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The Response of Gamma Interferon Activation Factor Is under Developmental Control in Cells of the Macrophage Lineage

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Gamma interferon activation factor (GAF) rapidly induces transcriptional activation of gamma interferon (IFN-γ)-responsive genes. Conversion of the GAF from a latent cytoplasmic to an activated, DNA-binding form is an immediate step in the cellular response to IFN-γ. The amount of IFN-γ-activated GAF, measured by exonuclease III protection or gel shift assays, increased strongly upon monocytic differentiation of U937 cells. Activated GAF contained the IFN-responsive 91-kDa protein as its DNA-binding activity in gel shift or exonuclease III assays could be inhibited through direct addition of specific antiserum, and it was not present in p91-immunodepleted extracts. There was a differentiation-induced increase in the amount of nonphosphorylated (latent) p91. Transcription rate measurement demonstrated a strong induction of the p91 gene during monocytic differentiation of U937 cells. The amount of p91 which was rapidly phosphorylated in response to IFN-γ was found to be much higher in the differentiated cells and suggested a differentiation-controlled increase in the signaling leading to p91 phosphorylation. Concomitantly with a better GAF response, transcriptional activation of IFN-γ-induced genes and the expression of GAF-dependent, transfected reporter plasmids increased in differentiated U937 monocytes. The promonocyte-monocyte transition also affected the IFN-α-responsive transcription factor ISGF-3. Differentiated U937 cells contained more of both the α-component p91 and the γ-component p48, which constitutes the DNA-binding subunit of the complex. Our study thus provides evidence that the synthesis of specific transcription factors can be a regulated event to control the cytokine responsiveness of cells during development.

Interferons (IFNs) comprise a family of cytokines that is subdivided into two distinct types, the alpha and beta IFNs (IFN-α/β, type I) and gamma IFN (IFN-γ, type II), that each act via distinct cell surface receptors (3, 76). The two IFN types share certain biological activities such as conferring an antiviral state upon cells or inhibiting the growth of many types of malignant cells (19).

IFN-γ is secreted by activated T lymphocytes during the immune response. Once released, IFN-γ participates in the regulation of both nonspecific and specific immunity by promoting cellular activation and the expression of major histocompatibility complex (MHC) surface proteins (74). The primary target of IFN-γ in the immune response is the macrophage. This cell shows the most pronounced phenotypic changes following exposure to IFN-γ. Macrophages progress into a state of activation that is accompanied by an altered expression of surface receptors, by secretion of cytokines and nonpeptide mediators, and by the acquisition of complex physiological attributes such as extra- or intracellular cytotoxicity against microbial or tumor targets (reviewed in references 1, 2, and 53). Most of the functional attributes of a mature, activated macrophage are acquired only upon terminal differentiation and are not associated with earlier stages of the myeloid lineage.

The induction of specific genes is an important step in IFN-γ-mediated cellular activation. Several genes have been reported to be transcriptionally stimulated in response to the interaction between IFN-γ and its receptor (9, 10, 16, 21, 22, 25, 37, 50, 51, 55, 57, 61, 77, 83, 85). Most of these genes can be induced in all investigated cell types, but some appear to be restricted to certain lineages (21, 22, 50, 51, 55, 77, 83, 85). We have recently described a DNA element, the IFN-γ activation site (GAS), that mediates inducibility by IFN-γ (18, 46). The GAS binds a cellular protein termed the IFN-γ activation factor (GAF) (18). The GAF is activated in the cytoplasm immediately after binding of IFN-γ to its receptor. Activation is brought about by an IFN-γ-regulated tyrosine kinase (68). The phosphorylated GAF translocates to the nucleus and initiates transcriptional induction (18, 52, 68).

It has been suggested that the decisive step in GAF activation is the IFN-γ-regulated phosphorylation of a single polypeptide with a molecular mass of 91 kDa (66, 68). The same p91 is also phosphorylated in response to IFN-α and participates in the formation of the IFN-α-regulated, heterotetrameric protein complex termed ISGF-3 (29, 38, 65). ISGF-3, the transcriptional activator of IFN-α-stimulated genes (ISGs), exerts its effect through association with another DNA element termed the interferon stimulation response element (ISRE) (44, 58, 59). Like the GAF, activated ISGF-3 is formed in the cytoplasm from preexisting proteins (p48, p84, p91, and p113) that associate upon signaling from the IFN-α receptor (14, 29, 38, 43, 66; reviewed in references 70 and 82). The formation of ISGF-3 requires p84, p91, and p113 to be phosphorylated on tyrosine (28, 66). The signal from the IFN-α receptor is transmitted by a protein tyrosine kinase termed tyk-2 or IFN-tyk (24, 39,

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79). These intriguing results demonstrate that the signals from the IFN-α and IFN-γ receptors can target identical proteins and physically link the transcriptional responses to the two IFN types. Moreover, IFN-γ can also influence the availability of latent ISGF-3 by inducing the synthesis of its DNA-binding subunit (45). An example of how interactive effects between cytokine responses can be coordinated is thus provided. Such interactive effects occur in the case of IFN-α and IFN-γ on the levels of both transcription and the induced cellular functions (16, 47, 86).

In this study, we sought to determine whether GAF activation and the accompanying transcriptional response to IFN-γ might contribute to the phenotype of an IFN-γ-activated macrophage. To address this question, we investigated the IFN-γ responsiveness of U937 cells before and after differentiation. These cells are committed to the macrophage branch of the myeloid lineage and can be induced by a variety of agents to mature from a promonocytic into a monocytic stage of development. This differentiation process leads to the acquisition of a number of morphological and functional attributes that are associated with mature macrophages (34). In particular, an activated state of immunocompetence can now be reached upon treatment with IFN-γ. This state includes the above-mentioned cellular cytotoxicity, the expression of MHC class II molecules, and the secretion of peptide and nonpeptide mediators.

