Endogenous interferon γ (IFNγ) promotes the host response to primary tumors, and IFNγ-insensitive tumors display increased tumorigenicity and can evade tumor surveillance mechanisms. Here we demonstrate that activating mutations of Ki-ras are sufficient to inhibit the expression of STAT1 and STAT2, transcription factors required for signaling by IFNs, providing a potential mechanism for the insensitivity of tumors to IFNs. We demonstrated that colon cancer cell lines with Ki-ras mutations display reduced expression of IFN-responsive genes compared with the cell lines that have retained wild type Ras and that inactivation of the mutant Ki-ras allele in the HCT116 colon cancer cell line is sufficient to restore the expression of STAT1, STAT2, and IRF-9. Accordingly, the expression of 27 interferon-inducible genes was reduced in HCT116 cells compared with the isogenic clones with targeted deletion of the mutant Ki-ras allele, Hkh2 and Hke-3. The expression of IFNγ receptors did not differ among the isogenic cell lines. IFNγ stimulated transcription of a STAT1-dependent reporter gene was impaired by RasV12, demonstrating a transmodulation of IFN/STAT signaling by activated Ras. Finally, we demonstrated that the expression of RasV12 in 293T cells is sufficient to inhibit the endogenous expression of STAT1 and STAT2, confirming the negative regulation of IFN signaling by oncogenic Ras. Our data demonstrate that the signaling initiated by activated Ki-ras interferes with the IFN/STAT signaling pathway and modulates the responsiveness of cancer cells to interferons. Furthermore, the data suggest that tumors harboring activating Ki-ras mutations may escape tumor surveillance mechanisms due to reduced responsiveness to IFNγ.

Oncogenic Ki-Ras Inhibits the Expression of Interferon-responsive Genes through Inhibition of STAT1 and STAT2 Expression*

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Point mutations that activate the Ki-ras protooncogene are present in up to 50% of sporadic colorectal tumors (1). The majority of the Ki-ras mutations are gain-of-function mutations at codon 12 and 13, and activated Ki-ras has been shown to synergize with both APC (2) and p53 mutations (3) in the transformation of colonic epithelial cells. In addition, recent reports suggested that the acquisition of an activated Ki-ras mutation may be sufficient for transformation of epithelial cells (4, 5). Oncogenic forms of Ras are locked in their active state and thereby transduce signals for transformation, angiogene-
sis, and metastasis. Dimers translocate to the nucleus and thereby induce transcription of target genes which harbor GAS (gamma-activated site) sites in their promoter region (reviewed in Refs. 13 and 14). Binding of IFN α/β to the type I receptor results in transcriptional activation of the Jak1/Tyk 2 kinase (15, 16). The activated kinases elicit tyrosine phosphorylation and dimerization of STAT1 and STAT2, which subsequently recruit the DNA-binding protein subunit, p48 (IFR-9) (17, 18). STAT1, STAT2, and p48 form a heterotrimeric tran-

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cDNA Microarray Analysis—We used microarray slides produced by the Albert Einstein College of Medicine, containing 29,216 and 27,224 sequences and identified sequences that are differentially expressed in HCT116 cells compared with the two clones with a disrupted mutant Ki-Ras allele. Only genes with a signal/background ratio > 1.25 were further analyzed. Using these criteria, on average 70% of sequences were included in the analysis. We considered sequences as potential Ki-Ras target genes if the ratio of the signals between HCT116 and either Hkh2 or Hke-3 cells was greater than 1.5 (Ki-Ras up-regulated genes) or less than 0.6 (Ki-Ras down-regulated genes).

Total RNA was isolated using the RNeasy Midi kit (Qiagen) as suggested by the manufacturer. 100 μg of RNA from HCT116 cells was labeled with Cy5 (channel 1) and RNA from Hke-3 or Hkh2 cells with Cy3 (channel 2) using fluorescent dye. After hybridization of labeled slides, slides were scanned (532 nm/Cy3 and 633 nm/Cy5), and the two images were superimposed and quantified using Scanalyze 1.41 software. Data were imported into an Access data base for further analysis. The expression profile for HCT116 cells is presented as the intensity of channel 1 corrected for the background (ch1/chB), and expression data for Hkh2 or Hke-3 cells as the intensity of channel 2 corrected for the background (ch2/B). Only genes with a signal/background ratio > 1.25 in both Hkh2 and Hke-3 clones were further analyzed. Results are expressed as the ratio between intensity of signal in HCT116 and Hke-3 cells or between HCT116 and Hkh2 cells.

The expression level of the IFN target genes was similarly assessed by a message analysis in a panel of 12 colorectal carcinoma cell lines and compared with reference RNA, representing a pool of RNA isolated from a panel of 12 colorectal cancer cell lines. cDNA microarray analysis was performed using microarray slides comprising 9216 cloned sequences produced by the Microarray Facility at the Albert Einstein College of Medicine. Microarray experiments were repeated twice and the data shown in Table II represent the average gene expression. For detailed analysis of the entire data base, see Mariadason et al. (79).

