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Involvement of mitogen-activated protein kinase in the cytokine-regulated phosphorylation of transcription factor GATA-1

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Gene-targeting experiments in transgenic mice have revealed an essential role for GATA-1 in the normal differentiation and development of erythroid cells. GATA-1 is phosphorylated *in vivo* on seven of its serine residues; the regulation and function of GATA-1 phosphorylation, however, is not understood. Here we demonstrate a role for MAP kinase (MAPK) signalling in the control of GATA-1 phosphorylation. We show that EGF-induced MAPK signalling results in the phosphorylation of ectopically expressed GATA-1 in COS cells. This phosphorylation can be positively or negatively regulated by genetic manipulation of the MAPK pathway through expression of constitutively activated, or dominant-negative, mutants of MAPK kinase (MAPKK), an upstream regulator of MAPK activity. *In vitro* phosphorylation experiments using purified MAPK and either recombinant GATA-1 or synthetic GATA-1 peptides suggest that GATA-1 is a MAPK substrate with MAPK phosphorylation occurring primarily on Ser26 and Ser178. We also show that GATA-1 is phosphorylated in factor-dependent haemopoietic progenitor cells in response to cytokine-induced signalling. Through the further use of a dominant-negative MAPKK mutant as well as chemical inhibitors of specific MAPKs, we identify ERK as an *in vivo* GATA-1 kinase. Finally, we demonstrate that mutation of serines 26 and 178 compromises the ability of GATA-1 to interact with the LIM-only protein LMO2 when both proteins are expressed in COS cells. These data implicate receptor-mediated signalling through the MAPK pathway as a control point in the regulation of transcription factor GATA-1. *The Hematology Journal* (2004) **5,** 262–272. doi:10.1038/sj.thj.6200345

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Introduction

GATA-1 is a member of an extended family of GATA transcription factors that share a conserved DNA-binding domain. GATA-1 is expressed in erythroid cells[1,2] as well as in megakaryocytes, bone marrow-derived mast cells,[3,4] basophils and eosinophils.[5] GATA-1 is also expressed, at least at low level, in progenitor cells.[6–8] Targeted disruption of the murine GATA-1 locus has revealed a critical role for GATA-1 only in the maturation of erythroid cells.[9–13]

GATA factors are subject to post-translational modifications. Both GATA-1 and GATA-2 have been shown to exist as phosphoproteins in erythroid[14] and multipotent progenitor cells, respectively.[15] GATA-1 is acetylated *in vivo* and the residues involved in acetylation appear to be important in GATA-1 function.[16,17] More recently, GATA-3 has been shown to be acetylated, with acetylation being important for GATA-3 function in the T-cell compartment.[18] The functional relevance of phosphorylation of GATA proteins is less clear. Through detailed two-dimensional phosphopeptide mapping analysis of wild-type and *in vitro*-mutagenised GATA-1 proteins, Crossley and Orkin have demonstrated that phosphorylation of GATA-1 occurs on seven specific serine residues.[14] Functional testing of COS cell-expressed GATA-1 proteins in which these serines were mutated to alanine, either individually or collectively, failed to reveal any effect upon DNA-binding, DNA-bending or transcriptional transactivation.[14]

While these experiments conducted in heterologous cells do not identify a function for GATA-1 phosphorylation, they do not exclude a role for phosphorylation

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in the regulation of GATA-1 function in a normal haemopoietic setting. Partington and Patient have explored this possibility using transformed haematopoietic cell lines. Their results suggest firstly that GATA-1 phosphorylation increases during the early phases of erythroid differentiation and, secondly, that phosphorylation of GATA-1 enhances its DNA-binding capacity.[19] This latter observation is consistent with data previously obtained by Taxman *et al.*,[20] examining the DNA-binding affinity of recombinant GATA-1 expressed in baculovirus. Against this general background, evidence for the regulation of GATA-1 phosphorylation by extracellular signals such as haemopoietic growth factors, which are ultimately involved in the survival growth and differentiation decisions of haematopoietic cells,[21,22] would provide additional support for functional relevance. Additionally, such information may give clues as to how other kinds of post-translational modifications are regulated. Mitogen-activated protein kinase (MAPK), also known as extracellular-signal regulated kinase (ERK), is thought to function as a key molecule in cytokine-induced signal transduction (reviewed in Denhardt[23]). Depending on cellular context, MAPK-dependent signalling may elicit either a proliferative or a differentiative response.[24] The fact that MAPKs can enter the nucleus when activated[25,26] supports the idea that phosphorylation of transcription factors by MAPKs provides the main cytoplasmic link between receptor-mediated events and changes in gene expression in the nucleus. The ubiquitously expressed transcription factors c-myc,[27] c-jun[28] and Elk-1[29] have been shown to be probable MAPK substrates; few tissue-specific transcription factors have been identified as MAPK targets.

We have previously shown that a component of GATA-2 phosphorylation in multipotential cells is regulated by the MAPK pathway in response to mitogenic signalling induced by the haemopoietic cytokine interleukin-3 (IL-3).[15] *In vitro* phosphorylation experiments using purified recombinant GATA-2 and MAPK proteins further suggested that MAPK itself functions directly as a GATA-2 kinase,[15] and more recently a docking site for erk has been identified within GATA-2.[30] Given the compartmentalised roles of GATA transcription factors in haematopoiesis,[31] it would be interesting to know whether the phosphorylation of GATA-1 is also regulated by the same signal transduction pathway. Identifying the kinases responsible for this phosphorylation may give us further clues both as to the exact mechanism of phosphorylation and provide clues as to the possible function. In this study, we show that one of the kinases acting on GATA-1 is an MAPK, and identify Ser26 and Ser178 as phosphorylation sites for the kinase. Mutation of these two residues abrogates the ability of GATA-1 to complex with one of its interaction partners, namely LMO2.[32,33] We also present evidence that GATA-1 is phosphorylated in response to mitogenic signalling by haemopoietic growth factors through the MAPK pathway.