We report here that the monocytic differentiation of U937 cells affected p91 phosphorylation and therefore activation of the GAF by IFN-γ. Concomitantly, we observed an enhanced transcriptional response to the cytokine. We discuss these findings with respect to a possible role for the GAF in the phenotype of an activated macrophage. In addition, profound changes occurred in the activation of the IFN-α-responsive transcription factor ISGF-3. We thus provide additional evidence for the physical link between the responses to the two IFN types and demonstrate how macrophage differentiation establishes a molecular basis for the interactions of cytokines in the overall response to external stimuli.

MATERIALS AND METHODS

Cells and reagents. U937 cells (ATCC CRL 1593, kindly provided by K. Resch, Hannover Medical School) and HeLa S3 cells (ATCC CCL 2.2) were cultured in RPMI medium supplemented with 10% fetal calf serum. For differentiation, U937 cells were transferred from petri dishes to tissue culture dishes at a concentration of 5×10^5 cells per ml and treated with a final concentration of 5 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) for 48 h unless otherwise indicated. IFNs were kindly provided by P. von Wussow and J. Roesler (Hannover Medical School). Recombinant human IFN-α_{2a} and recombinant human IFN-γ were used. IFN-α was added to a final concentration of 1,000 U/ml, and IFN-γ was used at a final concentration of 1 ng/ml. Incubation times are indicated for the individual experiments. Cycloheximide (CHX; Sigma) was used at a final concentration of 50 μg/ml. For cotreatment with IFN, cells were incubated with CHX 10 min prior to the addition of IFN.

Antibodies. The antiserum against a C-terminal peptide of the 91-kDa protein of ISGF-3 (termed a57) and the antiserum with specificity for the 113-kDa protein have recently been described (30, 65).

cDNAs and oligonucleotides. The cDNAs used to measure transcription rates in the nuclear run-on assay are described in references as follows: pGem (used as a negative control; Promega), chicken β-actin (12), rat glyceraldehyde 3-phosphate dehydrogenase (26), human guanylate-binding protein 1 (GBP-1) (11), human IFP 53 (9), human IP10 (50), human 9/27 (60), human ISGF-2/IFN-γ-regulatory factor 1 (IRF-1) (57), human HLA-DR (8), human oligo(A) synthetase (64), human IFN-β (a gift from E. Knight, du Pont), and human p91 (65). Double-stranded oligonucleotides used as probes for gel shift assays or for competition in gel shift and exonuclease (Exo) III assays have been described previously. They corresponded to the GBP GAS (18, 46) or the ISRE of ISG 15 (59). The plasmid used to measure GAS-dependent chloramphenicol acetyltransferase (CAT) expression (4XGAS-HC) has also been described previously (17). Expression of CAT is regulated through the basal promoter of the human immunodeficiency virus long terminal repeat (HIV LTR) and an array of four copies of a GAS oligonucleotide ligated one to another.

Cellular extracts. The simultaneous generation of nuclear and cytoplasmic extracts from cells after hypotonic lysis, based on the method of Dignam et al. (20), has been described elsewhere (43). Alternatively to breaking cells in hypotonic buffer by Dounce homogenization, we used the method of Lee et al. (41), in which the cells are lysed by forcing them repeatedly through a 25-gauge hypodermic needle. The microsome-organelle pellet was obtained from hypotonic extracts by centrifugation of the postnuclear supernatant at 20,000 × *g* for 30 min. If separation into nuclear and cytoplasmic extracts was not required, whole cell extracts (WCE) were prepared as described previously (5). Detergent-solubilized WCE were prepared by lysing the cells in the presence of 0.1% Triton X-100. To deplete extracts of p91, WCE were dialyzed against a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 100 mM KCl, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid], 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% (vol/vol) glycerol. They were then incubated for 2 h at 4°C with the indicated dilutions of anti-p91 or control antiserum. The immunocomplexes were subsequently removed by incubation with protein A-Sepharose (Pharmacia) (1 h at 4°C) and centrifugation. To control for the nonspecific adsorption of protein, control extracts were incubated with protein A-Sepharose without prior treatment with antiserum.

For inactivation of the ISGF-3 γ component (see reference 43 and Results), extracts were treated with a final concentration of 5 mM *N*-ethylmaleimide (NEM) for 15 min at room temperature, followed by inactivation of the alkylating agent by 20 mM dithiothreitol. The resulting extract is also referred to as α-components in Fig. 8b. For reconstitution of ISGF-3, the appropriate extracts were mixed and left at room temperature for 10 min prior to the binding reaction with labeled ISRE oligonucleotide for the gel shift assay.

Exo III protection assay. The assay was performed as described previously (18, 71, 84). Briefly, cellular extracts were incubated with a fragment of the GBP promoter spanning nucleotides −216 to −142, which was uniquely labeled at the 5′ end at position −216, thus revealing protection of the top strand. This region includes the GAS (−121 to −106). After the binding reaction, the DNA was digested with 90 U of Exo III for 15 min at 32°C. Binding of protein is revealed through the generation of specific Exo III stops. Specificity is controlled by performing the binding reaction in the presence of an excess of unlabeled GAS oligonucleotide. To inhibit the formation of the GAF Exo III stop, either anti-p91

antiserum or control antiserum in a final dilution of 1:50 was added to the binding reaction or the binding reaction was performed with extracts immunodepleted with p91 antiserum.

Gel shift assay. To measure the binding of factors to the GBP GAS, a double-stranded oligonucleotide comprising promoter sequences −125 to −101 (46) was used. The binding reaction was in a buffer containing 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 10% (vol/vol) glycerol, and poly(dI-dC) (250 ng/ml); 0.5 ng of ^{32}P-labeled double-stranded GAS oligonucleotide (about 10^5 cpm) was added. Protein-DNA complexes formed after 30 min at room temperature were analyzed on a 6% acrylamide gel at 4°C in 0.2× Tris-borate-EDTA as a running buffer. To block the formation of complexes, anti-p91 antiserum or control serum was added to the binding reaction in a final dilution of 1:100. The assay conditions for the binding of ISGFs to the ISRE were as described elsewhere (27, 31), modified as described by Kessler et al. (38). A labeled oligonucleotide comprising the ISRE of the ISG 15 promoter (59) was used to detect the binding of the proteins ISGF-1, -2, and -3 to this element.

