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Gamma Interferon Induces Expression of Mad1 Gene in Macrophage, Which Inhibits Colony-Stimulating Factor-1–Dependent Mitogenesis

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Abstract Gamma interferon (IFNγ) has long been known as an antiproliferative cytokine. The mechanism of its action, however, remains elusive. Monocytes and macrophages are primary targets of IFNγ. To understand the antiproliferative signaling of IFNγ, we studied the effect of IFNγ on expression of c-Myc, Mad1, Max, cyclin D1, and cyclin D2 genes in both a macrophage cell line and in primary bone marrow–derived macrophages (BMM) in response to colony-stimulating factor-1 (CSF-1). We found that whereas IFNγ inhibits CSF-1–stimulated c-Myc gene expression, it induces Mad1 expression. Induction of Mad1 mRNA could be detected as early as 90 min following IFNγ treatment and was maintained for at least 15 h. These results suggest that IFNγ treatment could shift the Myc–Max complex to the Mad1–Max complex in cells. The levels of Max, cyclin D1, and cyclin D2, however, remained unchanged. Enforced ectopic expression of Mad1 in the cells results in inhibition of [^{3}H]thymidine incorporation and proliferation in response to CSF-1. This study suggests a mechanism by which IFNγ inhibits CSF-1–stimulated proliferation of macrophages, i.e., by elevating the Mad1 level in the cells. J. Cell. Biochem. 72:232–241, 1999.

Key words: growth factor; cytokine; oncogene; tumor suppressor gene; antiproliferation; signal transduction

Type II/immune/γ-interferon (IFNγ) is the most important immunomodulatory IFN. It induces major histocompatibility complex class I and class II regions, regulates isotype expression of immunoglobulins, activates monocytes and macrophages by enhancing Fc receptor expression, and exerts antiviral and antitumor effects [Pestka et al., 1987; De Maeyer and De Maeyer-Guignard, 1988]. IFNγ has also been known for its antiproliferative effect on various types of cells, which has been utilized in combination with other cytokines or chemotherapies in the treatment of cancer [Schreiber and Celada, 1985]. In contrast to the well-characterized signaling mechanisms by which growth-stimulating peptides such as growth factors stimulate cell proliferation, the underlying mechanism of growth-inhibitory action by IFNγ remains largely unclear. Binding of IFNγ to its cell surface receptor causes hetero-oligomerization of the α and β receptor subunits ($\alpha_2\beta_{1or2}$), which in turn activates two preassociated protein tyrosine kinases, JAK1 and JAK2 [Darnell et al., 1994]. Activated JAK kinases phosphorylate the cytoplasmic domain of the IFNγ receptor, thereby providing a binding site for the SH2 domain of p91STAT1α. p91STAT1α then gets tyrosine phosphorylated by the JAK1 kinases, dissociates from the receptor, forms homodimers, and subsequently translocates to the nucleus, leading to transcriptional activation [Darnell, 1997; Suai et al., 1994]. Involvement of these immediate early events of IFNγ signaling antiproliferative action has been supported by complementation experiments using somatic cells mutants. For instance, the mutant cell line γ1A, deficient in response to IFNγ, and mutant U4A, deficient in response to both IFNγ and IFNα, could be complemented by introducing JAK2 and JAK1 cDNA, respectively, into these cells [Watling et al., 1993; Müler et al., 1993]. Mutant U3A cells, which do not respond to the antiproliferative effect of IFNγ, could be rescued by reintroducing p91STAT1α into these

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cells [Chin et al., 1996; Bromberg et al., 1996]. The downstream targets of the JAKs/STAT1α pathway, which are directly involved in antiproliferation, remain unknown, although the cell cycle inhibitor $p21^{WAF1/CIP1}$ was implicated [Chin et al., 1996; Hobeika et al., 1997].

Colony-stimulating factor-1 (CSF-1) or macrophage colony-stimulating factor (M-CSF) ensures survival and selectively stimulates cell cycle progression of monocytes and macrophages [Stanley et al., 1978; Tushinski et al., 1982]. It acts by binding to and activating a single class of high-affinity cell surface receptor (CSF-1R), which has an intrinsic protein tyrosine kinase activity [Sherr, 1990]. Upon ligand binding CSF-1R forms a dimer, which is believed to serve as the mechanism of cross-membrane signaling and activating the receptor tyrosine kinase [Downing et al., 1989; Li et al., 1991b]. CSF-1R autophosphorylation on tyrosine residues provides binding sites for signaling molecules containing Src-homology 2 (SH2) and/or phosphotyrosine-binding (PTB) domains [Rohrschneider et al., 1997; Roussel, 1997]. For example, mutation at the tyrosine-697 in the mouse CSF-1 receptor, a binding site for adapter protein GRB2, inhibits CSF-1–stimulated mitogenesis in Rat-1 fibroblasts [van der Geer and Hunter, 1993]. Mutation at tyrosine-721, a binding site for both phosphatidylinositol 3-kinase and phospholipase Cγ2, blocks CSF-1–induced differentiation of myeloid progenitor cells FDC-P1 in culture [Bourette et al., 1997].

