-1 protein in response to N-rasG13R expression, and this was decreased back to baseline level in K562 cells and below baseline levels in U937 cells by the expression of IRF-1 antisense. In U937 cells p21^{CIP1/WAF1} protein levels closely mirrored IRF-1 protein levels, both in the presence and absence of N-rasG13R, demonstrating the dependence of p21^{CIP1/WAF1} expression on IRF-1 expression in these cells. A similar expression pattern was observed in K562 cells, although the antisense suppression of IRF-1 seemed slightly less effective than in U937 cells and p21^{CIP1/WAF1} expression in response to N-rasG13R was only partially decreased by IRF-1 antisense expression. This decreased level of p21^{CIP1/WAF1} suppression by IRF-1 antisense in K562 cells may reflect the decreased efficacy of the antisense in this cell line (as evidenced by baseline level of IRF-1 in K562 cells expressing the antisense construct alone, Fig. 5) or may be indicative of an alternative tumour suppressive pathway in this cell line. The fact that there is up-regulation of p21^{CIP1/WAF1} in K562 cells in response to N-rasG13R even in the absence of detectable IRF-1 up-regulation (Fig. 5, lane 4) favors the latter explanation. This shows the existence of an alternative pathway acting in parallel to IRF-1 mediated up-regulation of p21^{CIP1/WAF1} in response to N-rasG13R in these cells. Thus, N-rasG13R induced growth suppression seemed completely dependent on IRF-1 in U937 cells and partially dependent on IRF-1 in K562 cells. These data further support the notion that IRF-1 mediates the tumor suppressive response to N-rasG13R in these cells.

Discussion

In these expression profiling experiments, a variety of genes showed altered expression in response to N-rasG13R in K562 (25.3%), TF-1 (6.8%), and U937 (12.4%) cells. In the growth suppressed cell lines K562 and U937, many of these genes were associated with myeloid differentiation. The higher percentage in K562 cells relative to both TF-1 and U937 may be due to the gross changes in morphology in these cells in response to N-rasG13R expression. In terms of these percentages, it should be noted that the gene sets on these arrays are specifically related to oncogenic transformation and regulation of proliferation.

Three of the experiments in this study used a decrease in the proportion of L9NrlGFP expressing U937 or K562 cells as a measure of growth suppression (Figs. 1 and 4). It is possible that
this decrease in the proportion of cells expressing GFP reflects a loss of GFP expression (possibly caused by an expression machinery or cellular metabolism mechanism) rather than growth suppression of cells expressing L9NrIGFP. However, such transgene silencing mechanisms would have to be specific (i) for the L9NrIGFP transcript (because expression of the control vector remained stable) and (ii) for K562 and U937 cells (because expression of the L9NrIGFP vector in TF-1 cells leads to stable GFP expression; ref. 15). Because activated ras genes have been shown to inhibit proliferation of K562 and U937 cells, but not TF-1 cells (7, 8, 15), growth suppression would seem to be the most likely explanation for this decrease in GFP expression observed in U937 and K562 cells. Additionally, in each case where a decrease in GFP expression was used as a measure of growth suppression, we have employed a confirmatory technique (cell cycle analysis in Fig. 1 and colony assays in Fig. 4). Thus, we are confident that the decrease in GFP expression observed is a reflection of growth suppression.

To the best of our knowledge, up-regulation of IRF-1 expression in response to ras signaling has not previously been reported, although IRF-1 inactivation cooperates with activated H-ras and myc genes to transform primary murine fibroblasts (34). IRF-1 is involved in the process of cellular growth arrest in response to DNA damage (35) and can directly activate the p21CIP1/WAF1 promoter (21). Thus, IRF-1 seems to be a tumor suppressor gene, which provides a mechanism for induction of p21CIP1/WAF1 in response to oncogenic signaling or DNA damage in the absence of p53. In support of this, IRF-1 is commonly inactivated in AML and myelodysplastic syndrome (33, 36).

IRF-1 plays an important role during myeloid differentiation. IRF-1 is transcriptionally up-regulated during the primary response of myeloid cells to differentiation stimuli (23) and inhibition of IRF-1 is sufficient to prevent phorbol ester induced differentiation and p21CIP1/WAF1 induction in myeloid cells (16). It seems plausible to conclude, therefore, that the physiologic role of IRF-1 in inducing growth suppression is part of the myeloid differentiation process. A “two-hit” model of leukemogenesis has been proposed in which gain of function mutations in genes which promote proliferation (e.g., N-ras) cooperate with loss of function mutations in hematopoietic transcription factors, generating the highly proliferative and undifferentiated blasts that characterize leukemia (37). The present finding that N-ras mutation can up-regulate IRF-1 expression fits well with this hypothesis, since it provides a specific example of how an oncogene and a hematopoietic transcription factor (acting as a tumor suppressor) may interact during leukemogenesis.

