Thrombopoietin (TPO) induces tyrosine phosphorylation and activation of STAT5 and STAT3

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Abstract The growth and differentiation of megakaryocytes are regulated by thrombopoietin (TPO), a recently characterized cytokine which exerts its effects via a member of the hematopoietin receptor superfamily, c-Mpl. Since many cytokines which bind hematopoietin receptors activate the STAT family of transcription factors, we investigated whether STAT proteins were activated by TPO. TPO induced the formation of a DNA-binding complex recognizing a known STAT-binding sequence. STAT5 was a major component of this DNA-binding complex, and STAT5 was tyrosine phosphorylated in response to TPO. Additionally, TPO-induced the tyrosine phosphorylation and DNA-binding activity of STAT3. Together with the recent demonstration of JAK2 activation in response to TPO, the data presented here define a rapid signaling pathway likely to be important in TPO-induced gene regulation.

Key words: Thrombopoietin; Signal transduction; Signal transducers and activators of transcription (STAT); Tyrosine phosphorylation

1. Introduction

Megakaryocytopoiesis and platelet production are regulated at many levels by thrombopoietin (TPO) a recently characterized cytokine that both supports the proliferation of megakaryocyte progenitors and induces their differentiation into large polyploid megakaryocytes [1–3]. The receptor for TPO is cMpl, a member of the hematopoietin receptor superfamily encoded by the normal cellular homologue of the retroviral oncogene v-mpl [4–6]. Like many other cytokines, TPO induces the rapid tyrosine phosphorylation of a number of intracellular proteins [7–9]. However, the cytoplasmic domains of hematopoietin receptors, including c-Mpl, lack intrinsic kinase activity [4,7]. Instead, hematopoietin receptors associate with and activate members of the Janus (JAK) family of cytoplasmic protein tyrosine kinases [10,11]. Indeed, we and others have recently demonstrated that TPO stimulates the rapid activation of JAK2, implicating JAK2 in the immediate biochemical response to TPO [8,9].

Many cytokines that activate JAK kinases induce activation of members of a family of latent cytoplasmic transcription factors known as STATs (Signal Transducers and Activators of Transcription) [10–12]. The first described members of this family, STAT1 and STAT2, are mediators of interferon (IFN)-induced gene activation [11], STAT3 is activated by interleukin (IL) -6 and IFNα [13,14], STAT4 by IL-12 [15], STAT5 by IL-2, prolactin, growth hormone and erythropoietin [16,17], and STAT6 by IL-4 [18]. Upon ligand–receptor interaction, STAT proteins become tyrosine phosphorylated within the receptor complex and then translocate to the nucleus where they regulate gene transcription through the binding of conserved DNA sequences within the promoters of cytokine responsive genes [11]. Such sequences are typified by the GRR (IFNγ-response region) derived from the promoter of the FcγR1 gene [19].

In this study we investigated whether STAT proteins were activated by TPO. TPO induced the tyrosine phosphorylation and DNA-binding activity of both STAT5 and STAT3, but not of other known STAT family members. STAT5 and STAT3 are therefore likely to play important roles in the regulation of gene expression by TPO.

2. Materials and methods

2.1. Cytokines, antibodies and cell culture

The following recombinant human cytokines were generously provided by the indicated sources: TPO (Kirin Brewery Co. Ltd., Tokyo, Japan); IFNα (Hoffmann-LaRoche Inc., Nutley, NJ); IL-2 (Cetus Oncology Corp., Emeryville, CA); granulocyte-macrophage colony stimulating factor (GM-CSF) and stem cell factor (SCF) (PeproTech Inc., Rocky Hill, NJ). Antisera recognizing STAT1 [20], STAT2 [21] and STAT5 [16] were kindly provided by Dr. Chris Schindler (Columbia University). Antisera against STAT1α [22], and specific C-terminal regions of STAT3 and STAT5 were generously provided by Dr. Andrew C. Larner (CBER, FDA). Monoclonal antibodies against STAT3 and STAT5 were purchased from Transduction Laboratories Inc. (Lexington, KY). Monoclonal antiphosphotyrosine antibody, 4G10, was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The human megakaryoblastic leukemia cell line Mo7e [23], generously provided by Genetics Institute (Cambridge, MA), was maintained as described [9] and cultured in the absence of growth factors for 18–40 h before any experiment.