Western immunoblot. Cytoplasmic extracts containing the indicated amounts of total protein were boiled in sodium dodecyl sulfate (SDS) sample buffer and subjected to Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) on a 7.5% acrylamide gel. The proteins were transferred to nitrocellulose (Hybond C Super; Amersham) by standard procedures. Blots were blocked with 0.5% gelatin and incubated for 1 h at room temperature with a 1:3,000 dilution of preimmune or a57 serum. For detection, blots were incubated for 1 h at room temperature with goat anti-rabbit affinity-purified biotinylated antibodies (1:3,000; Bio-Rad) and then incubated with streptavidin-biotinylated alkaline phosphatase complexes (Bio-Rad). Antigen-antibody complexes were visualized by incubation in 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl, 5 mM $MgCl_2$, 330 μg of 5-bromo-4-chloro-3-indolylphosphate per ml, and 150 μg of nitroblue tetrazolium per ml for 5 to 30 min.

Determination of p91 phosphorylation in vivo. For in vivo ^{32}P labeling, 5×10^6 cells per sample were incubated for 90 min in phosphate-free Dulbecco modified Eagle medium and subsequently labeled by incubation for 3 h in phosphate-free medium containing 1/10 normal $NaHCO_3$ and 2 mCi of carrier-free $^{32}P_i$ (9,000 Ci/mmol; Amersham) per ml. Labeling media were supplemented with 25 mM HEPES buffer and 10% dialyzed fetal calf serum. Cells were lysed by addition of 1 ml of ice-cold lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate [pH 8.0]). Insoluble material was removed by centrifugation (15,000 rpm, 30 min, 4°C), and lysates were normalized on the basis of protein determinations carried out by the method of Bradford (8a). Normalized volumes of lysates were incubated for 6 to 12 h at 4°C with a57 antibodies. Immunocomplexes were collected following incubation (1 to 2 h at 4°C) with protein A-Sepharose beads. Immunoprecipitates were washed once with lysis buffer, twice with 0.5 M LiCl–0.1 M Tris-HCl (pH 7.4), and once with 10 mM Tris-HCl (pH 7.4). The beads were eluted by boiling in Laemmli sample buffer. ^{32}P-labeled proteins were resolved by SDS-PAGE (7.5% acrylamide gel) and visualized by autoradiography at −70°C on preflashed Kodak XAR 5 films.

Nuclear run-on assay. The assay and its quantitative evaluation have recently been described (16, 40). In brief, nuclei of U937 cells after various pretreatments were isolated after hypotonic lysis and incubated in a reaction mixture containing [α-^{32}P]UTP. The labeled RNA was extracted with hot phenol and phenol-chloroform and then precipitated with ethanol. After a second precipitation with 5% trichloroacetic acid, the RNA was hybridized to denatured plasmid DNA immobilized on nitrocellulose.

Transfection of U937 cells and CAT assay. A total of 2×10^7 U937 cells were transfected with 20 μg of the 4XGAS-HC reporter plasmid by electroporation at 250 V and 960 μF in HeBS (140 mM NaCl, 25 mM HEPES, 0.75 mM Na_2HPO_4 [pH 7.05]), using a Bio-Rad Gene Pulser. Cells from four cuvettes were pooled to ensure equal transfection efficiency and divided into four culture dishes for differentiation with or without subsequent IFN-γ treatment. Half of the cells were then differentiated with TPA for 40 h and either left without further treatment or treated with IFN-γ during the last 18 h. The other half were treated in an identical manner except that the whole 40-h culture period was in medium without TPA. The procedure could be varied as follows with no change in results. A total of 4×10^7 cells were incubated for 24 h with TPA and differentiated as the initial step. Identical numbers of differentiated and undifferentiated cells were then electroporated in parallel, and each group was pooled and divided into separate dishes for control and IFN treatment. IFN-γ was then added 2 h after electroporation for a total of 20 h. During this period, TPA was again added to the differentiated cells.

Preparation of cell extracts and the assay for CAT activity were carried out according to standard methods (63).

RESULTS

Differentiation of U937 cells strongly increases the amount of IFN-γ-activated GAF. We tested the activation of GAF by IFN-γ in undifferentiated (promonocytic) and TPA-differentiated (monocytic) U937 cells. The promoter of the IFN-inducible GBP gene contains a GAS that allows us to assay for the activated, DNA-binding form of the protein by an Exo III protection assay. We will show below that a corresponding GAS-binding activity can also be measured in gel shift assays. To maintain the original definition of the factor, however, all experiments have been done either by Exo III assay alone or by both Exo III and gel shift assays.

Promonocytic U937 cells showed virtually no activation of GAF at all time points after IFN-γ addition (Fig. 1). In contrast, in U937 monocytes, high levels of activated factor were formed in a transient fashion early after IFN-γ addition. We consistently observed elevated levels of activated GAF even in cells treated for brief periods with a combination of IFN-γ and CHX (data not shown). Our results thus indicate that the activation of the GAF is part of the immediate-early response to IFN-γ in monocytic U937 cells but not or much less so in the promonocytic form.

