Regulation of c-myc expression by IFN-γ through Stat1-dependent and -independent pathways

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Interferons (IFNs) inhibit cell growth in a Stat1-dependent fashion that involves regulation of c-myc expression. IFN-γ suppresses c-myc in wild-type mouse embryo fibroblasts, but not in Stat1-null cells, where IFNs induce c-myc mRNA rapidly and transiently, thus revealing a novel signaling pathway. Both tyrosine and serine phosphorylation of Stat1 are required for suppression. Induced expression of c-myc is likely to contribute to the proliferation of Stat1-null cells in response to IFNs. IFNs also suppress platelet-derived growth factor (PDGF)-induced c-myc expression in wild-type but not in Stat1-null cells. A gamma-activated sequence element in the promoter is necessary but not sufficient to suppress c-myc expression in wild-type cells. In PKR-null cells, the phosphorylation of Stat1 on Ser727 and transactivation are both defective, and c-myc mRNA is induced, not suppressed, in response to IFN-γ. A role for Raf-1 in the Stat1-independent pathway is revealed by studies with geldanamycin, an HSP90-specific inhibitor, and by expression of a mutant cdc37 that is unable to recruit HSP90 to the Raf-1 complex. Both agents abrogated the IFN-γ-dependent induction of c-myc expression in Stat1-null cells. Keywords: c-jun/cell proliferation/geldanamycin/p50cdc37/PKR

Introduction

Interferons (IFNs) are pleiotropic cytokines that mediate anti-viral responses, inhibit proliferation and participate in immune surveillance and tumor suppression (Farrar and Schreiber, 1993; Stark et al., 1998). Transcriptional regulation in response to IFNs is mediated by the Jak–Stat pathway (for recent reviews, see Leaman et al., 1996; Darnell, 1997; Stark et al., 1998). Negative as well as positive regulation of gene expression in response to IFN-γ has been reported (Det et al., 1998; Sharma and Ioizzo, 1998). IFN-α and -β activate Stat1 and Stat2, which, with p48, form the transcription factor ISGF3, which binds to IFN-stimulated response elements. Activated Stat1 dimers translocate into the nucleus and bind to gamma-activated sequence (GAS) elements. All IFNs cause phosphorylation of Stat1 on Tyr701 and Ser727 (Wen et al., 1995) and both phosphorylations are required for maximal transactivation (Wen et al., 1995; Wen and Darnell, 1997). Stat1 dimers function through interaction with transcriptional co-activators such as CBP/p300, Nmi and MCM-5, as well as with other transcription factors, including p48 and SP1 (Look et al., 1995; Horvath et al., 1996; Zhang et al., 1996, 1998; Zhu et al., 1999). In addition to the IFNs, many growth factors and cytokines also activate Stat1 (Schindler and Darnell, 1995).

Targeted disruption in mice has confirmed that Stat1 is obligatory for signaling in response to all IFNs and has revealed that Stat1 is also involved in immune surveillance and tumor suppression (Durbin et al., 1996; Meraz et al., 1996; Kaplan et al., 1998). Some tumor cells and tumor-derived cell lines express little or no Stat1 mRNA or protein (L.H.Wong et al., 1997; Abril et al., 1998; Sun et al., 1998) or fail to activate Stat1 following treatment with IFNs (Lucas et al., 1998). IFNs inhibit the growth of many cell types (Balkwill and Taylor-Papadimitriou, 1978; Lin et al., 1986; Kimchi, 1992), and Stat1 that is fully active transcriptionally is required for this effect (Bromberg et al., 1996). The inhibition of cell growth correlates with the regulation of several cell cycle regulatory genes by IFNs. mRNAs encoding cyclin D and cdc25A decrease in response to IFN-α and -β, and expression of the cyclin-dependent kinase (CDK) inhibitor p21waf1 is up-regulated by IFN-γ in epidermal carcinoma and glioblastoma cell lines (Chin et al., 1996; Tiefenbrun et al., 1996; Kominsky et al., 1998). In contrast, the inhibition by IFN-γ of the growth of the colon carcinoma cell line HCT116 is independent of p21 (Sharma and Ioizzo, 1998). IFN-γ can stimulate rather than suppress the growth of certain cells (Caux et al., 1992; Shiohara et al., 1993), but the basis of this paradoxical activity is unclear. Stat1, and indeed other Stats, can also mediate negative regulation of gene expression in response to effectors other than the IFNs. For example, EGF-induced proliferation correlates with the transient activation of Stat1, whereas EGF-mediated growth suppression correlates with its sustained activation (Bromberg et al., 1998).