2.2. Electrophoretic mobility shift assay (EMSA)

Whole cell extracts were prepared from cytokine stimulated cells as previously described [24]. EMSAs were performed as previously described [15,25] using a ^{32}P-end-labeled double-stranded oligonucleotide (5′-AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAA-G-3′) corresponding to the GRR of the human FcγR1 gene [19]. Oligonucleotide competition was performed by incubating 100- fold excesses of unlabeled GRR or the IFNα-stimulated response element (ISRE) of ISG15 [26] with cell extracts for 10 min at 4°C before addition of probe.

When used, antibodies were incubated with cell extracts for 1 h at 4°C before addition of the probe.

2.3. Oligonucleotide affinity purification and immunoblotting of STAT proteins

Double-stranded GRR oligonucleotide was synthesized with biotin covalently attached to the 5′-end of the sense strand. This biotinylated GRR was bound to streptavidin-agarose (Sigma Chemical Co., St. Louis, MO) resulting in approximately 1.5 μg of DNA per μl of packed agarose. Cytokine stimulated cells were lysed in buffer containing 0.1% Triton X-100, and lysates incubated for 2 h at 4°C with GRR-bound agarose. The precipitates were then washed, resolved by SDS-PAGE, transferred to PVDF membranes (Immobilon, Millipore Corp., Bedford, MA) and probed with monoclonal anti-STAT antibodies according to the manufacturer's instructions.

2.4. Immunoprecipitation and antiphosphotyrosine immunoblotting

Cytokine-stimulated cells were lysed in 1% Triton X-100 buffer as previously described [27], immunoprecipitated with anti-STAT antisera coupled to protein A-Sepharose and separated by 8% SDS-PAGE. Resolved proteins were transferred to Immobilon and immunoblotted with anti-phosphotyrosine antibody, 4G10 as previously described [27].

3. Results

3.1. TPO induces a GRR-binding complex containing tyrosine phosphorylated components

We have previously demonstrated that TPO stimulates proliferation and ligand-dependent activation of JAK2 in the megakaryoblastic cell line Mo7e [9]. We therefore used these cells to investigate the activation of STATs by TPO. Cytokine-activated STAT proteins bind to a series of palindromic DNA

Fig. 1. TPO induces a DNA-binding complex that recognizes the GRR of the FcγR1 gene. Mo7e cells were incubated with medium alone, 200 ng/ml TPO or 20 ng/ml GM-CSF for the indicated times and whole cell extracts prepared. 10 μg of each extract was analyzed by EMSA using a radiolabeled GRR oligonucleotide probe. Competition was performed by adding 100-fold molar excess of unlabeled GRR (lanes 5–7) or ISRE (lanes 8–10) oligonucleotides to the DNA-binding reaction prior to electrophoresis.

