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Colony-stimulating factors and interferon-γ activate a protein related to MGF-Stat 5 to cause formation of the differentiation-induced factor in myeloid cells

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Abstract The Jak-Stat pathway of intracellular signals is used by growth factor- and cytokine receptors to induce gene transcription. We have recently reported that differentiation of myeloid cells, induced by phorbol ester, interferon-γ (IFN-γ) or colony-stimulating factor-1 (CSF-1) is accompanied by the activation of the differentiation-induced factor (DIF). Activated DIF specifically associates with a subclass of gamma-interferon activation site (GAS)-like DNA elements. We now report that GM-CSF, which like CSF-1 promotes the generation of mature macrophages, activates DIF. No activation was observed after treatment with the granulocyte growth and differentiation factor G- CSF. Antibodies raised against a Stat family protein, designated mammary gland factor-Stat 5 (MGF-Stat 5), reacted with DIF induced by either CSF-1, GM-CSF or IFN-γ. Antisera to other known Stats were without effect on the DIF complex in electrophoretic mobility shift assays (EMSA). A 112 kDa protein could be isolated from either GM-CSF- or IFN-γ-treated cells by GAS oligonucleotide precipitation. This protein reacted with antibodies to both MGF-Stat 5 and phosphotyrosine. MGF-Stat 5 and closely related proteins thus define a subfamily of Stat transcription factors that are present in a variety of cell types and are required for the onset of immediate gene expression in response to differentiating stimuli.

Key words: Myeloid differentiation; Macrophage; MGF-Stat 5; Gene expression; Transcription factor

1. Introduction

Rapid transcriptional induction of specific genes is an essential part of the cellular response to growth factors or cytokines. A recently discovered family of transcription factors, designated signal transducers and activators of transcription (Stat), plays a central role in regulating gene expression in response to signals from almost all classes of receptors for growth factors and cytokines [1–5]. These proteins participate in the signal transfer to the nucleus because they move from their site of activation in the cytoplasm, most likely in close vicinity to the plasma membrane, to the nucleus, where binding to specific DNA sequences occurs. Activation of Stats occurs through the activity of the Jak family of protein tyrosine kinases (ptk). Phosphorylation occurs at a single tyrosine residue in those Stat proteins in which the phosphorylation site has been mapped [6–8]. Thus far, six distinct members of the Stat protein family, Stat 1, Stat 2, APRF-Stat 3, Stat 4, MGF-Stat 5, IL-4-Stat, have been cloned [2,9]. Among the domains conserved between all family members are those that constitute the Src homology (SH) domains 2 and 3. The SH2 domain appears to mediate the dimerization or oligomerization of activated Stat proteins that is necessary for DNA binding [10].

Most Stats bind to a class of elements designated gamma-interferon activation site (GAS)-like elements. The common denominator of these elements is their ability to bind a dimer of Stat 1, the gamma-interferon activation factor (GAF), [11]. However, GAS-like elements differ slightly in their DNA sequence and are thus capable of specific association with other different Stat proteins [12–18]. One of these GAS-like elements has originally been found in the promoters of genes that encode milk proteins and that are induced upon prolactin stimulation of mammary gland cells during lactation [19]. The cognate transcription factor was characterized in rat as a 89 kDa protein and designated mammary gland factor (MGF); [20]. Two forms of MGF (84 and 92 kDa), that apparently result from a single gene, exist in sheep. The ovine MGF cDNA has been cloned and encodes a Stat family protein. It was renamed MGF-Stat 5 [2,21]. The MGF-Stat 5 cDNA gives rise to a 90 kDa protein when translated in vitro.

We have recently observed that stimulation of myeloid cells with cytokines that have the potential of promoting differentiation, colony-stimulating factor-1 (CSF-1) and IFN-γ, activate a transcription factor that binds to GAS-like elements [14]. This factor was also activated during the process of TPA-induced macrophage differentiation and it was therefore designated differentiation-induced factor (DIF). The protein(s) in the DIF–DNA complex did not react with Stat 1 antisera, but DNA specificity and tyrosine phosphorylation suggested that DIF might be another Stat family member. In this report we show that DIF is related to MGF-Stat 5. Our findings suggest that MGF-Stat 5 and related Stats have widespread functions in cellular differentiation processes.

