ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 31 (2003) 398–405

Erythropoietin-induced serine 727 phosphorylation of STAT3 in erythroid cells is mediated by a MEK-, ERK-, and MSK1-dependent pathway

Albertus T.J. Wierenga[a], Irma Vogelzang[a], Bart J.L. Eggen[b], and Edo Vellenga[a]

[a]*University Hospital Groningen, Department of Hematology, Groningen, The Netherlands;*
[b]*Developmental Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Haren, The Netherlands*

(Received 26 August 2002; revised 14 January 2003; accepted 18 February 2003)

***Objective*. Erythropoietin (EPO) is a key regulator of erythropoiesis, playing a role in both the proliferation and differentiation of erythroid cells. One of the signal transduction molecules activated upon EPO stimulation is signal transducer and activator of transcription (STAT) 3. Besides tyrosine 705 phosphorylation of STAT3, serine 727 phosphorylation has been described upon EPO stimulation. In the present study, we investigated which molecular pathways mediate the STAT3 serine 727 phosphorylation and the functional implications of this phosphorylation.**

***Methods*. The EPO-dependent erythroid cell line ASE2 was used to investigate which signaling routes were involved in the STAT3 serine 727 phosphorylation. Western blotting using phosphospecific antibodies was used to assess the phosphorylation status of STAT3 molecules. Transfection analysis was performed to investigate the transactivational potential of STAT3, and quantitative RT-PCR was used to study the in vivo gene expression of STAT3-responsive genes.**

***Results*. Western blotting of extracts of cells exposed to various chemical inhibitors revealed that the MEK inhibitors PD98059 and U0126 abrogated the EPO-mediated STAT3 serine 727 phosphorylation without an effect on tyrosine phosphorylation. Further analysis showed that MSK1 is activated downstream of ERK, and retroviral transductions with kinase-inactive MSK1 revealed that MSK1 is necessary for STAT3 serine phosphorylation. Furthermore, the STAT3-mediated transactivation was reduced by blocking the STAT3 serine phosphorylation with the MEK inhibitor U0126 or by expression of kinase-inactive MSK1.**

***Conclusions*. The EPO-induced STAT3 serine 727 phosphorylation is mediated by a pathway involving MEK, ERK, and MSK1. Furthermore, serine phosphorylation of STAT3 augments the transactivational potential of STAT3.** © **2003 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Hematopoiesis is regulated by a number of cytokines and growth factors, to accomplish a balanced production of mature blood cells. A number of lineage-restricted growth factors has been described, each of them playing a distinct role in the proliferation and differentiation of specific cell lineages. The most important growth factor for the erythroid lineage is erythropoietin (EPO) [1,2]. EPO is necessary to stimulate proliferation of erythroid precursor cells, as well as to induce differentiation of these cells to mature erythrocytes [3]. EPO exerts its effect by binding to the EPO receptor (EPOR) [4], which is composed of two identical subunits. Upon ligand binding, the two subunits dimerize and Janus kinases (JAK's) are recruited to the receptor complex resulting in the phosphorylation of several tyrosine residues on the receptor. These phosphorylated tyrosine residues form docking sites for several molecules, including the signal transducer and activator of transcription (STAT) transcription factors. The STAT molecules are phosphorylated on a single tyrosine residue by JAK kinases, leading to dimerization and subsequent translocation to the nucleus where they act as transcription factors [5,6]. Although STAT5 is the most prominent STAT molecule activated by EPO [7], STAT3 (and STAT1) is also, albeit to a lesser extent, activated by EPO stimulation [8–10]. The role of STAT3 activation in the biological function of EPO is not clear. Recent studies however, suggest that STAT3 can replace STAT5 in some

Offprint requests to: Edo Vellenga, M.D., Ph.D., University Hospital Groningen, Department of Hematology, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands; E-mail: E.Vellenga@int.azg.nl

0301-472X/03 $–see front matter.
doi: 10.1016/S0301-472X(03)00045-6

circumstances, implicating that the two STAT molecules might be functionally redundant [11,12]. Indeed, although STAT5A and STAT5B double-knockout mice show ineffective erythropoiesis, especially under stress conditions [13], only a modest reduction of the number of circulating erythrocytes is observed in these mice, suggesting that STAT5 is not totally indispensable for correct erythropoiesis [14]. This is in contrast to EPO receptor (EPOR) knockout mice, which die before birth from defective erythropoiesis in the fetal liver [15].

Besides tyrosine phosphorylation of STAT3, leading to nuclear translocation, STAT3 molecules can also be phosphorylated on a single serine residue, serine 727 [16,17]. There are conflicting data regarding the significance of this serine phosphorylation. It has been shown that serine 727 phosphorylation can reduce the transctivational potential of STAT3, by diminishing the tyrosine phosphorylation and subsequent binding to its target sequence in promoters [18–21]. On the other hand, phosphorylation of serine 727 of STAT3 has also been shown to enhance activation of transcription by STAT3 [16,22–24].

Much effort has been made to identify the kinase(s) responsible for the serine 727 phosphorylation of STAT3. Serine 727 in STAT3 (and STAT1 and 4) is situated in a conserved PMSP motive which resembles the consensus PxS/TP motive for mitogen-activated protein kinase (MAPK) targets [25]. Several MAPK family members have been identified as kinases capable of phosphorylating serine 727 of STAT3, including extracellular regulated kinase (ERK), p38 MAPK, and Jun N-terminal kinase (JNK) [20,21,26–29]. Unrelated kinases such as protein kinase C (PKC) δ have also been described to be able to phosphorylate STAT3 serine 727 [24,30]. In addition, mitogen and stress-activated protein kinase 1 (MSK1) was recently described to phosphorylate serine 727 of STAT3 in response to ultraviolet irradiation. The activation of MSK1 was found to be mediated by ERK as well as p38 in this report [31]. The actual kinase responsible for serine phosphorylation of STAT3 in a given cell type seems to be dependent on the cellular setting and the stimulus applied.