Among the immediate early genes induced by CSF-1, c-Myc has been demonstrated to play a role in CSF-1–induced G1 to S transition, at least in the cellular context of NIH3T3 cells [Roussel et al., 1991]. Function of c-Myc protein is regulated both positively by heterodimerizing with Max, which activates transcription of target genes, and negatively by Mad, which competes for binding to Max, [Grandori and Eisenman, 1997], thereby suppressing transcription. Mad1, the first identified member of Mad family genes (also including Mxi1, Mad3, and Mad4), belongs to transcription factors of the basic helix-loop-helix-leucine zipper (bHLHZip) family [Ayer et al., 1993]. Mad1 and c-Myc, whose cellular levels change in different stages of cells, compete for binding to Max, which always maintains a constant level in cells [Cerni et al., 1995]. The Mad1–Max complex binds to the same DNA sequence as the Myc–Max complex but suppresses the transcription of target genes [Ayer et al., 1993]. Therefore, the relative concentrations of Mad1 and c-Myc proteins in cells determine the ratio of Mad1–Max versus Myc–Max complexes and determine cell growth versus G1 arrest.

Mad1 has been shown to block cotransformation of cells by c-Myc and Ras and to arrest certain human tumor cells in the G1 phase of cell cycle [Cerni et al., 1995; Ayer et al., 1996; Koskinen et al., 1995; Lahoz et al., 1994]. It is possible that Mad1 might play a role in antiproliferative signaling by cytokines, such as IFNγ. Here, we have studied the antiproliferative effect of IFNγ in macrophages in response to CSF-1, focusing on c-Myc, Max and Mad1, as well as cyclin D1 genes. We report that IFNγ increases Mad1 expression while inhibiting c-Myc expression in the same cells. Ectopic expression of Mad1 in Bac1.2F5 macrophages inhibits CSF-1–stimulated mitogenesis. This study suggests a novel mechanism by which IFNγ blocks growth factor–triggered mitogenesis.

MATERIALS AND METHODS

Cell Culture

Murine Bac1.2F5 macrophages were grown in alpha medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (50 units/ml), streptomycin sulfate (50 μg/ml), and L-cell conditioned medium (10% final, v/v) as the source of murine CSF-1. Approximately 80% confluence of the cells was employed for all experiments. Cells were arrested in G1 by depriving CSF-1 for 16–20 h. To restimulate the cells, human recombinant CSF-1 (4.2 nM) or 10% L-cell conditioned medium was added for indicated times. Murine recombinant IFNγ (Gibco-BRL) was used at indicated concentrations.

Bone marrow–derived macrophages (BMM) were extracted and cultured as described elsewhere [Tushinski et al., 1982]. Briefly, femurs, after being removed aseptically from mice, were flushed out with 23-gauge needles with ice-cold alpha medium (5 ml/two femurs). To disperse the cells, the marrow plug was gently passed through 22-gauge needle twice and were centrifuged at 913*g* (2,000 rpm in GS-6R Beckman) for 5 min at 4°C. Cells were resuspended, counted, and plated in L-cell conditioned medium at a density of 1 million cells/ml. After

24 h, nonadherent cells were pelleted and cultured in fresh medium for 2 days. On day 3, nonadherent cells were resuspended in fresh medium following centrifugation and incubated for 2 additional days. The adherent cells, considered as day 5 BMM, were pooled and either frozen in liquid nitrogen for further use or continued in culture for 6 days. For experiments, the day 11 BMM cells were deprived for CSF-1 for 16 h and restimulated with murine CSF-1 for indicated times.

Subcloning and Viral Infection

Murine Mad1 cDNA (913 bp) in PGEM-7Zf(+), a gift from Dr. R. Eisenman (Fred Hutchinson Cancer Research Center), was released by Xho I digestion, filled in by DNA polymerase to blunt ends, and subcloned into retroviral vector pBPSTR1 by ligation. Transient transfection of pBPSTR1-Mad1 retroviral construct (5 μg/5 $\times$ 10^5 cells) or pBPSTR1 alone into Bosc-23 cells was carried out using lipofectamine. Cells were washed 24 h following transfection, and fresh Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS was added in the absence or presence of tetracycline. The medium was removed 48 h postinfection and was centrifuged at 500*g* to pellet any floating cells. The supernatant containing Mad1-gene ecotropic retrovirus was aliquoted and frozen at −80°C.

Bac1.2F5 cells, plated at a density of 2 million/10-cm tissue culture dish in the presence of 10% L-cell conditioned medium, were cultured overnight and infected with 4 ml of the retroviral stock in presence of 5 μg/ml of polybrene. Following 4 h incubation, medium was disposed, and cells were washed once with the same medium. Fresh CSF-1–containing medium was added without selective antibiotic (puromycin) and incubated for 2 days without or with tetracycline as described previously [Paulus et al., 1996]. Infected Bac1.2F5 cells from each 10-cm dish were pooled in 5 ml medium, and 1 ml of the pooled cells was plated in a 10-cm dish with 9 ml of alpha medium with L-cell conditioned medium in the presence of puromycin (1.5 μg/ml). Colonies were observed following 4–5 weeks of the selection. Individual clones were isolated and maintained in the same medium containing puromycin (1.5 μg/ml).