Quantitative RT-PCR Analysis—The differential expression level of 4 genes selected from Table II was confirmed by quantitative real-time RT-PCR analysis. RNA was isolated from HCT116, Hkh2, and Hke-3 cells that were either left untreated or were treated with IFNγ (1000 U/ml) for 1, 3, or 6 h. 1 μg of RNA was reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems). cDNA was amplified using specific primers for IRF-9, β-actin, p53, PKR, and IFNα and IFN-β, and c-myc. Primer sequences were as follows: IFNα/IFN-β (forward), 5’-TCTCCATATCGCTGGTTTCA-3’; IFNα/IFN-β (reverse), 5’-GGACCTCAAGCTTTCCAGAT-3’. Experiments were done in triplicate and the expression of each gene was standardized using glyceraldehyde-3-phosphate dehydrogenase as a reference. Amplification reactions were run using a 7900HT real-time PCR instrument (Applied Biosystems, Foster City, CA). The results were expressed as the ratio between the expression of the IFN target gene and the expression of glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

Expression of IFN-responsive Genes Is Repressed in a Panel of Colon Cancer Cell Lines That Harbor a Ki-Ras Mutation.—To identify profiles of gene expression that predict the response of colon cancer cells to chemotherapeutic drugs, we performed genome-wide analysis of 30 colorectal cell lines. A detailed statistical analysis of the entire data base has been published elsewhere (79), and data can be found on our website: sequence.aecom.yu.edu/bioinf/Augenlicht/default.html.

To study the mechanism whereby activating mutations of Ki-ras promote tumorigenesis we selected from the data base 12 cell lines with diverse Ki-ras status and compared their gene expression profile. Six of these cell lines harbor an oncogenic mutation in the Ki-ras protooncogene (D1d1 (G13D), HCT15 (G13D), HCT116 (G13D), LoVo (G12D), SW480 (G12V), and SW620 (G12V)) and six cell lines have wild type (WT) Ki-ras (Caco-2, Colo201, HT29, RKO, T84, and WiDr). CDNA microarray analysis was performed as described under "Exper-
Table I  
The expression of IFN-responsive genes in colon cancer cell lines with diverse Ki-ras status

| Accession no. | Gene         | Mean expression/standard RNA | MT/WT ratio |
|--------------|--------------|------------------------------|-------------|
|              | WT Ki-ras    | MT Ki-ras                    |             |
| AA419251     | IFN-9-27     | 2.12                         | 0.50        | 0.23        |
| AA479795     | IFN-20       | 2.30                         | 0.60        | 0.36        |
| AA157813     | IFN-37       | 1.16                         | 1.02        | 0.88        |
| AA409020     | IFN-15       | 1.98                         | 0.86        | 0.45        |
| AA491191     | IFN-16       | 2.67                         | 0.31        | 0.11        |
| AA630800     | IFN-IP 30    | 0.96                         | 0.92        | 0.95        |
| W24246       | IFNRA-58     | 1.05                         | 0.90        | 0.85        |
| W77997      | GBP2         | 1.22                         | 0.45        | 0.37        |
| AA489743     | IFNtetrat1   | 5.49                         | 0.74        | 0.15        |
| AA444878     | IFN-6-16     | 2.70                         | 0.50        | 0.18        |
| A017240      | Bag-1        | 1.28                         | 1.00        | 0.77        |
| AA291577     | p48          | 2.11                         | 0.80        | 0.37        |
| AA454813     | IRF-2        | 1.18                         | 0.86        | 0.73        |
| T62627       | IFN-75IS100  | 2.13                         | 0.80        | 0.37        |
| W42587       | IFN-PKR      | 2.59                         | 1.06        | 0.40        |
| AA456886     | Mxy-1        | 7.43                         | 0.71        | 0.09        |
| AA286908     | Mxy-2        | 2.91                         | 0.75        | 0.25        |
| AA44657      | MHC class I, A | 1.77                       | 1.20        | 0.67        |
| T63324       | MHC class II, Dqa1 | 1.18             | 0.82        | 0.69        |
| AA486072     | A5           | 1.20                         | 0.85        | 0.71        |

Mean: 2.27 ± 0.35  Mean: 0.78 ± 0.05  Mean: 0.34, p = 0.0002

*p < 0.05, Mann-Whitney nonparametric test.

The mean expression of 20 genes was calculated from six cell lines with WT Ki-ras and six cell lines with MT Ki-ras as described under “Experimental Procedures.” Bag-1, Bel-2-associated athanogene 1; p48, interferon-stimulated transcription factor 3; Myx-1 and Myx2, myxovirus (influenza) resistance 1 and 2; IFN-PKR, protein kinase, interferon-inducible double-stranded RNA-dependent; IFNRA-58, retinoid acid- and interferon-inducible protein, 58 kDa; GBP2, guanylate-binding protein 2, interferon-inducible; A5, small inducible cytokine A5, IFNtetrat1, interferon-induced protein with tetratricopeptide repeats 1.