c-myc, a transcription factor that helps to regulate proliferation, is induced rapidly and transiently by many growth factors and cytokines (Spencer and Groudine, 1991; Bouchard et al., 1998; Dang, 1999). The expression of c-myc is aberrant in a variety of human tumors (Marcu et al., 1992). Its ectopic expression overrides both the G1 and S check points, promoting genomic instability and tumorigenesis (Chernova et al., 1998; Felsher and Bishop, 1999). c-myc regulates the G1→S transition by activating
rapidly by IFN-γ whereas c-myc is induced transiently and rapidly in response to growth factor complexes on the E2F site of the c-myc promoter and suppresses c-myc expression in both Daudi and M1 cells (Resnitzsky and Kimchi, 1991; Melamed et al., 1993). The constitutive expression of ectopic c-myc overcomes IFN-γ-mediated arrest of macrophages and vascular smooth muscle cells, indicating that c-myc is likely to be involved in the inhibition of proliferation mediated by IFN-γ (Bennett et al., 1994; Vairo et al., 1995). We now find that IFN-γ inhibits the expression of c-myc in wild-type cells, an effect that is mediated by consensus GAS elements in the c-myc promoter to which Stat1 homodimers bind. Furthermore, in Stat1-null cells, both c-myc and c-jun are induced transiently and rapidly by IFNs, revealing a novel signaling pathway. In IFN-γ-treated PKR-null mouse cells, serine phosphorylation of Stat1 is defective, transactivation is impaired and c-myc mRNA is induced, not suppressed. Furthermore, inhibitors of Raf-1 activation abrogate the IFN-dependent induction of c-myc in Stat1-null cells, indicating that Raf-1 is important in Stat1-independent signaling.

Results

Regulation of c-myc gene expression by IFN-γ in wild-type and Stat1-null mouse embryo fibroblasts (MEFs)

To determine if c-myc is a target of IFN-γ-mediated signaling, we examined c-myc mRNA levels in wild-type and Stat1-null MEFs. In wild-type cells that were serum-starved for 36 h, IFN-γ treatment decreased c-myc mRNA expression by 4-fold in 3 h (Figure 1C). In contrast, c-myc mRNA was induced 6-fold by IFN-γ in Stat1-null cells, rapidly and transiently (Figures 1A, B and 2B). Treatment with IFN-β also suppressed the induction of c-myc by platelet-derived growth factor (PDGF) in wild-type cells and induced c-myc expression in Stat1-null cells (Figure 2C). These results are in accord with the suppression by IFN-γ of cell growth in wild-type cells and with the loss of growth inhibition in Stat1-null cells (Bromberg et al., 1996). Since other immediate-early genes are also induced transiently and rapidly in response to growth factors such as PDGF (Greenberg and Ziff, 1984), we investigated the induction by IFN-γ of genes in the fos and jun families in Stat1-null cells. c-jun was induced rapidly by IFN-γ in Stat1-null but not wild-type MEFs, whereas c-fos and jun-B were not induced in either Stat1-null or wild-type cells (Figure 1D).

Regulation of PDGF-dependent induction of c-myc by IFNs in wild-type and Stat1-null MEFs

PDGF, a major mitogen in serum, induces c-myc rapidly and transiently (Greenberg and Ziff, 1984). Treatment with IFN-α and -β has been found to abrogate the induction by PDGF of c-myc and entry into S-phase in mouse fibroblasts (Einat et al., 1985). We investigated the effect of IFN-γ on cell cycle progression in wild-type and Stat1-null MEFs. Cells at 20% confluence were serum-starved in 0.1% serum for 48 h, subsequently returned to medium with 10% serum, with or without IFN-γ, and examined after 24 h (their approximate doubling time) for cell cycle distribution. Only 29% of the wild-type cells were in S-phase with serum and IFN-γ, whereas 52% of the Stat1-null cells were in S-phase under the same conditions (Figure 2A). These results indicate that the effect of IFN-γ in limiting cell cycle progression depends on Stat1. Next, we examined the effect of IFN-γ or IFN-β on the PDGF-dependent induction of c-myc in wild-type and Stat1-null MEFs. PDGF induced c-myc expression in both types of cells. However, the induction was ~2-fold higher in Stat1-null cells than in wild-type cells. Simultaneous treatment with IFN-γ or IFN-β abolished the induction by PDGF of c-myc in wild-type but not in Stat1-null cells (Figure 2B and C). These results indicate that suppression of the PDGF-dependent induction of c-myc by IFNs depends on Stat1. Although PDGF or IFNs induced c-myc expression independently in Stat1-null cells, simultaneous treatment was not additive or synergistic, indicating that these