Northern Blot Analysis

Total RNA was extracted from cells using the RNeasy kit from Qiagen (Chatsworth, CA), following the manufacturer's protocol. Twenty micrograms of RNA was resolved in a 1% agarose-formaldehyde gel, transferred to Duralon-UV membrane (Stratagene, La Jolla, CA), prehybridized, and hybridized following standardized protocols [Molecular Cloning, CSH, 1989]. cDNA probes of Mad1, Max, c-Myc, Cyclin D1, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were labeled using Prime It-II Random Primer labeling kit (Stratagene) in the presence of [α-^{32}P]-dCTP (Amersham, Buckinghamshire, UK). The average specific activity of purified probes was $\sim 1 \times 10^6$ cpm/2 ng DNA. In hybridization reactions 2×10^6 cpm/ml (3×10^5 cpm/ml for GAPDH) was used. Membrane was washed sequentially with 2 $\times$ standard saline citrate (SSC) at 50°C, 0.2 $\times$ SSC, and 0.1% sodium dodeyl sulfate (SDS) at 60°C. A final wash was done at 65°C for 1 h in 0.1 $\times$ SSC and 0.1% SDS. To strip off the probe, membrane was subjected to boiling (in 0.1 $\times$ SSPE/0.5% SDS) for 2 min, shaking at room temperature for 10 min, and washing in 2 $\times$ SSPE for 10 min, prior to re-hybridization with a different probe.

Western Blot Analysis

Cells were solubilized in lysis buffer [Li et al., 1991b] with some modifications (20 mM Tris, pH 7.4, 50 mM NaCl, 50 mM sodium pyrophosphate, 30 mM sodium fluoride, 0.1 mM sodium orthovanadate, 5 μM zinc chloride, 2 mM iodoacetic acid, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A) and homogenized with 30 strokes in an all-glass Dounce homogenizer on ice. Following centrifugation, protein concentrations of the cell extracts were measured by Bio-Rad Micro-Assay. Aliquots were resolved in 12% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes (MSI), and incubated with polyclonal rabbit anti-human Mad1 antibody (Sc222, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Following washing, the membrane was incubated with horseradish peroxidase (HRP) linked to goat anti-rabbit whole IgG antibody (Amersham Life Science). Immunoreactive bands were visualized by using an ECL kit (Amersham Life Science). Purified recombi-

nant Mad1 protein (40 ng, Santa Cruz Biotechnology Inc.) was included as a control.

[^{3}H]Thymidine Incorporation and Cell Proliferation

Cells were seeded per well in triplicates in 12-well plates and incubated for 2 days. Cells were starved in CSF-1–free medium for 20 h prior to restimulation with CSF-1 (4.2 nM) for 12 h. Five microcuries of [^{3}H]thymidine (ICN, specific activity: 6.7 Ci/mmol) was added to each well and incubated for an additional 3 h. Cells were washed twice with ice-cold PBS and lysed in alkaline solution, as described previously [Li et al., 1991a]. Samples were subjected to filter assay, and retained radioactivity was measured in a Packard 2200 CA liquid scintillation counter. To determine cell doubling time, cells were seeded in triplicates in six-well tissue culture plates in CSF-1–containing medium for 6 days. Cell numbers were counted every 24 h with a hemocytometer. Doubling times of the cells were calculated as a mean of three independent cell doubling periods.

RESULTS

IFNγ Blocks CSF-1–Stimulated Proliferation of Bac1.2F5 Cells

The mouse macrophage cell line Bac1.2F5 requires the continued presence of CSF-1 for survival and proliferation [Morgan et al., 1987]. When CSF-1 is removed from the culture medium, the cells undergo growth arrest in G1 and subsequently die. If they are transiently starved in CSF-1–free medium and then restimulated with CSF-1, these cells re-enter synchronously through G1 into S phase [Matsushime et al., 1991]. It has been previously indicated that IFNγ blocks CSF-1–stimulated proliferation of Bac1.2F5 cells [Matsushime et al., 1991; Roussel et al., 1996]. Since Bac1.2F5 cells have different subclones and different laboratories use the cells with very different in vitro generations, we first tested the ability of IFNγ to block CSF-1–dependent proliferation in Bac1.2F5 cells. Cells were cultured in the absence or presence of CSF-1 with or without various concentrations of IFNγ, and cell numbers were counted each day in triplicate for 6 days. It is shown in Figure 1A that in the absence of CSF-1 the cells did not proliferate, and cell numbers declined over the entire period of culture (open symbols). CSF-1 stimulation, however, caused a steady-state proliferation of the cells (solid symbols) until the culture reached confluence, and the cell number then dropped (between day 5 and day 6). These data indicate that the cells did not acquire anchorage-independent growth even in the presence of CSF-1. In the presence of both CSF-1 and IFNγ, proliferation of the cells was completely blocked (hatched symbols). Inhibition of the proliferation of Bac1.2F5 cells by IFNγ could be detected in the presence of as low as 10 units/ml of mouse IFNγ (Fig. 1B). Thus, Bac1.2F5 cells in our laboratory can be used as a macrophage culture system for studying antiproliferative signaling by IFNγ.