The augmented potential to activate GAF is not a general TPA response. TPA has previously been reported to influence the immediate transcriptional response to IFN-α (56). However, the potential to activate high levels of GAF was truly a differentiation-associated phenomenon and not a rapid TPA effect by several criteria (data not shown). (i) It was not detected before the U937 cells had completely differentiated by morphological criteria (microscopic observation in culture and after Giemsa staining). This required a 48-h period. (ii) The augmented response remained stable once it was established and did not require the continuous presence of TPA. (iii) In HeLa cells, TPA did not affect the GAF response, but in the myeloid HL60 line, both TPA- and

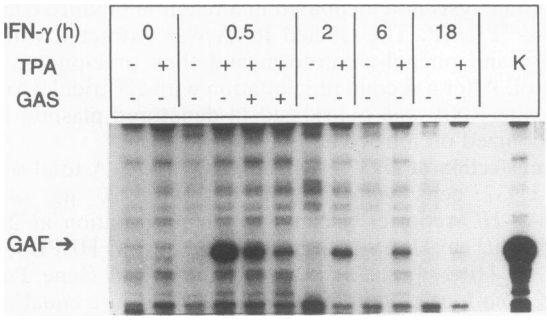

FIG. 1. Activation of the GAF in promonocytic and monocytic U937 cells. TPA-differentiated or untreated cells were incubated with IFN-γ for the indicated periods. Levels of GAF in nuclear extracts were then assayed by their ability to cause a characteristic Exo III stop on the GAS of the GBP promoter (see Materials and Methods for details). Lane K shows the position of the Exo III stop caused by partially purified GAF from HeLa cells as a control. GAS indicates competition with 20- and 50-fold molar excesses of unlabeled oligonucleotide corresponding to the sequence of the GBP GAS.

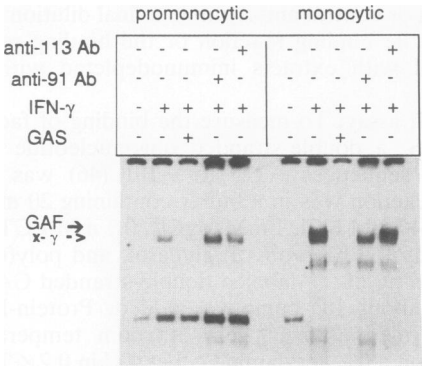

FIG. 3. Analysis of GAS-binding proteins in a gel shift assay. WCE from U937 promonocytes or monocytes were used for the binding reaction with a GAS oligonucleotide corresponding to the sequence in the GBP gene promoter. Where indicated, unlabeled GAS oligonucleotide was present in a 100-fold molar excess or antiserum to p91 or p113 was added to a final dilution of 1:100. Ab, antibody.

dimethyl sulfoxide-induced differentiation led to much higher activation of GAF after IFN-γ treatment.

Antibodies recognizing the IFN-responsive 91-kDa protein bind to the differentiation-responsive GAF. To assess the role of p91 in the formation of the differentiation-dependent U937 GAF, we used antiserum to p91 and, as a control, preimmune serum to block the binding of GAF to the GBP promoter. Figure 2 shows that GAF binding as detected in the Exo III assay was specifically blocked by the addition of p91 antibodies to the binding reaction. This result was confirmed by p91 immunodepletion experiments in which extracts of IFN-γ-treated U937 cells were incubated with either anti-p91 antibody and protein A-Sepharose, anti-p113 control antibody and protein A-Sepharose, or protein A-Sepharose alone prior to Exo III analysis. GAF activity was specifically removed only in the case of p91 antibody.

The observation of several GAF complexes in gel shift experiments (35) raised the possibility that other complexes not containing p91 and undetectable as separate entities in the Exo III assay play a role in the differentiation-induced augmentation of the GAF response in U937 cells. Therefore, we used the GAS DNA corresponding to the GBP promoter element in gel shift assays (Fig. 3). These experiments revealed a GAF DNA-binding activity that was differentiation dependent and could be blocked specifically by the use of p91 antibodies. The gel shift experiments thus confirm the results obtained in the Exo III assay and provide additional evidence for the role of p91 in the regulation of GAF activity.

A second IFN-γ-inducible protein-DNA complex, x-γ, with slightly higher mobility was also observed in our gel shift experiments. The respective protein existed also in extracts from promonocytic U937 cells, and complex formation with the GAS was not blocked by the addition of p91 antibodies. Its presence in U937 monocytes supports the assumption that more than one protein may be involved in regulating the activation of GAS elements in response to IFN-γ.

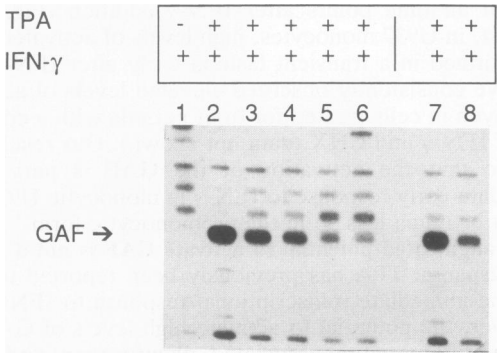

FIG. 2. Presence of p91 in GAF as detected by the Exo III assay. Dialyzed WCE from undifferentiated (lane 1) or differentiated (lanes 2 to 8) U937 cells were used. Lane 3, after depletion with protein A-Sepharose alone; lane 4, after depletion with a 1:300 dilution of preimmune serum; lane 5, after depletion with a 1:300 dilution of anti-p91 antibodies; lane 6, after depletion with a 1:100 dilution of anti-p91 antibodies. In lanes 7 and 8, a 1:50-diluted antiserum to p91 (lane 8) or preimmune serum (lane 7) was added to the binding reaction.

Differentiation induces p91 transcription, and U937 monocytes contain elevated levels of p91. To ascertain whether the amount of p91 was the same in promonocytic and monocytic U937 cells, we performed Western blot analysis probing with antibodies to p91. Detergent-solubilized WCE of differentiated U937 cells contained about five times more p91 than did their undifferentiated counterparts (Fig. 4a, lanes 1 and 2). Interestingly, the amounts of cytosolic p91, extracted in the absence of detergents, were comparable in undifferentiated and differentiated cells (lanes 3 and 4), whereas a major increase could be detected in differentiated cells after detergent solubilization of the organelle-microsome fraction (lanes 5 and 6), suggesting that p91 exists in a membrane-associated form prior to IFN treatment. Our data support a previous report that anti-p91 immunoreactivity was associated with plasma membrane fractions of untreated cells (55a).