When we analyzed the data base generated from the 12 cell lines with diverse Ki-ras status, we noticed that the expression of 20 IFN-responsive genes was reduced in six cell lines that harbor activating mutation of Ki-ras (79). The average ratio of expression of all 20 IFN-responsive genes was 2.27, compared with 0.78 in the six cell lines with a mutant Ki-ras, a highly statistically significant difference (p = 0.0002, Mann-Whitney test). In addition, the mean expression of individual IFN-responsive genes was markedly lower in cell lines containing MT Ki-ras than in cell lines containing WT Ki-ras. The differences were statistically significant (p < 0.05, Mann-Whitney test) for 35% of the individual genes examined (Table I).

When we compared the expression of the 20 IFN-responsive genes in cell lines with WT p53 (HCT116, Lovo, LS174T, RKO, and SW48) and MT p53 (16E, 19A, Colo205, HCT15, HT29, KM12, SW480, and SW620), we did not detect any significant differences in the expression of IFN-responsive genes between the two groups (ratio MTp53/WTp53 = 1.1, p = 0.42, data not shown), suggesting that the expression of IFN-dependent genes is not under major regulatory control of p53.

These results demonstrated that the effect of mutant Ras on the expression of interferon responsive genes is sufficiently strong to be detected in a population of cell lines that differ in a number of other genetic determinants. In addition, they suggested that oncogenic Ki-Ras interferes with the expression of IFN-responsive genes.

Targeted Inactivation of Mutant Ki-ras in Colon Cancer Cells Is Sufficient to Restore the Expression of IFN-responsive Genes—To further determine the role of oncogenic Ras in IFN signaling, we utilized a well defined system of isogenic cell lines that differ only in the presence of the mutant Ki-ras allele. The HCT116 cell line harbors an activating mutation in codon 13 of the Ki-ras protooncogene, and the Hkh2 and Hke-3 clones were generated by the targeted deletion of the mutant Ki-ras allele (11). Inactivation of the mutant Ki-ras allele in HCT116 cells resulted in reduced proliferation of cells, reduced tumorigenicity in vivo, and reduced capacity for anchorage-independent growth (11). We performed cDNA microarray analysis on the isogenic cell lines as described under “Experimental Procedures.” Experiments were performed twice in Hke-3 cells and twice in Hkh2 cells (Table II). The genes shown in Table I and Table II overlap only partially, because different cDNA microarray slides were used in the experiments (see “Experimental Procedures”).

Consistent with our hypothesis that mutant Ras interferes with the expression of IFN responsive genes, we found that a cluster of IFN-responsive genes included on the array was
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RNA isolated from HCT116 cells was labeled with Cy3 (ch1) and RNA from Hke-2 or Hkh2 cells with Cy5 (ch2) fluorescent dye. Results are expressed as the ratio between intensity of signals in HCT116 and Hkh2 cells or HCT116 and Hke-3 cells. Experiments were performed twice with each cell line. Results of each experiment are shown in the separate columns. ND, not determined; the sequence was not present on slide or the expression may be due to production of low endogenous levels of IFN.

| Accession no. | Gene       | HCT116/Hkh2 Ratio | HCT116/Hke-3 Ratio |
|--------------|------------|-------------------|--------------------|
| R82716       | IFN-15/17  | 0.12              | 0.3 (0.2)          |
| AA419251     | IFN-9–27   | 0.4               | 0.3 (0.3)          |
| AA058323     | IFN-1–8U   | 0.6               | 0.28              |
| AA489743     | IFN-56K    | 0.3               | 0.23              |
| AA187365     | IFN-1–SO   | ND                | 0.28              |
| W49768       | IFN-6–16   | ND                | 0.3               |
| AA160539     | IFN-30     | ND                | 0.2               |
| AA489743     | IFN/tetra1 | ND                | 0.5 (0.6)         |
| T97408       | Bax        | ND                | 0.2               |
| BFS29563     | p48        | 0.7               | 0.4 (0.8)         |
| AA210708     | STAT2      | 0.6               | 0.3               |
| W90777       | IRF-2      | 0.5               | 0.3               |
| AA478043     | IRF-1      | 0.5               | 0.3               |
| N46151       | IFN-PKR    | 0.5               | 0.5               |
| RT2444       | 2′-5′ A synthetase | 0.5   | 0.5                 |
| AA447481     | Nuclear antigen SP100 | 0.5 | 0.7           |
| AA458688     | Myc-1      | 0.6               | 0.4 (0.2)         |
| AA268690     | Myc-2      | 0.4               | 0.3               |
| R23541       | 2M         | ND                | 0.1               |
| AA464246     | MHC class I | 0.6              | 0.4 (0.5)         |
| W00378       | MHC class I, B | 0.4              | ND                |
| R96664       | MHC class, IDDR2 | 0.15    | ND                |
| H50622       | MHC class, DR7 | 0.38           | ND                |
| T50854       | MHC class, IDDP8 | 0.5           | ND                |
| RT2489       | MHC class, IDG | 0.4              | ND               |
| NM_001223    | Caspase 1  | 0.5               | 0.5 (0.8)         |
| R14760       | Caspase 3  | 0.7               | 0.5 (0.5)         |

Down-regulated in HCT116 cells, compared with the two isogenic clones with a targeted deletion of the mutant Ki-ras allele, Hkh2 and Hke-3 (Table II).