IFNγ Decreases c-Myc and Increases Mad1 Expression in Bac1.2F5 and BMM Macrophages

It has previously been shown that c-Myc is necessary for CSF-1–stimulated G1- to S-phase transition of the cell cycle and mitogenesis [Roussel et al., 1991]. It is conceivable that IFNγ inhibits the CSF-1–induced mitogenesis by either downregulating c-Myc and its partner Max, or by upregulating Mad1, a negative competitor of c-Myc in binding to Max, or both. To test the effect of IFNγ on c-Myc, Max, and Mad1 gene expression in the presence or absence of CSF-1, total RNA was isolated from Bac1.2F5 cells untreated or treated with IFNγ in the absence or presence of CSF-1, resolved in an agarose gel, transferred to a nylon membrane, and hybridized independently with ^{32}P-labeled c-Myc, Max, and Mad1 cDNA probes. It is shown in Figure 2A that CSF-1 treatment alone significantly induced expression of c-Myc gene (lane 2 vs. lane 1, upper panel). However, the CSF-1–induced c-Myc expression was blocked in cells by IFNγ in a time-dependent fashion (lanes 3–5, upper panel). Equal RNA loading of the RNA samples was indicated by levels of GAPDH gene expression (lower panel). To determine whether IFNγ also affects the expression of two important regulators of c-Myc function, Max and Mad1, total RNA was isolated from cells, either untreated or treated with IFNγ for different periods of time, and analyzed for the Mad1 and Max mRNA levels. It is shown in Figure 2B that, while untreated cells showed no expression of Mad1 (lanes 1), IFNγ treatment induced expression of Mad1 gene in a time-dependent manner (lanes 2–4). The induction of Mad1 mRNA by IFNγ could be detected as early as 90 min (lane 2), and continued increases were still