To determine the molecular basis for the increase in p91 levels during differentiation, we investigated the transcription rate of the p91 gene. The nuclear run-on assay presented in Fig. 4b shows that nuclei from monocytic U937 cells transcribe the p91 gene at a 10-fold-higher rate than do those from promonocytic cells (determined by densitometry and normalization to the actin signal).

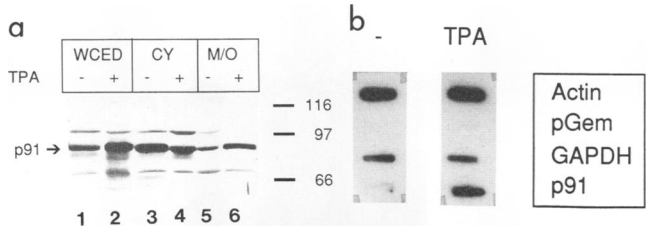

FIG. 4. Amounts of p91 and p91 gene transcription in U937 promonocytes and monocytes. (a) Extracts containing equal amounts of protein were subjected to SDS-PAGE and Western blot analysis. The blots were incubated with anti-p91 serum and developed by using a biotinylated second antibody-alkaline-phosphatase-streptavidin conjugate. WCED, WCE with Triton X-100; CY, cytoplasmic extracts; M/O, microsome-organelle pellet extracted in the presence of Triton X-100. Sizes are indicated in kilodaltons. (b) p91 gene transcription as determined by nuclear run-on analysis. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Monocytic U937 cells phosphorylate much higher levels of p91 after IFN-γ treatment. The participation of p91 in the formation of active GAF and its augmented levels in differentiated U937 cells suggested that this IFN-regulated protein was a target of differentiation-controlled signals. However, this assumption required more p91 to be phosphorylated after IFN-γ treatment in monocytic U937 cells. Figure 5 shows that ^{32}P-labeled promonocytic U937 cells (lanes 1 and 2) contained much less phosphorylated p91 after a 5-min treatment with IFN-γ than did the monocytic form (lanes 3 and 4). It has recently been documented that the de novo phosphorylation in response to IFN-γ is entirely in tyrosine (66, 68). The data strengthen our assumption of p91 and its phosphorylation as a major component in the differentiation-induced augmentation of the GAF response. The amounts of p91 phosphorylated in response to IFN-γ did not correlate with the amounts of p91 present in the cells (Fig. 4a). It is therefore likely that the amount of p91 is not the only factor determining the higher level of phosphorylated p91 in differentiated cells. A dephosphorylation step has been proposed in the activation mechanism of the GAF (35). The experiment in Fig. 5 demonstrates that the specific phosphorylation, i.e., the amount of phosphorylation in relation to total p91, is much lower in untreated monocytes than in untreated promonocytes. It remains to be shown whether this finding is related to the better GAF response after IFN-γ treatment.

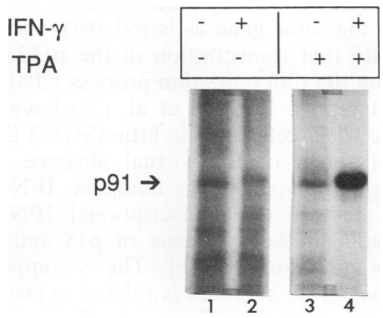

FIG. 5. p91 phosphorylation in promonocytic and monocytic U937 cells. The amount of protein phosphorylated in vivo was determined after labeling of cells with P_i. p91 was immunoprecipitated and subjected to SDS-PAGE prior to autoradiographic measurement of its phosphate content.

Differentiation changes the transcriptional response of U937 cells to IFN-γ. To address the question of whether the differentiation process affected transcription in response to IFN-γ in general or whether only certain genes are selected for an increased expression in IFN-γ-treated monocytes, we performed nuclear run-on assays using nuclei from IFN-γ-treated promonocytic and monocytic U937 cells. The cells had been either left untreated, treated with IFN-γ for 2 h or, to ensure measurement of immediate-early transcription only, treated with IFN-γ in the presence of CHX. The results are shown in Fig. 6a and have for sake of simplicity of evaluation been quantitated densitometrically and normalized to the actin signal in Fig. 6b.

Several genes induced by IFN-γ were studied: GBP (11, 16), IFP 53 (9), IP10 (50), 9/27 (60), ISGF-2/IRF-1 (57), and oligo(A) synthetase (64). In addition, we investigated transcription of the p34^{cdc2} kinase to determine whether it would change with differentiation or IFN-γ treatment and of the IFN-β gene as a control for differentiation as well as CHX treatment through its response to the elevated levels of NF-κB (42, 67). Some of the investigated genes (IP10, ISGF-2/IRF-1, and IFN-β) were induced by differentiation without further IFN-γ treatment. In the case of the ISGF-2 gene, this result is most likely due to the presence of an NF-κB site within its promoter as is also the case with the IFN-β gene (69). Most IFN-γ-inducible genes (GBP, IFP 53, IP10, and ISGF-2/IRF-1) showed a more pronounced transcriptional increase after IFN-γ treatment when the U937 cells were in the monocytic form. This was particularly true for the GBP gene and confirmed that GAF-responsive genes were expressed at higher levels in the differentiated cells. The IFP 53 gene is also regulated through the GAF, but additional factors bind to its GAS and may contribute to its higher levels of transcription in promonocytic U937 cells (our unpublished results and reference 71a). The ISGF-2 gene also contains a GAS consensus sequence (69).