The expression of IFN-responsive genes is normally induced in response to IFNs and several of them harbor a STAT1 binding site in their regulatory regions (37). Their basal level of expression may be due to production of low endogenous levels of IFN (38, 39). Although IFN-responsive genes represent a hitherto unrecognized group of Ras target genes, the expression of the IFN-9–27 gene has been shown to be strongly down-regulated (>100-fold) in fibroblasts upon transformation with Ha-Ras (12). Likewise, Ras transformation has been shown to down-regulate MHC class I expression in fibroblasts (40, 41). The expression of some of the IFN-responsive genes shown in Table II, such as MHC class I, MHC class II, and β2M, have been shown to be lost or reduced in colorectal cancers (42–44). Our data demonstrate that activating mutations of Ki-Ras are sufficient for the down-regulation of the MHC class I and II expression in epithelial cells and that through reduction of β2-microglobulin expression Ras signaling also interferes with the surface expression of MHC class I (45, 46). We have included caspase 1 and caspase 3 in Table II, because STAT1 has been shown to be required for their constitutive expression (47).

Significantly, among the IFN-responsive genes whose expression was reduced in cells harboring an activating Ki-Ras mutation were also STAT2 and p48/IRF-9 (Table II), transcription factors that associate with STAT1 to form ISGF3, a multimeric transcription factor required for signaling by type I IFNs. IRF-9 has been shown to be overexpressed in breast cancers but down-regulated compared with the normal adjacent mucosa in 40% of primary colon tumors (48). Whether these tumors carry mutations in Ki-Ras is not known.

Next we confirmed the differential expression of IFN/TETRA1, IRF-9, β2M, and IFN-PKR (Table II) in the isogenic cell lines by real-time RT-PCR analysis. RNA was isolated from HCT116, Hkh2, and Hke-3 cells that were either left untreated or were treated by IFNγ (10 ng/ml) for 1, 3, or 6 h. The expression of each gene was standardized using glyceraldehyde-3-phosphate dehydrogenase as a reference, and the data were expressed as the fold change relative to HCT116 cells. Consistent with data in Table I, the basal expression of IFN-TETRA1, IRF-9, β2M, and IFN-PKR in untreated cells (time 0) was higher in both clones with targeted deletion of mutant Ki-ras compared with the parental HCT116 cell line (Fig. 1, note log scale). Furthermore, the expression of these genes remained higher in Hkh2 and Hke-3 cells compared with the parental cell line, upon treatment with IFNγ for 1, 3, or 6 h (Fig. 1). Using the same cDNA we demonstrated that the expression of c-myc was lower in Hkh2 and Hke-3 than in HCT116 cells (Fig. 1), consistent with c-myc up-regulation by an activated Ras (11, 49). The expression of IFN target genes was consistently higher in the Hkh2 clone compared with Hke-3 clone, a difference that was not apparent from the microarray data, and may reflect clonal variations between Hkh2 and Hke-3 cells (see below).

These results confirmed that oncogenic Ki-Ras mutations interfere with the expression of IFN-responsive genes and demonstrated that targeted deletion of the mutant Ki-ras allele is sufficient to restore the expression of IFN-dependent genes.

Oncogenic Ki-Ras Inhibits the Expression of Transcription Factors That Are Required for IFN Signaling—As shown in Table II, the expression of STAT2 and p48, transcription factors required for signaling by IFNs and IFNβ, was reduced in cells harboring an activating Ki-ras mutation. We have confirmed by real-time RT-PCR analysis that the basal and inducible expression of p48/IRF-9 is reduced in HCT116 cells, compared with the two clones with a targeted deletion of the activated Ki-ras allele (Fig. 1). Next we confirmed the differential expression of STAT2 in the isogenic cell lines by Western
Because the expression of STAT2 is regulated by IFNs, we also compared the ability of IFN/H9253 to induce its expression in the isogenic cell lines. Cell lysates were prepared from cells that were either left untreated or were treated with sodium butyrate (3 mM), IFN/H9253 (50 ng/ml), or the combination of butyrate and interferon, for 24 h and examined by immunoblotting for STAT2 expression. We examined the effect of butyrate on the expression of STAT2, because we have recently demonstrated that this physiological regulator of homeostasis of colonic epithelial cells could modulate STAT1 signaling (80).

We confirmed that the basal expression of STAT2 was markedly reduced in HCT116 cells, compared with the Hkh2 cells, suggesting that the oncogenic Ki-ras mutation inhibits the expression of STAT2 (Fig. 2, A and B, “0”). The basal expression of STAT2 was also elevated in the Hke-3 clone compared with the HCT116 cells, although the levels of STAT2 in Hke-3 cells were lower than in Hkh2 cells. Similarly, the expression of IRF-9, PKR, IFN/TETRA1, and β2M was higher in Hkh2 than in Hke-3 cells (Fig. 1). Clonal variations between the Hkh2 and Hke-3 cells may be due to the differences in the expression of other factors that control the expression of these genes. The two clones have been recently shown to differ significantly in their response to HGF (50), confirming the existence of clonal variations between them.