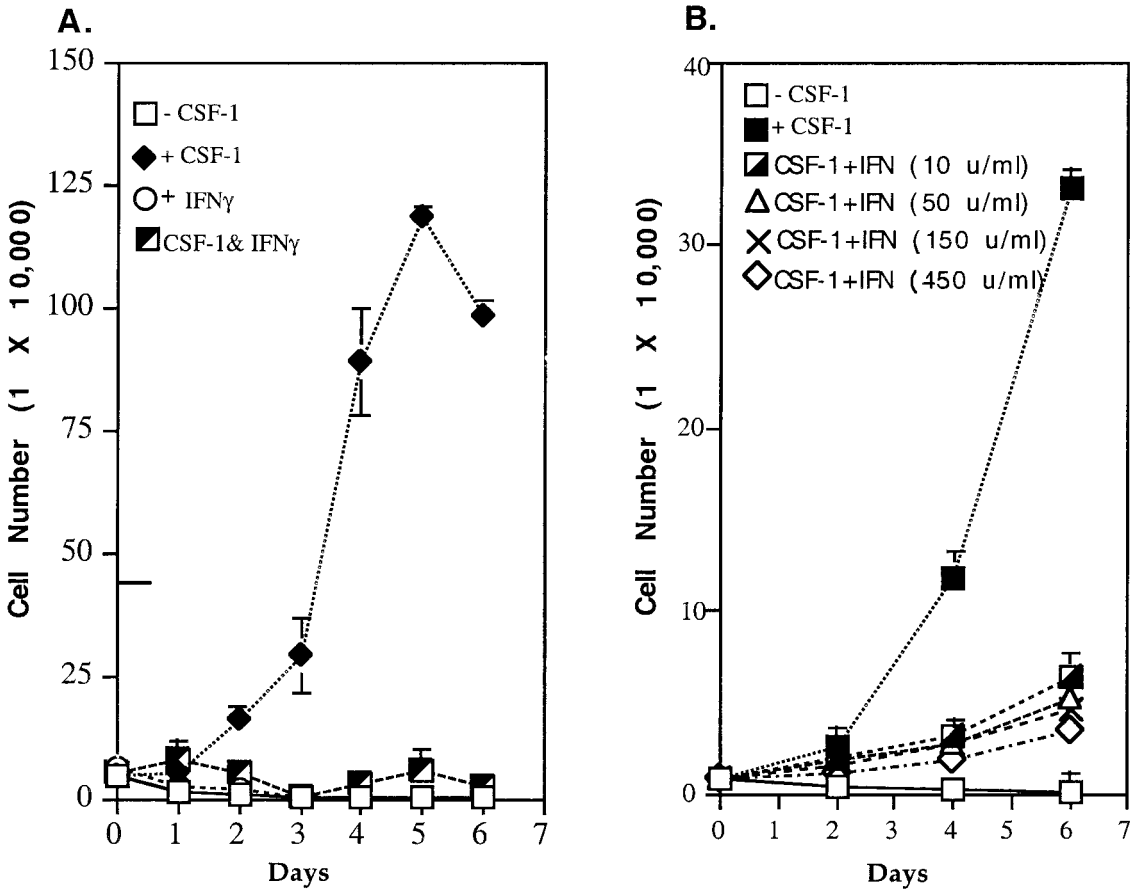


Fig. 1. IFNγ blocks CSF-1–stimulated proliferation in Bac1.2F5 cells. Bac1.2F5 cells were seeded into six-well tissue culture plates (1–5 × 10^4 cells/well) and cultured in the absence or presence of CSF-1 and with or without different concentrations of IFNγ for 6 consecutive days. Cell numbers were counted each day in triplicate for each of the conditions. **A:** Bac1.2F5 cells were cultured in the absence or presence of CSF-1 (4.2 nM), IFNγ (100 units/ml), or CSF-1 together with IFNγ for 6 days. **B:** A similar experiment was carried out except that the effects of different concentrations (10–450 units/ml) of IFNγ on CSF-1–stimulated cell proliferation were tested. Independent experiments were performed for three (A) and two (B) times, respectively. All values were means of triplicate samples (± SEM). Some of the error bars were too small to indicate.

seen following 15 h of IFNγ treatment (lane 4). Similar amounts of total RNA used were indicated by GAPDH levels (lower panel). To test if CSF-1 would interfere with the Mad1 induction by IFNγ, we carried out a similar experiment, in which cells were incubated in the presence of both CSF-1 and IFNγ. It is shown in Figure 2C (upper panel) that neither the untreated (lane 1) nor the CSF-1 alone–treated cells (lane 2) showed any increase in the Mad1 mRNA level. The presence of CSF-1 did not interfere with IFNγ-induced expression of the Mad1 gene (lane 3). Similar amounts of the total RNA were used (lower panel). To confirm this finding in primary macrophages, a similar experiment was carried out using bone marrow–derived macrophages (BMM). It is shown in Figure 2D (upper panel) that no Mad1 message was detectable in BMM cells in either the absence (lane 1) or presence (lane 2) of CSF-1. Incubation of the cells with IFNγ for 5 h significantly enhanced expression of the Mad1 gene (lane 3). The presence of CSF-1 did not interfere with induction (lane 4). Expression of GAPDH gene was included as the RNA loading control (lower panel). On the other hand, as shown in Figure 2E, a constitutive level of Max mRNA was detected in both the untreated (lane 1) and IFNγ-treated (lanes 2–4) cells. The slight decrease at the 15-h treatment of IFNγ (lane 4) was not reproducible in independent experiments. Expression of GAPDH gene was included as the RNA loading control (lower panel). We also examined the effect of IFNγ on expression of two other important cell cycle control genes, cyclin D1 and cyclin D2. As expected, CSF-1 stimulation triggered a dra-