Materials and methods

Cell lines

BA/F3 cells[34] were maintained in RPMI 1640 containing 10% foetal calf serum (FCS) and 2% mo-IL-3,[35] in the case of BA/F3 cells transfected with neomycin resistance expression vectors harbouring either the murine G-CSF receptor[35] or the dominant-negative MAPKK mutant, the medium was further supplemented with G418. Murine erythroleukaemia (MEL) cells and COS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. GATA-1 ts SCF cells were maintained at 32°C in RPMI 1640 containing 10% FCS and either mouse SCF (20 ng/ml), or mouse IL-3 (10 ng/ml) or human erythropoietin (0.2 U/ml).

Cell labelling and immunoprecipitation

Cell labelling was performed as follows: cells were incubated in phosphate-free medium containing dialysed FCS and [^{32}P] orthophosphate (1 mCi/ml, Amersham Corp.) for 6 h. The cells were washed with ice-cold medium and lysed in RIPA buffer (10 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) containing 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM benzamidine and 1 mM phenylmethylsulphfonyl fluoride (PMSF). Following incubation for 1 h at 4°C, an equal volume of RIPA buffer without NaCl was added to the extracts which were then centrifuged at 17 000 *g* for 20 min. The GATA-1 protein was then isolated from the supernatants by immunoprecipitation using 5 μg of rat monoclonal anti-GATA-1 antibody (N6) (Santa Cruz Biotechnology Inc.). The immunoprecipitates were washed five times with 1 ml of RIPA buffer containing 0.2 M NaCl. The samples were then boiled in Laemmli's sample buffer for 3 min, and fractionated on 10% SDS-PAGE gels.

PAGE and immunoblotting

Standard Laemmli-type SDS-PAGE gels were made using an acrylamide mix that contained a 29:1 ratio of acrylamide to *N*, *N*′ methylenebisacrylamide (bisacrylamide). SDS-PAGE gels used for 'shift' assays to separate phosphorylated from nonphosphorylated GATA-1 and MAPK contained an acrylamide/bisacrylamide ratio of 30:0.165, as described previously.[15] For Western analysis, gels were electroblotted onto PVDF membranes using a semi-dry transfer apparatus. Transfected MAPK was detected using monoclonal antibody 9E10,[36] which is directed against the myc tag present in the myc-ERK2 expression vector. Endogenous GATA-1 was detected by monoclonal antibody N6 (kind gift of D Engel). Transfected GATA-1 was detected using a monoclonal antibody directed against the flag tag (Kodak/IBI).

Transient expression in COS cells

Transfection of COS cells and preparation of whole-cell lysates were performed exactly as described previously.[15] pEF-flag-GATA-1 was constructed from a full-length murine GATA-1 cDNA clone (kind gift of SH Orkin) as follows: An *Nco*1(end-filled)–*Xho*1 fragment from the mGATA-1 cDNA clone (an *Nco*1 site spans the initiator methionine) was inserted into *Sma*1–*Xho*1 cleaved pBFT3 (kind gift of J Licht) producing an in-frame 5′-flag/GATA-1-3′ fusion. This fusion gene was subsequently transferred into the eukaryotic expression vector pEF-BOS-XC (kind gift of G Evan). The reporter plasmid pαD6 (kind gift of G Felsenfeld), which comprises concatamerised GATA-motif-oligomers linked to an αD-globin promoter-driven, CAT transcription unit, was used for CAT assays. Cell extracts for CAT assays were prepared, and CAT assays were performed as described previously,[15] except that 1-Deoxy (dichloroacetyl-1-14 C) chloramphenicol was used as a substrate. The total amount of DNA transfected in each experiment was kept constant by using carrier plasmid DNA. Quantitative estimates of CAT activity (% acetylation) were determined by analysis of chromatograms using a Molecular Dynamics PhosphorImager TM . CAT activities were normalised relative to β-galactosidase activity derived from a cotransfected β-gal expression vector which served to control for the efficiency of transfection.

In vitro *phosphorylation assays*

The coding region of the mouse GATA-1 cDNA was isolated from pEF-flag-GATA-1 by restriction endonuclease digestion with *Nco*I, 3′-end-filled by Klenow fragment, and cloned into the *Sma*I site of pGEX-30 (Pharmacia LKB Biotechnology Inc.) containing glutathione S-transferase (GST). The integrity of the final construct, pGEX-30-GATA-1, was confirmed by sequencing. Expression of GST-GATA-1 in *E. coli* (DE3) was induced for 3 h with 0.1 mM isopropylthiogalactoside and the GST-GATA-1 fusion protein purified using a GST column (Amersham), according to the manufacturer's instructions. A measure of 4 μg of each GST fusion protein was used for *in vitro* phosphorylation assays, which were performed as previously described.[15] Purified MAPK and MAPKAP kinase-1 were generous gifts of Dr P Cohen and Dr CJ Marshall, respectively. Reaction products were analysed by SDS-PAGE and transferred to a PVDF membrane prior to autoradiography or phosphopeptide mapping. Synthetic peptides were purified by HPLC and mass spectrometry. *In vitro* phosphorylated peptides were analysed by fractionation on tricine SDS-PAGE gels.

Phosphopeptide mapping

To generate a tryptic digest, the Immobilon fragment containing the labelled protein was incubated with 1 mg/ml of α-chymotrypsin for 18 h at 37°C, followed by oxidisation by performic acid. Phosphopeptide mapping was performed by two-dimensional separation on cellulose thin layer plates. The first dimension was electrophoresis at 1000 V for 30 min at pH 1.9, and the second dimension was chromatography in the vertical dimension using 1-butanol:pyridine:acetic acid:water (75:50:15:60, v/v).