2. Materials and methods

2.1. Cell culture, reagents

U937 or BAC 1.2F5 cells were cultured as described [14]. The cells were stimulated for the indicated times with the following recombinant human cytokines: IFN-γ (5 ng/ml), GM-CSF (500 U/ml), CSF-1 (30,000 U/ml) or G-CSF (500 U/ml). Production of antibodies to the N-terminus of ovine MGF-Stat 5 in chicken will be described elsewhere (H. Wakao and B. Groner, in preparation). Rabbit antibodies to Stat 2 were kindly provided by Chris Schindler (Columbia University,

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New York). Antibodies to Stat 3 and Stat 4, also produced in rabbit, were a kind gift of Zhong Zhong and James E. Darnell Jr. (The Rockefeller University, New York). Monoclonal antibodies to phosphotyrosine (clone 4G10) were purchased from UBI (Lake Placid, USA). The sequence of the IFP53-GAS oligonucleotide has been reported elsewhere [22]. For precipitation experiments one strand of a dimerized binding site was biotinylated on the 5′ end through the use of biotinylated phosphoamidites during synthesis.

2.2. Cellular extracts

Nuclear extracts were prepared as described by Dignam et al. [23]. Whole cell extracts were either prepared by salt extraction (WCE) or by lysis with Triton X-100 (WCED) as recently reported [24].

2.3. Electrophoretic mobility shift assay (EMSA)

The assay was performed under our standard conditions using an end-labelled, double-stranded IFP53-GAS oligonucleotide as a probe [14].

2.4. Precipitation with biotinylated GAS oligonucleotides

Biotinylated oligonucleotides were coupled to streptavidin-agarose (Sigma, Deisenhofen, Germany) by mixing 100 μl of agarose beads with 200 μl of oligonucleotide (1 mg/ml) and shaking overnight at 4°C. After several washes with 1 M KCl and NEB buffer (100 mM KCl, 20 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 5% glycerol) the coupled oligonucleotides were resuspended in 1 μl of NEB per μl sedimented Sepharose. 10 μl of the resulting suspension were mixed with 200 μl of cell extract (about 1 mg of protein) and incubated at 4°C overnight on a rotating wheel. Bound proteins were sedimented by centrifugation (30 s, 1700 × *g*) and washed several times in NEB with 300 mM KCl prior to elution by boiling in SDS sample buffer and SDS-PAGE analysis.

2.5. Western blotting

Electrotransfer of proteins after SDS-PAGE to nitrocellulose membranes was performed as recently reported [24]. Blotted proteins were detected by using a horseradish peroxidase-coupled second antibody and chemiluminescence reagents kit according to the manufacturer's instructions (Amersham, Braunschweig, Germany).

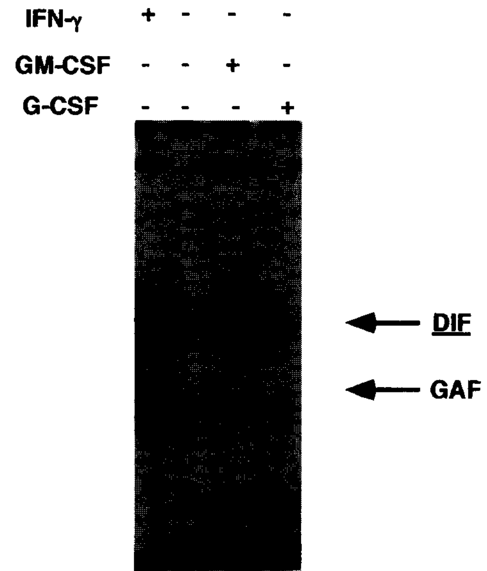


Fig. 1. DIF is activated by IFN-γ and GM-CSF, but not G-CSF in U937 cells. Nuclear extracts were prepared after treatment of the cells with the indicated cytokines for 10 min. The extracts were analyzed by EMSA using the IFP-53 GAS as a probe.

3. Results

3.1. Activation of DIF by CSFs and reactivity with Stat antisera

We have reported DIF activation in response to CSF-1 in murine BAC 1.2F5 macrophages [14]. In U937 promonocytes DIF activation occurs either in response to IFN-γ or during TPA-mediated differentiation. U937 cells do not express receptors for CSF-1 but those for G-CSF and GM-CSF are present