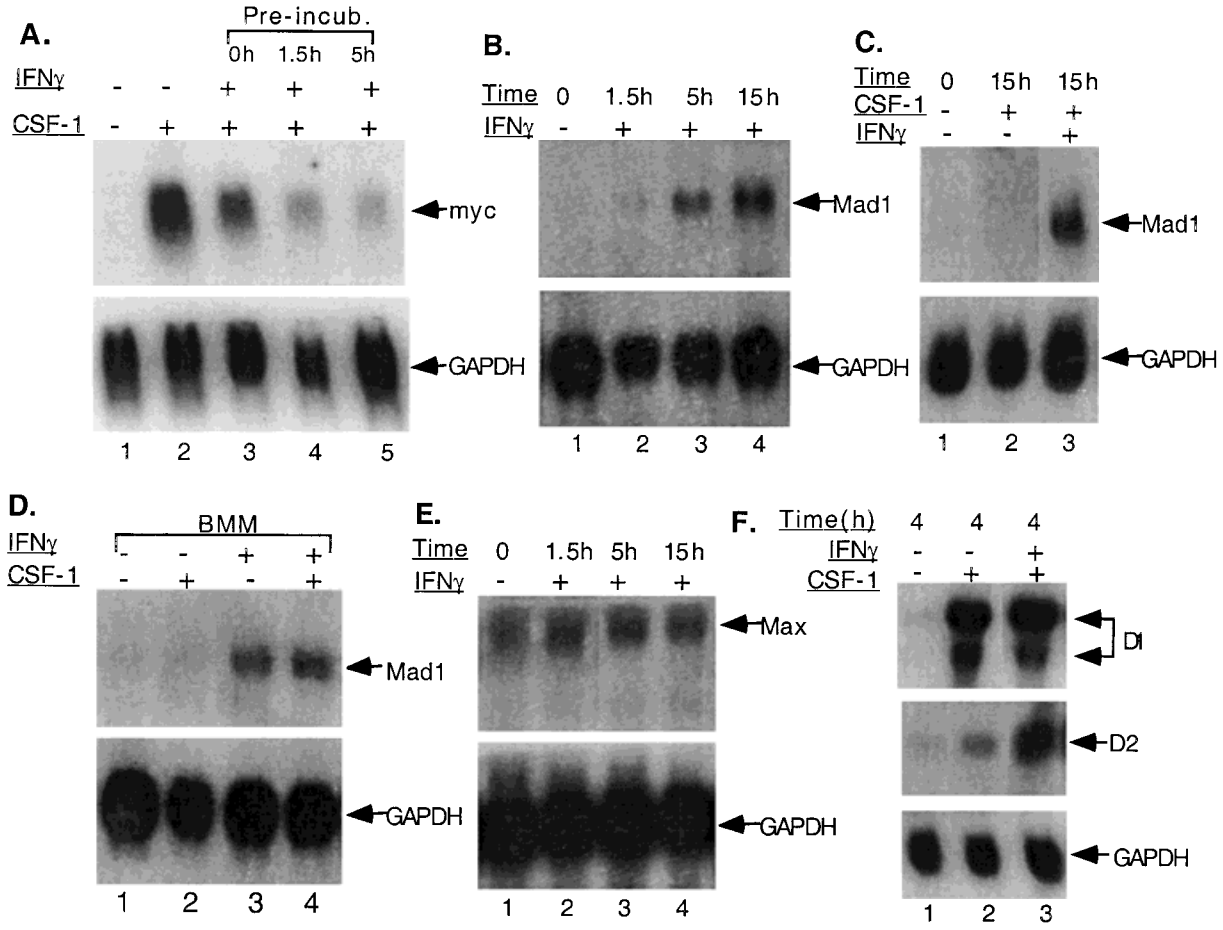


Fig. 2. IFNγ blocks c-Myc and induces Mad1 expression in macrophages. Total cellular RNA was isolated from either Bac1.2F5 cells or bone marrow–derived macrophages (BMM) and treated with the indicated conditions. Twenty micrograms of each RNA sample was resolved in agarose gels, transferred to membranes, and hybridized with corresponding ^{32}P-labeled cDNA probes. Following washing, the results were visualized by autoradiography. **A:** Effect of IFNγ (100 units/ml) on CSF-1(4.2 nM)–induced c-Myc expression. **B:** IFNγ induces Mad1 gene expression in the absence of CSF-1. **C:** Effect of IFNγ on Mad1 in the presence of CSF-1. **D:** IFNγ induces Mad1 expression in BMM macrophages. **E:** Effect of IFNγ on Max. **F:** Effect of IFNγ on cyclin D1 and D2 gene expression. These results were reproducible in every four to six repeated experiments.

matic increase in cyclin D1 (Fig. 2F, upper panel, lane 2 vs. lane 1) and cyclin D2 (Figure 2F, middle panel, lane 2 vs. lane 1). IFNγ did not interfere with the induction of cyclin D1 or cyclin D2 by CSF-1 (lanes 3). Instead, IFNγ enhanced the expression of cyclin D1 and cyclin D2 genes. The reason for this effect is unclear.

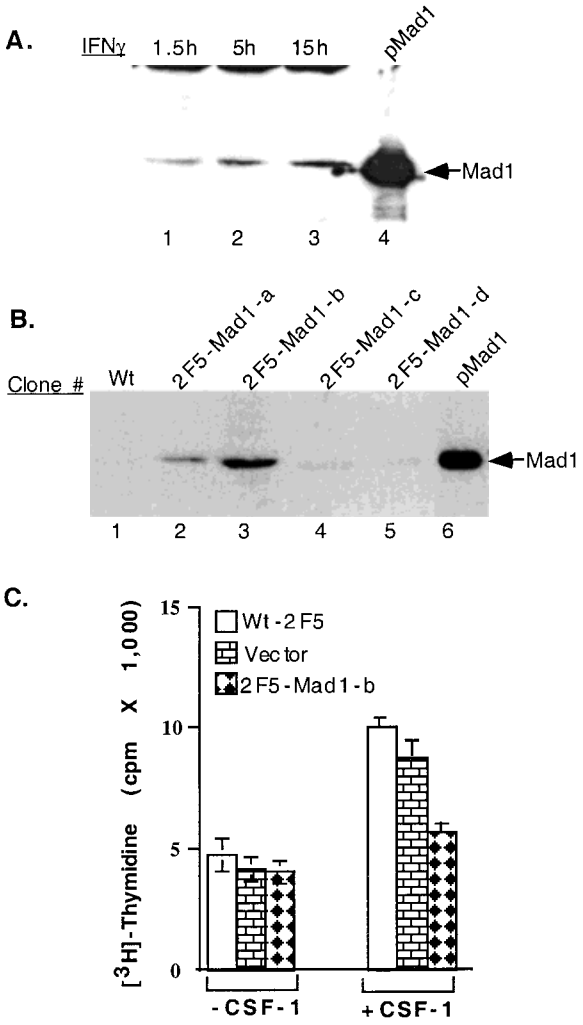
To confirm the induction of Mad1 by IFNγ, we examined the Mad1 protein levels in cells treated with IFNγ for different periods of time. Total lysates of the cells treated with IFNγ for 1.5 h, 5 h, and 15 h were resolved in SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-Mad1 antibody. In proliferating Bac1.2F5 cells, little Mad1 protein was detected (Fig. 3B, lane 1). IFNγ treatment increased the level of Mad1 protein in a time-dependent manner (Fig. 3A, lanes 1 to 3). Purified recombinant Mad1 protein (50 ng) was included as a control (lane 4).