oligonucleotides with the consensus sequence TTNCNNNAA [11]. Such sequences are typified by the GRR of the FcγRI gene [19], which has been shown to bind STAT-containing complexes induced by a number of different cytokines [12]. Since no TPO-activated genes have yet been defined, we performed EMSAs using a radiolabeled GRR probe to analyze the DNA-binding proteins induced by TPO. Extracts from TPO-stimulated Mo7e cells (Fig. 1, lanes 2 and 3), but not from unstimulated cells (lane 1) contained a GRR-binding complex (indicated by an arrow). A similarly migrating complex was also strongly induced by GM-CSF (lane 4). In addition, a second, more slowly migrating GRR-binding complex was clearly induced by GM-CSF, but only very weakly by TPO. This difference may possibly result from differences in signal intensity since TPO typically induced weaker GRR-binding complexes than did GM-CSF. The binding of these cytokine-induced complexes to the GRR probe was inhibited by competition with unlabeled GRR oligonucleotide (Fig. 1, lanes 5–7) but not by unlabeled ISRE (lanes 8–10), demonstrating their sequence specificity. The assembly of STAT-containing DNA-binding complexes requires that certain components be tyrosine phosphorylated [12,28]. Accordingly, incubation of extracts from TPO-stimulated cells with monoclonal antiphosphotyrosine antibodies prior to the addition of radiolabeled GRR probe markedly inhibited the formation of the TPO-induced GRR-binding complex (data not shown), suggesting that this complex contained proteins phosphorylated on tyrosine residues, possibly STAT proteins.
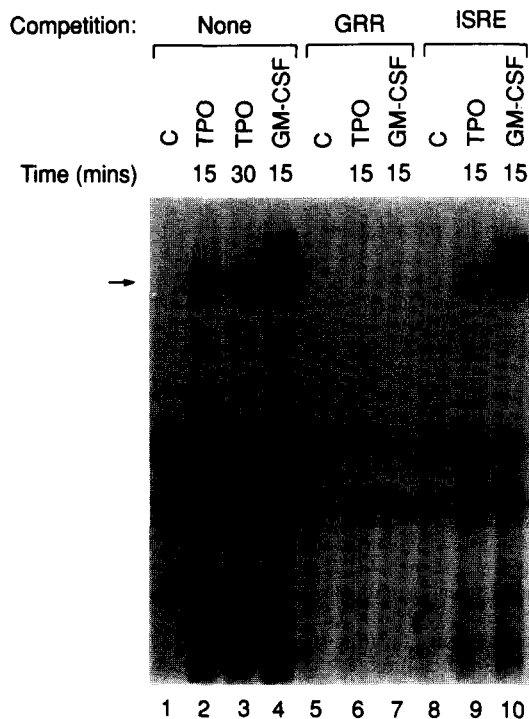
3.2. Tyrosine phosphorylation of STAT proteins in response to TPO

To identify the STAT proteins activated by TPO we performed immunoprecipitation with specific anti-STAT antisera followed by antiphosphotyrosine immunoblotting. Treatment of Mo7e cells with IFNα induced tyrosine phosphorylation of STAT1 (Fig. 2A, lane 4) and STAT2 (Fig. 2B, lane 4) which co-immunoprecipitated from IFNα-stimulated cells as previously described [11,29]. However, neither TPO nor GM-CSF induced tyrosine phosphorylation of STAT1 (Fig. 2A, lanes 2 and 3) or STAT2 (Fig. 2B, lanes 2 and 3). Equal protein loading was confirmed by reblotting each membrane with the immunoprecipitating antibody (data not shown). We also did not detect inducible tyrosine phosphorylation of STAT4 in response to TPO (data not shown).