In the present study, the pathway leading to serine phosphorylation of STAT3 in erythroid cells is studied. It is shown that serine 727 of STAT3 is phosphorylated by a signal transduction cascade including MEK, ERK, and MSK1. Moreover, it is demonstrated that serine phosphorylation of STAT3 augments the transactivational potential of STAT3 in erythroid cells.

Materials and methods

Cell culture, reagents, and antibodies

ASE2 cells (kindly provided by Dr. M. Tomonaga, Dept. of Hematology, Nagasaki University School of Medicine, Nagasaki, Japan) were cultured in IMDM with additives (ICN, Costa Mesa, CA, USA) supplemented with 20% fetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands), 2U/mL rh-EPO (Eprex, Jansen Cilag, The Netherlands), and penicillin-streptomycin (ICN) [32]. Cell concentration was kept between 0.2 and 1.0×10^6 cells/mL. Stimulation was performed with 10 U/mL rh-EPO, 25 ng/mL interleukin (IL)-6 (kind gift from Immunex Corp., Seattle, WA, USA), 100 ng/mL stem cell factor (SCF; gift from Immunex Corp.), or 50 nM phorbol myristate acetate (PMA) (Sigma, Zwijndrecht, The Netherlands). Phoenix amphotropic packaging cells were a kind gift of Dr. P. Coffer (Dept. Of Pulmonology, University Medical Center, Utrecht, The Netherlands), and were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. Antibodies against phospho-tyrosine 705 of STAT3, phospho-serine 727 of STAT3, phospho-serine 376 of MSK1, and phospho-threonine 202/tyrosine 204 ERK were obtained from Cell Signaling (Beverly, MA, USA). Antibodies against STAT3 (F2) and ERK1 were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibodies against MSK1 and phospho-tyrosine 694 STAT5 were obtained from Upstate Biotechnology (Lake Placid, NY, USA). An antibody against the FLAG-tag (M2) was obtained from Sigma. HRP-conjugated secondary antibodies were obtained from DAKO (Glostrup, Denmark). PD98059, SB203580, AG490, and Rottlerin were obtained from Biomol (Plymouth Meeting, PA, USA) and used at concentrations of 50 μM, 10 μM, 100 μM, and 20 μM, respectively. U0126 (10 μM) was obtained from Promega (Leiden, The Netherlands). LY284002 (20 μM) was obtained from Alexis (San Diego, CA, USA). All other reagents and chemicals were obtained from Sigma.

cDNA constructs

A flag tagged MSK1(D565A) construct cloned in pCMV5 were obtained from D. Alessi (MRCP, University of Dundee, Scotland, UK). A STAT3 luciferase reporter was constructed by ligating three copies of the ICAM-1 STAT3 response element (5′-AGCTTAGGTTTCCGGGAAAGCAC-3′) into the pGL3ti vector, which was a kind gift from Dr. L. Jonk [33]. Exression vectors for STAT3 wild-type and STAT3 (Ser727 to ala) were gifts from Dr. J.J. Schuringa (Sloan-Kettering Memorial Institute, New York, NY, USA). An expression vector for STAT3 with tyrosine 705 mutated to alanine was a kind gift of Dr. M. Saunders (Devgen NV, Ghent-Zwijngaarde, Belgium) [34]. pDM2lacZ, constitutively expressing β-galactosidase, is described elsewhere [35]. A retroviral vector named pBabe-IRES-EGFP-puro was constructed by insertion of the IRES-EGFP cassette from pLZRS into pBabe-puro (both vectors kindly provided by Dr. P. Coffer, Dept. Of Pulmonology, University Medical Center, Utrecht, The Netherlands). To obtain pBabe-MSK1(D565A)-IRES-EGFP-puro, the flag-tagged MSK1(D565A) cDNA was cloned into pBabe-IRES-EGFP-puro. This vector expresses the MSK1 protein and EGFP from the same mRNA molecule.

Preparation of protein extracts and Western blotting

Before stimulation, ASE2 cells were washed three times with IMDM and incubated for 16 hours in the presence of 10% FBS without EPO. To prepare protein extracts, 3×10^6 EPO-deprived cells per well were plated in 12-well cell culture plates, stimulated, washed, and collected in phosphate-buffered saline (PBS). Whole-cell lysates were prepared by lysing the cells directly in Laemmli sample buffer. After SDS-PAGE, proteins were transferred to PVDF membrane (Millipore, Bedford, MA, USA),

blocked with Tris-buffered saline (TBS) buffer containing 0.1% Tween-20 and 5% nonfat milk prior to incubation with antibodies, and diluted in TBS containing 5% bovine serum albumin. Binding of each antibody was detected by the appropriate HRP-conjugated secondary antibodies using Super Signal (Pierce, Perbio Science, Etten-Leur, The Netherlands) according to the manufacturer's recommendations.