Enforced Ectopic Expression of Mad1 Inhibits DNA Synthesis and Proliferation of Bac1.2F5 Cells in Response to CSF-1

The important question then was, Would increased Mad1 expression by IFNγ inhibit CSF-1-induced mitogenesis in Bac1.2F5 cells? Bac1.2F5 cells stably expressing Mad1 were established (Materials and Methods) and used for testing the problem. First, individual clones of the Mad1-expressing Bac1.2F5 cells were examined for Mad1 protein levels by Western immunoblot analysis using an antibody against

Mad1. Equal amounts (50-μg proteins) of Triton-X-100 extracts of either the wild-type (wt) or Mad1-expressing Bac1.2F5 cells (2F5-Mad1) were resolved in an SDS gel, transferred to nitrocellulose membrane, and immunoblotted with the anti-Mad1 antibody. It is shown in Figure 3B that there was no detectable Mad1 protein in the wt Bac1.2F5 cells (lane 1) and in vector-alone cells (data not shown). However, various amounts of Mad1 protein were detected in all four clones (a–d, lanes 2–5). Among them, the clones 2F5-Mad1-a and 2F5-Mad1-b showed relatively higher Mad1 expressions (lanes 2 and 3). Purified recombinant Mad1 protein (40 ng, Santa Cruz) was included as a control (lane 6).

We then measured [^{3}H]thymidine incorporation to detect differences among the wt Bac1.2F5, Bac1.2F5 with the vector alone, and 2F5-Mad1-b cells in mitogenic response to CSF-1. It is shown in Figure 3C that CSF-1 stimulation caused more than a twofold increase in [^{3}H]thymidine incorporation in wt Bac1.2F5 cells (open bars) and in the 2F5 cells containing the vector alone (middle bars). In 2F5-Mad1-b cells, however, CSF-1–stimulated [^{3}H]thymidine incorporation was significantly inhibited (diamond bars). Because the 2F5-Mad1-a clone showed significantly less inhibition than 2F5-Mad1-b cells, the degrees of inhibition by Mad1 correlated with the levels of Mad1 proteins expressed in the cells (data not shown). It should be pointed out that complete inhibition was not expected because these cells would not be able to be recovered. To confirm this finding, we compared doubling times between the wt Bac1.2F5, Bac1.2F5-vector, and 2F5-Mad1-b cells. Cells were cultured in CSF-1–containing medium for 5 consecutive days, and two 24-h periods (day 2–3 and day 4–5)



D.

Cell lines	Doubling time
WT 2F5	18.3 h
v-2F5	17.7 h
2F5-Mad1-b	23.5 h

Fig. 3. Ectopically expressed Mad1 inhibits CSF-1–stimulated proliferation of Bac1.2F5 cells. **A:** Bac1.2F5 cells were treated with IFNγ (100 units/ml) for the indicated times. Total lysates (40 μg/each) of the cells were resolved in an SDS-PAGE, transferred to nitrocellulose membrane (equal transfer of the proteins was seen by Ponceau S staining), and immunoblotted with anti-Mad1 antibodies (Santa Cruz). Purified recombinant Mad1 protein (40 ng, Santa Cruz) was included as a positive control. **B:** Bac1.2F5 cells were infected with virus preparations of either pBPSTR1 vector alone or pBPSTR1 containing the full-length coding region of Mad1 cDNA. Stable cells were selected under puromycin, and individual clones were isolated and expanded (see Materials and Methods). Western blot analysis of the total cell lysates using anti-Mad1 antiserum. wt, wild-type Bac1.2F5; 2F5-Mad1-a to 2F5-Mad1-d, independent Mad1-expressing clones; pMad1, purified recombinant Mad1 protein (40 ng, Santa Cruz). **C:** [^{3}H]Thymidine incorporation in the wt, vector alone, and 2F5-Mad1-b clone in response to CSF-1. 8×10^3 cells of either wt or 2F5-Mad-b clone were seeded in each well of 12-well tissue culture plates and G1-arrested in CSF-1–free medium for 20 h, prior to addition of CSF-1 (4.2 nM). Addition of [^{3}H]thymidine, washing and lysing of cells, and measurment of radioactivity are described in Materials and Methods. The data represent means of triplicate samples (± SEM) from one of three independent experiments. **D:** Cells were cultured in the presence of CSF-1 for 5 consecutive days, and cell numbers were counted every 24 h in triplicate. Cell doubling times were calculated by taking the means of two different counting (day 2–3 and day 4–5) periods.

were selected for calculating doubling time of the cells. It is shown in Figure 3D that comparable doubling times were obtained for wt (top column) and vector alone (middle column) cells. However, a significantly extended doubling time was seen in the 2F5-Mad1-b cells (bottom column). Again, the partial inhibition of CSF-1–stimulated cell doubling by Mad1 was due to the fact that cells expressing higher levels of Mad1 completely blocked CSF-1 signaling and would not survive selection. Thus, results of these experiments indicate that Mad1 can inhibit CSF-1–stimulated proliferation in macrophages.