Therefore, we next examined the phosphorylation of STAT5 and STAT3 in response to TPO. In agreement with previous reports [16,17] STAT5 was inducibly phosphorylated in response to both GM-CSF (Fig. 2C, lane 3) [17] and IL-2 (lane 4) [16]. Importantly, treatment of the cells with TPO also strongly induced the tyrosine phosphorylation of STAT5 (Fig. 2C, lane 2). Likewise, TPO readily induced tyrosine phosphorylation of STAT3 (Fig. 2D, lane 2). In contrast however, the growth-factor GM-CSF (at concentrations that induced readily detectable phosphorylation of JAK2; data not shown) induced much less tyrosine phosphorylation of STAT3 (Fig. 2D, lane 3) than did TPO. As expected, STAT3 was also inducibly phosphorylated in response to IFNα (Fig. 2D, lane 4) [14]. Again, the presence of equal amounts of STAT5 and STAT3 in each lane was demonstrated by immunoblotting with monoclonal antibodies (data not shown). Together these data indicate that STAT5 and STAT3, but not STAT1, STAT2, or
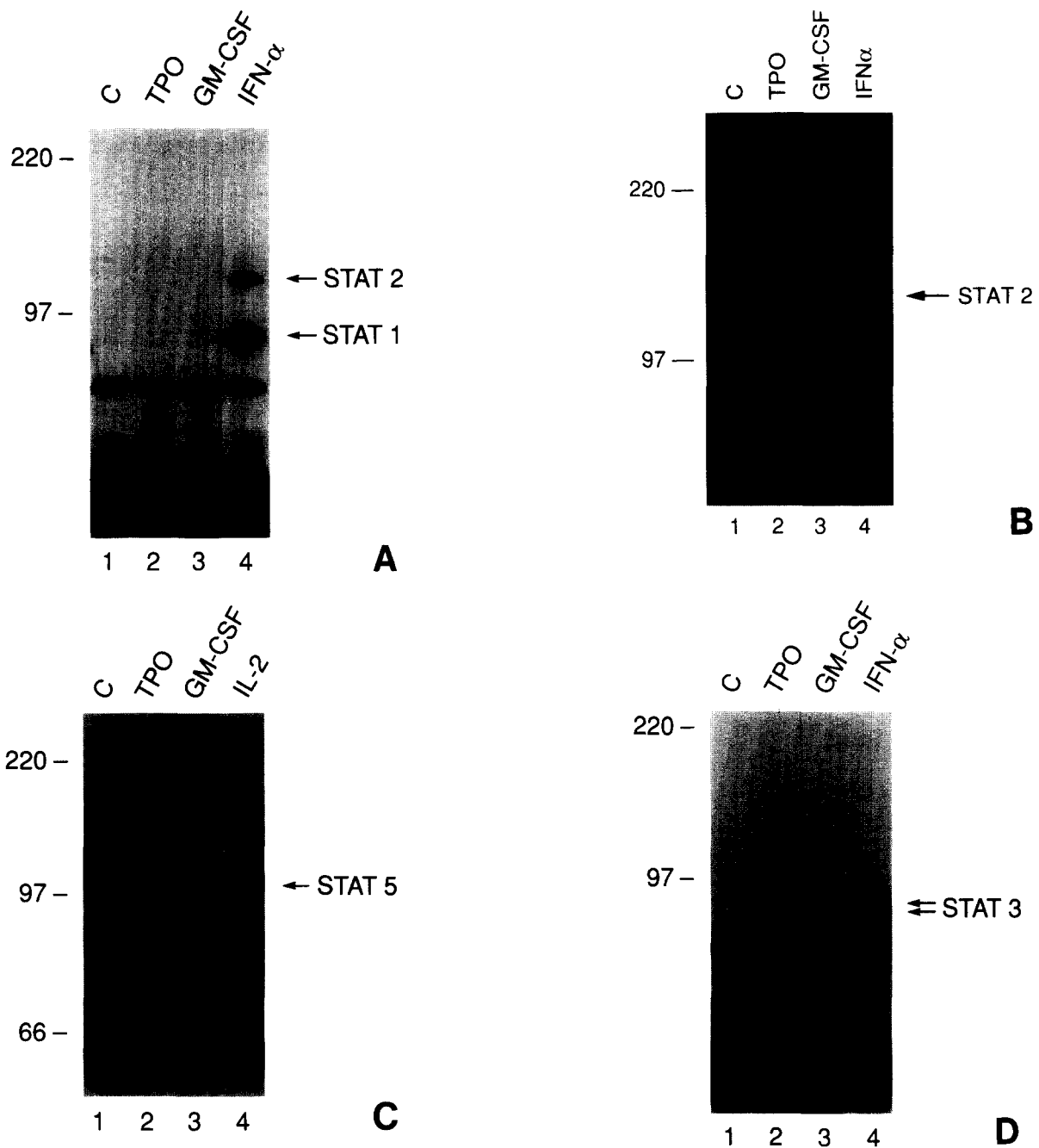
Fig. 2. TPO induces tyrosine phosphorylation of STAT5 and STAT3, but not STAT1 or STAT2. Mo7e cells were stimulated for 15 min as described, and lysates then immunoprecipitated with anti-STAT antisera, resolved by SDS-PAGE, and immunoblotted with the antiphosphotyrosine antibody, 4G10. (A) Anti-STAT1: lane 1 = medium alone; lane 2 = TPO (200 ng/ml); lane 3 = GM-CSF (50 ng/ml); lane 4 = IFNα (1000 U/ml). (B) Anti-STAT2: lane 1 = medium alone; lane 2 = TPO; lane 3 = GM-CSF; lane 4=IFNα. (C) Anti-STAT5: lane 1=medium alone; lane 2 = TPO; lane 3 = GM-CSF; lane 4 = IL-2 (1000 U/ml). (D) Anti-STAT3: lane 1 = medium alone; lane 2 = TPO; lane 3 = GM-CSF; lane 4 = IFNα.