Some of the investigated IFN-γ-inducible genes appeared relatively unaffected by the differentiation process. The 9/27 gene was transcribed at a very low rate irrespective of how the cells had been treated. We have recently shown that the ISRE in the promoter of this gene is blocked by silencing sequences and cannot be transcribed efficiently in IFN-γ-treated HeLa cells (71). This transcriptional block seems to operate also in U937 cells. The oligo(A) synthetase gene was also transcribed at a very low rate, and the data do not allow us to judge whether the slight increase in the IFN-γ response observed after differentiation is significant. Finally, the HLA-DR gene was transcribed at a fairly high constitutive rate in promonocytic or monocytic U937 cells. Again, this transcription rate was not affected by either differentiation or IFN-γ treatment. Since the transcriptional induction of MHC class II genes has been described as slow and protein synthesis dependent in some cells (4, 8), we also investigated HLA-DR transcription after 18 h of IFN-γ treatment but could not observe a significant effect caused by the promonocyte-monocyte transition of U937 cells (data not shown). Thus, while there is a clear tendency toward a general enhancement of IFN-γ-induced transcription in monocytic U937 cells, individual genes are quite heterogeneous in the details of their differentiation-dependent response.

Expression of the p34^{cdc2} gene was reduced by both differentiation and IFN-γ treatment. It remains to be investigated whether this effect contributes to the G_1 arrest of differentiating U937 cells.

IFN-γ inducibility of genes can be controlled by more than

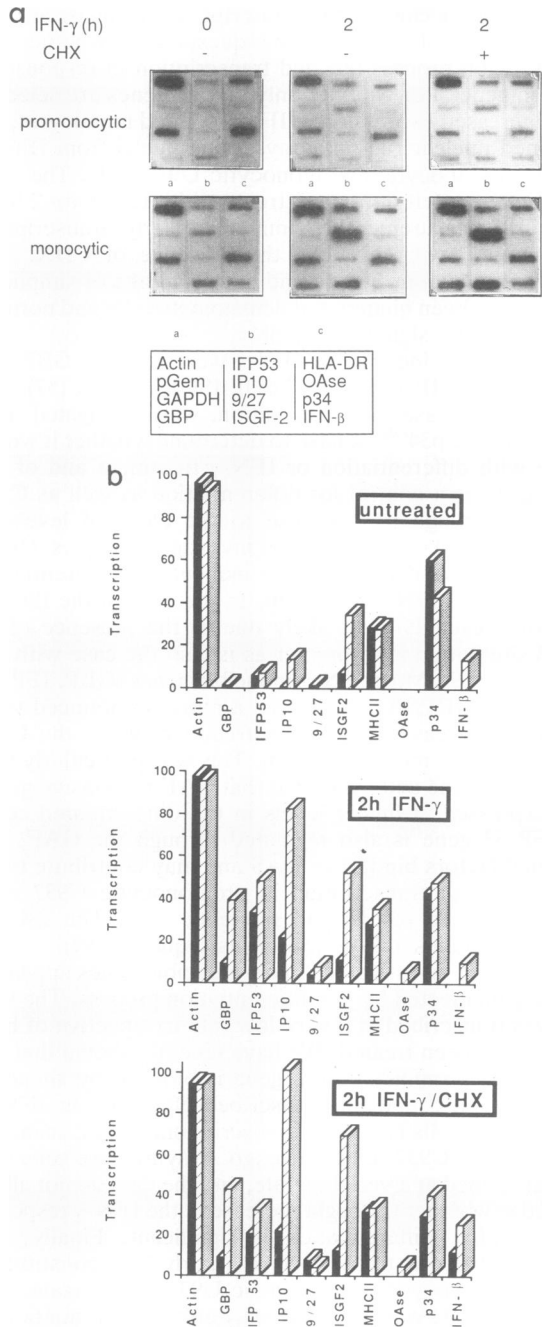

FIG. 6. IFN-γ-induced transcription in U937 promonocytes and monocytes. Nuclei from cells before or after differentiation and treatment with IFN-γ alone or in combination with CHX as indicated were subjected to nuclear run-on assays. (a) Autoradiograph obtained after exposure of the hybridized nuclear RNA to film. (b) Quantitative evaluation of the autoradiograph by scanning densitometry and normalization to the actin signal. Dark bars show transcription rates in untreated U937 cells; striped bars represent transcription rates in TPA-differentiated U937 cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OAse, oligo(A) synthetase.

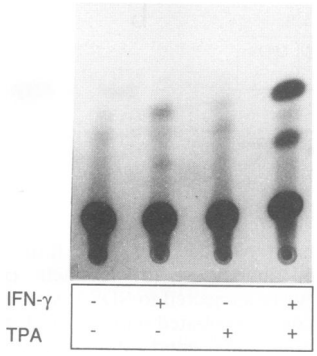

FIG. 7. GAF-dependent transcription in undifferentiated and differentiated U937 cells. The cells were transfected with a CAT reporter plasmid containing basal promoter elements from the HIV LTR and four copies of a GAS oligonucleotide. Samples of undifferentiated and TPA-differentiated cells were then treated with IFN-γ for 18 h. Finally, CAT activity was determined in cellular extracts, using standard methods. In a quantitative evaluation of the autoradiograph by scanning densitometry, the IFN-γ-induced level of CAT was 2-fold over control values in undifferentiated cells and 10-fold over control values in the differentiated cells.

one IFN-responsive element. For example, the GBP gene's response to IFN-γ depends on both the ISRE and the GAS (46). To ensure that the increased levels of GAF actively contribute to the augmented, IFN-γ-induced transcription, we tested the ability of U937 promonocytes and monocytes to induce expression of the CAT gene linked to basal promoter elements from the HIV LTR and four copies of an oligonucleotide comprising the GAS sequence from the GBP promoter (4XGAS-HC). The experiment shown in Fig. 7 reveals a strong, IFN-γ-dependent increase of 4XGAS-HC transcription in TPA-differentiated U937 cells. This increase was not due to different efficiencies of transfection because the cells were pooled after electroporation and subsequently split for the individual treatments (see Materials and Methods for details). The results prove that GAF-dependent transcription is augmented by differentiation and that the factor can contribute to the enhanced transcriptional response of the genome to IFN-γ in mature macrophages.