DISCUSSION

Identification and characterization of target genes have been major experimental approaches toward understanding the underlying mechanism of antiproliferative action by IFNs. In this study, we have shown that IFNγ executes two functionally related sequential events to inhibit CSF-1-stimulated mitogenesis in macrophages: 1) to block CSF-1–induced c-Myc expression and 2) to elevate Mad1 level, a negative regulator of c-Myc function in transcriptional activation. Under these conditions, the constitutive cellular level of Max and induction of cyclin D1 and cyclin D2 gene expression by CSF-1 remain unaffected. The IFNγ-stimulated induction of Mad1 occurred in both G1-arrested (in the absence of CSF-1) cells, in which c-Myc level remains low, and cells cultured in the continued presence of CSF-1. The earliest induction of Mad1 by IFNγ was around 1.5 h, consistent with its blockade of cell cycle during the G1- to S-phase transition [Cocks et al., 1992; Matsushime et al., 1991; Roussel et al., 1996]. Enforced ectopic expression of Mad1 in Bac1.2F5 inhibited DNA synthesis and significantly extended the doubling time of macrophage cells in response to CSF-1. Therefore, this study suggests that induction of Mad1 may represent a mechanism by which IFNγ executes its antiproliferative action.

Downregulation of growth-promoting genes and upregulation of growth-inhibitory genes by IFNs (IFNα/β and IFNγ) have been reported in a variety of cell types [Kimchi, 1987, 1992]. Cocks et al. reported that IFNγ inhibits CSF-1–induced cyclin D1 expression in BMM cells [Roussel et al., 1996], which is different from our results in the Bac1.2F5 cell line (Fig. 2F). Tiefenbrun et al. showed that IFNα downregulates cyclin D3 and CDC25A genes, which correlates with G_o-like arrest in Burkitt's lymphoma cells [Tiefenbrun et al., 1996]. Harvat and Jetten reported inhibition by IFNγ of expression of cyclin A and cdc2 kinase [Harvat and Jetten, 1996]. Similar to inhibition of c-Myc gene expression, downregulation of these cell cycle–promoting factors can arrest cells at various stages of cell cycle even in the presence of serum factors. Increased expression of cyclin-dependent kinase inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ has also been shown in cells treated by IFNα/β and IFNγ [Chin et al., 1996; Bromberg et al., 1996; Kuniyasu et al., 1997]. At a post-transcriptional level, both IFNα/β and IFNγ block serum-stimulated hyperphosphorylation of retinoblastoma protein (Rb) [Renitzky et al., 1992; Vastrik et al., 1995], which would prevent dissociation of Rb from Rb–E2F complexes. These findings were conceptually similar to the effects of IFNγ on the c-Myc, Max, and Mad pathway observed in this study; i.e., IFNs hit multiple components in a given mitogenic signaling pathway.

It has been well documented in many cell types that the level of Mad1 is kept low in proliferating cells and increased during cell differentiation. Inhibition of cell growth and transformation by ectopically expressed Mad1 protein has also been indicated in mouse keratinocytes [Vastrik et al., 1995], human astrocytes [Chen et al., 1995], primary rat embryo fibroblasts [Ayer et al., 1998], and human erythrocytes [Cultraro et al., 1997]. Furthermore, Roussel et al. have shown that overexpression of Mad1 inhibited CSF-1 signaling in NIH3T3 cells expressing transfected human CSF-1 receptor [Cocks et al., 1992]. The inhibitory effect of Mad1 requires its basic region, the leucine zipper and the N-terminal protein interacting domain, and correlates with decreased Myc-Max and increased Mad1–Max complexes in cells [Ayer et al., 1996; Roussel et al., 1996; Cultraro et al., 1997]. Our data support the notion that replacing the Myc–Max complex with the Mad–Max complex serves as a switch between proliferation and antiproliferation and/or differentiation [Ayer and Eisenman, 1993]. It has recently been shown by independent groups that Mad1–Max complexes repress transcription by recruiting histone deacetylases (HDAC1 and HDAC2) via the corepressor mSin3 [Kasten et al., 1997; Laherty et al., 1997; Sommer et al., 1997]. This suggests that an

alteration in chromatin structure may be the inhibitory mechanism. It remains to be studied whether IFNγ also stimulates formation of such transcription-inhibitory complexes.

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