STAT4 are tyrosine phosphorylated following stimulation of Mo7e cells with TPO.

3.3. STAT3 and STAT5 are components of the TPO-induced DNA-binding complex

In order to confirm the activation of STAT5 and STAT3 by TPO, and to show that these proteins were indeed components of the TPO-induced DNA-binding complex, we first used the combined techniques of oligonucleotide affinity purification

and Western blotting. TPO-induced GRR-binding complexes were affinity purified by incubation of whole cell extracts with GRR-conjugated agarose. GRR-bound proteins were resolved by SDS-PAGE and immunoblotted with specific anti-STAT antibodies. Whereas GRR precipitates from unstimulated Mo7e cells contained neither STAT5 (Fig. 3A, lane 1) nor STAT3 (Fig. 3B, lane 1), STAT5 was detected in GRR-precipitates from cells stimulated with TPO (Fig. 3A, lane 2) and GM-CSF (Fig. 3A, lane 3). Similarly, STAT3 was present in
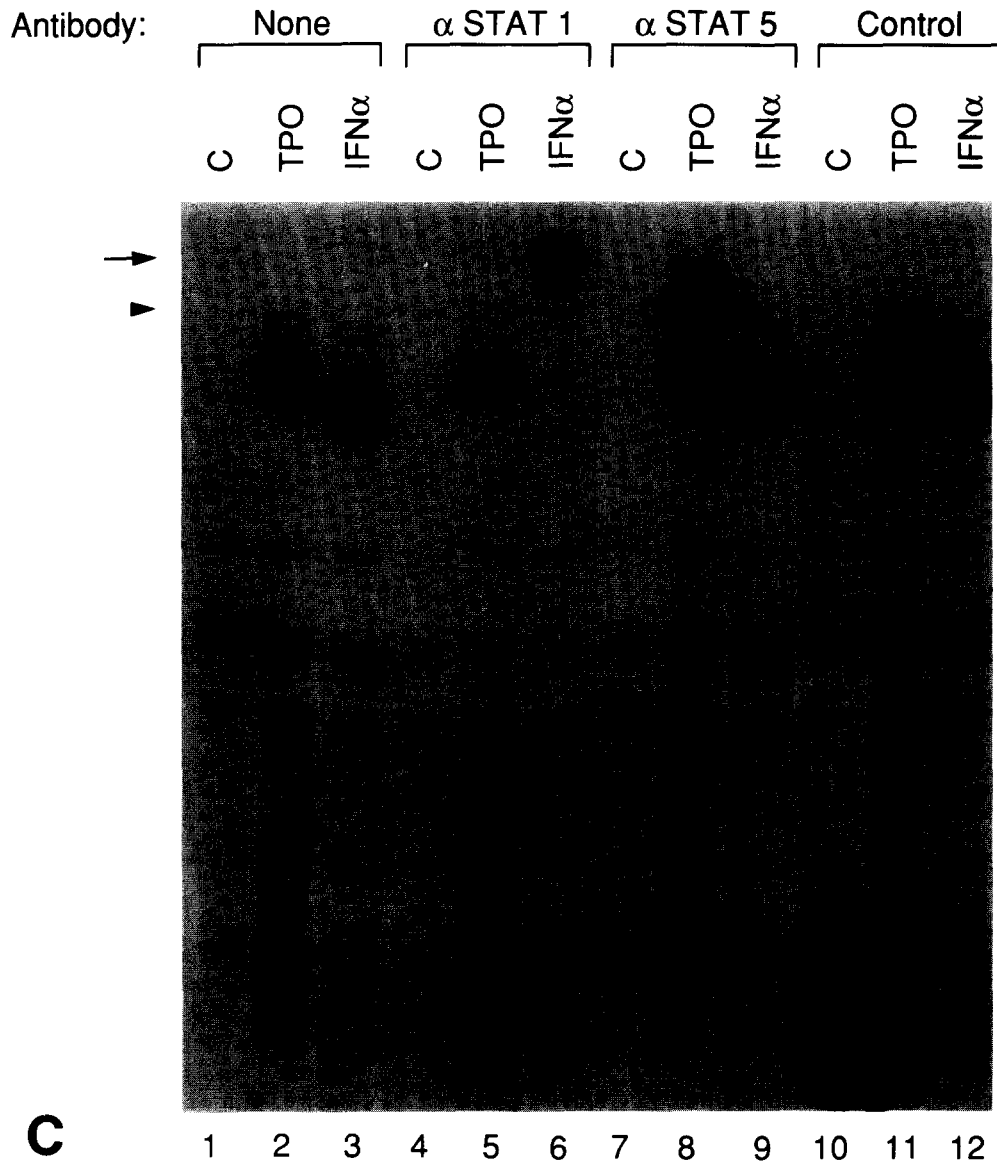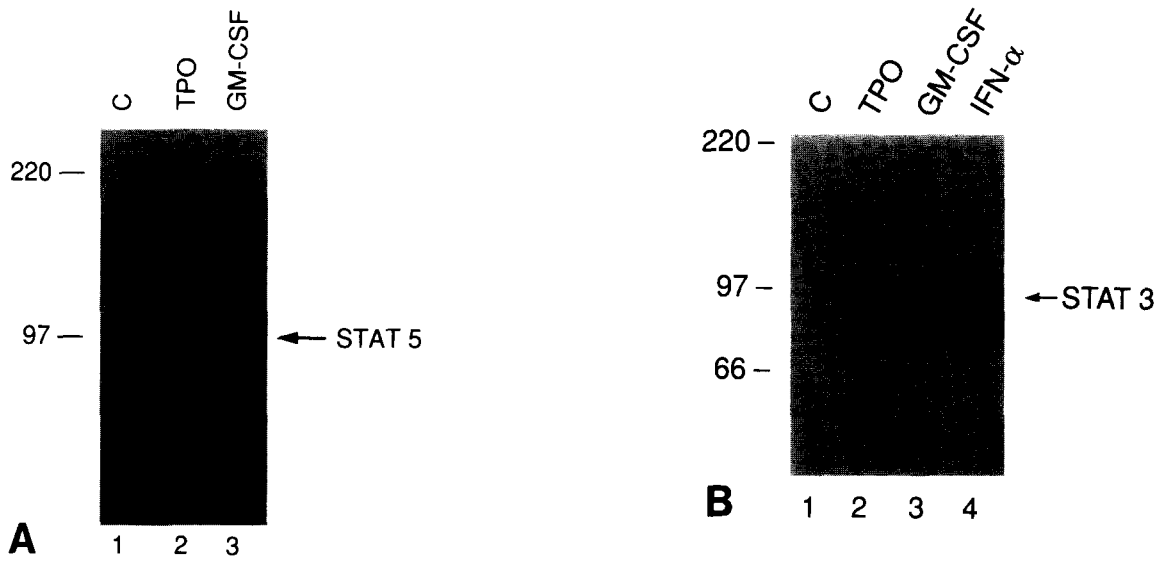
GRR-precipitates from cells stimulated with TPO (Fig. 3B, lane 2) and IFNα (Fig. 3B, lane 4), but not in precipitates from cells treated with GM-CSF (Fig. 3B, lane 3). The inability to detect GRR-bound STAT3 in response to GM-CSF is consistent with the comparatively low level of STAT3 tyrosine phosphorylation induced by this cytokine (Fig. 2D).