Transient transfections

ASE2 cells were transfected by electroporation. After three washes with IMDM medium without FBS, 2×10^7 cells were resuspended in 175 μL IMDM, 25 μg DNA was added, and the mixture was transferred to a 0.4-cm-gap-width electroporation cuvette. The cells were pulsed using a Bio-Rad gene pulser electroporation device (Bio-Rad, Sunnyvale, CA, USA) with 240 V and a capacity setting of 960 μF. Immediately after pulsing, the cells were transferred to fresh IMDM media containing 10% FBS and incubated overnight. For reporter studies, the cells were stimulated for 8 hours and harvested. Cell extracts were made and luciferase expression was measured according to the manufacturer's protocol (Promega, Leiden, The Netherlands). β-galactosidase expression was measured to correct for differences in transfection efficiency.

Retroviral transductions

MSK1(D565) retroviral particles were produced by transient transfection of pBabe-MSK1(D565A)-IRES-EGFP-puro into Phoenix amphotropic packaging cells using Fugene6 (Roche, Almere, The Netherlands) according to the manufacturer's instructions. After one day, medium was replaced with IMDM containing 20% FCS. Two days later, supernatant containing retroviral particles was collected, filtered through a 0.45-μm filter, and added to ASE2 cells in the presence of 8 μg/mL polybrene. After one week, GFP^+ cells were sorted using MoFlo equipment. GFP^+ cells were further expanded in growth medium and used for Western blotting and reverse transcriptase polymerase chain reaction (RT-PCR) experiments.

Quantitative RT-PCR

For RT-PCR, total RNA was extracted from 3×10^6 cells, with TriZol Reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Two μg of total RNA was reverse transcribed using random hexamer priming in a total volume of 20 μL with 200 U MMLv reverse transcriptase (Invitrogen). The obtained cDNA was diluted to 100 μL, and 2 μL of this solution was used in a 20-μL PCR reaction with the DNA Master SYBR Green1 kit (Roche, Almere, The Netherlands) and the indicated primers using the LightCycler equipment (Roche), according to the manufacturer's recommendations. Serially diluted cDNA was amplified in parallel with the samples to facilitate quantification of the amount of specific cDNA present in each sample. The relative amount of cDNA was calculated using LightCycler software provided by Roche. All PCR reactions were performed in triplicate. The sequence of the primers used were (5′→3′): HPRT, forward: TGGCGTCGTGATTAGTGATG, reverse: GATGTAATCCAGCAGGTCAG, c-fos forward: AGCGCAGAGCATTGGCAGGA, reverse: TCGGTGAGCTGCCAGGATGA, c-myc forward: ACCAGCAGCGACTCTGAGGA, reverse: GACGTGGCACCTCTTGAGGA.

Results

EPO induces phosphorylation of STAT3 and STAT5 in erythroid cells

To investigate which STATs are activated upon EPO stimulation in erythroid cells, Western blotting using different phospho-specific anti-STAT3 antibodies was performed. After deprivation of EPO for 16 hours, ASE2 cells were stimulated with EPO for different time points. Figure 1A demonstrates a time-dependent phosphorylation on tyrosine 705 of STAT3. A similar time course was shown for tyrosine phosphorylation of STAT5. The amount of tyrosine phosphorylation of STAT3 and STAT5 was maximal at 15 minutes of stimulation and decreased thereafter. Serine phosphorylation of STAT3 was also induced by EPO stimulation and peaked at about 15 to 30 minutes. The serine phosphorylation was not specific for ASE2 cells, as similar results were obtained with the EPO-dependent UT7-EPO cell line (data not shown).

Serine phosphorylation of STAT3 is mediated by an ERK-dependent pathway

Since different signal transduction pathways are described to be involved in the serine 727 phosphorylation of STAT3, inhibitor studies were performed to discriminate between some major pathways. Inhibitors were used to block the MEK (PD98059 and U0126), p38 (SB203580), PI3K (LY294002), JAK2 (AG490), and PKCδ (Rottlerin) pathways, respectively. ASE2 cells were incubated for 1 hour with the inhibitors at concentrations known to inhibit their respective kinases and subsequently stimulated with EPO for 15 minutes. As shown in Figure 2A, blocking the JAK2 activation by AG490 resulted in a general suppression of EPO-induced signaling in ASE2 cells, judged by the strongly reduced phosphorylation of STAT3 and 5, as well as the absence of phosphorylated ERK. The EPO-induced serine phosphorylation of STAT3 was specifically blocked by the MEK inhibitors U0126 and, to a lesser extent, PD98059, whereas the inhibitors for p38, PI3K, and PKCδ had no

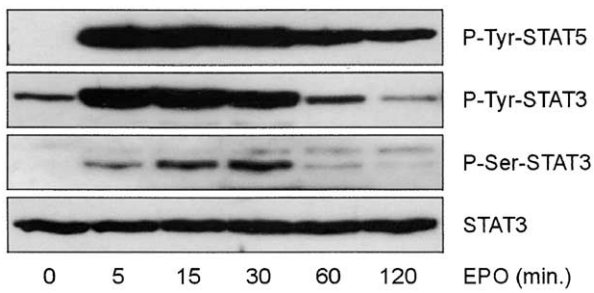

Figure 1. Phosphorylation of STAT molecules by EPO. ASE2 cells were deprived of EPO overnight, stimulated with EPO (10 U/mL) for different time points, and whole-cell extracts were blotted against phospho-tyrosine STAT5, phospho-tyrosine STAT3, and phospho-serine STAT3. The lower panel shows equal amounts of STAT3 in each sample. Experiments were performed at least three times; one representative experiment is shown.