Results

While *in vivo* labelling with orthophosphate and subsequent immunoprecipitation reveals the phosphorylated nature of GATA-1 in murine erythroleukaemia cells (see Figure 1a and Crossley and Orkin[14]), the assay does not readily afford a comparison between phosphorylated and nonphosphorylated forms of the protein. The different forms of the protein can, however, be distinguished by a 'shift' assay using polyacrylamide gels which contain lower than normal amounts of bis-acrylamide (Figure 1b). The left-hand track shows an immunoprecipitation of ^{32}P-labelled COS cells transfected with a GATA-1 expression clone; by definition, only phosphorylated GATA-1 protein is revealed. Western blotting using an anti-GATA-1 antibody reveals two bands, the upper one corresponding to the form revealed by ^{32}P-labelling. That these two forms represent phosphorylated and nonphosphorylated forms

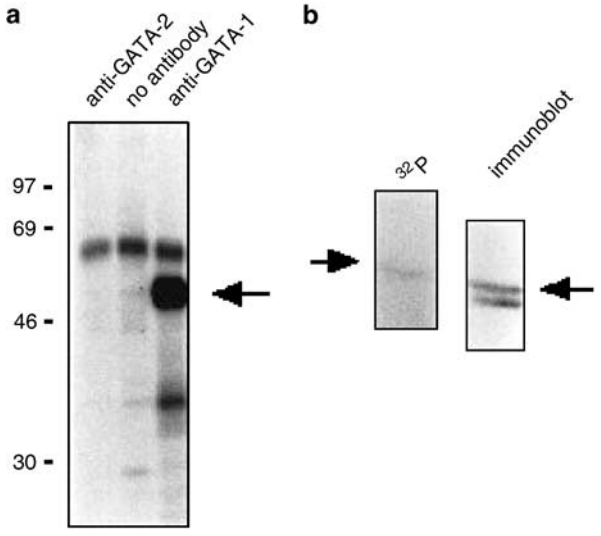

Figure 1 Assays for GATA-1 phosphorylation. (a) *in vivo* labelling: Cell lysates prepared from ^{32}P orthophosphate-labelled MEL cells were immunoprecipitated with the antibodies indicated. Immunoprecipitates were fractionated on standard SDS-PAGE gels; autoradiography reveals the presence of phosphorylated GATA-1, indicated by the arrow. The numbers next to the autoradiographs indicate relative molecular weight in kDa. (b) Shift assay: GATA-1 immunoprecipitated from ^{32}P-labelled COS cells transfected with pEF-flag-mGATA-1 (see methods) were fractionated on SDS-PAGE gels containing only 0.165% bis-acrylamide. After transfer to PVDF membrane, direct autoradiography reveals the migration position of phosphorylated GATA-1 (left lane). Subsequent immunoblot analysis of the same membrane reveals the differential migration of phosphorylated (upper band) *versus* nonphosphorylated (lower band) forms of GATA-1.

was further confirmed by phosphatase treatment (not shown). Thus, the shift assay provides an important and useful analytical tool to monitor relative levels of GATA-1 phosphorylation.

Involvement of the MAPK pathway in GATA-1 phosphorylation

In the first instance, we examined the role of MAPK in GATA-1 phosphorylation using the COS cell system because (i) MEL cell GATA-1 and COS cell-expressed GATA-1 have identical 2D-phosphopeptide maps,[14] and (ii) MAPK activity can be manipulated relatively easily in COS cells. Thus, MAPK activity can be suppressed in COS cells by serum starvation, and then rapidly stimulated by the addition of epidermal growth factor (EGF). MAPK activity in COS cells can also be modulated using functional mutants of MAPKK,[37] an upstream regulator of MAPK function. Glutamic acid substitutions at Ser217 and Ser221 residues produce a constitutively activated MAPKK mutant, termed CA-MAPKK, that constitutively phosphorylates and thereby activates MAPK.[24,37] Alanine substitutions at the same serine residues produce a dominant-negative form of MAPKK, termed DN-MAPKK, which blocks the activation of MAPK.[24,37]

GATA-1 and MAPK were coexpressed in COS cells; MAPK activity was manipulated by EGF stimulation and/or expression of functional mutants of MAPKK, as indicated in Figure 2. The phosphorylation status of GATA-1 and MAPK are shown in the upper and lower panels, respectively. Phosphorylation of MAPK leads to a shift in its mobility, as described above for GATA-1. Under conditions of serum starvation, where MAPK is not phosphorylated (Figure 2a, left lane), the bulk of GATA-1 also appears unphosphorylated (Figure 2a, left lane). Activation of MAPK by cotransfection of CA-MAPKK (Figure 2a, right lane) or EGF stimulation (Figure 2b, left lane) results in an increase in the abundance of the phosphorylated form of GATA-1 (Figure 2a, right lane & Figure 2b, left lane). Cotransfection of DN-MAPKK, however, blocks EGF-dependent activation of MAPK and GATA-1 phosphorylation (Figure 2b, right lanes). These results implicate components of the MAPK pathway, downstream of MAPKK, in the regulation of GATA-1 phosphorylation.

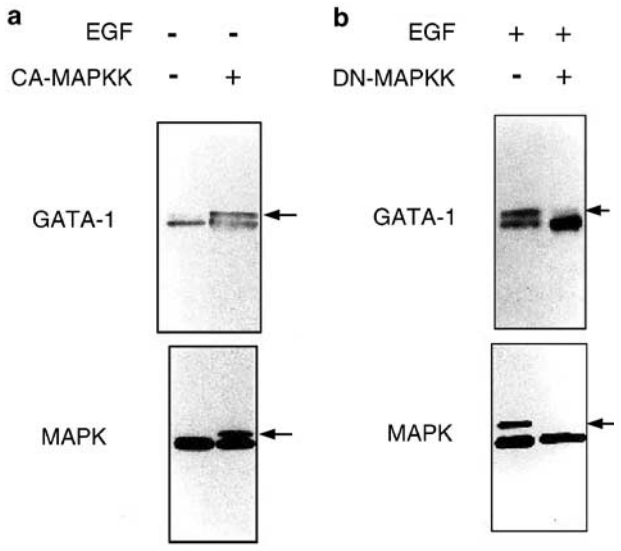

Figure 2 Regulating GATA-1 phosphorylation by manipulating the MAPK pathway in COS cells. COS cells were cotransfected with flag-tagged mouse GATA-1 and myc-tagged MAPK expression vectors. MAPK activity in these cells was modulated by cotransfection of either constitutively activated (CA-) or dominant negative (DN-) MAPKK. At 24 h post-transfection, the culture medium was replaced with serum-free DMEM, and the cells were incubated for a further 24 h. EGF was then added to the samples indicated, for 5 min, at a concentration of 10 ng/ml. The cells were lysed in RIPA buffer, fractionated by modified SDS-PAGE and immunoblotted using a rat anti-GATA-1 monoclonal antibody. An equivalent amount of lysate was analysed for MAPK activity by Western blotting using antibody 9E10 directed against the myc tag; the results of this analysis are presented in the corresponding lower panels.