Differentiation affects the activation of IFN-α-responsive ISGF-3. Since p91 is one of the proteins constituting the heterotetrameric transcriptional activator ISGF-3, we tested whether differentiation would have an effect also on its IFN-α-induced activation. Figure 8a shows a strongly augmented response of ISGF-3 DNA-binding activity after differentiation. Therefore, the elevated levels of p91, which is one of the ISGF-3 α-components, most likely also contribute to the stronger response of this factor to IFN-α in monocytic cells. The other α-components may be regulated in much the same manner by differentiation as is p91. p84 is derived from the same gene as is p91 (65), and preliminary results indicate that transcription of the p113 gene is also induced during the differentiation process (20a).

In a recent report, Improta et al. (36) have shown that promonocytic U937 cells contain little ISGF-3 DNA-binding activity as a result of the virtual absence of p48, the DNA-binding component of the complex. IFN-γ treatment was able to restore the transcriptional IFN-α response because it induced the synthesis of p48 and the ISGF-3 complex was now formed (36). The γ-component has a molecular mass of 48 kDa and is related in structure to the IRF family of proteins (78). The levels of p48 can be assayed by a reconstitution assay. First the α-component extract is made by NEM alkylation of cytoplasm from IFN-treated cells, which selectively inactivates p48 but leaves the α-components intact. In a second step, the α-component extract is

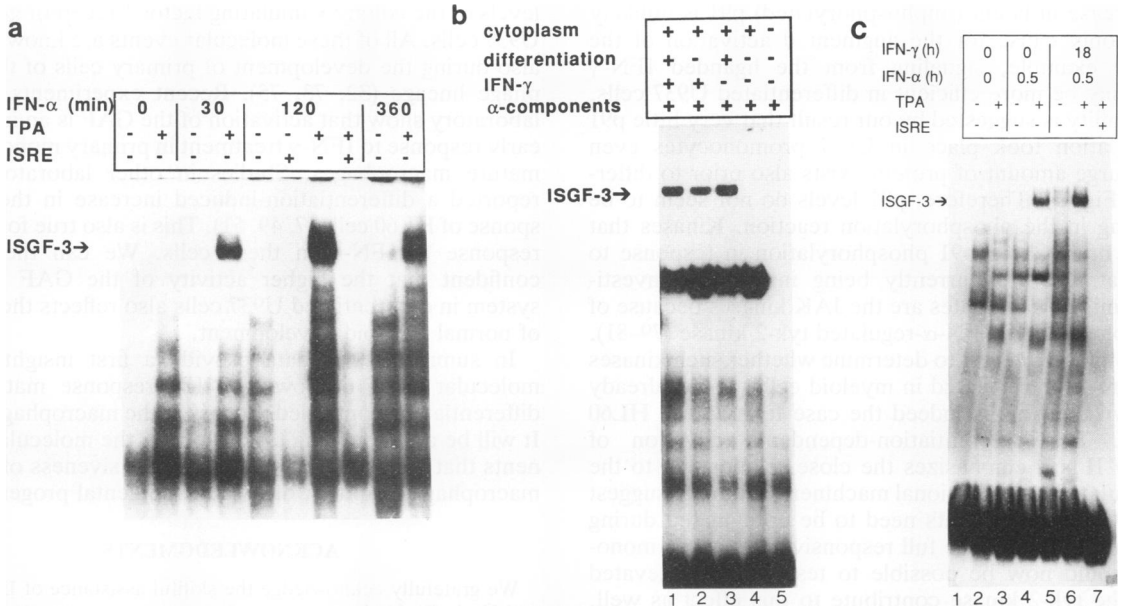

FIG. 8. Effect of U937 differentiation on ISGF-3. The amount of factor present in nuclear extracts after the indicated treatments was assayed in a gel shift experiment using an oligonucleotide corresponding to the ISG 15 ISRE as a labeled probe. ISRE indicates competition by a 100-fold molar excess of unlabeled oligonucleotide. (a) Induction of ISGF-3 in TPA-differentiated and control cells by IFN-α. (b) Levels of the ISGF-3 γ-component (p48) in U937 cells. The protein was assayed by reconstitution of ISGF-3 binding activity in an NEM-treated, superinduced HeLa extract (α-components) with cytoplasmic extracts from U937 cells that had been treated with TPA (48 h) and/or IFN-γ (30 min) as indicated (see text for details). (c) Effect of IFN-γ pretreatment (priming) on ISGF-3 induction by IFN-α in undifferentiated and TPA-differentiated U937 cells.

reconstituted with a p48-containing extract that does not contain any activated α-components itself to yield the DNA-binding form of ISGF-3. The reconstitution assay is quantitative because α-components are in excess over p48 (43).

We investigated whether a differentiation-induced synthesis of p48 contributed to the increased levels in ISGF-3 DNA-binding activity. Figure 8b shows that when an NEM-treated, α-component-containing cytoplasm from HeLa cells was reconstituted with cytoplasm from undifferentiated U937 cells, no ISGF-3 was observed (lane 4). After the promonocytes had been treated with IFN-γ, ISGF-3 was reconstituted (lane 2). Cytoplasm from monocytic cells contained more p48 than did the IFN-γ-treated promonocytes (lane 3), and this amount of p48 was not further increased by additional IFN-γ treatment (lane 1).

To assess the relative contribution of p48 to the differentiation effect, we compared the amount of ISGF-3 activated in response to IFN-α after a long pretreatment of U937 promonocytes with IFN-γ and after monocytic differentiation. Figure 8c shows that IFN-γ pretreatment in promonocytes increases the amount of ISGF-3 (lane 5), but the levels of factor are substantially lower than after differentiation (lane 4). Changes in the levels of α-components and, possibly, signal transduction molecules thus contribute strongly to the elevated ISGF-3 response after monocytic differentiation.