![Fig. 1. c-myc mRNA expression in response to IFN-γ in Stat1-null and wild-type MEFs. (A) Subconfluent, serum-starved MEFs were either untreated or treated with 1000 IU/ml of murine IFN-γ for 15 or 30 min. c-myc and GAPDH mRNA levels were analyzed by Northern blotting. (B) Stat1-null cells were treated with murine IFN-γ(1000 IU/ml). c-myc and GAPDH mRNA levels were determined as above. (C) Wild-type cells were treated with murine IFN-γ(1000 IU/ml). c-myc and GAPDH mRNA levels were determined as above. (D) Subconfluent, serum-starved fibroblasts were either untreated or treated with 1000 IU/ml of murine IFN-γ for 30 min. Northern blot analyses were conducted with the probes indicated.](image-url)
Regulation of c-myc by Stat1

Fig. 2. Effects of growth factor and IFN treatment on c-myc regulation. (A) MEFs were grown to 20% confluence in DMEM with 10% FCS. The cells were serum-starved in DMEM with 0.1% FCS for 48 h. Cells were either untreated (–) or treated with 10% FCS, alone (FCS) or with 1000 IU/ml of murine IFN-γ (FCS/H11001 IFN-γ). Twenty-four hours later, the cells were stained with propidium iodide and the DNA content was analyzed by flow cytometry. The percentage of cells in the G1, S and G2/M parts of the cell cycle are indicated in each histogram. (B) Stat1-null or wild-type MEFs were either untreated or treated with 1000 IU/ml of IFN-γ alone, 200 ng/ml of PDGF alone, or PDGF plus IFN-γ. Northern transfers were hybridized with c-myc or GAPDH probes. (C) Stat1-null or wild-type MEFs were either untreated or treated with 30 min with 1000 IU/ml of IFN-β alone, 200 ng/ml of PDGF alone, or PDGF plus IFN-β. Northern transfers were hybridized with c-myc or GAPDH probes.

IFN-γ regulates the expression of c-myc but not the CDK inhibitor p21 in human fibrosarcoma cells

Human fibrosarcoma cells expressing Stat1 (2fTGH), lacking Stat1 (U3A) or reconstituted with Stat1 (U3A variants) have been used extensively to study the roles of Stat1 in a variety of biological responses (Horvath et al., 1996; Kumar et al., 1997b). We investigated the IFN-γ-dependent regulation of c-myc expression in these cell lines. As in MEFs, the expression of c-myc was suppressed by IFN-γ in 2fTGH and induced in U3A cells (Figure 3A). Since the cyclin–CDK inhibitor p21\textsuperscript{waf1} has been suggested to mediate growth arrest in response to IFN-γ in epidermal carcinoma and glioblastoma cells (Chin et al., 1996; Kominsky et al., 1998), we investigated the response of the p21 gene to IFN-γ in 2fTGH and U3A cells. Northern and Western blot analyses revealed that p21 expression was not significantly increased by IFN-γ treatment in either cell line (Figure 3).

Both tyrosine and serine phosphorylation of Stat1 are required to suppress c-myc expression

We examined the effects of IFN-γ in U3A cells reconstituted with Stat1 variants lacking the tyrosine phosphorylation site 701 or the serine phosphorylation site 727, which are in the transactivation domain of Stat1. c-myc expression was induced, not suppressed, by IFN-γ in these cell lines, indicating that these two amino acid residues, required for Stat1-dependent transactivation, are also required to suppress c-myc expression (Figure 4). In control U3A cells reconstituted with wild-type Stat1, the expression of c-myc was suppressed similarly to parental 2fTGH cells (Figure 4). In U4A or γ2A cells, lacking Jak1 and Jak2, respectively, neither induction nor suppression of c-myc was observed in response to IFN-γ, indicating that both of these kinases are required for both Stat1-dependent and Stat1-independent regulation of c-myc expression (data not shown).