Phosphorylation of GATA-1 by MAPK in vitro

MAPKK is a dual-specificity protein kinase that activates MAPK by phosphorylating a threonine and a tyrosine residue.[38,39] The other downstream component of the classical MAPK pathway is MAP kinase-activated protein kinase-1 (MAPKAP kinase-1), previously designated p90rsk, reviewed in (Su and Karin)[40]. Inspection of the amino-acid sequence of GATA-1 reveals that two serine residues (Ser26 and Ser178) out of the seven serine residues known to be phosphorylated in MEL cells and GATA-1-expressing COS cells[14] lie within a consensus sequence [P/L-X-(X)-S/T-P] for MAPK.[38,39] On the other hand, the seventh serine residue Ser310 lies within a recognition motif for MAPKAPK-1 [R-X-X-S].[40] To determine whether GATA-1 is a substrate for either of these kinases *in vitro*, we performed *in vitro* phosphorylation assays of bacterially expressed recombinant GATA-1 and chemically synthesised GATA-1 peptides using purified MAPK and MAPKAPK-1. Figure 3a shows the results of *in vitro* phosphorylation experiments using purified MAPK, which had been chemically activated by thiophosphorylation. GST-GATA-1 is phosphorylated by MAPK, as is GST-GATA-2 which serves as a positive control substrate in this analysis; no phosphorylation is observed in the GST alone or no extract lanes which serve as negative controls. However, neither GST-GATA-1 nor a chemically synthesised peptide incorporating the Ser310 region was phosphorylated *in vitro* by MAPKAPK-1; a synthetic peptide incorporating a canonical MAPKAPK-1 site provided a positive control (data not shown).

We then performed two-dimensional phosphopeptide mapping of MAPK-phosphorylated GST-GATA-1. The 2D-pattern obtained after chymotrypsin digestion (Figure 3b) was compared to the chymotryptic maps reported for *in vivo* labelled GATA-1,[14] and found to be consistent with phosphorylation of GATA-1 at Ser26

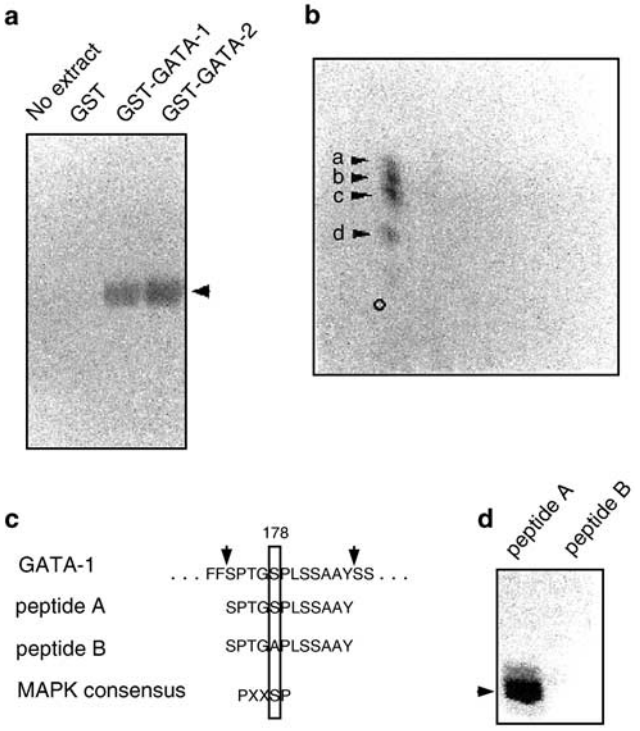

Figure 3 Phosphorylation of GATA-1 by MAPK *in vitro*. (a) *In vitro* kinase assays performed using chemically activated (thiophosphorylated) MAPK. The reaction mixture in each lane contains, from the left: no extract, 2 μg of GST protein, 2 μg of GST-GATA-1, and 2 μg of GST-GATA-2 protein, respectively. The reaction products were fractionated on a 10% polyacrylamide gel. (b) 2-D phosphopeptide map of GST-GATA-1 labelled by MAPK *in vitro*. Electrophoresis and chromatography were conducted in the horizontal and vertical dimensions, respectively. 'O' marks the origin and labelled phosphopeptides are arrowed. The data were collected by the Molecular Dynamics PhosphorImager™. (c) Two peptides corresponding to the predicted tryptic peptide (amino acids 174–178) spanning serine 178 in mouse GATA-1 were chemically synthesised. Peptide A contains the wild-type sequence, while serine 178 which lies within a MAPK consensus site is substituted by alanine in peptide B. The arrowheads indicate sites of tryptic cleavage. (d) Synthetic peptides A and B were used as substrates in *in vitro* kinase reactions with purified MAPK. The reaction products were fractionated by tricine-SDS-PAGE.

and Ser178. To confirm this, we chemically synthesised a peptide corresponding to the predicted chymotryptic peptide incorporating Ser178 (peptide A in Figure 3c). This peptide was effectively phosphorylated by MAPK *in vitro* (Figure 3d). To confirm that phosphorylation was occurring specifically and exclusively at Ser178, a second peptide (peptide B in Figure 3c) was synthesised, in which an alanine substitution at Ser178 destroys the MAPK motif; peptide B was not phosphorylated by MAPK (Figure 3d). Taken together with the previous data obtained in COS cells and the experiments of Crossley and Orkin,[14] we conclude that GATA-1 is phosphorylated by MAPK at these two serine residues.