We noted on short exposures of our experiments such as the one shown in Fig. 8c that two ISGF-3–DNA complexes can be resolved in U937 cells, whereas only one such complex is seen in HeLa cells or fibroblasts (44, 45). This finding could be explained by a potential of the proteins that form ISGF-3 to assemble complexes of either different composition or different stoichiometry. The U937 cell line may represent a useful tool for investigating this possibility.

DISCUSSION

The differentiation of cells along a given lineage is accompanied by an alteration of their response to environmental stimuli. In the case of cells belonging to the immune system, this process largely involves the acquisition of full responsiveness to cytokines (48). These are the molecules that mediate communication between cells in immune or inflammatory processes and help to achieve a state of immune competence that allows an appropriate response to situations that endanger the host organism (6, 15). It has been documented that totipotent embryonic cells are unable to produce type I IFNs (IFN-α/β) in response to virus (33), indicating that cellular interactions through cytokines mature on the levels of both synthesis and response.

Macrophages play an important and indispensable role in immune responses. To function properly, they must be able to respond to the activating signal provided by IFN-γ. In this study, we addressed the question of whether the altered response to IFN-γ upon differentiation of macrophages involves an altered transcriptional response to the cytokine and whether we could define molecules that are targets of differentiation-induced changes and mediate this alteration. We show here that activation of the GAF as part of the immediate-early response to IFN-γ is such a developmentally controlled step and that the transcription of IFN-γ-responsive genes increased concomitantly with a better GAF response. The IFN-responsive p91 is very likely to be a key molecule in the altered GAF response. The factor contains p91, and levels of the protein were increased through transcriptional activation of its gene during differentiation. More importantly, much more p91 was phosphorylated in the differentiated cells in response to IFN-γ, stressing the role of the protein in the transcriptional response to IFN-γ.

The increase in latent (unphosphorylated) p91 is unlikely to be the only cause for the augmented activation of the GAF. For example, signaling from the liganded IFN-γ receptor may be more efficient in differentiated U937 cells. This possibility is suggested by our result that very little p91 phosphorylation took place in U937 promonocytes even though a large amount of protein exists also prior to differentiation (Fig. 5). Therefore, p91 levels do not seem to be rate limiting in the phosphorylation reaction. Kinases that ultimately bring about p91 phosphorylation in response to IFN-γ treatment are currently being intensively investigated. Promising candidates are the JAK kinases because of their homology to the IFN-α-regulated tyk-2 kinase (79–81). It will be of great interest to determine whether such kinases are differentiation regulated in myeloid cells. It has already been shown that this is indeed the case for tyk-2 in HL60 cells (24). The differentiation-dependent activation of ISGF-3 by IFN-α emphasizes the close relationship to the IFN-γ-regulated transcriptional machinery. Our data suggest that all ISGF-3 components need to be upregulated during differentiation to reach the full responsiveness of the monocyte. It should now be possible to test whether elevated levels of the tyk-2 kinase contribute to this effect as well. Such studies are under way in our laboratory.

What is the overall importance of the observed alterations in p91 in the context of the entire transcriptional response to IFN-γ? To date, not many GAF-regulated genes have been described. Our own unpublished data and those of other laboratories have revealed at least three more genes with IFN-γ-responsive DNA elements that compete for GAF binding in our experiments. These are the gene encoding the Fc receptor gamma (7a), the IFP 53 gene (9, 71a), and the gene for the Ly6E/A surface antigen (13, 67a). We may thus expect p91 involvement in transcriptional gene regulation by IFN-γ to be not a singular feature of the GBP promoter but rather a more common phenomenon.

Although our experiments underline the importance of GAF and its constituent p91, they do not allow us to draw general conclusions about the transcriptional response to IFN-γ. Future work will have to clarify the biochemical nature and role of all GAS-binding proteins detected by us (Fig. 6) and other laboratories (35). Also, the ISRE has been shown to contribute to the IFN-γ responsiveness of the GBP gene (46) and may have a function in the IFN-γ responsiveness of other genes as well. Therefore, the relative contributions of the GAS and ISRE need to be addressed. Genes encoding MHC class II proteins are regulated by IFN-γ but do not contain promoter sequences with obvious GAS homologies (37). Current evidence thus makes p91 involvement in the regulation of these genes rather unlikely and suggests that unrelated factors are involved in this part of the transcriptional response to IFN-γ (23).

Another important aspect to consider in evaluating our data is the amenability of U937 cells as a model for macrophage differentiation. The cell line was originally described as a histiocytic lymphoma (72), but subsequent analyses have shown the monoblast/promonocyte character of the cells. A plethora of investigations have demonstrated and confirmed the phenotype of the differentiated cells to resemble a monocyte stage of macrophage differentiation. Among the typical macrophage attributes of TPA-differentiated U937 cells are phagocytosis, extracellular cytotoxicity, and the production of reactive oxygen intermediates (34). Molecular studies have demonstrated a differentiation-associated induction of constitutive NF-κB (32, 73), an induction of the transcription factor NF-IL-6 (54), and elevated mRNA levels of the colony-stimulating factor 1 receptor gene (62) in U937 cells. All of these molecular events are known to occur also during the development of primary cells of the macrophage lineage (32, 73, 75). Recent experiments from our laboratory show that activation of the GAF is an immediate-early response to IFN-γ treatment in primary monocytes and mature macrophages. Studies in other laboratories have reported a differentiation-induced increase in the IFN response of HL60 cells (7, 49, 51). This is also true for the GAF response to IFN-γ in these cells. We can therefore be confident that the higher activity of the GAF activation system in differentiated U937 cells also reflects the situation of normal myeloid development.

In summary, our data provide a first insight into the molecular basis of how the IFN response matures in a differentiation-controlled process in the macrophage lineage. It will be an interesting task to define the molecular components that distinguish the IFN-γ responsiveness of a mature macrophage from that of its developmental progenitors.

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