IFNγ was a poor inducer of STAT2 in the parental HCT116 cells, compared with Hkh2 and Hke-3 clones (Fig. 2), suggesting that Ki-ras mutations interfere with signaling by IFNγ. Butyrate was a weak inducer of STAT2 expression in HCT116, Hkh2, and Hke-3 cells. The levels of Rac1, which served as a loading control, were not affected by the treatment of cells with IFN or butyrate.

Next we compared the dose-dependent induction of STAT2 by IFNγ in HCT116 and Hkh2 cells. Cells were treated with increasing concentrations of IFNγ as indicated for 24 h, and cell lysates were examined for STAT2 and Rac1 expression by immunoblotting (A). The intensity of signal for STAT2 was determined by densitometry (B).
and Hkh2 cells upon treatment with IFN$_\gamma$ (Fig. 3B); however, the levels of STAT2 in HCT116 cells that were treated with 100 ng/ml of IFN$_\gamma$ remained lower than the levels of STAT2 in untreated Hkh2 cells (Fig. 3B). The blots were reprobed with an anti-Rac antibody to control for equal loading, and the intensity of the STAT2 signal in HCT116 and Hkh2 cells was determined by densitometry (Fig. 3B). IFN$_\gamma$ induced STAT2 expression also in Hke-3 cells in a concentration-dependent manner (data not shown), although the basal expression of STAT2 as well as IFN-induced STAT2 accumulation in Hke-3 cells were lower than in the Hkh2 cells, consistent with results shown in Fig. 2.

The cDNA for another obligatory transcription factor in IFN signaling, STAT1, was not present on the microarray. Because STAT1 is also an interferon inducible gene (51), we tested by immunoblotting whether oncogenic Ki-Ras interferes with expression and/or IFN-induced activation of this transcription factor. Cell lysates were isolated from untreated HCT116, Hkh2, and Hke-3 cells and from cells that were treated with increasing concentrations of IFN$_\gamma$ for 24 h. Immunoblotting was performed using a STAT1 specific antibody and reprobed with an anti-Rac antibody to control for equal loading. As shown in Fig. 4A, the basal expression of STAT1 (“0” and inset in Fig. 4B) was lower in HCT116 cells than in the two clones with the deleted mutant Ki-ras allele, suggesting that the oncogenic activation of Ki-Ras in HCT116 cells results in the inhibition of STAT1 expression. In addition, IFN$_\gamma$ failed to induce significant STAT1 accumulation in HCT116 cells at concentrations lower than 50 ng/ml. In contrast, in both Hkh2 and Hke-3 cells, concentrations of IFN$_\gamma$ as low as 0.1 ng/ml were sufficient for the activation of STAT1 expression (Fig. 4A and B), demonstrating that the Ki-ras mutation reduced the sensitivity of cell to IFN$_\gamma$. In contrast to STAT2, IFN$_\gamma$ induced STAT1 expression with higher efficiency in the Hke-3 clone than in Hkh2 clone.

Thus, although the quantitative effect of the targeted deletion of oncogenic Ki-ras allele on STAT1 and STAT2 expression differs in the two isogenic clones due to clonal variations, elimination of constitutive Ras signaling by targeted deletion of the mutant Ki-ras allele is sufficient to elevate the basal and inducible expression of STAT1 and STAT2.

**Activated Ki-Ras Inhibits IFN-stimulated Transcription of a STAT-1-dependent Reporter Gene**—Because the expression of the majority of IFN target genes depends on functional STAT1 signaling, we examined the possibility that Ras inhibits STAT1 transcriptional activity. First we determined whether STAT1-dependent transcriptional activity differs in the isogenic cells. We transiently transfected HCT116, Hkh2, and Hke-3 cells with a STAT1-dependent reporter plasmid. In this construct luciferase gene expression is under the control of a promoter that contains eight GAS binding sites (8×GAS-LUC). Cells were cotransfected with TK-renilla to control for transfection efficiency and results were expressed as the ratio of luciferase and Renilla activity.

HCT116, Hkh2, and Hke-3 cells were transiently transfected with the 8×GAS-LUC reporter plasmid and were either left untreated or were treated with IFN$_\beta$ (100 units/ml) or IFN$_\gamma$ (100 ng/ml) for 24 h. The basal activity of the STAT-1-responsive reporter gene was significantly lower in HCT116 cells than in Hkh2 and Hke-3 cells (inset in Fig. 5). This result is consistent with our data demonstrating that the basal levels of STAT1 are lower in HCT116 cells than in Hkh2 and Hke-3 cells (Fig. 4). IFN$_\beta$ did not activate STAT1-dependent transcription, while IFN$_\gamma$ induced STAT1-dependent transcriptional activity.
In HCT116, Hkh2, and Hke-3 cells. However, IFNγ-induced STAT1 transcriptional activity was markedly lower in HCT116 than in Hkh2 and Hke-3 cells (Fig. 5). These results are consistent with data shown in Fig. 1, which demonstrated that IFNγ-induced expression of several IFN target genes is reduced in cells harboring an activating Ras mutation.