Cytokine dependence of GATA-1 phosphorylation

In light of the fact that a number of haematopoietic growth factors activate the ras-raf-MAPK pathway,[41,42] we investigated whether haematopoietic growth factors stimulate the phosphorylation of GATA-1, and, if so, whether it is effected through MAPK activation. For these experiments, we used the murine IL-3-dependent progenitor cell line BA/F3.[34] BA/F3 cells are generally considered to be representative of the pro-B lymphocyte compartment, although recent evidence suggests that they may represent an earlier progenitor with less restricted lineage potential. In addition to expressing GATA-2,[15] the passage of BA/F3 cells used here expresses low levels of both GATA-1 message, as determined by Northern blot analysis, and GATA-1 protein, as determined by GMSA and Western blotting (May, Gale and Enver, unpublished observations). In addition, we also used a novel multipotent progenitor derived from a previously described cell line.[43] This cell line is termed GATA-1 ts SCF, and is responsive to IL-3, or Epo or SCF.[44]

We compared the phosphorylation status of GATA-1 in IL-3-deprived *versus* IL-3stimulated BA/F3 cells. Cells were deprived of IL-3 for 6 h (−IL-3) and then re-exposed to IL-3 for 5 min (+IL-3). Figure 4a shows Western blot analysis of cell lysates using anti-GATA-1 and anti-MAPK antibodies. The results demonstrate that IL-3-induced activation of MAPK is accompanied by an increase in the abundance of phosphorylated GATA-1. Further evidence for MAPK-dependent phosphorylation of GATA-1 in BA/F3 cells, was obtained using BA/F3 cells, which had been engineered to ectopically express the G-CSF receptor,[35] BA/F3-[G-CSF-R]; the G-CSF receptor also signals through the MAPK pathway. The results presented in Figure 4b show that exposure of cytokine-deprived BA/F3-[G-CSF-R] cells to G-CSF also results in MAPK activation and GATA-1 phosphorylation. Finally, direct evidence for the role of cytokine-induced, MAPK-dependent signalling in the regulation of GATA-1 phosphorylation in haemopoietic progenitor cells was obtained using the interfering mutant of MAPKK. Thus, IL-3 stimulation of factor-deprived BA/F3 cells expressing this interfering mutant, BA/F3-[DN-MAPKK], did not produce MAPK activation or GATA-1 phosphorylation (Figure 4c).

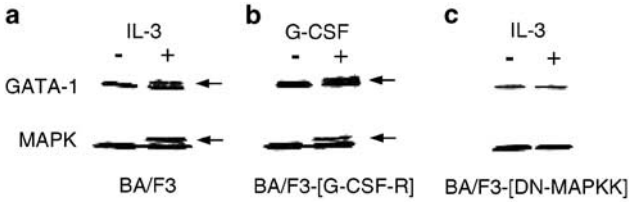

Figure 4 Cytokine-dependent stimulation of GATA-1 phosphorylation involves the MAPK pathway. Wild-type BA/F3 cells (panel (a)), or BA/F3 cells transfected with expression vectors for either the G-CSF receptor (panel (b)), or the dominant-negative mutant of MAPKK (panel (c)), were analysed by shift assays for GATA-1 (upper panels) and MAPK (lower panels) phosphorylation using anti-GATA-1 and anti-MAPK monoclonal antibodies, respectively. In all cases, cells were deprived of IL-3 for 6 h (lanes) and then stimulated for 5 min by addition of either IL-3 (panels (a) and (c)) or G-CSF (panel (b)).

To evaluate the ability of cells to phosphorylate GATA-1 in response to other growth factors, we further examined the GATA-1 ts SCF cell line. The GATA-1 ts SCF cell line derives from mouse bone marrow cells (GATA-1 ts Epo) of transgenic mice conditionally immortalised by a thermosensitive T-antigen gene placed under the transcriptional control of GATA-1 promoter and enhancer sequences.[43] From these cells which were originally responsive to erythropoietin, we further selected a line that responds well to either SCF, IL-3 or Epo, and that can be reversibly passaged between these factors. This line, when grown in SCF, expresses very low levels of globin RNA and megakaryocytic markers; upon treatment with IL-3 or Epo, or alternatively, GM, G and M-CSF, these cells are induced to express globin RNA or myeloid markers, respectively.[44] GATA-1 ts SCF cells, grown in Epo for several days, then deprived of Epo for 6 h, were stimulated with IL-3, Epo or SCF. Under these conditions, there is a strong increase (within 30′) of the upper (phosphorylated) GATA-1 band in the presence of IL-3 or Epo, but not SCF (Figure 5a, see lanes 9, 11 and 13 *versus* lanes 1, 2). On the other hand, when cells are grown in SCF, thereby increasing the level of SCF-receptor c-kit, and then starved, addition of SCF causes an early increase of the phosphorylated GATA-1 band; IL-3 has a weaker effect and Epo has none (Figure 5b, see lanes 3, 5 and 7 *versus* lanes 1 and 2). These effects are paralleled by an increase of the upper (phosphorylated) band or erk-2 upon IL-3 or Epo (Figure 5a, lanes 9, 11) or SCF (Figure 5b, lane 7) stimulation.

As both Epo and IL-3 are able to induce haemoglobinisation of GATA-1 ts SCF cells,[44] the experiment was also carried out in the presence of the p38 inhibitor SB203580, that is known to inhibit Epo-dependent haemoglobinisation in SKT6 cells.[44,45] Paradoxically, the p38 inhibitor further stimulated Epo- or IL-3-induced erk-2 phosphorylation (Figure 5a, lanes 10, 12) and SCF-induced erk-2 phosphorylation (Figure 5b, lane 8), while not increasing GATA-1 phosphorylation. As erk-2 is a substrate for signals mediated by different growth factor-activated pathways, we investigated the effects of several chemical inhibitors of these pathways. As shown in Figure 5c, the MEK inhibitor U0126 abolishes both the upper GATA-1 and erk-2 bands, whereas the p38 inhibitor SB203580 and jak-2 inhibitor AG490 are ineffective. Thus, the data obtained indicate that an MEK, as opposed to p38 or jak-2, related pathway is involved in MAPK and GATA-1 phosphorylation in GATA-1 ts SCF cells under the experimental conditions used.