A consensus GAS element in the c-myc promoter is necessary but not sufficient for negative regulation of expression in response to IFN-γ

A series of promoter deletions linked to a luciferase reporter was used to identify the elements responsible for suppression of c-myc expression in 2fTGH cells. Sequences between –1138 and –1100 were required to mediate a 5-fold reduction in luciferase expression in response to IFN-γ (Figure 5A). Sequence analysis revealed a consensus GAS element at –1107 to –1099, with strong homology to other GAS elements (Table I). A concatamer
containing seven copies of the c-myc GAS element trans-activated the expression of the same reporter by 70-fold, showing that this GAS element lacks intrinsic repressor activity (Figure 5B). Therefore, this element is necessary but not sufficient for c-myc suppression. It is likely that suppression involves the interaction of Stat1 bound to the GAS element with a co-repressor bound elsewhere in the c-myc promoter. A 1.7 kb c-myc promoter fragment linked to the cell surface marker cd2 was stably transfected into NIH 3T3 fibroblasts. Treatment with PDGF induced cd2 expression, whereas IFN-γ abrogated this induction of expression by PDGF (Figure 5C), in accord with the results of Northern analysis in MEFs (Figure 2).

Electrophoretic mobility shift assays (EMSAs) indicate that Stat1 bound as a homodimer to the c-myc GAS element in extracts of IFN-γ-treated 2fTGH but not U3A cells (Figure 6A). Binding was abolished by pre-incubation with either anti-Stat1 or unlabeled competitor oligonucleotide, indicating that the interaction is specific (Figure 6B). EMSAs with extracts of NIH 3T3 cells revealed that IFN-γ stimulated the binding of Stat1 homodimers to the c-myc GAS element, whereas PDGF did not generate any complex (Figure 6C). In response to PDGF, Stat1 and Stat3 homodimers and Stat1–Stat3 heterodimers are formed on a high-affinity GAS element such as the SIE, and the complexes with Stat3 predominate (Vignais et al., 1996). Apparently the relatively small amount of Stat1 dimer formed in response to PDGF was not sufficient for us to observe binding to the c-myc GAS element under the conditions employed.

**Table I.** Comparison of functional GAS elements

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>GAS sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc</td>
<td>mouse</td>
<td>T T C T G G G A A</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>human</td>
<td>T T C C G G G A A</td>
</tr>
<tr>
<td>IRF-1</td>
<td>mouse</td>
<td>T T C C C G G A A</td>
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<tr>
<td>ICSBP</td>
<td>human</td>
<td>T T C C C C G A A</td>
</tr>
<tr>
<td>FcγRI</td>
<td>human</td>
<td>T T C C C C C A A</td>
</tr>
<tr>
<td>IFP 53</td>
<td>human</td>
<td>T T C C C C C A A</td>
</tr>
</tbody>
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*The c-myc sequence is from Roussel et al. (1994) and the others are from Schindler and Darnell (1995).*

anti-proliferative responses to the IFNs (Williams, 1995; Clemens and Elia, 1997). A dominant-negative variant of PKR can abrogate the IFN-α-mediated inhibition of c-myc expression and the inhibition of cellular proliferation in M1 myeloid leukemia cells (Raveh et al., 1996). PKR-null cells are defective in activating the transcription factors IRF-1 and NF-κB in response to double-stranded RNA and in signaling in response to IFN-γ (Yang et al., 1995; Kumar et al., 1997a). Furthermore, the activation of the GBP and IRF-1 promoters in response to IFN-γ is
defective in PKR-null cells (Kumar et al., 1997a). Since
these promoters are complex, requiring NF-κB and IRF-1
in addition to Stat1, we analyzed transcription driven by an
oligomeric GAS element alone to determine the response to
IFN-γ in PKR-null and wild-type cells. Stat1-dependent
transactivation was 4-fold lower in PKR-null cells than in
wild-type cells (Figure 7A). EMSAs revealed that the
binding of Stat1 to DNA was not defective in PKR-null
cells, either in the absence (Figure 7B) or in the presence
of serum (Kumar et al., 1997a). However, the IFN-γ-
stimulated phosphorylation of Stat1 on Ser727 was defec-
tive in serum-starved PKR-null cells (Figure 7C). Also
consistent with a defect in Stat1 activation in PKR-null
cells, treatment with IFN-γ led to the induction of c-myc
mRNA, rather than to the suppression seen in wild-type
cells, with kinetics similar to those observed in Stat1-null
cells (Figure 7D). These results reveal that both Stat1 and
PKR are required for IFN-γ to suppress c-myc expression.