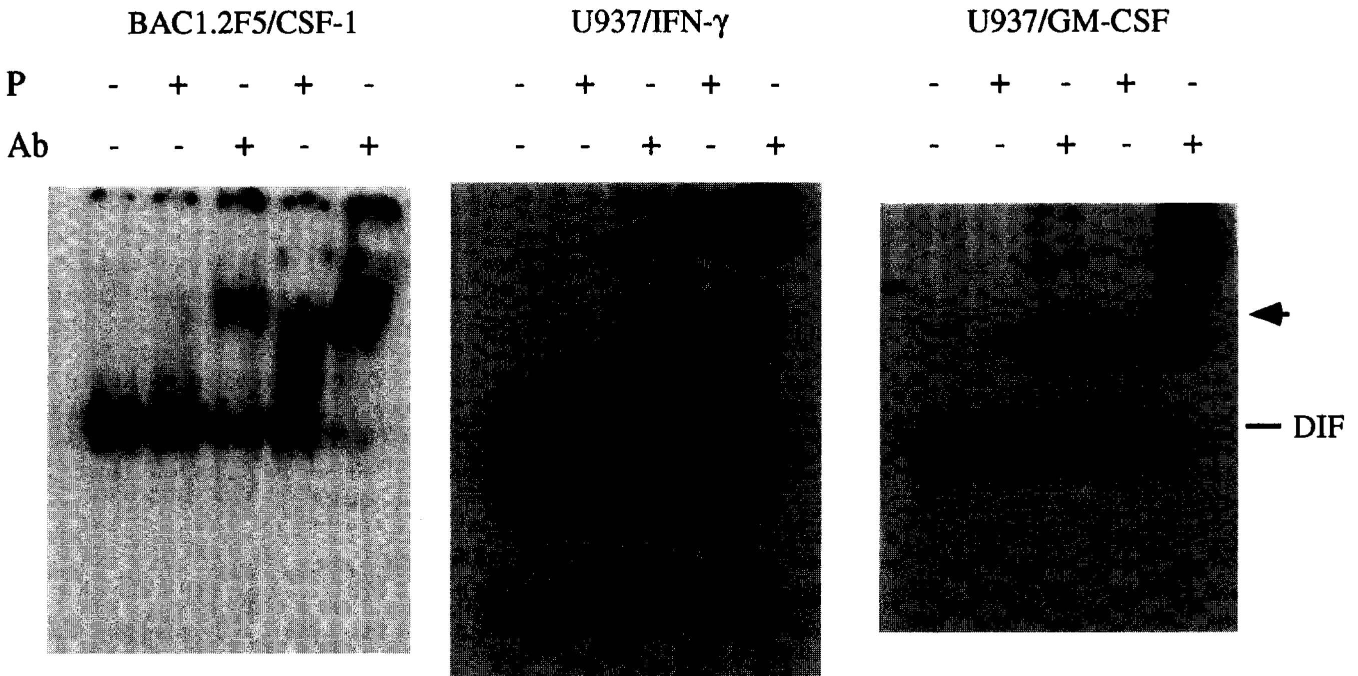
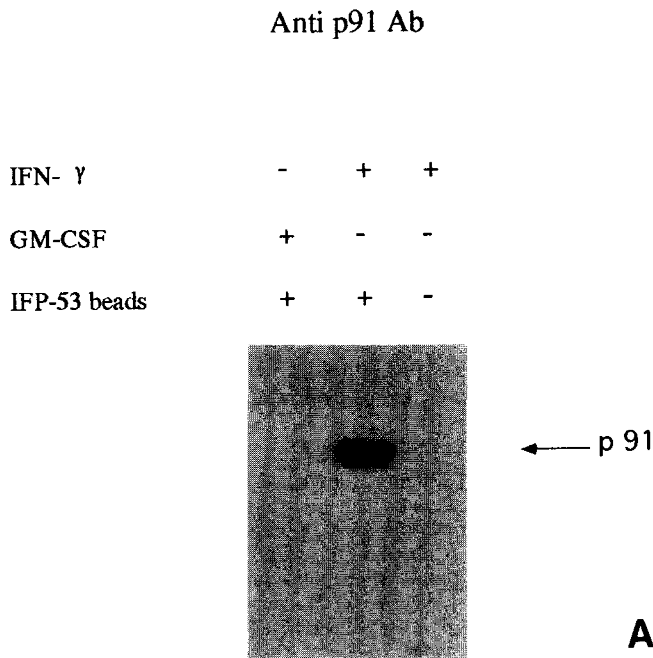
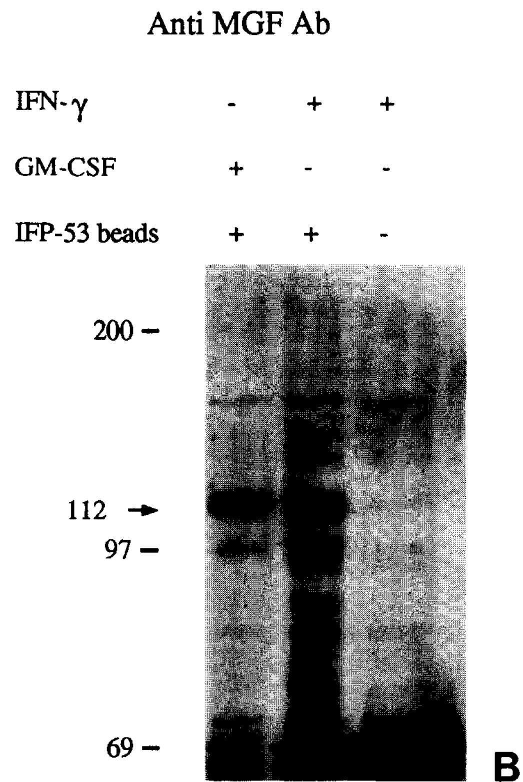