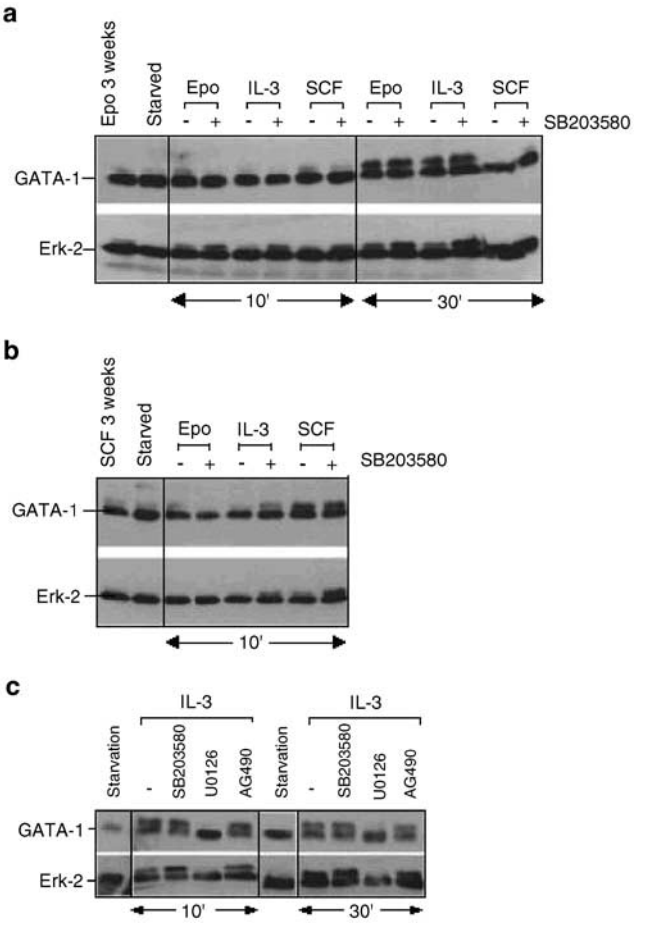

Figure 5 Several growth factors stimulate GATA-1 phosphorylation in a mouse multipotent haematopoietic cell line. (a) GATA-1 ts SCF cells were grown for 3 weeks or more under standard conditions in the presence of 0.2 U/ml of human erythropoietin; following starvation for erythropoietin for 6 h, the cells were stimulated for 10–30 min by addition of either recombinant IL-3 (10 ng/ml), Epo (0.5 U/ml) or SCF (20 ng/ml), in the presence or absence of the p38 inhibitor SB203580 at 10 μM, as indicated above the figure. (b) GATA-1 ts SCF cells were grown in the presence of 20 ng/ml of SCF for at least 3 weeks and stimulated as above for 10 min (no effects were seen at 30 min, not shown). (c) Cells grown in Epo as above were stimulated with IL-3 in the presence or absence of the following inhibitors: p38 inhibitor SB203580 (10 μM), MEK inhibitor U0126 (10 μM), jak-2 inhibitor AG490 (5 μM).

Function of MAPK-dependent phosphorylation

Since Ser26 and Ser178 lie in the region responsible for transactivation, we examined the influence of the phosphorylation by MAPK on the transcriptional transactivation potential of GATA-1. These experiments were performed in COS cells using a GATA-dependent chloramphenicol acetyl transferase (CAT) reporter gene. COS cells were chosen so as to allow comparability with previous studies, and also to avoid complication by endogenous GATA-1 expression in haemopoietic cells. Representative data are presented in Figure 6a, and collective data obtained from four independent experiments have been plotted in Figure 6b. While expression of GATA-1 markedly increases CAT activity, as expected, manipulation of MAPK activity using CA-MAPKK and DN-MAPKK mutants does not appear to markedly positively or negatively modulate the total level of GATA-1-dependent transactivation observed. These results, which are similar with those previously reported for GATA-1[14] and GATA-2,[15] suggest that MAPK-dependent phosphorylation of GATA-1 does not significantly alter its

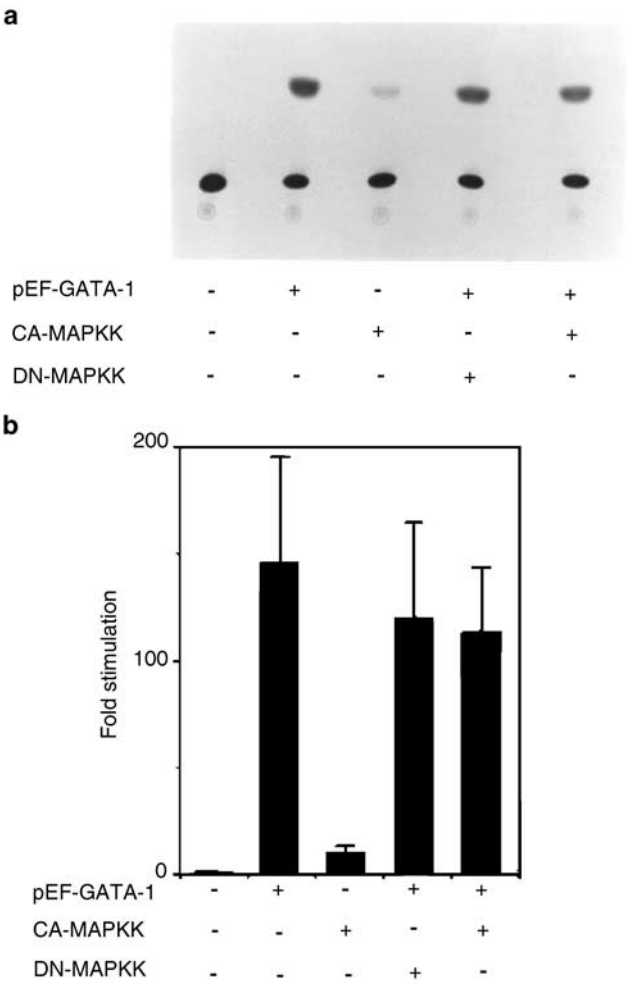

Figure 6 Analysis of transactivation potential of GATA-1. A GATA-dependent CAT reporter gene was cotransfected together with the various combinations of GATA-1, CA-MAPKK or DN-MAPKK expression vectors indicated. A chromatogram from a typical experiment is shown in panel (a). (b) Quantitative data obtained from five independent experiments is collectively presented in the graph. CAT activities were quantitated by scanning on a Molecular Dynamics PhosphorImager. The data are expressed as fold stimulation relative to the basal activity of the GATA-CAT reporter construct, with standard errors indicated by the vertical bars.