**IFN-dependent induction of c-myc expression in Stat1-null cells is inhibited by geldanamycin or dominant-negative p50cdc37.**

Raf1 activation is critical for the mitogen-induced sig-
naling pathways that mediate the induction of c-myc

Fig. 6. Binding of Stat1 to the c-myc GAS. (A) EMSAs were
performed with whole-cell extracts prepared from U3A or 2TGH
cells, not treated or treated for 15 or 30 min with 1000 IU/ml of
IFN-γ. (B) EMSAs were performed with whole-cell extracts from
2TGH cells treated with 1000 IU/ml of IFN-γ. The extracts were pre-
incubated with anti-Stat1 or with a 100-fold molar excess of the
unlabeled SIE (m67) GAS. (C) EMSAs were performed with whole-
cell extracts from NIH 3T3 cells, treated for 30 min with 200 ng/ml of
PDGF, 1000 IU/ml of murine IFN-γ, or not treated.

Fig. 7. Defective Stat1 activation and serine phosphorylation in PKR-
null cells. (A) 8× con GAS linked to luciferase was transiently
transfected into wild-type or PKR-null MEFs. Luciferase activity was
determined with or without murine IFN-γ (1000 IU/ml) for 6 h.
Results are presented with standard deviations from three independent
experiments. (B) Whole-cell extracts were prepared from serum-
starved wild-type or PKR-null cells, with or without treatment with
1000 IU/ml of murine IFN-γ. Stat1 binding to the SIE (m67) GAS was
determined by EMSA. (C) Extracts of serum-starved cells, either
untreated or treated with IFN-γ (1000 IU/ml for 20 min) were
immunoprecipitated with anti-Stat1. The transfer was probed first with
an antibody specific for a Stat1 peptide that includes phosphorylated
Ser727 and then reprobed with anti-Stat1. (D) RNA from MEFs
untreated or treated with murine IFN-γ (1000 IU/ml) was analyzed by
the Northern procedure.
expression (Kerkoff et al., 1998; Aziz et al., 1999). p50\textsuperscript{ΔN,2} and HSP90 are critical partners in the activation of Raf-1 in mammalian cells (Grammatikakis et al., 1999). Pre-treatment with geldanamycin, an HSP90-specific inhibitor, or overexpression of dominant-negative p50\textsuperscript{ΔN,2} prevents the association of Raf-1 with p50\textsuperscript{ΔN,2} and HSP90, and the activation of Raf-1 and MAPK in response to growth factors (Grammatikakis et al., 1999). Pre-treatment with geldanamycin prevented the induction of c-myc expression by IFN-γ, IFN-β or PDGF in Stat1-null cells (Figure 8A). Similarly, expression of dominant-negative p50\textsuperscript{ΔN,2} also prevented the induction of c-myc expression by IFN-γ in Stat1-null cells (Figure 8B). These results suggest that Raf-1 activation is critical for the induction of c-myc expression by IFNs in Stat1-null cells.

**Discussion**

c-myc and c-jun, required for cell cycle progression (Grandori and Eisenman, 1997; Obaya et al., 1999; Wisdom et al., 1999), are important targets of Stat1-independent responses to IFN-γ. c-jun is required for progression through G1 and for trans-activation of the cyclin D1 gene in fibroblasts (Wisdom et al., 1999), thus helping to provide a link between the response to growth factors and cell cycle regulation. Deregulated expression of c-myc and c-jun is likely to be important in the abnormal proliferation of Stat1-null cells in response to IFN-γ, which can serve as a growth factor for some cells (Caux et al., 1992; Shiohara et al., 1993). IFN-γ suppressed the expression of c-myc in wild-type cells and is likely to be important in regulating the switch between growth arrest and proliferation. IFN-γ or -β abrogated the induction by PDGF of c-myc expression in wild-type cells but not in Stat1-null cells, suggesting that Stat1 is required for this response. IFN-α also abrogated the induction of c-myc by PDGF in NIH 3T3 fibroblasts and in Kaposi’s sarcoma cells (Eina et al., 1985; Koster et al., 1996), although the direct involvement of Stat1 in these responses was not demonstrated. Our results also indicate that c-myc, but not the CDK inhibitor p21\textsuperscript{α,2}, is a target of regulation by IFN-γ in human fibrosarcoma cells. p21 has been implicated as a mediator of IFN-γ-dependent growth arrest in epidermal carcinoma and glioblastoma but not colon carcinoma (HCT116) cell lines (Chin et al., 1996; Kominsky et al., 1998; Sharma and Iozzo, 1998). p21 is induced by IFN-γ in tumor cell lines harboring mutated p53 but not in cell lines expressing wild-type p53 such as HT1080 (from which 2fTGH and U3A cells are derived), where its basal expression is high, indicating that the regulation of p21 by p53 is dominant over IFN-γ-mediated regulation of this gene. Studies in U3A cell variants indicate that tyrosine and serine phosphorylation sites in the C-terminal transactivation domain of Stat1 are required to suppress c-myc expression. These results are consistent with previous data suggesting that transcriptionally competent Stat1 is required for the anti-proliferative effect of IFNs (Bromberg et al., 1996).