We next examined the possibility that reduced STAT1-dependent transcriptional activity in HCT116 cells is due to reduced expression of IFNγ receptors in these cells compared with the Hkh2 and Hke-3 cells. As shown in Fig. 6A, the levels of IFNγRα and IFNγRβ protein expression did not differ between the HCT116, Hkh2, and Hke-3 cells. We analyzed the surface expression of the ligand binding α chain by FACS analysis (Fig. 6B). The mean fluorescence intensity (MFI) was 8.38 in HCT116 cells, 9.7 in Hkh2 cells and 8.52 in Hke-3 cells, demonstrating that the surface expression of the IFNγRα chain did not differ among the isogenic cell lines with different Ras status. Thus, the reduced STAT1 transcriptional activity in HCT116 cells is not due to reduced expression of the IFNγ receptor in these cells compared with Hkh2 and Hke-3 cells.

We next examined whether transient transfection of cells with the vector directing the expression of mutated Ras modulates the transcriptional activity of the STAT1 dependent promoter. Hke-3 cells were transiently transfected with the 8×GAS-LUC reporter gene in the presence of the empty vector (CMV-neo) or cotransfected with the vector directing the expression of mutant Ras (CMV-RasV12). Cells were either left untreated or were treated with IFNγ (0.1 or 1 ng/ml) for 8 h, and results were expressed as the activity of LUC/µg of cellular protein. Expression of mutant Ras inhibited the basal activity of the STAT-1 reporter gene (Fig. 7A) and markedly inhibited the responsiveness of the reporter gene to IFNγ (Fig. 7B).

These data demonstrate that activated Ras interferes with STAT1-dependent transcription. This is likely to contribute to the decreased expression of IFN-dependent genes in cells harboring an activated Ki-ras mutation (Tables I and II and Fig. 1).

Expression of RasV12 Inhibits the Endogenous Expression of STAT1 and STAT2—To confirm the effect of RasV12 on the expression of endogenous STAT1 and STAT2, we transiently transfected human kidney epithelial cells 293T with a vector directing the expression of the mutant RasV12. (The experiments were performed in 293T cells because of their high transfection efficiency.) As expected, RasV12 induced strong activation of the ERK1/ERK2 pathway in 293T cells (Fig. 8A). Cells were either left untreated or were treated with IFNγ (10 ng/ml) for 24 h, and the levels of STAT1 and STAT2 were determined by immunoblotting. IFNγ was a potent inducer of STAT1 expression in parental 293T cells. In cells transfected with RasV12, the basal levels of STAT1 and IFNγ-induced accumulation of STAT1 were reduced by ~50%. Similarly, cells transfected with RasV12 had reduced basal levels of STAT2, and inducibility of STAT2 by IFNγ was abrogated (Fig. 8A).

These data verified that oncogenic activation of Ras negatively regulates the expression of STAT1 and STAT2.

IRF-1 is a transcriptional target of STAT1 but is also an important transcriptional activator of STAT1 expression (52), suggesting that the two proteins form a feedback loop that regulates responsiveness of cells to IFNs. We examined the expression of IRF-1 in 293T cells transfected with an empty vector or transiently transfected with an activated Ras. Cells were left untreated or were treated with IFNγ (10 ng/ml) for 24 h, and cell lysates were tested for the expression of IRF-1 by immunoblotting. The basal levels of IRF-1 were low in uninduced cells and treatment of cells with IFNγ resulted in strong accumulation of IRF-1 in cells transfected with an empty plasmid. The activation of the IRF-1 expression by IFNγ was markedly inhibited by an activated Ras (Fig. 8B).

Two major signaling pathways activated by oncopgenic Ras are the Raf/ERK1/ERK2 pathway and the PI3K/AKT pathway. To dissect the contribution of these Ras effector pathways, we treated 293T/RasV12 cells with a chemical inhibitor of the Raf pathway (Raf inhibitor I, Calbiochem) or the inhibitor of the PI3K signaling (LY294002) for 1 h prior to the addition of IFNγ. Inhibition of the Raf kinase had no effect on the basal or inducible expression of STAT1 or IRF-1 (Fig. 9). Treatment of Ras-transfected 293T cells with LY294002 significantly enhanced IFNγ-induced expression of both STAT1 and IRF-1 but did not affect the basal expression of STAT1 and IRF-1. Inhibition of the PI3K pathway enhanced the inducibility of STAT1 and IRF-1 by IFNγ also in HCT116 cells (Fig. 9), further establishing the role of PI3K in IFN signaling. These results strongly suggest that Ras inhibits responsiveness to IFNγ (and the expression of IFN dependent genes), at least in part, through activation of the PI3K pathway. The downstream effectors of the PI3K pathway involved in repression of IFN signaling remain to be determined.

These data demonstrate that expression of oncogenic Ras is
sufficient to restore the expression of STAT1 and STAT2, and their downstream targets, confirming a role of Ras signaling in the expression of transcription factors required for IFN signaling.