To further analyze the composition of the TPO-induced DNA binding complex, we performed EMSAs in which antisera recognizing specific STAT proteins were added to the DNA-binding reaction before addition of the ^{32}P-labeled probe. Antiserum against STAT1 supershifted the GRR-binding complex induced by IFNα (Fig. 3C, lane 6), but not the TPO-induced complex (lane 5), confirming that TPO does not activate STAT1 in these cells. Similarly, anti-sera against STAT2 and STAT4 also had no effect on the TPO-induced DNA-binding complex (data not shown). Addition of anti-STAT3 antiserum also did not alter the GRR-binding complex induced by TPO (data not shown). Importantly however, using Mo7e cells, the antiserum also failed to alter the complexes induced by a number of other cytokines previously shown to induce STAT3 binding to the GRR, including IL-2 and IFNα [16,30] (data not shown), suggesting that the antiserum was unable to supershift any STAT3 containing DNA-bound complex from these cells.

However, in support of the data presented above, addition of antiserum recognizing STAT5 produced a distinct super-shifted complex in extracts from TPO-treated (Fig. 3C, lane 8), but not IFNα-treated (lane 9) cells, indicating the presence of STAT5 in the complexes induced by TPO but not by IFNα. Control antiserum had no effect on the GRR-binding complexes induced by either TPO (lane 11) or IFNα (lane 12). These data clearly confirm that STAT5 and STAT3 are components of the TPO-induced DNA binding complex, and suggest an important role for these proteins in TPO-induced gene activation.

4. Discussion

In this report we demonstrate by both oligonucleotide affinity purification and by EMSA that TPO induces the rapid assembly of a DNA-binding complex containing STAT5, which is tyrosine phosphorylated in response to TPO. In addition, TPO induces the tyrosine phosphorylation and DNA-binding of STAT3. Together with the recent finding that TPO activates JAK2 [8,9], these data identify a rapid and direct signaling pathway by which TPO-induced gene activation might occur.

TPO induces both the proliferation of megakaryocyte progenitor cells and their differentiation into mature, polyploid

megakaryocytes [1–3,6]. While the genes activated by TPO during these two processes have not been defined, our studies suggest that the activation of STAT5 and STAT3 might be important in the induction of megakaryocyte growth and differentiation. STAT5 is activated by a number of other cytokines in addition to TPO, including prolactin, growth hormone, GM-CSF, erythropoietin [17], and IL-2 [16], and may therefore be a common element in the growth and differentiation signals transduced by these cytokines. The identification of STAT5 regulated genes could provide valuable insight into the regulation of cell proliferation and differentiation. Moreover, disregulation of STAT5 activity could play a role in disorders of cell growth and maturation such as cancer, suggesting that STAT5 might be a novel target for pharmacological treatment of such diseases.

We have recently demonstrated that, like TPO, the T cell growth factors IL-2, IL-7, IL-9 and IL-15 (cytokines whose receptors each utilize the common γ chain (γc)) also activate STAT5 and STAT3 [16]. This is intriguing since these cytokines each activate JAK1 and JAK3 [16], whereas TPO activates JAK2 [9], indicating that the ability of a cytokine to activate specific STATs cannot be correlated with the activation of a given JAK. Instead, recent evidence suggests that the selective utilization of STATs by cytokines is determined by specific STAT binding motifs within the receptor itself [31].

Despite the fact that they all activate STAT5 and STAT3, IL-2, IL-7, IL-9 and TPO each trigger a range of different biological effects in different cell types suggesting that factors other than STATs themselves may important determinants of cytokine specificity. STATs often bind to DNA as complexes with other STATs or with other non-STAT proteins (for example members of the IRF-1 family) [32]. The combinatorial association of these factors may be one mechanism by which signal diversity is achieved. In addition, many other signaling molecules such as the MAP kinase family are activated by cytokines. Such pathways also regulate cytokine-induced gene expression, and may even directly modulate STAT activation. By interacting with other signal transduction pathways to induce expression of distinct sets of genes, STAT3 and STAT5 may subserve roles in both the growth and differentiation of megakaryocytes. Elucidation of the exact composition of the STAT complexes formed in response to TPO and identification of other signaling pathways activated by TPO may lead to a better understanding of the molecular mechanisms for the many biological effects of TPO.

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Fig. 3. The TPO-induced DNA-binding complex contains STAT5 and STAT3. (A and B) Mo7e cells were incubated with medium alone (lanes 1), TPO (lanes 2), GM-CSF (lanes 3), or IFNα (Fig. 3B, lane 4) lysed and incubated with GRR-coupled agarose. GRR-bound proteins were resolved by SDS-PAGE, and immunoblotted with antibody against (A) STAT5 or (B) STAT3. (C) Extracts from Mo7e cells treated with medium alone (lanes 1, 4, 7, 10), TPO (lanes 2, 5, 8, 11) or IFNα (lanes 3, 6, 9, 12) were incubated for 1 h with buffer alone (lanes 1–3), with anti-STAT1 antiserum (lanes 3–6), with antiserum recognizing STAT5 (lanes 7–9), or with control antiserum (lanes 10–12). Extracts were then incubated with GGR probe and analyzed by EMSA.

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