Transient transfection of a c-myc promoter fragment linked to luciferase revealed that a consensus GAS element −1107 to −1099 (relative to the P1 promoter) is required for c-myc suppression. This element differs from the previously identified GAS element, which binds to Stat3 preferentially, overlaps the E2F element and functions in the IL-6- and gp130-mediated transactivation of c-myc (Kiuchi et al., 1999). Stat1 binds to the upstream GAS as a homodimer in extracts of IFN-γ-treated wild-type cells. The upstream element is necessary but not sufficient for suppression of c-myc since it lacks intrinsic repressor activity. Therefore, Stat1 is likely to interact with a co-repressor bound to another site in the c-myc promoter to inhibit expression. A likely candidate is Blimp-1, a member of the Groucho family of co-repressors that binds to the PRF site of the c-myc promoter and mediates repression (Lin et al., 1997; Ren et al., 1999). Since Blimp-1 is expressed exclusively in B-lymphocytes, other Groucho family members may participate in repressing c-myc expression in other cell types. Another candidate is MBP-1, which represses c-myc expression when bound to the E2F site (Ray and Miller, 1991). IFN-γ inhibits the transcription of several genes, including those encoding perlecan, bullous pemphigoid antigen 1 and cyclin A (Tamai et al., 1995; Sharma and Iozzo, 1998; Sibinga et al., 1999). Transcriptional repression of the perlecan gene by IFN-γ requires functional Stat1 and a promoter region containing multiple GAS elements. However, the binding of Stat1 to these GAS elements has not been reported and thus the mechanism of repression is not known (Sharma and Iozzo, 1998).

In NIH 3T3 cells stably expressing cd2 under the control of the 1.7 kb c-myc promoter, treatment with PDGF induced cd2 expression, and simultaneous treatment with IFN-γ abrogated this induction. The induction of gene expression by PDGF depends on several signal transduction pathways, among which is the ras/MAPK
pathway, and involves the E2F site of the c-myc promoter (Sacca and Cochran, 1990; Claesson-Welsh, 1994). ras/MAPK-activated Ets factors have been proposed to mediate c-myc expression in response to growth factor stimulation (Roussel et al., 1994; Aziz et al., 1999; Cheng et al., 1999). The abrogation by IFN-γ of c-myc induction in response to PDGF might involve a competition between promoter-bound Ets factors and Stat1 dimers for co-activators such as CBP/p300 (Horvai et al., 1997). PDGF activates the formation of complexes involving Stat1, Stat5 and Stat3 on the SIE element of the c-fos gene (Vignais et al., 1996). However, PDGF did not induce the formation of Stat complexes on the c-myc GAS element, indicating that the abrogation by IFN-γ of c-myc induction in response to PDGF does not involve a competition between different Stat dimers for the GAS site.

PKR is involved in regulating anti-viral, anti-proliferative and tumor suppressor functions (Clemens and Elia, 1997), and PKR-null cells are defective in activating IRF-1 and NF-kB in response to double-stranded RNA (Kumar et al., 1997a). The defective activation of the GBP and IRF-1 promoters by IFN-γ in PKR-null cells can be rescued by expressing wild-type but not mutant PKR, indicating that PKR is also required in IFN-γ-dependent signaling (Kumar et al., 1997a). In extracts of IFN-γ treated cells, a decrease in the mobility of PKR in SDS–PAGE gels was observed, consistent with its phosphorylation (Kumar et al., 1997a). A dominant-negative derivative of PKR abrogated both the IFN-α-mediated downregulation of c-myc expression and the inhibition of cell growth (Raviv et al., 1996). Our results indicate that PKR-null cells are defective in phosphorylating Stat1 on Ser727, and that Stat1-dependent transactivation is 4-fold lower in PKR-null than in wild-type cells, an effect comparable to the reduction observed for the S727A mutant of Stat1 (Zhang et al., 1998). Furthermore, in PKR-null cells, c-myc mRNA is induced transiently and rapidly in response to IFN-γ, just as it is in Stat1-null cells. Therefore, both Stat1 and PKR are required to suppress the expression of c-myc in wild-type cells. PKR associates with Stat1 both in vitro and in vivo, although it does not phosphorylate Stat1 directly (A.H. Wong et al., 1997). Therefore, PKR may be part of a kinase cascade involved in phosphorylating Stat1 on Ser727 in response to IFN-γ. The phosphorylation of Stat1 on Ser727 is required for maximal transactivation and recruitment of the transcription co-factor MCM-5 (Wen et al., 1995; Zhang et al., 1998), also a component of the DNA replication licensing factor. The recruitment of MCM-5 from origins of DNA replication to the transcriptional machinery, mediated by Stat1 in response to IFN-γ, has been suggested as a mechanism for suppression of proliferation (Zhang et al., 1998). Nmi, originally identified because it interacts with N-myc, was later shown to be inducible by IFN-α and also to enhance Stat1-dependent transactivation (Bao and Zervos, 1996; Lebrun et al., 1998; Zhu et al., 1999). Whether PKR is involved in regulating Nmi or MCM-5 is not known at present.