Fig. 2. DIF reacts with antibodies to MGF-Stat 5. BAC 1.2F5 or U937 cells were treated for 5 min (CSF-1) or 10 min (GM-CSF, IFN-γ) with the indicated cytokines. Whole cell extracts (BAC 1.2F5, CSF-1) or nuclear extracts (U937, IFN-γ, GM-CSF) were prepared and reacted with a final dilution of 1 : 50 (lanes 2 and 3) or 1 : 15 (lanes 4 and 5) of either preimmune serum (P, lanes 2 and 4) or specific anti-MGF-Stat 5 antibodies (Ab, lanes 3 and 5). After a 10 min incubation on ice the reaction products were analyzed by EMSA with the IFP53-GAS as a probe.





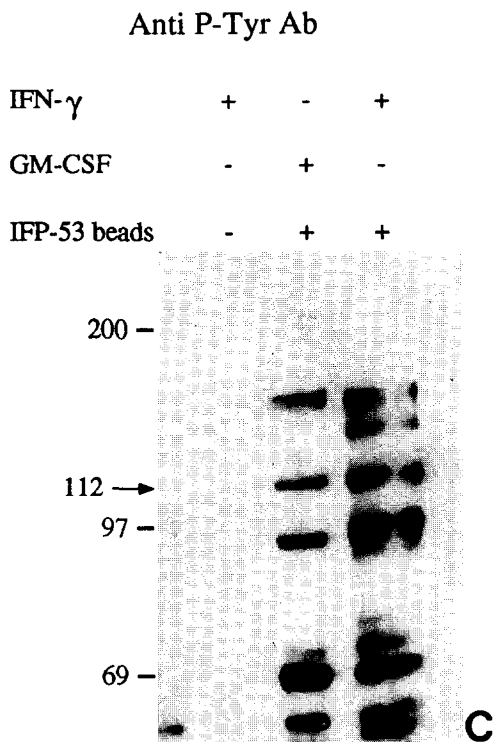


Fig. 3. Precipitation of DIF and GAF with IFP53-GAS oligonucleotide and analysis of precipitated proteins with specific antibodies. Whole cell extracts or nuclear extracts from U937 cells were reacted with biotinylated oligonucleotide coupled to streptavidin-Sepharose. Bound protein was analyzed by Western blotting and staining with antibodies. (A) Analysis with antibodies specific for the Stat 1a C-terminus [37]. (B) Analysis with MGF-Stat 5 antibodies. (C) Analysis with 4G10 antiphosphotyrosine antibody.

[25,26]. DIF activation was observed in U937 cells after stimulation with GM-CSF, but not G-CSF (Fig. 1) indicating that this factor might take part in differentiation events specific for, or restricted to, the monocyte-macrophage lineage. In contrast to IFN-γ, which activates DIF almost exclusively in the promonocytic stage of U937 [14], GM-CSF was effective in the promonocytic as well as the monocytic stage (data not shown). To test whether DIF might be related to known Stats, we probed extracts containing the factor with antisera against these proteins and analyzed them by EMSA. As recently reported, no reaction with different antisera to Stat 1 was observed [14]. Similarly, antisera to Stats 2, 3 and 4 caused no change in the abundance or mobility of the DIF retarded band in EMSA (data not shown). In contrast, antibodies to the MGF-Stat 5 N terminus produced a strongly supershifted DIF complex when compared to preimmune serum (Fig. 2). These results were obtained with DIF activated by either IFN-γ or GM-CSF in U937 promonocytes or by CSF-1 in BAC 1.2F5 macrophages. No reaction of the MGF-Stat 5 antibodies was seen with the Stat 1 dimer (GAF) present in IFN-γ-treated extracts (Fig. 2, middle panel).