STAT1 promotes apoptosis and negatively regulates cell growth, metastasis, and angiogenesis (35, 56, 57). STAT1 has been shown to be required for the inhibition of c-myc expression by IFN and thus for the anti-proliferative effect of IFN (54). In the absence of functional STAT1, IFN induced rather than repressed c-myc expression (21, 22, 58), which is likely to contribute to the enhanced proliferation of STAT1 deficient cells in the presence of IFN (23). Thus, although IFN can modulate gene expression and exert biological activity in a STAT-1-independent manner (21), the biological outcome of IFN signaling critically depends on the presence of intact STAT1 signaling. Our data therefore suggest that tumors harboring Ki-ras mutations may display reduced or altered responsiveness to IFNs due to reduced levels of STAT1.

The mechanisms responsible for the inhibition of STAT1 expression in transformed cells have not been determined. A study by Karpf et al. (59) demonstrated that in HT29 colon carcinoma cells (which have WT Ki-ras), STAT1, STAT2, and STAT3 are silenced by DNA methylation, resulting in inhibition of the IFN-responsive genes and, importantly, in reduced sensitivity of cells to IFN. Treatment of HT29 cells with 5-aza-2-deoxycytidine restored the expression of STAT1, STAT2, and STAT3, which coincided with transcriptional induction of IFN-responsive genes and increased sensitivity to IFNα (59). Several tumorigenic viruses have been shown to modulate IFN signaling; microarray analysis has identified STAT-1 and IFN-inducible genes and as a major transcriptional target of the human tumorigenic Papillomavirus type 31 (60). Likewise, cells transformed with the adenoviral E1A oncoprotein also exhibit reduced IFN signaling and display impaired IFN-driven gene expression due to reduced cellular levels of STAT1 and p48 (61–63).

Our results revealed an additional mechanism for modulation of IFN target genes; we demonstrated that acquisition of an oncogenic Ki-ras mutation is sufficient to inhibit STAT1 expression and to impair the expression of IFN target genes. IFN/STAT signaling has been shown to be inhibited during prostate tumor progression (64), which is frequently driven by oncogenic Ras activation (65). Because ras mutations are found in ~30% of human cancers, it is likely that they represent a frequent mechanism for the deregulation of STAT1 and STAT2 expression and subsequent reduced sensitivity of tumor cells to IFNs. Indeed, a study by Huang et al. (66) demonstrated that colon cancer cell lines with WT Ki-ras display higher sensitivity to IFN than cell lines harboring a mutant Ki-ras (66).

In accord with reduced expression of STAT1, STAT2, and IRF-9 in cells harboring an activating Ki-ras mutation, we demonstrated in these cells a uniform down-regulation of IFN-inducible genes that mediate anti-proliferative, proapoptotic,
angiostatic, and immune functions of IFNs (67, 68). For example, we showed that interferon-inducible double-stranded RNA-dependent protein kinase PKR, which has potent growth inhibitory and potential tumor suppressor properties (69, 70), is reduced in cells harboring an activated Ras (Fig. 1). Activation of Ras in NIH3T3 cells did not modulate the levels of PKR, but it inhibited the viral-induced activation of PKR, resulting in enhanced permissiveness of Ras transformed cells to Herpes simplex virus infection (71). Whether activation of Ras in epithelial cells modulates the activity of PKR remains to be determined. IFN-inducible genes have been mapped mainly to two chromosomally regions, 10q23–26 and 17q 21. Coregulation of IFN-inducible genes has been described by Fambrough (72), raising the possibility that these genes may be a part of a coregulated module. Intriguingly, that report also indicated that attenuation of Ras signaling might release this gene module from inhibition in a STAT1-dependent manner.

We demonstrated that oncogenic Ras inhibits the expression of IRF-1 (Fig. 8D), a transcription factor with tumor suppressor properties that has been shown to revert the transformed phenotype of Ras-transformed cells in vitro and in vivo (73) and plays a major role in transcriptional activation of STAT1 (52). Mouse embryonic fibroblasts homozygous for IRF-1 deficiency undergo transformation upon expression of an activated form of Ha-Ras and no longer require a cooperating oncogene, such as c-myc (74). This suggests that Ras can oviate or minimize the need for cooperation with other oncogenes through inhibition of IRF-1 expression. Ki-ras mutations have been shown to cooperate with both p53 and Apc mutations in the development of colon cancer. In this regard it is interesting that mice deficient in both STAT1 and p53 develop spontaneous tumors earlier than p53–/– mice and, unlike mice lacking p53 alone, form non-lymphoid tumors that include teratomas, hemangiosarcoma, and chordomasarcoma (24). Whether STAT1–/- deficiency promotes Ras-induced transformation and/or Apc-initiated tumor formation remains to be determined. IFNα/β has recently been shown to induce p53 expression through transcriptional activation of the gene by ISGF3, suggesting a role of IFNα/β in p53-mediated tumor suppression (25). As all the components of the ISGF3 complex, STAT1, STAT2, and IRF-9, are reduced in p53-mediated tumor suppression (25). As all the components of the ISGF3 complex, STAT1, STAT2, and IRF-9, are reduced in

repression of IFN signaling remain to be determined. Activation of the PI3K pathway negatively regulates IFN signaling (76); however, our data suggest that constitutive activation of the PI3K pathway negatively regulates IFN signaling. The downstream effectors of PI3K that mediate the repression of IFN signaling remain to be determined.