The kinases directly responsible for phosphorylating Stat1 on Ser727 are not known. Stat1 serine phosphorylation is enhanced by treatment with IFN-γ or LPS, or by serum stimulation (Wen et al., 1995; Kovarik et al., 1998; Takaoka et al., 1999). The Ser727 phosphorylation site lies within an MAPK consensus sequence and the MAPK Erk2 has been proposed to phosphorylate this residue (David et al., 1995). The IFN-γ-induced activation of Erk2, serine phosphorylation of Stat1 and Stat1-dependent transactivation are strongly inhibited by overexpression of a dominant-negative form of the protein tyrosine kinase Pyk2 in a Jak2-dependent manner (Takaoka et al., 1999). Evidence for a Stat1 serine kinase that depends on Jak2 and is distinct from MAPK has also been presented (Zhu et al., 1997). These results indicate that the phosphorylation of Stat1 on serine is likely to be regulated by kinase cascades rather than by a single kinase.

Genetic analyses in yeast and Drosophila have shown that p50(26,37) functions both in the cell cycle and in the ras/raf/MAPK pathway in close cooperation with its partner HSP90 (Reed, 1980; Cutforth and Rubin, 1994). Recent studies have shown that p50(26,37) is the primary determinant of HSP90 recruitment to Raf-1 and of the activation of Raf-1 by serum and growth factors in mammalian cells (Grammatikakis et al., 1999). Co-expression of p50(26,37) strongly potentiated the v-src-mediated activation of Raf-1 and, conversely, dominant-negative p50(26,37) unable to recruit HSP90 into the Raf-1 complex, abrogated the activation of Raf-1 (Grammatikakis et al., 1999). Inhibition of Raf-1 activation by pre-treatment with geldanamycin or expression of dominant-negative p50(26,37) abrogated the induction of c-myc by IFN-γ in Stat1-null cells, suggesting that Raf-1 activation is critical for this pathway. Jak1 and Jak2 are also implicated in the regulation of c-myc expression in response to IFN-γ. Conditional dimerization of Jak1 or Jak2, leading to their activation, can stimulate the c-myc promoter (Mizuguchi and Hatakeyama, 1998; Moli et al., 1998). It is likely that, in the absence of Stat1, IFN-γ-activated Jak1 and Jak2 phosphorylate as yet unknown signaling molecules that can activate c-myc expression through Raf-1. Both IFN-γ and IFN-β mediate the activation of c-myc in the absence of Stat1 and it remains to be determined whether the signals emanating from the two different receptors are the same or different.

A global expression study has shown that growth factor-dependent stimulation of a mutant PDGF receptor with a restored ras-GAP binding site promotes the induction of IFN-γ-responsive genes rather than of immediate-early genes (Fambrough et al., 1999), revealing a link between PDGF- and IFN-γ-dependent pathways. We have identified several additional genes repressed in wild-type cells or induced in U3A cells in response to IFN-γ (Der el al., 1998; C.V.Ramana and G.R.Stark, unpublished data). Characterization of the promoters of these genes and further analysis of the c-myc and c-jun promoters should also help to identify the components of this novel pathway.