transactivation potential in COS cells, at least under these experimental conditions. It is perhaps noteworthy that the basal level of GATA-1-independent reporter gene activity is increased in the presence of the constitutively active MAPKK, presumably reflecting increased phosphorylation of some basal transcriptional component. It may be appropriate to take this into account when considering the extent of GATA-1-dependent transactivation in the presence of CA-MAPKK.

GATA proteins are known to have several interaction partners. We therefore examined the possibility that phosphorylation by MAPK may regulate these interactions. We tested this using the LIM-only protein LMO2, a GATA-1 partner protein which participates in a complex in erythroid cells that involves GATA-1, SCL, E47 and LdB-1.[32,33] COS cells were transfected with

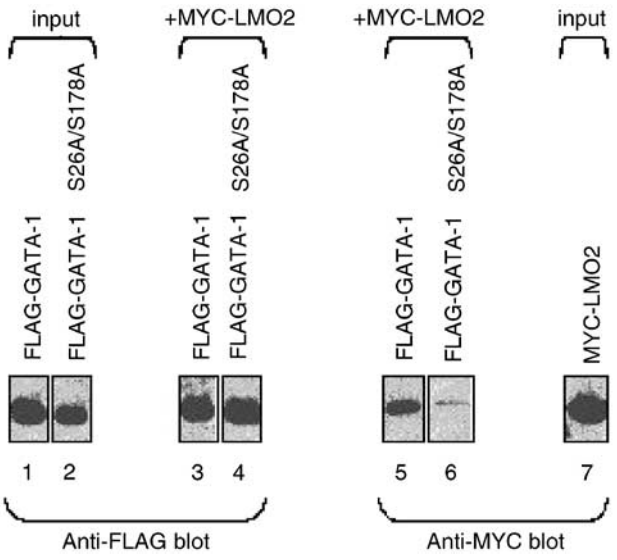

Figure 7 Mutation of serines 26 and 178 compromise the formation of GATA-1-LMO2 complex. COS cell nuclear extracts programmed with either wild-type GATA-1, or an S26A/S178A double-mutant GATA-1 were mixed with an LMO2-programmed extract were mixed and used in immunoprecipitation experiments. The GATA-1 and LMO2 molecules were tagged with FLAG and MYC epitopes, respectively. The input (lanes 1, 2 and 7) and immunoprecipitated material (lanes 3–6) was subjected to Western blotting using FLAG (lanes 1–4) or MYC (lanes 5–7) antibodies. Comparison of lanes 5 and 6 indicated that the mutated form of GATA-1 is significantly compromised in its ability to interact with LMO2.

FLAG-tagged versions of wild-type GATA-1, as well as a GATA-1 mutant in which both serines 26 and 178 had been mutated to alanine. Nuclear extracts were prepared and the levels of GATA-1 within them assessed by Western blotting (Figure 7, lanes 1 and 2). Nuclear extracts containing equivalent amount of GATA protein were mixed with COS nuclear extracts that had been programmed with a myc-tagged version of LMO2 (Figure 7, lane 7) and immunoprecipitated using an anti-FLAG antibody. Immunoprecipitates were analysed by Western blotting using both FLAG (Figure 7, lanes 3 and 4) and MYC antibodies (Figure 7, lanes 5 and 6). The data show that mutated form of GATA-1 is significantly compromised in its ability to interact with LMO2, and suggest that the residues of GATA-1 that are phosphorylated by MAPK may be involved in this interaction.

Discussion

We have provided evidence that the MAPK signal transduction pathway is involved in the phosphorylation of transcription factor GATA-1; in particular, we have shown that GATA-1 phosphorylation in haemopoietic cells can be regulated by cytokine-induced signalling in a MAPK-dependent manner. We have also shown that two of the seven serine residues in GATA-1 (S26 and S178) that have been reported to be phosphorylated *in vivo* are phosphorylated by purified

MAPK *in vitro*. Experiments conducted in heterologous COS cells failed to provide clear evidence for a function for MAPK-dependent phosphorylation in regulating the innate transactivation potential of GATA-1, but did provide evidence for the involvement of S26 and S178 in the interaction of GATA-1 with LMO2.

MAPK is ideally placed to effect transcription factor phosphorylation in response to receptor-mediated events at the plasma membrane, because it translocates from the cytosol into the nucleus upon activation.[25,26] While a number of ubiquitously expressed transcription factors have been shown to be targets for MAPK phosphorylation,[27–29] few transcription factors with tissue-restricted patterns of expression have been identified as MAPK substrates. A notable exception is transcription factor CEBPβ[46] (also known by a number of other names including NF-IL6). G-CSF-induced phosphorylation of CEBPβ by MAPK has the functional consequence of derepressing its transcriptional activation domain.[46] In our experiments, MAPK-dependent phosphorylation of GATA-1 did not appear to alter its ability to transactivate transcription from a GATA-dependent reporter gene in COS cells. Transcriptional transactivation experiments with GATA-2, also performed in COS cells, have similarly failed to reveal a function for GATA-2 phosphorylation.[15] These results are in agreement with those reported by Crossley and Orkin,[14] who examined the function of GATA-1 phosphorylation by systematic mutagenesis of *in vivo* phosphorylated serine residues; no gross qualitative effect was observed on transcriptional transactivation, DNA binding or DNA bending by GATA-1.[14] All of these experiments are in a sense compromised, since they are conducted in heterologous (COS) cells on episomal reporters and, probably, in conditions of excess expression. A point of potential interest is the data obtained with the CA-MAPKK, which showed increased reporter activity as a result of CA-MAPKK expression in the absence of GATA-1. If this is taken into account, one might argue that the additional activation seen in this situation in response to GATA-1 is somewhat reduced. Given that not all of the GATA-1 expressed is likely to be phosphorylated at any given moment, this may further mask any reduced activity of GATA-1 resulting from its phosphorylation. This scenario, however, would be hard to reconcile with the results of Partington and Patient,[19] who examined the effects of GATA-1 phosphorylation in erythroleukaemic cell lines and provided evidence for (i) differentiation-associated changes in phosphorylation, and (ii) increased DNA-binding activity as a result of phosphorylation.