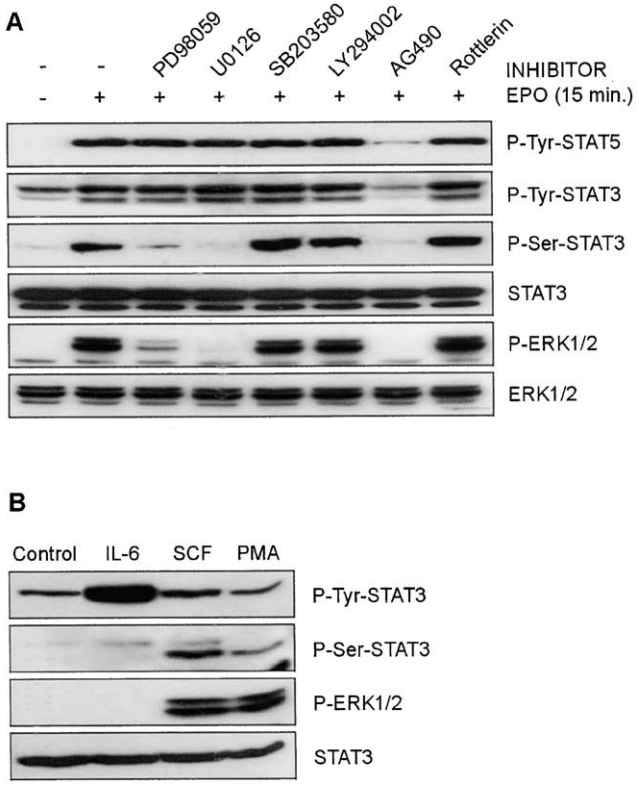

Figure 2. Serine phosphorylation of STAT3 is mediated by an ERK-dependent pathway. (**A**) ASE2 cells were stimulated with 10 U/mL EPO for 15 minutes in the absence or presence of the indicated inhibitors. Whole-cell extracts were prepared and blotted against phospho-tyrosine STAT5, phospho-tyrosine STAT3, phospho-serine STAT3, and phospho-ERK. The blots against STAT3 and ERK show equal amounts of protein in each sample. (**B**) ASE2 cells were stimulated with IL-6 (20 ng/mL), SCF (100 ng/mL), and PMA (50 ng/mL) for 15 minutes and whole-cell extracts were blotted against phospho-tyrosine STAT3, phospho-serine STAT3, and phospho-ERK. The lower panel, blotted against STAT3, shows equal amounts of protein in each sample. Of four experiments performed, one representative experiment is shown.

effect. Tyrosine phosphorylation of STAT3 and STAT5 was not affected by these inhibitors, excluding possible toxic effects of these compounds. As expected, the phosphorylation of ERK was also totally blocked by PD98059 and U0126. To strengthen the finding that the serine phosphorylation of STAT3 was mediated by an ERK-dependent pathway, two unrelated ERK inducing agents were tested for their ability to induce serine phosphorylation of STAT3. In Figure 2B, it is shown that both SCF and PMA induce serine phosphorylation of STAT3 in addition to phosphorylation of ERK. In contrast, although stimulation with IL-6 resulted in a clear induction of tyrosine phosphorylation of STAT3, no induction of ERK or STAT3 serine phosphorylation was observed.

MSK1 is activated downstream of ERK

Because STAT3 serine 727 phosphorylation has been described to be mediated by MSK1 in fibroblasts, the activation of MSK1 upon EPO stimulation was investigated. ASE2 cells were stimulated for different time points with EPO and the lysates were subsequently blotted with antibodies against phospho-MSK1. Figure 3A shows that MSK1 is activated after 5 minutes of stimulation and peaked at 15 minutes. To confirm that MSK1 is activated downstream of ERK, the MEK inhibitors U0126 and PD98059 were used to block the MEK-ERK pathway. As demonstrated in Figure 3B, EPO-induced MSK1 phosphorylation was clearly blocked by these inhibitors. In contrast, the inhibitors for p38, PI3K, and PKCδ (SB203580, LY294002, and Rottlerin, respectively) did not influence the phosphorylation of MSK1 (data not shown). Furthermore, stimulation with SCF and PMA, previously shown to induce phosphorylation of ERK and subsequent serine phosphorylation of STAT3, also resulted in the activation of MSK1 (data not shown).

MSK1 activation is necessary for STAT3 serine phosphorylation

To study whether MSK1 activation plays a role in the phosphorylation of STAT3 on serine 727, retroviral transductions with kinase-inactive MSK1 (MSK1-D565A) were performed. ASE2 cells were retrovirally transduced with empty vector (mock transduced) or with a vector expressing kinase-inactive MSK1 (D565A). As depicted in Figure 4, stimulation of mock-transduced cells with EPO resulted in serine phosphorylation of STAT3. Expression of kinase-inactive (D565A) MSK1, however, severely reduced the