Materials and methods

Reagents and cell culture

Recombinant human and mouse IFN-γ were purchased from Boehringer Mannheim. PDGF-BB and murine IFN-β were purchased from Gibco-BRL and PBL, respectively. The human fibrosarcoma cell lines 2TGH, U3A, U4A, 3A and derivatives of U3A reconstituted with Stat1 variants were described by Kumar et al. (1997b). The HT1080 parents of all these cells have wild-type p53, which drives high expression of p21 (our unpublished data). MEFs from littermates of Stat1-null, PKR-null and the corresponding wild-type mice were obtained as described by Meraz et al. (1996) and Kumar et al. (1997a). NIH 3T3 mouse fibroblasts
were obtained from M.Roussel (St Jude Children’s Research Hospital, Memphis, TN). All cells were maintained in Dulbecco’s modified Eagle’s medium (DME) supplemented with 5% fetal calf serum (FCS; Gibco-BRL). Subconfluent cells were serum-starved for 24–48 h in DMEM containing 0.1% serum before treatment with IFNs or PDGF, which were added directly to the serum-free medium.

RNA, protein and cell cycle analyses
Total RNA was prepared by using the TRIzol (Gibco-BRL) method according to the manufacturer’s instructions. Northern transfers were analyzed with c-myc, c-fos, c-jun, jun-B and p21 cDNA probes (Langer et al., 1992; el-Deiry et al., 1993). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was from Ambion (Austin, TX). The expression levels of c-myc and GAPDH were quantitated with a PhosphorImager (Molecular Dynamics). Western analyses were performed as described by Chernov et al. (1998). Antibodies to Stat1 and p21^WAF1 were purchased from Transduction Laboratories and Santa Cruz Biotechnology, respectively. Cell cycle analysis was performed according to Bromberg et al. (1996). The data were analyzed using the LYSIS II program.

Reporter constructs, transient transfections and luciferase assays
Luciferase constructs containing elements of the c-myc promoter (–1100 to +580 and –424 to +580, relative to the PI transcription site) have been described by Wong et al. (1995). The BglII fragment (–1138 to +580) was purified from M Bg CAT (Yang et al., 1986) and ligated into the BamHI site of pGL2-Basic Luc (Promega) or p3-cd2 (a gift from K.Kerr, ICRF, London) to generate c-myc promoter-driven luciferase and cd2 reporters. The 8× consensus (con) GAS–Luc was described by Horvai et al. (1997), and 7× c-myc–GAS–Luc was generated by replacing the 8× GAS in the above construct with a concanamer containing seven copies of the c-myc GAS/TTCTCGGAAA, using NheI and PstI linkers. Dominant-negative p50(9–37) was described previously (Grammatikakis et al., 1999). Transient transfections were performed using Fugene 6 (Boehringer Mannheim), according to the manufacturer. Twenty-four hours after transfection, the cells were serum-starved overnight and treated with human or murine IFN-γ (1500 U/ml) for 6 h. Luciferase assays were performed according to the manufacturer (Promega, Glo-Light), and the activities were normalized to total protein concentrations, determined in Bio-Rad assays. Each experiment was performed in duplicate and the results shown are the average of three independent experiments (standard deviations shown). The c-myc promoter-cd2 expression vector was transfected stably into NIH 3T3 cells, individual clones expressing cd2 were isolated, and the induction of cd2 by PDGF was confirmed by FACScan analysis. Sixteen hours after treating serum-starved cells with PDGF or PDGF plus IFN-γ, the expression of cd2 was determined by staining the cells with a phycoerythrin-conjugated monoclonal antibody against cd2 (Dako), followed by FACScan analysis using a Becton Dickinson instrument and the LYSYS II software package.

Immunoprecipitations and immunoblot analyses
Wild-type and PKR-null cells were untreated or stimulated with IFN-γ or IFN-τ (1000 U/ml) for 20 min and cell extracts were prepared as described by Kumar et al. (1997a). Equal amounts of extracts were immunoprecipitated with anti-Stat1 (Transduction Laboratories), and the immune complexes were collected on Gamma-bind G–Sepharose beads. Samples were run on SDS–7.5% polyacrylamide gels, transferred to polyvinylidene difluoride membranes and probed with the immunoprecipitating antibody or with an antibody to a Stat1 peptide containing phosphorylated Ser727 (from Upstate Biotechnology) followed by anti-rabbit IgG coupled to horsedarshid peroxidase (Bio-Rad). The transfers were analyzed by using the Renaissance chemiluminescence reagent (DuPont NEN).

Electrophoretic mobility shift assays (EMSAs)
Nuclear extracts were prepared and EMSAs were performed as described by Kumar et al. (1997a), using as probe a double-stranded oligonucleotide containing the c-myc promoter sequence 5′-CCCTTGGTTGATGCCGGGTT-3′ (the homology to GAS sequences is underlined). The high-affinity Stat binding site, SIE (m67), was described by Vignais et al. (1996).

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References

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