The function of phosphorylation, however, need not be restricted to regulating the innate transactivation potential or absolute DNA-binding activity of GATA-1, and could rather alter GATA activity in any of a number of other ways. For example, phosphorylation may modulate the binding site preference of GATA-1 or its ability to interact with itself or other transcriptional regulators; evidence suggests a functional role for homotypic GATA-1 interactions and the involvement of GATA-1 in multiprotein complexes has been reported.[32,33,47] Such modulations could alter the genes targeted by GATA-1, and/or its transactivation ability in the context of a native transcriptional complex. Our data which show that mutation of serines 26 and 178, which are phosphorylated by MAPK, alters the ability of GATA-1 to interact with LMO2 are relevant in this regard, and raise the possibility that extracellular signalling via the MAPK pathway may regulate the formation or activity of GATA-1-containing multiprotein complexes. Intriguingly. these residues lie outside the zinc-finger regions of GATA-1 where many of the described protein–protein interactions take place. To our knowledge, the regions of GATA-1 and LMO2 that are involved in the interaction of these two proteins is not yet known, and this information will be valuable in determining the mechanism by which mutation of these two serines abrogates protein–protein interaction.

If phosphorylation of GATA-1 is important in regulating the activity of multiprotein complexes such as those that include LMO2, then cell fate could prove a more sensitive and relevant assay than transient transfection systems, particularly those such as the ones we have used here which comprise isolated GATA motifs. BAF-3 cells are strictly dependent on IL-3 signalling for survival and proliferation; on the other hand, GATA-1 ts SCF cells require SCF for survival and proliferation, but in the presence of IL-3 and Epo induce haemoglobinisation, while continuing to actively proliferate.[44] Thus, the results obtained with the latter cell line (Figure 5) hint at possible roles of GATA-1 phosphorylation not only in cell replication-related events, but also in aspects of cell differentiation, that is, haemoglobinisation. Intriguingly, in this model, we were able to distinguish a MEK as opposed to p38 or jak-2-related pathway as being responsible for GATA-1 phosphorylation. It will be interesting to see if this finding extends into other cell systems where GATA factors (eg GATA-2) are subject to cytokine-mediated phosphorylation.

Several cell fate assays for GATA factor function have been employed, including the rescue of the erythroid differentiation and maturation potential of GATA-null ES cells[12] and GATA-null erythroid cell lines[13] by ectopic GATA expression, as well as the transgenic expression of GATA-1 cDNA in GATA-1 knockdown mice.[48] GATA-1 has also been shown in certain instances to be capable of reprogramming the lineage output of both committed and multipotential haematopoietic progenitor cell lines,[49,50] as well as primary GM progenitor cells.[51] To date, however, the function of GATA-1 phosphorylation has only been addressed in one cell fate-based assay, namely the induction of megakaryocytic differentiation of the myeloid progenitor cell line 416B. Enforced expression of GATA-1 results in differentiation of 416B cells into acetylcholinesterase-positive megakaryocytes.[52] Surprisingly, dephos-GATA-1 was as efficient as wild-type GATA-1 in eliciting megakaryocyte differentiation of 416B cells. However, the interpretation of GATA-1-induced megakaryocyte differentiation in this assay requires the consideration of a number of key points.

First, the phosphorylation pattern of GATA-1 in megakaryocytes is not known, and the mutation of all possible phosphorylation sites may obscure potentially positive or negative functions of individual sites. Second, targeted disruption of the GATA-1 locus in mice does not significantly disrupt initial differentiation down the megakaryocyte pathway.[53] Third, ectopic expression of GATA-2 (which is already reasonably expressed in 416B cells) as well as GATA-3 (which is not normally expressed in megakaryocytes) also elicits megakaryocytic differentiation in this system.[54] Finally, expression of just the C-terminal finger regions of either GATA-1 or GATA-2 is sufficient for megakaryocytic differentiation.[55] These results, like those obtained in the ES system, suggest a degree of redundancy and overlap in GATA function that will undoubtedly complicate further experimental approaches aimed at elucidating the possible functional relevance of GATA-1 phosphorylation.

Finally, such experiments may need to take particular care with respect to levels of expression of wild-type and mutant GATA proteins which may themselves be altered by post-translation modification. Several recent reports have provided evidence for the importance of precise GATA-factor levels.[56–59] Overexpression systems may therefore mask important functions of post-translational modifications of GATA proteins.

These caveats aside, our report provides the first clear evidence for regulation of the post-translational modification status of GATA-1 in response to extracellular signals, and delineates the signalling pathway involved in the modification of two specific residues which are subject to phosphorylation *in vivo*. Furthermore, through mutation of these two residues, we provide evidence for their involvement in the interaction of GATA-1 with LMO2. These results therefore raise the possibility that MAPK-dependent extracellular signalling may regulate the interaction of GATA-1 with its partner proteins. Thus, while the complete functional relevance of GATA-1 phosphorylation remains to be understood, the key observation reported in this paper, that is, its regulation by receptor-mediated MAPK signalling, is intriguing, particularly in light of the fact that the phosphorylation of GATA-2 is also in part regulated by this pathway.[15] MAPK-dependent extracellular signalling may therefore provide one mechanism for regulating crosstalk between GATA-1 and GATA-2.

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