It has been shown that IFN regulatory factor-2 (IRF-2) antagonizes IRF-1–mediated transcription (38). IRF-2 is a known

Figure 4. Expression of IRF-1 antisense inhibits N-rasG13R associated growth arrest in U937 and K562 cells. U937 (A) or K562 (B) cells transduced with either the pcDNA3 control vector (○, ■) or the IRF-1 antisense vector pcDNA3-IRF-1A (○, □) were infected with the GFP control vector L9GFP (○, △, broken line) or the N-rasG13R expressing L9NrIGFP vector (■, ●, solid line) to generate mixed populations of GFP-positive and GFP-negative cells. GFP-positive cells were purified from these cultures by flow cytometry to obtain pure populations of cells stably expressing the L9NrIGFP or L9GFP vector. Growth of these pure populations (both K562 and U937 shown in C and D, respectively) was assessed in liquid culture (8 days after introduction of the retroviral vector) over a 72 hours time period as indicated. In K562 cells (C), IRF-1 antisense (●, □) partially offset the growth suppression induced by N-rasG13R (■, ●). In U937 cells (D), IRF-1 antisense (●, □) completely abrogated the growth suppression induced by N-rasG13R (■, ●). Growth of pure populations was also assessed using colony formation in semisolid media. In K562 cells, expression of N-rasG13R decreased the size of individual colonies in semisolid media without significantly affecting the number of colonies, and this was partially alleviated by expression of IRF-1 antisense (E). Colonies in semisolid media formed by U937 cells transduced with pcDNA3 and L9GFP (F), or pcDNA3-IRF-1A and L9GFP (H). In U937, cells transduced with pcDNA3 and L9NrIGFP colony formation is completely abolished (G) but is restored in cell expressing pcDNA3-IRF-1A and L9NrGFP (I). Representative examples.
IRF-1 Mediates N-ras Induced Growth Suppression

Figure 5. IRF-1 antisense suppresses WAF1 up-regulation in response to N-rasG13R. K562 or U937 cells selected for either the control (pcDNA3) or IRF-1 antisense expression (pcDNA3-IRF-1A) constructs were transduced with either the GFP control (L9IGFP) or N-rasG13R and GFP bicistronic (L9NrIGFP) retroviral vectors and GFP positive cells were isolated by flow cytometry. Analysis of these populations by Western blotting for IRF-1 and p21WAF1/WAF1 showed that expression of both IRF-1 and p21(CDKN1A) is increased in both K562 and U937 cells expressing the N-rasG13R gene. In U937 cells, this up-regulation was prevented by suppression of IRF-1 expression using the antisense construct. In K562 cells p21(CDKN1A) up-regulation is only partially inhibited by IRF-1 antisense expression despite abrogation of IRF-1 up-regulation in response to N-rasG13R. Western blots were done in triplicate.

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4. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogene and is implicated in the pathogenesis of myeloid leukemia (39). Furthermore, analysis of bone marrow samples from AML patients has revealed significantly lower IRF-1/IRF-2 ratios than normal (33), suggesting that the relative expression levels of these two genes is of greater phenotypic significance than the absolute level of either. Interestingly, our study showed that the N-rasG13R–induced growth suppression resistant TF-1 cell line showed stronger expression of IRF-2 than did the N-rasG13R–induced growth suppression sensitive U937 and K562 cell lines. This provides a likely explanation for the ability of TF-1 cells to proliferate despite relatively high IRF-1 expression.

The two most effective pharmacologic therapies for the treatment of AML at present are all-trans retinoic acid and arsenic trioxide, both of which are used in the treatment of AML M3 subtype (acute promyelocytic leukemia). Both of these agents induce differentiation of the leukemic blasts by releasing the block in differentiation which is caused by the PML-RARα oncoprotein (40). In view of this, therapeutic strategies aimed at inducing differentiation of leukemic blasts have significant potential, and oncogenes that block differentiation are attractive targets for such therapies. Thus, it may be possible to restore IRF-1 function in leukemic blasts by targeting the expression of IRF-2. Taken together with the results presented in this study, this suggests that IRF-2 may be a good target for therapies in which the ultimate goal is the induction of differentiation of the leukemic blasts.

In summary, our results show that the growth arrest of these myeloid cell lines in response to N-rasG13R expression is part of a differentiation response which is mediated by IRF-1. This novel tumor suppressive pathway activated by oncopgenic signaling may explain the high incidence of IRF-1 inactivation in AML and myelodysplastic syndrome, because inactivation of this growth suppressive pathway may be necessary for N-ras–associated leukemogenesis.

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N-Ras–Induced Growth Suppression of Myeloid Cells Is Mediated by IRF-1

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