In conclusion, our data demonstrate that signaling by activated Ras inhibits the expression of IFN target genes through inhibition of transcription factors required for signaling by IFNs, including STAT1, STAT2, IRF-1, and IRF-9. This indicates that oncogenic Ras modulates the responsiveness of cells to IFNs and suggests that Ras promotes tumorigenesis not only by mimicking the activity of growth factors but also by modulating the responsiveness of cells to negative regulators of cell growth, such as TGF-β (10) and interferons.

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REFERENCES

1. Kinzler, K. W., and Vogelstein, B. (1996) Cell 87, 159–170
2. D’Aubaco, G. M., Whitehead, R. H., and Burgess, A. W. (1996) Mol. Cell. Biol. 16, 884–891
3. Seygivan, C., Wlodarski, P., Kirillova, J., Mercer, W. E., Danielson, K. G., Iozzo, R. V., and Calabretta, B. (1998) J. Clin. Invest. 101, 1572–1580
4. Jansen, K. P., el-Maryus, F., Pinto, B., Sastre, X., Rouillard, D., Fouquet, C., Soussi, T., Louvard, D., and Robine, S. (2002) Gastronercology 123, 492–504
5. Smith, G., Carey, F. A., Beattie, J., Wilko, M. J., Lightfoot, T. J., Coxhead, J., Garson, R. C., Steele, R. J., and Wolf, C. R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9433–9438
6. Rebello, A., and Martinez, A. C. (1999) Blood 94, 2971–2980
7. Shields, J. M., Pruiti, K., McFall, A., Shaub, A., and Der, C. J. (2000) Trends Cell Biol. 10, 147–154
8. Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, 1395–1415
9. Malumbres, M., and Pellicer, A. (1999) Front. Biosci. 3, 4887–4912
10. Kretzschmar, M., Doody, J., Timolchka, I., and Massague, J. (1999) Genes Dev. 13, 804–816
11. Shirasawa, S., Furuse, M., Yokoyama, S., and Sasaki, T. (1993) Science 260, 85–88
12. Zuber, J., Thernitsna, O. I., Hinzmann, B., Schmitz, A. C., Grips, M., Hellriegel, M., Brenn, C., Essenthaler, A., and Schafer, R. (2000) Nat. Genet. 24, 144–152
13. Bie, J. N., and Kerr, I. M. (1995) Trends Genet. 11, 69–74
14. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421
15. Kisseleva, T., Bhattacharya, S., Braunstein, J., and Schindler, C. W. (2002) Gene (Amst.) 285, 1–24
16. Jelinek, R. (2000) Oncogene 19, 2460–2467
17. Blayssen, A. N., Durbin, J. E., and Levy, D. E. (1996) Cytokine Growth Factor Rev. 7, 11–17
18. Blayssen, H. A., and Levy, D. E. (1997) J. Biol. Chem. 272, 4600–4605
19. Dale, T. C., Rosen, J. M., Guilh, M. J., Lewin, A. R., Porter, A. G., Kerr, I. M., and Stark, G. R. (1989) EMBO J. 8, 831–839
20. Veisla, S. A., Schindler, C., Leonard, D., Fu, X. Y., Abersold, R., Darnell, J. E., Jr., and Levy, D. E. (1992) Mol. Cell. Biol. 12, 3315–3324
21. Ramana, C. V., Gil, M. P., Schreiber, R. D., and Stark, G. R. (2002) Trends Immunol. 23, 96–101
22. Ramana, C. V., Gil, M. P., Han, Y., Rasoschoff, R. M., Schreiber, R. D., and Stark, G. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6674–6679
23. Gil, M. P., Bohn, E., O’Guin, A. K., Ramana, C. V., Levine, B., Stark, G. R., Virgin, H. W., and Schreiber, R. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6680–6685
24. Kaplan, D. H., Shankarvan, V., Dighe, A. S., Stockert, E., Aguet, M., Old, L. J., and Schreiber, R. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7556–7561
25. Nakao, A., Hayakawa, S., Yanai, H., Stuber, D., Negishi, H., Kikuchi, H., Sasaki, S., Imai, K., Shibuie, T., Honda, K., and Taniguchi, T. (2003) Nature 424, 516–523
26. Vilecek, J. (1970) Science 168, 398–399
27. Sun, M. (1981) J. Biol. Chem. 256, 272, 831–835
28. Colamonici, O. R., Domanski, P., Platanias, L. C., and Diaz, M. O. (1992) Science 260, 1404–1409
29. Colamonici, O. R., Domanski, P., Platanias, L. C., and Diaz, M. O. (1992) Blood 80, 744–749
30. Billard, C., Signaux, F., Castagne, S., Valensi, F., Flandrin, G., Degos, L., Falcoff, E., and Aguet, M. (1986) Blood 67, 821–828
31. Abdiel, E., Reil, L. M., Serrano, A., Jimenez, P., Garcia, A., Canton, J., Trigo, I., Garrido, F., and Ruiz-Cabello, P. (1998) Cancer Immunol. Immunother. 47, 113–120
Oncogenic Ki-Ras Inhibits the Expression of Interferon-responsive Genes through Inhibition of STAT1 and STAT2 Expression

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