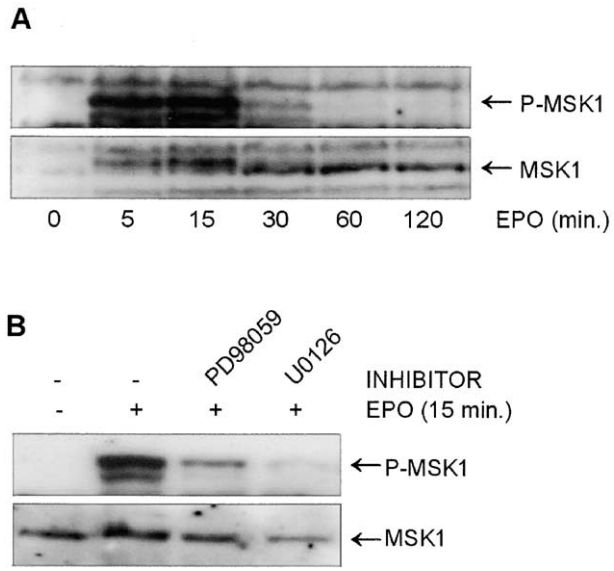

Figure 3. MSK1 is activated by EPO in an ERK-dependent manner. (**A**) ASE2 cells were stimulated with EPO (10 U/mL) for different time points and whole-cell extracts were blotted against phospho-MSK1. The lower panel shows equal amounts of MSK1 in each sample. (**B**) ASE2 cells were stimulated with 10 U/mL EPO for 15 minutes in the absence or presence of the indicated inhibitors. The lower panel shows equal amounts of MSK1 in each sample. Experiments were repeated three times and one representative experiment is shown.

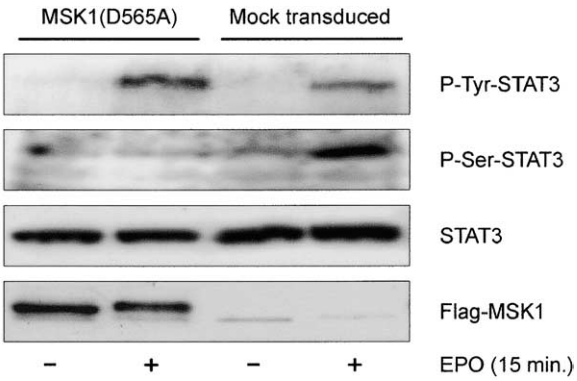

Figure 4. MSK1 activity is necessary for EPO-induced serine phosphorylation of STAT3. ASE2 cells were retrovirally transduced with empty vector (mock) and kinase-inactive MSK1 (D565A), sorted by FACS sorting on the basis of GFP expression to 99% purity, and used for Western blotting. Whole-cell lysates were blotted against phospho-serine STAT3, phosphotyrosine STAT3, and total STAT3. Expression of the FLAG-MSK1 construct was confirmed by blotting against the FLAG tag. One representative experiment out of three experiments performed is shown here.

serine phosphorylation of STAT3, showing that MSK1 activity is necessary for serine phosphorylation of STAT3.

Serine phosphorylation potentiates the transcriptional activity of STAT3

To address the question of whether serine phosphorylation influences the transcriptional activity of STAT3, transactivation studies were performed. ASE2 cells were transiently transfected with a luciferase reporter construct containing three multimerized STAT3 binding sites from the ICAM promoter. Preliminary experiments revealed that the STAT3 reporter used in these experiments was not sensitive enough to accurately measure the endogenous STAT3 activation. Therefore, wild-type STAT3 was cotransfected with the reporter in order to be able to quantify the transcriptional activity of STAT3 in this system. As shown in Figure 5A, stimulation with EPO resulted in an approximately twofold increase in luciferase activity. Blocking the ERK-induced serine phosphorylation of STAT3 with U0126, however, resulted in 50% reduction of transcriptional activity (1.9-fold induction vs 1.5-fold induction, respectively, $p < 0.05$). The EPO-induced STAT5 transactivation, measured with a STAT5-specific reporter, was not affected by U0126 (data not shown). Furthermore, as shown in Figure 5B, transfection of serine 727 to alanine-mutated STAT3 resulted in a similar reduction of STAT3-mediated transactivation (2.1-fold induction vs 1.6-fold, $p < 0.05$). Transfection of tyrosine 705 to phenylalanine-mutated STAT3 resulted in a lack of response to EPO, as expected. These data indicate that the EPO-induced STAT3 ser 727 phosphorylation by a signaling pathway involving ERK, is required for maximal transcriptional activation by STAT3.

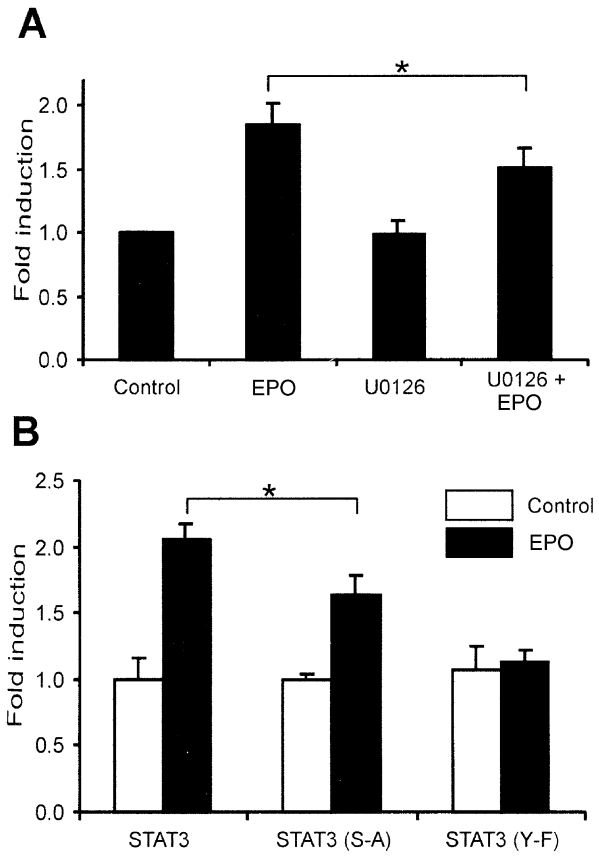

Figure 5. Transactivational potential of STAT3 is reduced by inhibition of STAT3 serine phosphorylation. **(A)** ASE2 cells were transiently transfected with a STAT3 reporter plasmid (pGL3-IRE) in the presence of wild-type STAT3. Cells were subsequently stimulated with EPO (10 U/mL) for 8 hours and luciferase expression was determined, corrected for transfection efficiency with lacZ expression, and expressed as fold induction compared to unstimulated samples. **(B)** ASE2 cells were transiently transfected with wild-type STAT3, ser 727 to ala–mutated STAT3, and tyr 705 to phe–mutated STAT3. Means and standard deviation of three experiments performed in duplicate are shown; asterisk indicates significant reduction ($p < 0.05$).