3.2. Characterization of DIF by oligonucleotide precipitation and Western blotting

This approach has been successfully used by other groups to characterize proteins binding to GAS-like sequences [12,27]. GAF, the Stat 1 dimer, is present in IFN-γ-treated U937 cells and binds to the IFP53-GAS (Fig. 1, lane 1). We detected the proteins obtained after precipitation with oligonucleotide-coupled streptavidin-agarose beads, or with beads alone, with antibodies to Stat 1. Western blot and staining with Stat 1 antibodies showed that large amounts of Stat 1 (p91) were precipitated from IFN-γ-treated nuclear extracts (NE) by the oligonucleotide (Fig. 3A, lane 2). In contrast, insignificant amounts were

precipitated if no GAS oligonucleotide was coupled to the agarose beads (lane 3) or if extracts from untreated cells were used (lane 1). A 2 h pretreatment with vanadate increases the amount of DIF activated after a subsequent treatment with IFN-γ, presumably due to the inhibition of tyrosine phosphatases [14]. Having demonstrated the specificity of the IFP53-GAS oligonucleotide precipitation we repeated the experiment with nuclear extracts from either vanadate/IFN-γ or GM-CSF-treated U937 cells and stained the Western blot with anti-MGF-Stat 5 antibodies. Both extracts produced a specific major band of 112 kDa, while a minor band of about 97 kDa was additionally present in vanadate/IFN-γ-treated extracts (Fig. 3B; see section 4). To prove that the 112 kDa protein represents DIF, we stained the blot of oligonucleotide-precipitated proteins with antibodies to phosphotyrosine: several bands were obtained. This was not unexpected because we have shown earlier that constitutive tyrosine-phosphorylated proteins bind to the IFP53-GAS under EMSA conditions [14]. However, the 112 kDa band produced a strong signal in both IFN-γ- and GM-CSF-treated extracts. A band of about 97 kDa also stained with p-tyr antibodies in both extracts, while a less prominent 93 kDa band, most likely the tyrosine-phosphorylated form of Stat 1, was only observed after IFN-γ treatment.

4. Discussion

An important task of future cytokine research will be the biochemical analysis of the Stat family transcription factors and the identification of their activation signals and biological functions. As a contribution to this research we have presented a first characterization of DIF. This GAS-binding factor is activated by CSF-1 and GM-CSF but not by G-CSF, and it might have a role in macrophage but not in granulocyte maturation. CSFs promote proliferation, differentiation and survival of myeloid cells [28]. It is unlikely that DIF plays a role in cell proliferation because it is activated by IFN-γ and TPA. Both treatments lead to growth arrest and differentiation of U937 cells. Our study provides strong evidence that the DIF complex contains a MGF-Stat 5-related protein with a molecular weight of 112 kDa. This protein was present in extracts from both GM-CSF- and IFN-γ-treated cells, bound specifically to the IFP53-GAS, reacted with anti-MGF-Stat 5 antibodies and contained p-tyr. DIF might either be the human MGF-Stat 5 counterpart or stem from the MGF-Stat 5 gene and its mRNA generated through a tissue-specific alternative splicing event. The latter assumption would account for the larger molecular weight of DIF compared to rat, mouse (C. Schindler, personal communication), or sheep MGF-Stat 5. Alternatively, DIF might be encoded by a MGF-Stat 5-related gene.

Western blots from IFP53-GAS-precipitated extracts also contained a tyrosine-phosphorylated protein of about 97 kDa which weakly reacted with the MGF-Stat 5 antiserum. This protein might be part of a yet different, minor IFP53-GAS–protein complex which is activated under similar conditions as DIF, migrates slightly faster in EMSA experiments and reacts weakly with the MGF-Stat 5 antibodies in EMSA (Figs. 1 and 2; most obvious in lane 5 of the middle panel in Fig. 2). We have recently detected this complex and named it y-γ [14]. Moreover, Larner and collegues have reported a GM-CSF-activated protein of 80 kDa that binds to the GAS-like element within the promoter of the FcγRI receptor gene in primary human monocytes [12]. Different Stats with distinct preferences for GAS-like elements may thus be activated by the same cytokine.

MGF-Stat 5 assumes DNA binding activity when reacted with the Jak 2 ptk in vitro [8]. The prolactin receptor activates Jak 2 in vivo [29–32]. GM-CSF and IFN-γ receptors are both capable of activating Jak2 [33–35]. DIF might thus be a substrate for this kinase as well. However, the CSF-1 receptor belongs to the family of tyrosine kinase receptors. These may activate the Jak 1 ptk rather than the Jak 2 ptk as demonstrated for the EGF receptor [36]. It may thus be possible that CSF-1 and GM-CSF activation of DIF occur through different kinases. Answering this question, together with the establishment of a DIF cDNA, will demonstrate the precise relationship to MGF-Stat 5.

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