To validate this finding, we determined the in vivo role of serine 727 phosphorylation in the activation of gene transcription. Quantitative RT-PCR analysis of two known targets of STAT3 was performed to determine the role of STAT3 serine phosphorylation in transcriptional activation of these endogenous target genes. ASE2 cells were stimulated for 1 hour (in the case of c-fos) or 2 hours (for c-myc) with EPO, either in the absence or presence of U0126, to inhibit serine 727 phosphorylation and the level of c-fos and c-myc mRNA was measured using quantitative RT-PCR analysis. Figure 6A demonstrates that stimulation with EPO resulted in a 26-fold induction of c-fos mRNA level, which was reduced to 15-fold in the presence of U0126 ($p < 0.05$). EPO-induced c-myc expression was approximately twofold, and was reduced to 1.2-fold by U0126 ($p < 0.05$). RT-PCR analysis of retrovirally transduced cells, as shown in Figure 6B,

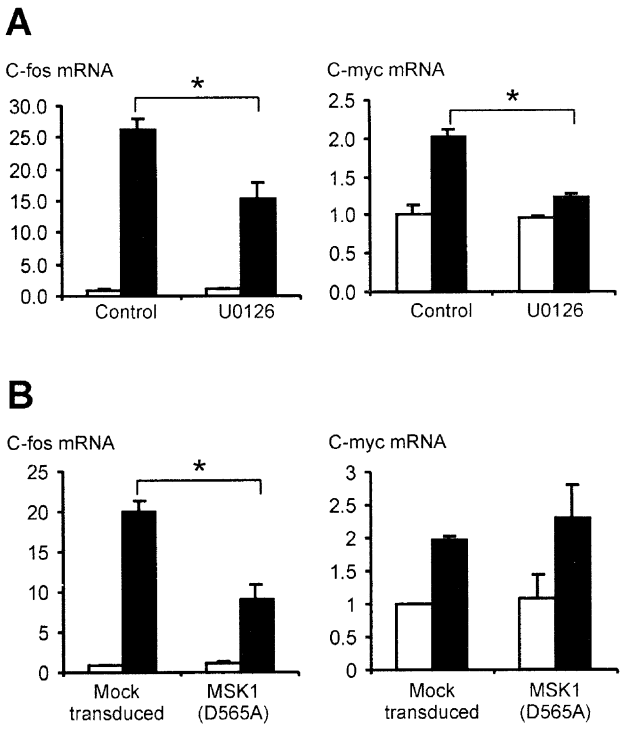

Figure 6. EPO-induced c-fos and c-myc mRNA expression is reduced by inhibition of STAT3 serine phosphorylation. (**A**) ASE2 cells were stimulated with EPO (10 U/mL) for 1 hour (c-fos) or 2 hours (c-myc) in the absence or presence of U0126. mRNA levels were measured by quantitative RT-PCR and corrected for amounts of cDNA by RT-PCR of HPRT mRNA levels. Results are expressed as fold induction compared to unstimulated samples. (**B**) ASE2 cells were retrovirally transduced with empty vector (mock) or kinase-inactive (D565A) MSK1. Cells were stimulated and mRNA expression was determined as in (A). Means and standard deviation of a representative experiment performed in triplicate is shown; asterisk indicates significant reduction ($p < 0.05$).

revealed that EPO-induced c-fos mRNA was similarly reduced in the presence of a kinase-inactive MSK1 (D565A), as expected. In the case of c-myc, however, no reduction of EPO-induced expression could be observed, suggesting that c-myc expression is not totally dependent upon STAT3 activity. In summary, these data underscore the observations using reporter genes, that serine 727 phosphorylation is required for a maximal EPO-induced transcriptional response of STAT3 target genes like c-fos and, to a lesser extent, c-myc.

Discussion

In this study, the molecular pathway leading to serine 727 phosphorylation of STAT3 in erythroid cells was explored. It was shown that EPO stimulation of ASE2 cells results in the rapid phosphorylation of STAT3 on both tyrosine 705 and serine 727. The EPO-induced phosphorylation of STAT3 on tyrosine 705 has been described before in other erythroid cells, such as UT-7/EPO [9]. In these cells, it was found that tyrosine residue 432 of the EPOR mediates the activation of STAT3 upon EPO stimulation, whereas STAT5 tyrosine phosphorylation was mediated by the three most proximal tyrosine residues. Apart from tyrosine phosphorylation, STAT3 was also phosphorylated on serine 727 by EPO stimulation. A number of different pathways have been implicated in serine 727 phosphorylation of STAT3. In this study, the MEK-ERK-MSK1 pathway was identified as the primary route leading to STAT3 serine 727 phosphorylation. Several lines of evidence point toward this conclusion. Using inhibitors for several pathways, only the MEK inhibitors PD98059 and U0126 specifically inhibited the serine 727 phosphorylation. Furthermore, SCF and PMA, two unrelated ERK-inducing agents, were also effective in mediating serine 727 phosphorylation of STAT3. During the preparation of this manuscript, Haq et al. described a similar induction of STAT3 serine 727 phosphorylation by EPO in BaF3 cells expressing the EPO receptor [36]. In these cells, the MEK pathway was also identified to be responsible for the serine 727 phosphorylation. Our study extends this finding further by identifying the downstream kinase responsible for the serine 727 phosphorylation. MSK1 is a nuclear kinase, identified as a target of ERK, JNK, and p38, and implicated in phosphorylation of CREB and ATF1. MSK1 is activated in an ERK-dependent manner in ASE2 cells upon EPO stimulation, as the inhibitors PD98059 and U0126 could block the MSK1 phosphorylation. In mouse JB6 Cl 41 cells, ultraviolet A irradiation resulted in activation of MSK1 and the subsequent serine 727 phosphorylation of STAT3 [31]. In this cell system, both the MEK inhibitor PD98059 and the p38 inhibitor SB202190 blocked the activation of MSK1. In contrast to the latter finding, in ASE2 cells the p38 inhibitor SB203580 did not show any effect on the phosphorylation of MSK1 (data not shown), so whether p38 is not activated by EPO in ASE2 cells, or p38 does not play a role in the activation of MSK1 in ASE2 cells, is at present unknown. We show in the present study that MSK1 is necessary for the serine 727 phosphorylation of STAT3, since expression of a mutant protein with an inactivated kinase domain abrogated the serine 727 phosphorylation. Zhang et al. [31] already identified MSK1 as a kinase capable of phosphorylating serine 727 in STAT3 by using an in vitro kinase assay. Therefore it is conceivable that in our cell system, MSK1 is responsible for the phosphorylation of STAT3 serine 727. However, we cannot rule out the possibility that another intermediate kinase is located between MSK1 and STAT3 and is actually the direct kinase for STAT3.

The role of serine 727 phosphorylation in the regulation of the transcriptional potential of STAT3 is not well understood. Some studies report a reduced tyrosine phosphorylation and binding of STAT3 to its target sequence upon serine phosphorylation. Additional studies describe an enhancing effect of STAT3 serine 727 phosphorylation on the transcriptional potential of STAT3. STAT3β, an alternatively spliced variant of STAT3 lacking serine 727, has been described to act as a dominant negative over wild-type STAT3, and

STAT3 molecules with serine 727 to alanine mutations have been described to have decreased transactivational potential [23]. In our study, serine 727 phosphorylation was shown to increase the transactivational potential of STAT3. With the use of a STAT3 reporter, we were able to show that blocking serine 727 phosphorylation by U0126 resulted in a diminished transactivation. In accordance with this, expression of a serine 727 to alanine-mutated STAT3 resulted in a diminished STAT3-mediated transactivation. By analyzing the EPO-induced expression of STAT3 target genes, we showed that expression of c-fos and c-myc were inhibited by U0126, underscoring the results with the reporter construct. Expression of c-fos mRNA was also reduced in the presence of a kinase-inactive MSK1 construct, as expected from the results with the MEK inhibitor U0126. EPO-induced expression of c-myc, however, was not affected by the expression of kinase-inactive MSK1. The cause of this discrepancy between the results with the kinase-inactive MSK1 and the MEK inhibitor U0126 is not clear. Probably other signal transduction pathways play a role in the regulation of c-myc expression. Interactions between AP-1 and STAT3 have been described, leading to enhanced STAT3-mediated transactivation [37]. This enhancement of STAT3 transactivation has been shown to be sensitive to the MEK inhibitor PD98059, possibly explaining the reduced c-myc expression upon incubation with U0126. This would suggest that the expression of c-myc is regulated by multiple factors, including STAT3, instead of STAT3 binding alone.

In conclusion, the present study describes the identification of the MEK-ERK-MSK1 pathway as the route responsible for the EPO-induced STAT3 serine 727 phosphorylation, and that this phosphorylation leads to an increase in transactivational potential of STAT3 in erythroid cells.

Acknowledgment

This work was supported by a grant from the Dutch Cancer Foundation (RUG 2000-2316).

References

1. Goodnough LT, Skikne B, Brugnara C. Erythropoietin, iron, and erythropoiesis. Blood. 2000;96:823–833.
2. Gabrilove J. Overview: erythropoiesis, anemia, and the impact of erythropoietin. Semin Hematol. 2000;37:1–3.
3. Bieber E. Erythropoietin, the biology of erythropoiesis and epoetin alfa. An overview. J Reprod Med. 2001;46:521–530.
4. Youssoufian H, Longmore G, Neumann D, Yoshimura A, Lodish HF. Structure, function, and activation of the erythropoietin receptor. Blood. 1993;81:2223–2236.
5. Mulcahy L. The erythropoietin receptor. Semin Oncol. 2001;28:19–23.
6. Tilbrook PA, Klinken SP. The erythropoietin receptor. Int J Biochem Cell Biol. 1999;31:1001–1005.
7. Smithgall TE, Briggs SD, Schreiner S, et al. Control of myeloid differentiation and survival by Stats. Oncogene. 2000;19:2612–2618.
8. Kirito K, Uchida M, Yamada M, Miura Y, Komatsu N. A distinct function of STAT proteins in erythropoietin signal transduction. J Biol Chem. 1997;272:16507–16513.
9. Kirito K, Nakajima K, Watanabe T, et al. Identification of the human erythropoietin receptor region required for Stat1 and Stat3 activation. Blood. 2002;99:102–110.
10. Kirito K, Uchida M, Takatoku M, et al. A novel function of Stat1 and Stat3 proteins in erythropoietin-induced erythroid differentiation of a human leukemia cell line. Blood. 1998;92:462–471.
11. Watowich SS, Mikami A, Busche RA, et al. Erythropoietin receptors that signal through Stat5 or Stat3 support fetal liver and adult erythropoiesis: lack of specificity of stat signals during red blood cell development. J Interferon Cytokine Res. 2000;20:1065–1070.
12. Millot GA, Svinarchuk F, Lacout C, Vainchenker W, Dumenil D. The granulocyte colony-stimulating factor receptor supports erythroid differentiation in the absence of the erythropoietin receptor or Stat5. Br J Haematol. 2001;112:449–458.
13. Socolovsky M, Nam H, Fleming MD, et al. Ineffective erythropoiesis in Stat5a$^{-/-}$5b$^{-/-}$ mice due to decreased survival of early erythroblasts. Blood. 2001;98:3261–3273.
14. Teglund S, McKay C, Schuetz E, et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell. 1998;93:841–850.
15. Yu X, Lin CS, Costantini F, Noguchi CT. The human erythropoietin receptor gene rescues erythropoiesis and developmental defects in the erythropoietin receptor–null mouse. Blood. 2001;98:475–477.
16. Wen Z, Darnell Jr. JE. Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3. Nucleic Acids Res. 1997; 25:2062–2067.
17. Decker T, Kovarik P. Serine phosphorylation of STATs. Oncogene. 2000;19:2628–2637.
18. Jain N, Zhang T, Fong SL, Lim CP, Cao X. Repression of Stat3 activity by activation of mitogen-activated protein kinase (MAPK). Oncogene. 1998;17:3157–3167.
19. Woetmann A, Nielsen M, Christensen ST, et al. Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and functional inhibition of STAT3. Proc Natl Acad Sci U S A. 1999;96:10620–10625.
20. Lim CP, Cao X. Serine phosphorylation and negative regulation of Stat3 by JNK. J Biol Chem. 1999;274:31055–31061.
21. Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol Cell Biol. 1997;17:6508–6516.
22. Wen Z, Zhong Z, Darnell Jr. JE. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell. 1995;82:241–250.
23. Schuringa JJ, Jonk LJ, Dokter WH, Vellenga E, Kruijer W. Interleukin-6-induced STAT3 transactivation and Ser727 phosphorylation involves Vav, Rac-1 and the kinase SEK-1/MKK-4 as signal transduction components. Biochem J. 2000;347:89–96.
24. Schuringa JJ, Dekker LV, Vellenga E, Kruijer W. Sequential activation of Rac-1, SEK-1/MKK-4, and protein kinase Cδ is required for interleukin-6-induced STAT3 Ser-727 phosphorylation and transactivation. J Biol Chem. 2001;276:27709–27715.
25. Gonzalez FA, Raden DL, Davis RJ. Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. J Biol Chem. 1991;266:22159–22163.
26. Gotoh A, Takahira H, Mantel C, et al. Steel factor induces serine phosphorylation of Stat3 in human growth factor–dependent myeloid cell lines. Blood. 1996;88:138–145.
27. Ng J, Cantrell D. STAT3 is a serine kinase target in T lymphocytes. Interleukin 2 and T cell antigen receptor signals converge upon serine 727. J Biol Chem. 1997;272:24542–24549.
28. Turkson J, Bowman T, Adnane J, et al. Requirement for Ras/Rac1-mediated p38 and c-Jun N-terminal kinase signaling in Stat3 transcriptional activity induced by the Src oncoprotein. Mol Cell Biol. 1999; 19:7519–7528.

29. Yokogami K, Wakisaka S, Avruch J, Reeves SA. Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR. Curr Biol. 2000;10:47–50.
30. Jain N, Zhang T, Kee WH, Li W, Cao X. Protein kinase C δ associates with and phosphorylates Stat3 in an interleukin-6-dependent manner. J Biol Chem. 1999;274:24392–24400.
31. Zhang Y, Liu G, Dong Z. MSK1 and JNKs mediate phosphorylation of STAT3 in UVA-irradiated mouse epidermal JB6 cells. J Biol Chem. 2001;276:42534–42542.
32. Miyazaki Y, Kuriyama K, Higuchi M, et al. Establishment and characterization of a new erythropoietin-dependent acute myeloid leukemia cell line, AS-E2. Leukemia. 1997;11:1941–1949.
33. Jonk LJ, Itoh S, Heldin CH, ten Dijke P, Kruijer W. Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-β, activin, and bone morphogenetic protein-inducible enhancer. J Biol Chem. 1998; 273:21145–21152.
34. Kaptein A, Paillard V, Saunders M. Dominant negative stat3 mutant inhibits interleukin-6-induced Jak-STAT signal transduction. J Biol Chem. 1996;271:5961–5964.
35. Boer PH, Potten H, Adra CN, et al. Polymorphisms in the coding and noncoding regions of murine Pgk-1 alleles. Biochem Genet. 1990;28: 299–308.
36. Haq R, Halupa A, Beattie BK, et al. Regulation of erythropoietin-induced STAT serine phosphorylation by distinct mitogen-activated protein kinases. J Biol Chem. 2002;277:17359–17366.
37. Schuringa JJ, Timmer H, Luttickhuizen D, Vellenga E, Kruijer W. c-Jun and c-Fos cooperate with STAT3 in IL-6-induced transactivation of the IL-6 response element (IRE). Cytokine. 2001;14:78–87.